

# Hepatocyte Nuclear Factor 4 alpha 2 Messenger RNA Reprograms Liver-Enriched Transcription Factors and Functional Proteins in End-Stage Cirrhotic Human Hepatocytes

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The only definitive therapy for end-stage liver disease is whole-organ transplantation. The success of this intervention is severely limited by the complexity of the surgery, the cost of patient care, the need for long-term immunosuppression, and the shortage of donor organs. In rodents and humans, end-stage degeneration of hepatocyte function is associated with disruption of the liver-specific transcriptional network and a nearly complete loss of promoter P1-driven hepatocyte nuclear factor 4-alpha (P1-HNF4 $\alpha$ ) activity. Re-expression of *HNF4 $\alpha$ 2*, the predominant P1-HNF4 $\alpha$ , reinstates the transcriptional network, normalizes the genes important for hepatocyte function, and reverses liver failure in rodents. In this study, we tested the effectiveness of supplementary expression of human *HNF4 $\alpha$ 2* messenger RNA (mRNA) in primary human hepatocytes isolated from explanted livers of patients who underwent transplant for end-stage irreversibly decompensated liver failure (Child-Pugh B, C) resulting from alcohol-mediated cirrhosis and nonalcoholic steatohepatitis. Re-expression of *HNF4 $\alpha$ 2* in decompensated cirrhotic human hepatocytes corrects the disrupted transcriptional network and normalizes the expression of genes important for hepatocyte function, improving liver-specific protein expression. End-stage liver disease in humans is associated with both loss of P1-HNF4 $\alpha$  expression and failure of its localization to the nucleus. We found that while *HNF4 $\alpha$ 2* re-expression increased the amount of P1-HNF4 $\alpha$  protein in hepatocytes, it did not alter the ability of hepatocytes to localize P1-HNF4 $\alpha$  to their nuclei. **Conclusion:** Re-expression of *HNF4 $\alpha$ 2* mRNA in livers of patients with end-stage disease may be an effective therapy for terminal liver failure that would circumvent the need for organ transplantation. The efficacy of this strategy may be enhanced by discovering the cause for loss of nuclear P1-HNF4 $\alpha$  localization in end-stage cirrhosis, a process not found in rodent studies. (*Hepatology Communications* 2021;5:1911-1926).

Over 1 million deaths are estimated per year worldwide due to complications of end-stage liver disease.<sup>(1)</sup> The most common causes include chronic infection by hepatitis viruses, alcohol-mediated cirrhosis, and nonalcoholic steatohepatitis (NASH); the final step in decompensated disease is the development of portal hypertension and hepatocellular failure.<sup>(2)</sup> Pathogenesis involves

*Abbreviations:* cDNA, complementary DNA; CEBP $\alpha$ , CCAAT/enhancer-binding protein alpha; CF VII, coagulation factor VII; DPBS, Dulbecco's phosphate-buffered saline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HMM, hepatocyte maintenance medium; HNF1/4 $\alpha$ , hepatocyte nuclear factor 1/4-alpha; mRNA, messenger RNA; NASH, nonalcoholic steatohepatitis; OTC, ornithine transcarbamylase; P1-HNF4 $\alpha$ , promoter P1-driven hepatocyte nuclear factor 4-alpha; PAH, phenylalanine hydroxylase; PBS, phosphate-buffered saline; PPAR $\alpha$ , peroxisome proliferator-activated receptor alpha; qPCR, quantitative polymerase chain reaction; TAT, tyrosine aminotransferase; UGT1A1, UDP glucuronosyltransferase family 1 member A1; UTR, untranslated region.

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progressive damage of liver parenchyma and replacement of healthy liver by scar tissue.<sup>(3)</sup> Unfortunately, the underlying mechanisms responsible for deterioration of hepatocyte function and consequent hepatic failure in humans are not fully understood. Chronic hepatic damage produces oxidative<sup>(4)</sup> and endoplasmic reticulum stress<sup>(5)</sup> that can reduce the proliferative capacity of hepatocytes<sup>(6)</sup> and induce cell death.<sup>(5,7,8)</sup> Ongoing injury may also generate intrinsic damage to resident hepatocytes, whose function may be compromised by the abnormal microenvironment of cirrhosis.<sup>(9)</sup> However, the extent to which each of these factors plays a role in hepatocyte survival and dysfunction remains unclear.

Some progress has been made using transcriptomic analysis of end-stage decompensated cirrhotic livers during the past decade.<sup>(9-11)</sup> In rat studies, progression

to hepatocyte failure has been associated with down-regulation of promoter P1-driven hepatocyte nuclear factor 4- $\alpha$  (P1-HNF4 $\alpha$ ) expression and suppression of its regulatory network.<sup>(12)</sup> Simple comparisons of gene expression levels in normal human hepatocytes compared to hepatocytes isolated from decompensated livers of patients have failed to produce such a simple correlation. This is not surprising because there is variability in the baseline expression of hepatocyte-specific genes between individual patients, in contrast to what occurs in inbred rodents. Thus, it has been difficult to elucidate the underlying mechanism of hepatic failure at the molecular level in humans. However, quantitative analysis of gene and protein expression in liver samples from a relatively large cohort of patients with cirrhosis and liver dysfunction showed diminished P1-HNF4 $\alpha$  activity, where loss of P1-HNF4 $\alpha$  expression strongly

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*Potential conflict of interest: Dr. Fox is the cofounder of and owns stock in Von Baer Wolff; he owns stock in Pittsburgh ReLiver Inc. and consults for Miromatrix; he is coinventor on a patent application that describes the use of transcription factors to treat chronic liver failure and on a patent application related to methods to enhance hepatic functions in human failing livers. Dr. Soto-Gutierrez is the cofounder and owns stock in Von Baer Wolff and Pittsburgh ReLiver Inc; he is coinventor on a patent application that describes the use of transcription factors to treat chronic liver failure and on a patent application related to methods to enhance hepatic functions in human failing livers. Dr. Ostrowska is the cofounder of and owns stock in Pittsburgh ReLiver Inc.; she is coinventor on a patent application related to methods to enhance hepatic functions in human failing livers. Dr. Bell is coinventor on a patent application that describes the use of transcription factors to treat chronic liver failure and on a patent application related to methods to enhance hepatic functions in human failing livers. Dr. Guzman-Lepe owns stock in Von Baer Wolff and is coinventor on a patent application related to methods to enhance hepatic functions in human failing livers. Dr. Tafaleng is coinventor on a patent application related to methods to enhance hepatic functions in human failing livers. Dr. Schultz owns stock in Moderna. Dr. Martini owns stock in and is employed by Moderna. The other authors have nothing to report.*

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correlated with worsening liver function.<sup>(10,11)</sup> In addition, P1-HNF4 $\alpha$  protein expression in hepatocytes isolated from patients with cirrhosis and liver dysfunction was not localized to the nucleus, a finding not observed in rat studies.<sup>(12,13)</sup>

As HNF4 $\alpha$  is the master transcription factor in the liver, it stabilizes the rest of the hepatic transcriptional network to ensure proper hepatocyte differentiation and function.<sup>(10,14,15)</sup> HNF4 $\alpha$  has several isoforms generated through two promoters and alternative splicing.<sup>(16-19)</sup> P2-HNF4 $\alpha$  isoforms produced from the distal P2 promoter are expressed in fetal liver and are involved in early liver development. P2-HNF4 $\alpha$  isoforms are not normally expressed in adult liver, but their aberrant expression has been implicated in the pathogenesis of hepatocellular carcinoma, colorectal carcinoma, and alcoholic hepatitis.<sup>(10,16-19)</sup> P1-HNF4 $\alpha$  isoforms produced from the proximal P1 promoter are highly expressed in adult liver and are involved in hepatocyte maturation and function.<sup>(16-19)</sup> HNF4 $\alpha$ 2 is the predominant P1-HNF4 $\alpha$  isoform in human adult liver.<sup>(20)</sup>

We previously showed that restoring HNF4 $\alpha$ 2 expression by exogenous delivery *in vitro* in end-stage rat hepatocytes and *in vivo* in decompensated cirrhotic animals with liver failure corrected hepatocyte function and phenotype.<sup>(12)</sup> We therefore hypothesized that restoration of HNF4 $\alpha$ 2 expression would correct hepatocyte-specific metabolic functions in hepatocytes from livers of patients with terminal liver failure, using messenger RNA (mRNA) as a prospective therapeutic modality to increase P1-HNF4 $\alpha$  protein expression. Supplemental HNF4 $\alpha$ 2 mRNA was generated *in vitro* by T7 RNA polymerase-mediated transcription. Similar to previous studies, HNF4 $\alpha$ 2 mRNA was initiated with a cap, followed by a 5' untranslated region (UTR), an open-reading frame encoding HNF4 $\alpha$ 2, a 3' UTR, and a polyadenylated tail. Uridine was also globally replaced with N1-methylpseudouridine.<sup>(21-24)</sup> These modifications have been shown to increase mRNA stability and translation efficiency, and prevent detection by toll-like receptors that activate foreign RNA-induced innate immune responses.<sup>(25-27)</sup> Unlike viral-based vectors, nucleoside-modified mRNA provides pulse-like immediate gene expression with no need for host-cell transcription, transient gene expression with no danger of integration into the host genome, and minimal activation of foreign RNA-induced innate immune responses.<sup>(22,28,29)</sup> Because of the inherent variability in the basal P1-HNF4 $\alpha$

expression in human hepatocytes, we used green fluorescent protein (GFP)-treated hepatocytes from each patient as their own controls, allowing us to attain statistical significance in our analysis. Our data indicate that supplemental mRNA-mediated re-expression of HNF4 $\alpha$ 2 in decompensated cirrhotic hepatocytes improves expression of liver-enriched transcription factors, reprograms hepatocyte-specific gene expression, and increases coagulation factor VII (CF VII), ornithine transcarbamylase (OTC), and albumin protein production. These findings indicate that re-expression of HNF4 $\alpha$ 2 may be effective, as was previously shown in rats, for treating patients with terminal liver failure as an alternative to organ transplantation in humans.

## Materials and Methods

### HUMAN HEPATOCYTE ISOLATION

The Institutional Review Board of the University of Pittsburgh has determined that the human hepatocyte isolation protocol employed in this study is exempt from further review. Liver donor information is shown in Supporting Table S1. Primary hepatocytes were isolated from explanted liver specimens obtained from patients receiving orthotopic liver transplantation for decompensated liver cirrhosis or for metabolic liver disease without end-stage liver disease. Liver tissue specimens were protected from ischemic injury by flushing with ice-cold University of Wisconsin solution immediately after vascular clamping and resection in the operating room, keeping the specimens on ice, and transporting the specimens immediately to the laboratory. Hepatocytes were isolated from encapsulated human liver segments (preferably the left lateral segment) by a modified three-step perfusion technique. Briefly, the livers were flushed under a sterile biosafety hood through the portal vein and/or hepatic vessels (recirculation technique) with 1 L of calcium-free Hank's balanced salt solution (Sigma, H6648-1L) supplemented with 0.5 mM ethylene glycol tetraacetic acid (Thermo Fisher, 50-255-956) prewarmed to 37°C and then with collagenase/protease solution (VitaCyte, 007-1010) prewarmed to 37°C until the tissue was fully digested. The digestion time for each preparation was in the range 45-60 minutes. The digested liver was removed and immediately cooled with ice-cold

Leibovitz's L-15 Medium (Invitrogen, 11415114) supplemented with 10% fetal bovine serum (FBS; Sigma, F4135). The final cell suspension was centrifuged twice at 65g for 3 minutes at 4°C, and the medium was aspirated. The yield and viability of freshly isolated hepatocytes were estimated by trypan blue staining.

## HUMAN HEPATOCYTE CULTURE

Final cell suspensions were centrifuged one more time at 65g for 3 minutes at 4°C, and the medium was aspirated. Cells were resuspended in basal medium plus SingleQuots (hepatocyte maintenance medium [HMM]; Lonza, CC-3197 and CC-4192) supplemented with 5% FBS. Cells were then dispensed into 12-well plates precoated with 50 µg/mL collagen (Corning, 354249) at a density of  $5 \times 10^5$ /well and incubated at 37°C and 5% CO<sub>2</sub> for 4 hours.

## TRANSFECTION WITH *HNF4α2* mRNA

After incubation in seeding medium for 4 hours, cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, 14190250). Culture medium in each well was then replaced with fresh 500 µL of HMM. A solution of 100 µL Opti-MEM (Invitrogen, 31985070) + 3 µL Lipofectamine MessengerMAX Transfection Reagent (Invitrogen, LMRNA015) + 0, 50, 100, or 500 ng *HNF4α2* (NM\_000457.5) or GFP mRNA (Moderna Inc., Cambridge, MA) was prepared according to Invitrogen's instructions and was added to each well. *HNF4α2* was chosen for transfection as it is the predominant P1-HNF4α isoform in the human adult liver.<sup>(20)</sup> Cells were incubated at least 10 hours at 37°C and 5% CO<sub>2</sub>. Wells were then washed twice with DPBS, and the medium was replaced with fresh 1mL HMM. Samples for RNA isolation, protein lysate isolation, and 4% paraformaldehyde (PFA) fixation were collected 24 hours posttransduction.

## TOTAL RNA EXTRACTION AND QUANTITATIVE POLYMERASE CHAIN REACTION

Information regarding Taqman gene expression assays is presented in Supporting Table S2. RNA was extracted from primary human hepatocytes using the miRNeasy Mini Kit (Qiagen, 217004), according to the

manufacturer's instructions. RNA quantity and integrity were evaluated using a NanoDrop 1000 spectrometer (Thermo Fisher). Complementary DNA (cDNA) was reverse transcribed from 0.5 to 1 µg total RNA, using the SuperScript IV cDNA Synthesis Kit (Invitrogen, 18-091-050). For gene expression studies, reactions were performed on MicroAmp fast Optical 96-well plates (Applied Biosystems, 4346906), using 1X TaqMan Fast Advanced Master Mix (Applied Biosystems, 4444556), 1X TaqMan gene expression assays (Applied Biosystems), 100 ng of cDNA, and nuclease-free water (Invitrogen, AM9937) to a final volume of 20 µL. Quantitative polymerase chain reaction (qPCR) was performed using the fast mode on an ABI StepOnePlus thermocycler (Applied Biosystems) set to autothreshold and autobackground. All reactions were performed with two or three technical replicates with passive reference dye 6-carboxy-X-rhodamine (ROX) normalization. Relative quantities were calculated using the delta-delta Ct method using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the reference gene and normalizing to GFP-treated samples from the same patient. Values are reported as mean ± SEM. Statistical significance was analyzed through GraphPad Prism. For differences between GFP- and *HNF4α2*-treated groups, a preliminary Shapiro-Wilk normality test was performed, followed by one-tailed ratio paired *t* tests. An  $\alpha$  of 0.05 was used as the cutoff for significance for all genes tested.

## ANTIBODIES

Catalogue numbers and dilutions of antibodies are listed in Supporting Table S3. Mouse monoclonal anti-HNF4α antibody (clone K9218) was used for detection of P1-HNF4α protein expression in liver tissue and isolated hepatocyte samples. This antibody was raised against a recombinant peptide fragment corresponding to the N-terminal domain of the human HNF4α protein and has been used to specifically detect human P1-HNF4α isoforms in human hepatocytes, human hepatocellular carcinoma tissues and cell lines, and human colorectal carcinoma tissues and cell lines.<sup>(10,16-19)</sup>

## IMMUNOHISTOCHEMICAL STAINING

Human liver tissues were cut into 5-10-mm-thick sections and fixed in 10% formalin overnight at 4°C.

After macroscopic examination, noncancerous tissue was trimmed, paraffin-embedded, and sliced into 4- $\mu$ m-thick sections. Samples were deparaffinized with xylene, rehydrated with decreasing concentrations of ethanol in water, and washed with water for 5 minutes. Samples were incubated in hot (95°C) sodium citrate buffer (pH 6.0) for 20 minutes and washed with phosphate-buffered saline (PBS) for 5 minutes. Samples were quenched with 3% hydrogen peroxide for 10 minutes, washed with PBS, and blocked with blocking buffer containing 2.5% normal serum for 20 minutes. Samples were then incubated with primary antibody in blocking buffer overnight at 4°C and washed with PBS for 5 minutes. Samples were incubated with diluted biotinylated anti-mouse antibody in blocking buffer for 30 minutes, washed with PBS for 5 minutes, incubated with diluted Vectastain Elite ABC-horseradish peroxidase (HRP) reagent for 30 minutes, and washed with PBS for 5 minutes, using the components of the Vectastain Elite ABC-HRP Mouse immunoglobulin G Kit (Vector Laboratories, PK-6102). Detection of peroxidase activity was performed by exposure to 3,3'-diaminobenzidine (Vector Laboratories, SK-4105) for 2-10 minutes. Samples were washed with water and counterstained with Richard-Allan Scientific Signature Series Hematoxylin (Thermo Scientific, 7211L).

## WESTERN BLOT ANALYSIS

Primary human hepatocytes samples were incubated with lysis solution containing NP-40 cell lysis buffer (Invitrogen, FNN0021), 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher, 78440), and 2  $\mu$ M phenylmethanesulfonyl fluoride (Sigma-Aldrich, 93482) with rocking for 30 minutes at 4°C. Samples were scraped off the wells using a cell scraper (Thermo Fisher, 08-100-241), transferred to a 1.5-mL microfuge tube, and centrifuged at 20,000g for 10 minutes at 4°C. The supernatant from each sample was then transferred to a new microfuge tube and was used as the whole-cell lysate. Protein concentrations were determined by comparison with a known concentration of bovine serum albumin using a Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher, 23227). Equal amounts of lysate (5  $\mu$ g protein for albumin blot, 20  $\mu$ g for all other antibodies) were loaded per lane into Novex WedgeWell 8%-16% Tris-Glycine Gel (Invitrogen, XP08162BOX). After

sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred onto iBlot Transfer Stack polyvinylidene fluoride membrane (Invitrogen, IB401001), using the iBlot Dry Blotting System (Invitrogen). Membranes were incubated with primary antibody solution overnight and then washed. Membranes were incubated for 1 hour in secondary antibody solution and then washed. Target antigens were finally detected using SuperSignal West Dura or Femto substrates (Thermo Fisher, 34075 or 34095). Images were scanned and analyzed using ImageJ software. All band density values were normalized to the band density for GAPDH. Relative band densities were normalized to GFP-treated hepatocytes from the same patient. Values are reported as mean  $\pm$  SEM. Statistical significance was analyzed through GraphPad Prism. For comparison of basal levels, a preliminary Shapiro-Wilk normality test was performed, followed by two-tailed unpaired *t* tests. For differences between GFP- and *HNF4 $\alpha$ 2*-treated groups, a preliminary Shapiro-Wilk normality test was performed, followed by one-tailed ratio paired *t* tests. An  $\alpha$  of 0.05 was used as the cutoff for significance for all proteins tested.

## IMMUNOFLUORESCENCE STAINING, IMAGING, AND IMAGE ANALYSIS

Primary human hepatocytes were washed with warm DPBS, fixed with 4% PFA for 15 minutes, and washed 3X with DPBS. Samples were washed 3X with wash buffer (PBS, 0.1% bovine serum albumin [BSA], and 0.1% Tween 20) for 5 minutes and then blocked and permeabilized in blocking buffer (PBS, 10% normal donkey serum, 1% BSA, 0.1% Tween 20, and 0.1% Triton X-100) for 1 hour at room temperature. Samples were then incubated with primary antibody in blocking buffer for 24 hours at 4°C. Samples were washed 3X with wash buffer for 5 minutes and incubated with secondary antibody in blocking buffer for 1 hour in the dark at room temperature. Samples were washed 3X with wash buffer for 5 minutes, 3X with DPBS, and then counterstained with 1  $\mu$ g/mL of Hoechst 33342 (Sigma Aldrich, B2261) for 1 minute at room temperature in the dark. Samples were washed 3X with DPBS and stored in 2 mL DPBS in the dark at 4°C. Incubation times were doubled for samples that were overlaid with collagen. Samples

were imaged using an Eclipse Ti inverted microscope (Nikon) and the NIS-Elements software platform (Nikon). For each sample, images were taken from three random fields at a magnification of 200 $\times$ . Analysis of images was done using ImageJ program (National Institutes of Health), using a standard automated cell counting of single-color images.<sup>(30)</sup> Values for threshold and particle size were adjusted depending on the analysis being performed. For counting the total number of cells and the total number of P1-HNF4 $\alpha$ -positive cells, the threshold was adjusted to 18-85 for the red channel (P1-HNF4 $\alpha$ ) and 9-85 for the blue channel (Hoechst). For counting the number of cells that stained intensely for P1-HNF4 $\alpha$ , the threshold for the red channel was set to 50-85  $\pm$  5 depending on the background. For determining the number of cells with nuclear or cytoplasmic staining, the particle size was limited to  $\sim$ 300 to infinity. For determining the number of cells with cytoplasmic-only staining, the particle size was limited to  $\sim$ 900 to infinity. Statistical significance was analyzed through GraphPad Prism. For differences between GFP- and *HNF4 $\alpha$ 2*-treated groups, a preliminary Shapiro-Wilk normality test was performed, followed by one-tailed ratio paired *t* tests. An  $\alpha$  of 0.05 was used as the cutoff for significance.

## Results

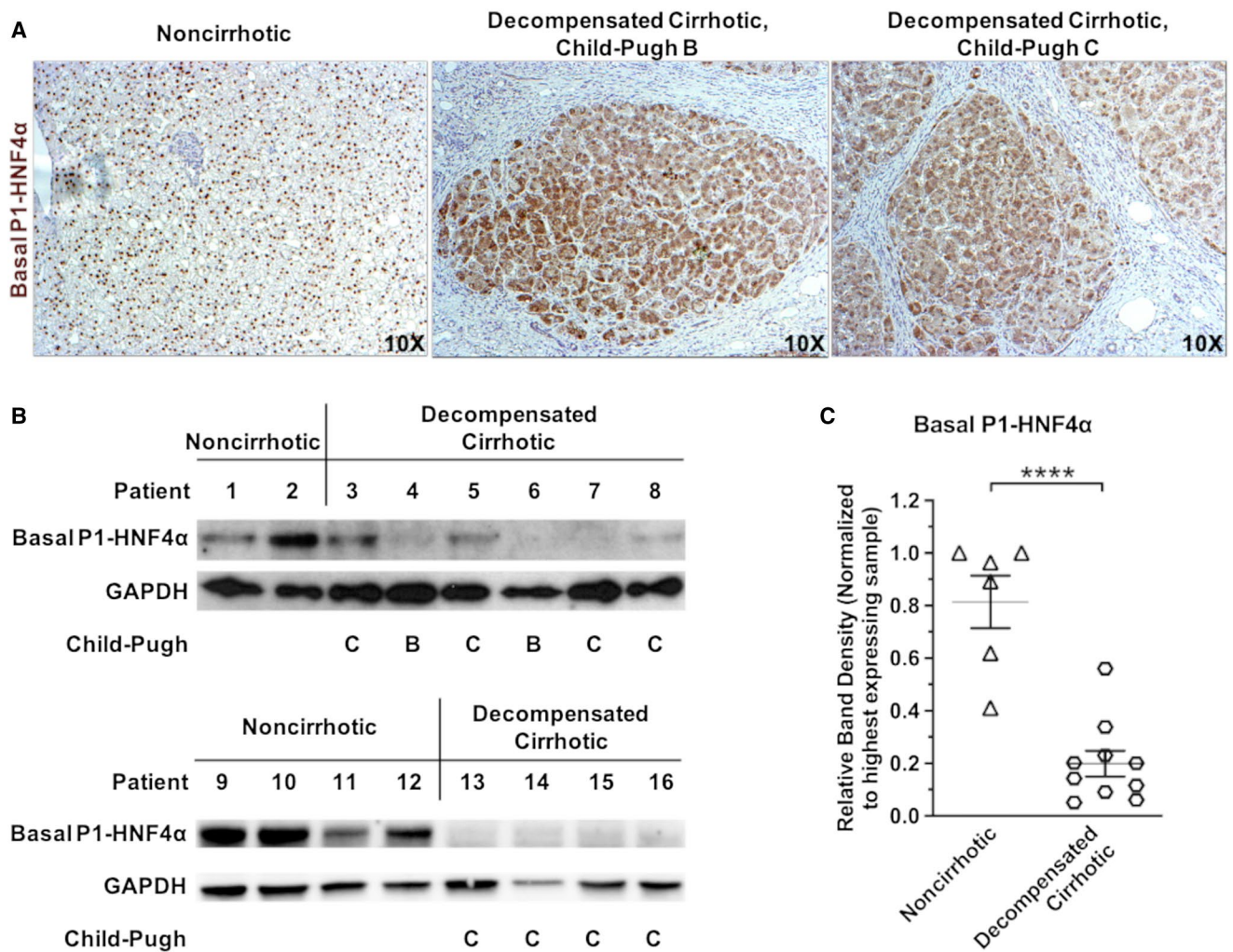
### HEPATOCTE-SPECIFIC P1-HNF4 $\alpha$ PROTEIN EXPRESSION IS CONSISTENTLY LOW IN END-STAGE LIVER DISEASE, ALTHOUGH THERE IS CONSIDERABLE VARIABILITY IN BASELINE EXPRESSION AMONG INDIVIDUALS

Studies in the rat have shown that the expression of the transcription factor P1-HNF4 $\alpha$  is down-regulated at the mRNA and protein level in decompensated hepatocytes compared to control hepatocytes.<sup>(12)</sup> We therefore compared the expression of P1-HNF4 $\alpha$  in hepatocytes from a cohort of patients that did not have cirrhosis and those that underwent liver transplant for end-stage decompensated cirrhosis due to alcohol-mediated cirrhosis and NASH (Child-Pugh

B, C) (Supporting Table S1). Immunohistochemical staining showed variability in the localization of P1-HNF4 $\alpha$  in tissue specimens from controls without cirrhosis compared to those from patients with cirrhosis (Fig. 1A). While noncirrhotic specimens exhibit P1-HNF4 $\alpha$  staining almost exclusively within the nucleus, the majority of cirrhotic specimens exhibit diffuse P1-HNF4 $\alpha$  staining throughout the cell. This difference in P1-HNF4 $\alpha$  distribution makes it difficult to measure and compare the absolute levels of P1-HNF4 $\alpha$  using this method. Western blot analysis was therefore performed to determine the levels of P1-HNF4 $\alpha$  in isolated hepatocytes from various individuals. The analysis revealed that P1-HNF4 $\alpha$  protein levels were significantly down-regulated in decompensated cirrhotic hepatocytes compared to noncirrhotic hepatocytes (Fig. 1B). However, there was considerable dispersion of patient data points in each group (Fig. 1C), indicating significant variability in baseline P1-HNF4 $\alpha$  expression in isolated hepatocytes obtained from various individuals. Thus, in order to properly test the effect of a treatment on gene and protein expression in primary hepatocytes and to attain statistical significance in our analysis, it was imperative to measure the effect of a treatment using untreated hepatocytes from the same individual as its control.

### TREATMENT OF PRIMARY DECOMPENSATED CIRRHOTIC HEPATOCYTES WITH 100 ng OF *HNF4 $\alpha$ 2* mRNA EFFECTIVELY UP-REGULATES PROTEIN LEVELS OF HEPATOCYTE-ENRICHED TRANSCRIPTION FACTORS AND DOWNSTREAM GENES

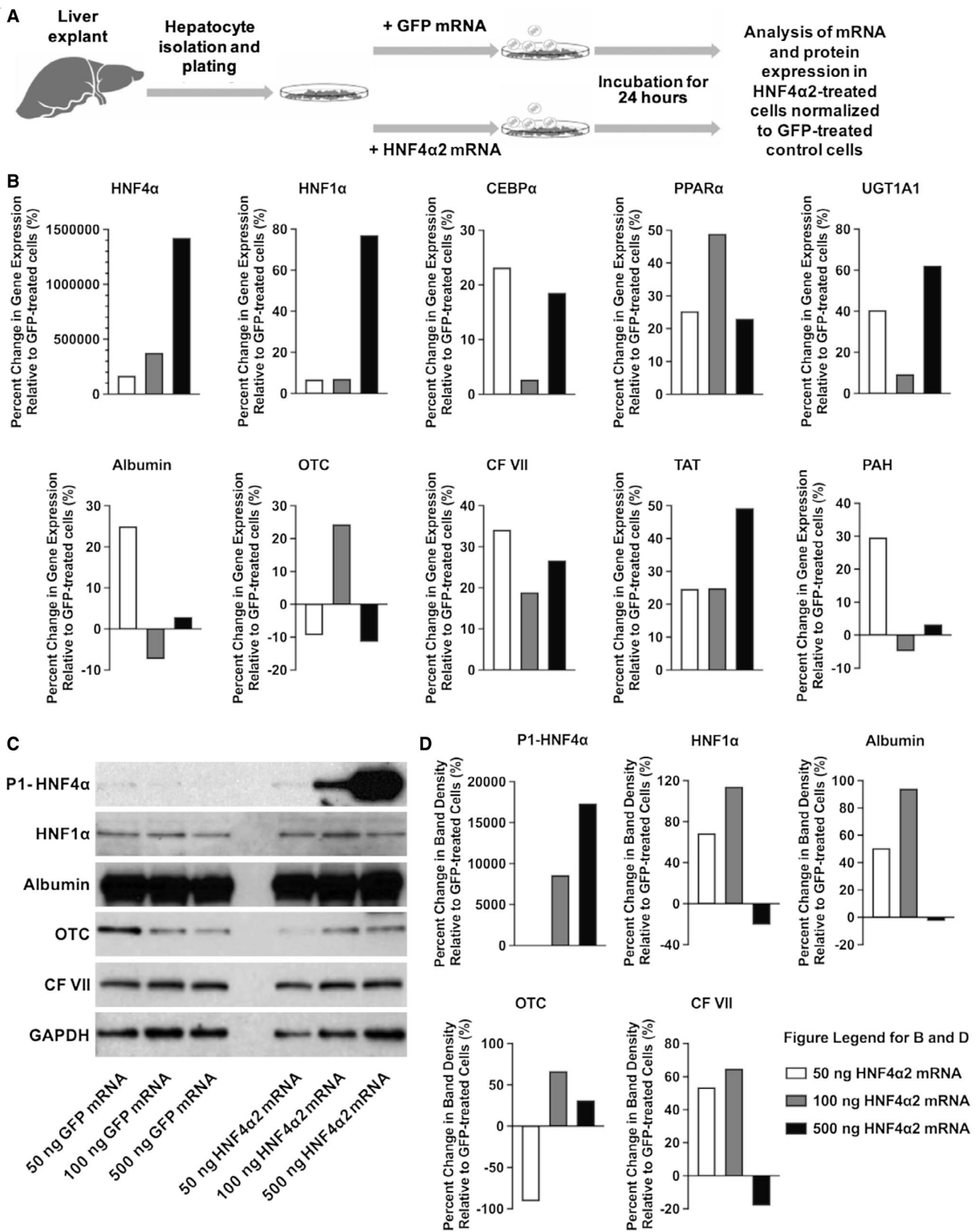
While several studies have described successful transgene delivery in healthy primary human hepatocytes or humanized mouse livers using a variety of methods,<sup>(31-37)</sup> equivalent studies in primary human hepatocytes isolated from decompensated cirrhotic livers have not been reported. Our past experience with the introduction of exogenous genes into primary decompensated cirrhotic hepatocytes isolated from livers using conventional methods has also proven difficult (data not shown). We therefore tested supplemental mRNA as a means of re-expressing *HNF4 $\alpha$ 2*



**FIG. 1.** Basal expression of P1-HNF4 $\alpha$  protein is variable in human hepatocytes isolated from livers. (A) Representative images of immunohistochemical staining for P1-HNF4 $\alpha$  in liver tissue sections from noncirrhotic livers (normal control livers or livers of patients with single-gene mutations possessing only metabolic disease characteristics) or from decompensated cirrhotic livers. Magnification  $\times 10$ . (B) Western blot analysis of baseline P1-HNF4 $\alpha$  protein levels in primary human hepatocytes isolated from noncirrhotic livers or from decompensated cirrhotic livers cultured for 24 hours on thin-layer collagen before sample collection. GAPDH was used as a gel-loading control. Top (patients 1-8) and bottom (patients 9-16) panels were blotted on separate membranes. (C) Quantification of bands shown in (B). P1-HNF4 $\alpha$ /GAPDH density ratios were normalized to the highest expressing sample and are shown as dot plots with mean  $\pm$  SEM ( $n = 6$  noncirrhotic,  $n = 10$  decompensated cirrhotic; each dot represents a patient). Statistical significance was determined by two-tailed unpaired  $t$  test; \*\*\*\* $P \leq 0.0001$ . Mean P1-HNF4 $\alpha$  protein level was significantly lower in decompensated cirrhotic hepatocytes, but there was considerable dispersion of patient data points within groups.

in decompensated cirrhotic hepatocytes using GFP as a control. Because basal levels of P1-HNF4 $\alpha$  in isolated human hepatocytes are highly variable (Fig. 1), results obtained from *HNF4 $\alpha$ 2*-treated hepatocytes were always normalized to those from GFP-treated hepatocytes isolated from the same patient (Fig. 2A). In a pilot study using cirrhotic hepatocytes from one patient, we transfected various amounts of mRNA (50, 100, and 500 ng) to determine a dose that would

be effective at up-regulating P1-HNF4 $\alpha$  as well as other hepatocyte-enriched transcription factors and downstream hepatocyte expressed proteins 24 hours posttransfection. As expected, there was increased up-regulation of P1-HNF4 $\alpha$  mRNA and protein expression with increasing amounts of transfected *HNF4 $\alpha$ 2* mRNA, and transfection with 500 ng *HNF4 $\alpha$ 2* mRNA resulted in the highest up-regulation of P1-HNF4 $\alpha$  mRNA and protein (Fig. 2B-D). There was,





**FIG. 2.** Pilot study to determine an effective dose of *HNF4 $\alpha$ 2* mRNA for *in vitro* studies. Transfection of decompensated cirrhotic hepatocytes with 100 ng *HNF4 $\alpha$ 2* mRNA leads to effective increase in the protein levels of HNF1 $\alpha$ , albumin, OTC, and CF VII. (A) Schematic illustration showing hepatocyte isolation from liver, plating, transfection, incubation for 24 hours, and analysis after *HNF4 $\alpha$ 2* versus GFP treatment. Isolated hepatocytes were transfected with *HNF4 $\alpha$ 2* or GFP mRNA and cultured for 24 hours on thin-layer collagen before sample collection. (B) qPCR analysis of *HNF4 $\alpha$* , *HNF1 $\alpha$* , *CEBPA*, *PPAR $\alpha$* , *UGT1A1*, albumin, *OTC*, *CF VII*, *TAT*, and *PAH* levels in decompensated cirrhotic hepatocytes 24 hours after transfection with 50, 100, or 500 ng *HNF4 $\alpha$ 2* or GFP mRNA. *GAPDH* was used as a reference gene. Relative gene expression of *HNF4 $\alpha$ 2*-treated samples were normalized to GFP-treated samples. Data are presented as percentage change in gene expression relative to GFP-treated cells. (C) Western blot analysis of P1-HNF4 $\alpha$ , HNF1 $\alpha$ , albumin, OTC, and CF VII protein levels in decompensated cirrhotic hepatocytes 24 hours after transfection with 50, 100, or 500 ng *HNF4 $\alpha$ 2* or GFP mRNA. *GAPDH* was used as a gel-loading control. (D) Quantification of bands shown in (C). Protein/*GAPDH* band densities of *HNF4 $\alpha$ 2*-treated samples were normalized to GFP-treated samples. Data are presented as percentage change in band density relative to GFP-treated cells. While transfection with various doses of *HNF4 $\alpha$ 2* mRNA did not lead to a singular trend in altering mRNA levels of analyzed downstream hepatocyte-enriched transcription factors and genes, transfection with 100 ng of *HNF4 $\alpha$ 2* mRNA resulted in the highest up-regulation of protein levels of HNF1 $\alpha$ , albumin, OTC, and CF VII and was therefore selected as the most effective dose for transfection in further studies. (B,D) White bar, 50 ng *HNF4 $\alpha$ 2* mRNA; gray bar, 100 ng *HNF4 $\alpha$ 2* mRNA; black bar, 500 ng *HNF4 $\alpha$ 2* mRNA.

however, no clear trend in the change of mRNA levels of other hepatocyte-enriched transcription factors, including *HNF1 $\alpha$* , CCAAT/enhancer-binding protein alpha (*CEBPA*), and peroxisome proliferator-activated receptor alpha (*PPAR $\alpha$* ), or of the downstream functional genes UDP glucuronosyltransferase family 1 member A1 (*UGT1A1*), *OTC*, albumin, *CF VII*, tyrosine aminotransferase (*TAT*), and phenylalanine hydroxylase (*PAH*), with increasing amounts of transfected *HNF4 $\alpha$ 2* mRNA (Fig. 2B). In contrast, transfection with 100 ng *HNF4 $\alpha$ 2* mRNA resulted in the highest increases in the protein levels of HNF1 $\alpha$ , albumin, OTC, and CF VII, with 115%, 95%, 70%, and 65% increases, respectively (Fig. 2B-D). Because 100 ng of *HNF4 $\alpha$ 2* mRNA resulted in effective reprogramming, subsequent experiments were performed using this dose.

## TREATMENT OF PRIMARY DECOMPENSATED CIRRHOTIC HEPATOCYTES WITH SUPPLEMENTAL *HNF4 $\alpha$ 2* mRNA INDUCES UP-REGULATION OF P1-HNF4 $\alpha$ PROTEIN

We then transfected 100 ng *HNF4 $\alpha$ 2* mRNA into a cohort of decompensated cirrhotic hepatocytes isolated from patients with Child-Pugh Class B or C with either alcoholic cirrhosis or NASH with Child-Pugh scores that range from 9 to 13 (Supporting Table S1). qPCR analysis of samples taken 24 hours posttransfection showed an average increase in P1-HNF4 $\alpha$  mRNA level by more than 2,700-fold in

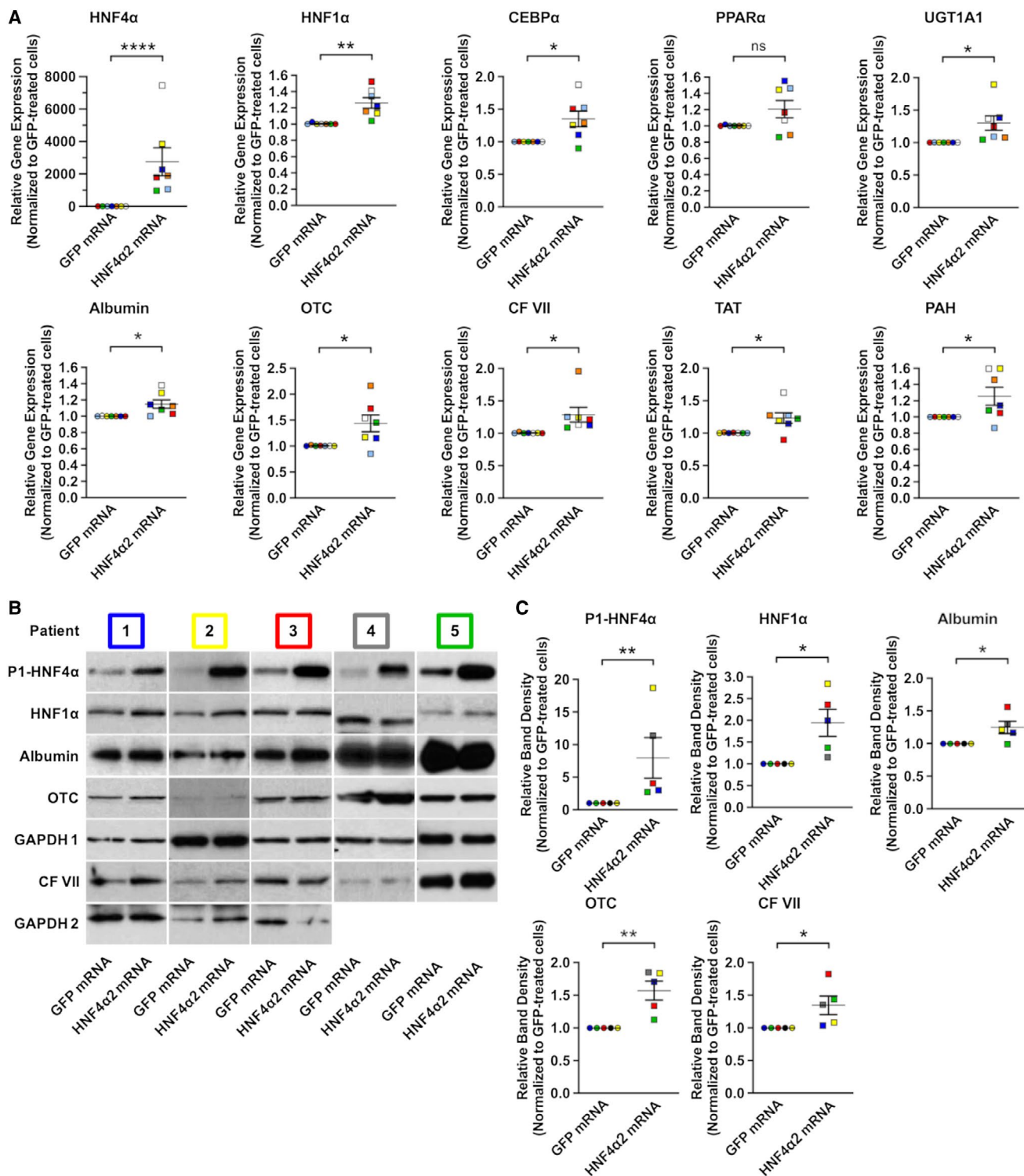
hepatocytes treated with *HNF4 $\alpha$ 2* mRNA compared to those that received GFP mRNA (Fig. 3A). More importantly, western blot analysis of samples obtained 24 hours posttransfection revealed that this increase in mRNA expression led to a significant up-regulation of P1-HNF4 $\alpha$  protein by an average of 8-fold in hepatocytes treated with *HNF4 $\alpha$ 2* mRNA (Fig. 3B,C). Similar results were obtained in transduction experiments with high-titer *HNF4 $\alpha$ 2* lentivirus, using a GFP lentivirus as control. However, the increases in P1-HNF4 $\alpha$  mRNA and protein expression as long as 72 hours after lentivirus transduction were not as pronounced compared to that observed after mRNA transfection (Supporting Fig. S1). We therefore selected mRNA transfection as the method for inducing *HNF4 $\alpha$ 2* re-expression for all subsequent studies.

## SUPPLEMENTAL mRNA-MEDIATED RE-EXPRESSION OF *HNF4 $\alpha$ 2* INDUCES REPROGRAMMING OF OTHER LIVER-ENRICHED TRANSCRIPTION FACTORS AND DOWNSTREAM HEPATOCYTE-SPECIFIC FUNCTIONAL GENES IN PRIMARY HEPATOCYTES FROM DECOMPENSATED CIRRHOTIC LIVERS

Re-expression of *HNF4 $\alpha$ 2* in isolated hepatocytes from CCl<sub>4</sub>-treated rats with decompensated cirrhotic liver failure induced expression of the other hepatocyte-expressed transcription factors and restored hepatocyte function.<sup>(12)</sup> We therefore sought

to determine whether this strategy would also rescue function in human hepatocytes from patients with end-stage degenerative liver disease. Similar to earlier rodent studies, re-expression of *HNF4 $\alpha$ 2* in

decompensated cirrhotic human hepatocytes led to significantly increased expression of the transcription factors *HNF1 $\alpha$*  and *CEBP $\alpha$*  and the downstream functional genes *UGT1A1*, *OTC*, albumin, *CF VII*, *TAT*,



**FIG. 3.** Supplemental mRNA-mediated re-expression of *HNF4 $\alpha$ 2* in decompensated cirrhotic hepatocytes increases expression of liver-enriched transcription factors and downstream hepatocyte-specific functional proteins. (A) qPCR analysis of *HNF4 $\alpha$* , *HNF1 $\alpha$* , *CEBP $\alpha$* , *PPAR $\alpha$* , *UGT1A1*, albumin, *CF VII*, *OTC*, *TAT*, and *PAH* mRNA expression in decompensated cirrhotic hepatocytes 24 hours after transfection with 100 ng *HNF4 $\alpha$ 2* mRNA. *GAPDH* was used as a reference gene. Relative gene expression of *HNF4 $\alpha$ 2*-treated samples were normalized to GFP-treated samples and are shown as dot plots with mean  $\pm$  SEM (n = 7; each dot represents a patient). Statistical significance was determined by one-tailed ratio paired *t* test; \**P*  $\leq$  0.05, \*\**P*  $\leq$  0.01, \*\*\*\**P*  $\leq$  0.0001. (B) Western blot analysis of P1-HNF4 $\alpha$ , HNF1 $\alpha$ , albumin, OTC, and CF VII protein levels in decompensated cirrhotic hepatocytes 24 hours after transfection with 100 ng *HNF4 $\alpha$ 2* mRNA. *GAPDH* was used as a loading control. P1-HNF4 $\alpha$ , HNF1 $\alpha$ , albumin, OTC, and *GAPDH* 1 were run and blotted on the same membrane, while CF VII and *GAPDH* 2 were run and blotted on a separate membrane for patients 1-3. All proteins were run and blotted on the same membrane for patients 4 and 5. (C) Quantification of bands shown in B. Protein/*GAPDH* band densities of *HNF4 $\alpha$ 2*-treated samples were normalized to the GFP-treated samples and are shown as dot plots with mean  $\pm$  SEM (n = 5; each dot represents a patient). Statistical significance was determined by one-tailed ratio paired *t* test; \**P*  $\leq$  0.05, \*\**P*  $\leq$  0.01. Although re-expression of *HNF4 $\alpha$ 2* in decompensated cirrhotic hepatocytes led to modest increases (15%-45%) in the mRNA levels of analyzed hepatocyte-enriched transcription factors and genes, it resulted in substantial increases (25%-95%) in protein levels of HNF1 $\alpha$ , albumin, OTC, and CF VII. Similarly colored circles and boxes denote data from the same patient sample. Abbreviation: ns, not significant.

and *PAH* 24 hours posttransfection (Fig. 3A). *PPAR $\alpha$*  was likewise up-regulated by *HNF4 $\alpha$ 2* re-expression, but because of the spread in data points, this trend was not statistically significant. The increased expression in hepatocyte-enriched transcription factors and downstream functional genes was observed only in primary hepatocytes from patients with decompensated cirrhosis but not in primary hepatocytes from patients without cirrhosis (Supporting Fig. S2). Western blot analysis also revealed a significant increase in protein expression after re-expression of *HNF4 $\alpha$ 2* in primary decompensated cirrhotic hepatocytes. On average, there was a 95% increase in HNF1 $\alpha$ , a 25% increase in albumin, a 60% increase in OTC, and a 35% increase in CF VII protein expression 24 hours after *HNF4 $\alpha$ 2* re-expression in decompensated cirrhotic hepatocytes (Fig. 3B,C).

### **SUPPLEMENTAL mRNA-MEDIATED RE-EXPRESSION OF *HNF4 $\alpha$ 2* INCREASES THE TOTAL AMOUNT OF P1-HNF4 $\alpha$ PROTEIN BUT DOES NOT SIGNIFICANTLY ALTER THE LOCALIZATION OF P1-HNF4 $\alpha$ PROTEIN IN PRIMARY DECOMPENSATED CIRRHOTIC HEPATOCYTES**

Human studies have described reduced nuclear localization of P1-HNF4 $\alpha$  protein in irreversible decompensated cirrhosis.<sup>(11,13)</sup> Because P1-HNF4 $\alpha$  is a transcription factor, its localization to the nucleus is essential for its biological function in hepatocytes. We

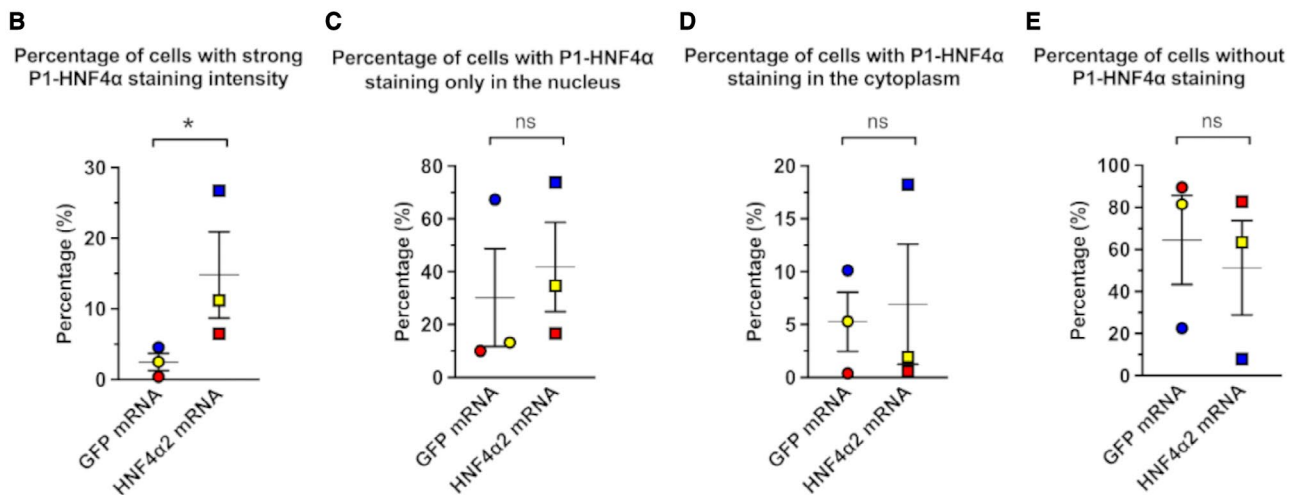
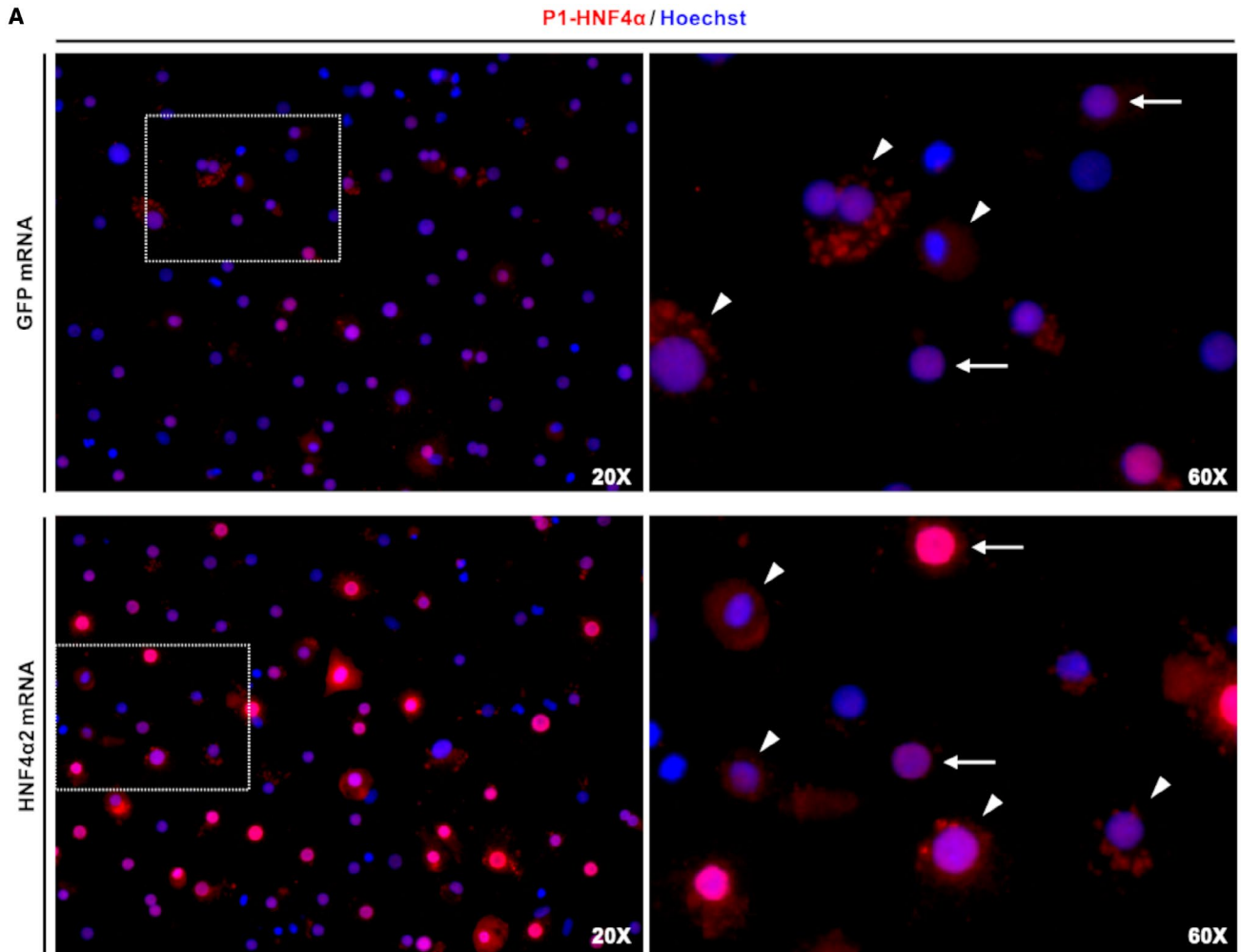
therefore sought to determine whether re-expression of *HNF4 $\alpha$ 2* in primary decompensated cirrhotic hepatocytes would lead to increased nuclear localization of P1-HNF4 $\alpha$ . Immunofluorescence staining of hepatocytes revealed a 6-fold increase in the percentage of cells with strong P1-HNF4 $\alpha$  staining after *HNF4 $\alpha$ 2* re-expression in decompensated cirrhotic hepatocytes (Fig. 4A,B; Supporting Figs. S3 and S4). Thus, *HNF4 $\alpha$ 2* re-expression led to an increase in the total amount of P1-HNF4 $\alpha$  protein. However, *HNF4 $\alpha$ 2* re-expression did not statistically alter the proportion of cells with nuclear-only P1-HNF4 $\alpha$ , the proportion of cells with cytoplasmic-only or cytoplasmic + nuclear P1-HNF4 $\alpha$ , or the proportion of cells that did not express P1-HNF4 $\alpha$  (Fig. 4C-E). These data indicate that *HNF4 $\alpha$ 2* re-expression did not affect the distribution of P1-HNF4 $\alpha$  within the cells. Taken together, the results show that *HNF4 $\alpha$ 2* re-expression increases the amount of P1-HNF4 $\alpha$  protein produced by hepatocytes but does not improve the ability of the subset of hepatocytes that do not express P1-HNF4 $\alpha$  in their nucleus to localize it to their nuclei.

## **Discussion**

P1-HNF4 $\alpha$ , an established master regulator of liver development and function, controls a large number of key genes essential for hepatocyte function. Previous work with a rat model of end-stage liver disease showed that down-regulation of P1-HNF4 $\alpha$  was associated with hepatocyte failure and that re-expression of *HNF4 $\alpha$ 2* normalized the

expression of other liver-enriched transcription factors and restored hepatocyte function.<sup>(12)</sup> A recent study of liver tissues from a large cohort of patients

with liver disease also found that down-regulation of P1-HNF4 $\alpha$  correlated with the extent of liver dysfunction.<sup>(11)</sup>



**FIG. 4.** Supplemental mRNA-mediated re-expression of *HNF4 $\alpha$ 2* in decompensated cirrhotic hepatocytes increases overall expression of P1-HNF4 $\alpha$  but does not significantly affect localization of the P1-HNF4 $\alpha$  protein. (A) Representative immunofluorescent images of decompensated cirrhotic hepatocytes from a patient 24 hours after transfection with 100 ng of either *HNF4 $\alpha$ 2* or GFP mRNA. P1-HNF4 $\alpha$  (red) and Hoechst (blue). Boxed areas on the left panels are shown at a higher magnification on the right panels. White arrows point to cells with nuclear-only P1-HNF4 $\alpha$  staining, while white arrowheads point to cells with cytoplasmic-only or cytoplasmic + nuclear P1-HNF4 $\alpha$  staining. Quantitative data from this patient sample are presented in (B-E) as blue circles and boxes. See Supporting Figs. S4 and S5 for representative images from 2 other patients (yellow or red circles and boxes). (B) Percentage of cells that exhibit strong P1-HNF4 $\alpha$  staining intensity in GFP-treated and *HNF4 $\alpha$ 2* mRNA-treated samples. The percentage of cells that stained strongly for P1-HNF4 $\alpha$  in *HNF4 $\alpha$ 2*-treated cells is 6-fold that observed in GFP-treated cells. (C) Percentage of cells that exhibit only nuclear P1-HNF4 $\alpha$  staining in GFP-treated and *HNF4 $\alpha$ 2* mRNA-treated samples. (D) Percentage of cells that exhibit cytoplasmic P1-HNF4 $\alpha$  staining in GFP-treated and *HNF4 $\alpha$ 2* mRNA-treated samples. (E) Percentage of cells that did not exhibit any P1-HNF4 $\alpha$  staining in GFP-treated and *HNF4 $\alpha$ 2* mRNA-treated samples. (B,E) The percentages of cells was obtained by analyzing multiple fields of stained samples from each of the 3 patients and are shown as dot plots with mean  $\pm$  SEM (n = 3; each dot represents a patient). Statistical significance was determined by one-tailed ratio paired *t* test; \**P*  $\leq$  0.05. While there was a significant increase in the overall expression of P1-HNF4 $\alpha$  protein in *HNF4 $\alpha$ 2*-treated cells, there was no significant difference in the distribution of P1-HNF4 $\alpha$  protein between GFP-treated and *HNF4 $\alpha$ 2* mRNA-treated samples. Similarly colored circles and boxes denote data from the same patient sample in Fig. 3. Abbreviation: ns, not significant.

In this study involving a relatively small cohort of end-stage patients with liver disease, we demonstrate that, similar to what has been shown in rats, the re-expression of *HNF4 $\alpha$ 2* in isolated hepatocytes from patients with decompensated cirrhosis restores the transcriptional network and normalizes hepatic function. This was accomplished using specimens from each patient as its own control, thereby circumventing the considerable variability in baseline P1-HNF4 $\alpha$  expression observed in isolated hepatocytes from different individuals. Re-expression of *HNF4 $\alpha$ 2* improved endogenous mRNA expression of liver-enriched transcription factors HNF1 $\alpha$ , CEBP $\alpha$ , and PPAR $\alpha$  as well as the downstream hepatocyte functional genes that are essential for amino acid metabolism (PAH, TAT), coagulation (CF VII), bilirubin conjugation (UGT1A1), osmotic balance (albumin), and urea metabolism (OTC). More importantly, this led to increased endogenous HNF1 $\alpha$ , albumin, OTC, and CF VII protein expression.

Our previous work with hepatocytes isolated from decompensated cirrhotic livers found decreased nuclear P1-HNF4 $\alpha$  and increased cytoplasmic P1-HNF4 $\alpha$  compared to that observed in control hepatocytes.<sup>(13)</sup> This finding is important because P1-HNF4 $\alpha$  is a nuclear transcription factor and its function relies on its nuclear localization. In this study, we found that re-expression of *HNF4 $\alpha$ 2* significantly increased the total amount of P1-HNF4 $\alpha$  expressed in hepatocytes but did not change the distribution of P1-HNF4 $\alpha$  within the hepatocytes. This indicates that while *HNF4 $\alpha$ 2* re-expression increased the total amount of P1-HNF4 $\alpha$  protein, it did not alter the ability of all hepatocytes

to localize P1-HNF4 $\alpha$  to their nuclei. This abnormal increase in P1-HNF4 $\alpha$  localization to the cytoplasm in decompensated cirrhotic hepatocytes is a phenomenon that was not observed in the rat model of end-stage liver disease.<sup>(12)</sup> Thus, it was not previously possible to determine whether *HNF4 $\alpha$ 2* re-expression would be able to correct this aberrant subcellular distribution of P1-HNF4 $\alpha$ . Our strategy has allowed analysis of P1-HNF4 $\alpha$  localization in decompensated hepatocytes from patients with cirrhosis and underscores its importance and utility in studying other mechanisms responsible for decreased P1-HNF4 $\alpha$  activity.

In a recent study, immunohistochemical analysis of liver tissues revealed that P2-HNF4 $\alpha$  expression, although barely detectable in the nucleus of normal hepatocytes, was dramatically up-regulated in hepatocytes from patients with alcoholic cirrhosis.<sup>(10)</sup> Moreover, knockdown of P2-HNF4 $\alpha$  resulted in the up-regulation of P1-HNF4 $\alpha$  mRNA and protein as well as P1-HNF4 $\alpha$  target genes in HepG2 cells.<sup>(10)</sup> Interestingly, P2-HNF4 $\alpha$  has been implicated in the induction of cytoplasmic localization of P1-HNF4 $\alpha$  through the repression of the clock gene brain and muscle ARNT-like 1 (*BMAL1*, also known as *ARNTL*) in human hepatocellular carcinoma.<sup>(19)</sup> In addition, the proto-oncogene tyrosine-protein kinase Src has also been shown to phosphorylate P1-HNF4 $\alpha$  but not P2-HNF4 $\alpha$ , and its activation correlated with cytoplasmic localization of P1-HNF4 $\alpha$  and poor prognosis in colorectal carcinoma.<sup>(18)</sup> These findings provide potential mechanisms for the mislocalization of P1-HNF4 $\alpha$  that need to be further studied in the context of decompensated cirrhotic hepatocytes.

Our results show that re-expression of *HNF4α2* is effective at normalizing endogenous expression of liver-enriched transcription factors and hepatocyte functional genes but only in the subset of decompensated cirrhotic hepatocytes that already had the capacity to localize P1-HNF4α to their nucleus. The fact that significant improvement in overall P1-HNF4α-driven gene expression and hepatocyte function was detected even though the analysis included the entire population of hepatocytes, including those that could not localize P1-HNF4α to their nucleus, suggests that the actual improvement resulting from *HNF4α2* re-expression is even more robust. While the up-regulation of nuclear P1-HNF4α in cells that are able to localize P1-HNF4α to the nucleus was adequate to correct disease *in vitro*, additional strategies aimed at enhancing the localization of P1-HNF4α to the nucleus should improve the efficacy of this strategy for correcting liver failure in end-stage disease. Because epigenetics may play a role in the down-regulation of P1-HNF4α and its target genes in end-stage cirrhosis, analysis of the chromatin landscape in decompensated cirrhotic hepatocytes may also uncover additional potential targets for therapeutic intervention.

mRNA technology has been used for protein replacement therapy,<sup>(21-24)</sup> cancer immunotherapy,<sup>(38,39)</sup> and the generation of vaccines against infectious diseases.<sup>(40-42)</sup> Most recently, its use as a strategy for vaccinating patients against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes coronavirus disease 2019 (COVID-19), has caused excitement in the scientific community.<sup>(43-48)</sup> The mRNA used in all of these applications retains most of the structure of natural mRNA but is engineered to contain alterations in the 5' cap structure, 5' and 3'-UTRs, and coding sequence<sup>(21-24)</sup> that improve stability and translation efficiency, and prevent detection by toll-like receptors.<sup>(25-27)</sup> Therapeutic applications using mRNA have emerged as an efficient strategy for protein expression because it is relatively cost effective and easy to scale up and it provides immediate, dose-dependent, and transient gene expression with minimal activation of foreign RNA-induced innate immune responses.<sup>(21-23,28,29)</sup> Our results now show that this strategy was successful at generating P1-HNF4α protein at levels that were effective for increasing downstream proteins important for liver function in decompensated cirrhotic human hepatocytes; it is significantly more effective than forced transgene expression using

lentiviral (Supporting Fig. S1) or adeno-associated viral vectors (data not shown). The use of this approach in the clinic will depend on the half-life of P1-HNF4α and downstream effector proteins. Future experiments might include delivery in nonhuman primates to better understand and optimize *HNF4α2* mRNA stability for therapeutic purposes.

This study confirms the principle and extends the work from our rodent study and strongly suggests that mRNA-mediated re-expression of *HNF4α2* can reverse hepatic failure by restoring the entire network of liver enriched transcription factors and downstream genes responsible for hepatocyte function. Because there is no entirely adequate animal model to further examine this hypothesis, the next step in assessing the efficacy of this strategy may require its application in a clinical trial.

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## Supporting Information

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