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Liver X receptor α (LXR α /NR1H3) regulates differentiation of hepatocyte-like cells via reciprocal regulation of HNF4 α

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Background & Aims: Hepatocyte-like cells, differentiated from different stem cell sources, are considered to have a range of possible therapeutic applications, including drug discovery, metabolic disease modelling, and cell transplantation. However, little is known about how stem cells differentiate into mature and functional hepatocytes.

Methods: Using transcriptomic screening, a transcription factor, liver X receptor α (NR1H3), was identified as increased during HepaRG cell hepatogenesis; this protein was also upregulated during embryonic stem cell and induced pluripotent stem cell differentiation.

Results: Overexpressing NR1H3 in human HepaRG cells promoted hepatic maturation; the hepatocyte-like cells exhibited various functions associated with mature hepatocytes, including cytochrome P450 (CYP) enzyme activity, secretion of urea and albumin, upregulation of hepatic-specific transcripts and an increase in glycogen storage. Importantly, the NR1H3-derived hepatocyte-like cells were able to rescue lethal fulminant hepatic failure using a non-obese diabetic/severe combined immunodeficient mouse model.

Conclusions: In this study, we found that NR1H3 accelerates hepatic differentiation through an HNF4 α -dependent reciprocal network. This contributes to hepatogenesis and is therapeutically beneficial to liver disease.

Introduction

Liver development depends on a complex network, requiring several growth factors, genetic homeostasis and cell-extracellular matrix interactions [1]. Identifying the hepatogenesis-promoting factors, favouring liver development, is thought to be essential for liver regeneration and could improve hepatocyte transplantation for end-stage liver disease [2]. Different sources of progenitor cells have been investigated as potential sources for hepatic differentiation, including human mesenchymal stem cells (MSCs) [3,4], embryonic stem cells (ESCs) [5] and induced pluripotent stem cells (iPSCs) [6], all of which hold the potential to differentiate into hepatocyte-like cells. However, unstable hepatic function and atypical morphology have limited the usefulness of present cell treatments. Thus, improved progenitor cells are needed in order to carry out further studies of the treatments of liver disease.

The human bipotent liver progenitor cell line HepaRG is an alternative source of cells that is used for toxicity screening during drug discovery [7,8], viral hepatitis research, hepatocyte differentiation and transplantation testing [9]. This cell line has several potential advantages. First, during differentiation, HepaRG cells evolve from a homogeneous depolarized epithelial phenotype showing no specific organization into a structurally well-defined and polarized monolayer that closely resembles those formed by primary human hepatocytes in culture [10]. Secondly, these cells show long-term stability in terms of various mature hepatocyte functions, including iron storage, cytochrome P450 enzyme activities and albumin secretion [11]; Thirdly, polarized hepatocyte-like cells differentiated from HepaRG cells mimic mature primary human hepatocytes (PHHs) [12], as evidenced by the fact that they can support the *in vitro* infection and replication of human hepatitis B virus (HBV) [13], a feature that has not been observed in hepatocyte-like cells generated from other stem cell systems. Thus, the HepaRG cell line has become a promising *in vitro* model to determine not only the basic regulation of hepatic cell fate but also the maturation into hepatocytes.

Keywords: Hepatogenesis; LXR α /NR1H3; HNF4A; HepaRG progenitor cell.

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Abbreviations: NR1H3, liver X receptor α ; HNF, hepatocyte nuclear factor; MSCs, mesenchymal stem cells; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; RT, reverse-transcription; ChIP, chromatin immunoprecipitation.

In order to screen the hepatic differentiation-inducing factors, genome-wide studies have been conducted in other stem cell systems to identify the regulatory circuitry involved in liver development and hepatic cell specification. For example, by genome-wide chromatin immunoprecipitation (ChIP) combined with promoter microarray analysis in liver and pancreas tissues, the transcriptional regulators HNF1 α , HNF4 α and HNF6 α were found to occupy the promoters of various tissue-specific genes and were also shown to be involved in the control of regulatory circuits that are necessary for normal hepatocyte function [14]. In addition, HNF4 α was found to be essential for the specification of hepatic progenitors from human pluripotent stem cells (iPSCs) [15]. The homeobox transcription factor Prox1 (prospero-related homeobox 1), an early specific marker during the development of liver and pancreas from the foregut endoderm, is a co-regulator of HNF4 α and human liver receptor homolog-1 (hLRH-1) [16,17]. Furthermore, combining genome-wide location and microarray analysis has also helped to reveal the targets of C/EBP beta (CCAAT enhancer-binding protein beta), a basic-leucine zipper transcription factor; these targets are critical to the regulation of numerous biological processes, including liver development [18]. These and other findings have demonstrated that a genomic approach is one of the key ways of screening for important candidates that are likely to control hepatocyte differentiation and liver development [19,20].

In this study we have identified the function of a novel transcription factor, liver X receptor α (NR1H3), and show that it is involved in the induction of the hepatic differentiation. This was done by establishing the genomic profile associated with the development of functional hepatocyte-like cells from undifferentiated HepaRG cells. Hepatocytes overexpressing NR1H3 showed significant increases in several adult liver cell markers and in the process gained a range of liver cell functions; moreover, these cells were able to rescue hepatic failure when transplanted into CCl₄-injured severe combined immunodeficiency (SCID) mice.

Materials and methods

HepaRG cell culture

HepaRG cells were cultured in William's E medium (Gibco) supplemented with 10% FCS, 100 mM glutamine, 5 mg/ml insulin, 50 μ M hydrocortisone hemisuccinate and 1% penicillin. The differentiated HepaRG cells were obtained as described [10].

RNA isolation, reverse-transcription PCR (RT-PCR) and quantitative RT-PCR

RNA was extracted using the RNeasy kit (Qiagen). 1 μ g RNA was reverse-transcribed into cDNA using the Moloney murine leukemia virus reverse-transcriptase (M-MLV Reverse Transcriptase, Promega). For the reverse-transcription polymerase chain reaction (RT-PCR), the following conditions were used: 94 °C for 40 s, 56 °C for 50 s, 72 °C for 60 s for 30 cycles, after an initial denaturation at 94 °C for 5 min. The primers used are listed in Table 1. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems), and normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to quantify the messenger RNA levels.

Periodic acid-Schiff (PAS) staining

HepaRG cells were fixed in 4% formaldehyde for 30 min and then permeabilized with 0.1% Triton X-100 for 10 min. The cells were next either treated or not

Table 1. Primers for RT-qPCR.

Primer	Sequence
<i>Albumin</i>	F: GGC ACA ATG AAG TGG GTA AC R: AGG CAA TCA ACA CCA AGG
<i>CK-18</i>	F: CTA CAT CAA CAA CCT TAG GC R: TCC ACA TCC TTC TTG ATG
<i>HNF4A</i>	F: CCA AGT ACA TCC CAG CTT TC R: TTG GCA TCT GGG TCA AAG
<i>TDO2</i>	F: GGA ACT ACC TGC ATT TGG R: TCT CTG AAG TCA TTG AAG TCC
<i>TAT</i>	F: ACT GTG TTT GGA AAC CTG CC R: GCA GCC ACT TGT CAG AAT GA
<i>CYP3A4</i>	F: CCT TAC ATA TAC ACA CCC TTT G R: GGT TGA AGA AGT CCT CCT AAG CT
<i>CYP2B6</i>	F: ATG GGG CAC TGA AAA AGA CTG A R: AGA GGC GGG GAC ACT GAA TGA C
<i>NR1H3</i>	F: GCT GCA AGT GGA ATT CAT CAA CC R: ATA TGT GTG CTG CAG CCT CTC CA
<i>ETV-1</i>	F: GTG CCT GTA CAA TGT CAG TG R: GGT AGC TGC TAT CTG GTA TG
<i>HHEX</i>	F: GGT AAG CCT CTG CTG TGG TC R: TCT TCT CCA GCT CGA TGG TT
<i>XBP-1</i>	F: ACA CGC TTG GGA ATG GAC AC R: CCA TGG GAA GAT GTT CTG GG
<i>GAPDH</i>	F: GAG TCC ACT GGC GTC TTC R: GAC TGT GGT CAT GAG TCC TTC

treated with diastase (Sigma-Aldrich) for 40 min, 37 °C. The samples were then oxidized in 1% periodic acid for 5 min, rinsed three times in deionized (d) H₂O, treated with Schiff's reagent (Sigma-Aldrich) for 15 min in the dark, and rinsed in dH₂O for 5 min. Finally, cells were visualized by light microscopy.

Measurement of cytochrome P450 enzyme activity

CYP3A4 activity was measured using a P450-Glo Assays kit (Promega) according to the manufacturer's instructions. Briefly, cells were washed with phosphate-buffered saline (PBS), which was then replaced with fresh medium containing luminogenic CYP3A4 substrate luciferin-PFBE, followed by a 3 h incubation at 37 °C. To determine CYP P450 enzyme activity, 50 μ l of medium was transferred and 50 μ l luciferin detection reagent was added to initiate the luminescent reaction for 30 min. The luminescence of the mixture was then read using an Infinite M1000 (TECAN Group Ltd.) luminometer. Cytochrome activity was stated as relative light units (RLU)/10⁶ cells/ml.

Gene expression microarray

Total RNA was isolated from HepaRG cells by RNeasy Mini kit (Qiagen). The expression profiles were performed by the National Research Progress for Genomic Medicine Microarray and Gene expression analysis Core Facility, National Yang-Ming University, Taiwan. Gene expression analysis was done as described in our previous publication [21].

Animal model and HepaRG cell transplantation

Five-week-old NOD-SCID mice were injected intrasplenically with 2.5×10^7 viable NR1H3-derived hepatocytes and HepaRG cells per kilogram body weight 24 h after administration of carbon tetrachloride (CCl₄), the procedure was modified based on previously publications [22,23]. Primary human hepatocytes were purchased from Life Technologies (HEP10, Gibco®) and cultured according to the manufacturer's instructions.

Urea production

Urea production by HepaRG cells was determined by the Urea Assay kit (BioVision) according to the manufacturer's instructions. Briefly, 25 μ l of culture medium was directly added into 96-well plates and incubated with 50 μ l of assay buffer for 1 h at 37 °C while protected from light. The amount of urea present was measured at OD 570 nm using a microplate reader.

Histopathology and immunochemical analysis

Samples were collected 8 days after transplantation. Organs were fixed with 4% paraformaldehyde and prepared in paraffin-embedded blocks for sectioning at 4-micrometer thickness. The sections were deparaffinized, rehydrated and epitope retrieval was performed in Trilogy™ solution (Cell Marque) by autoclaving for 10 min. The sections were then incubated with anti-human NR1H3 antibody (5 μ g/ml, Abnova), anti-human albumin antibody (2.5 μ g/ml, Abcam Inc.) and anti-cytokeratin 19 antibody (1 μ g/ml, Mybiosource Inc.) at 4 °C overnight, followed by staining with goat antibodies against rabbit IgG (Dako), or a goat anti-mouse IgG antibody (Dako) for 40 min at room temperature.

Western blotting

After HepaRG cell lysis, 50 μ g total protein was resolved by 10% SDS polyacrylamide gel electrophoresis, transferred onto NC membrane and analysed with antibodies against HNF4A (ABGENT) and NR1H3 (Abnova).

Human albumin assay

Human albumin production by the HepaRG cells was determined by the human serum albumin EIA Kit (ACE) according to the manufacturer's instructions. Briefly, 50 μ l mouse plasma was directly added into 96-well plates and incubated with Immunoassay buffer A for 1 h at 37 °C and the AChE Tracer, EIA monoclonal antibody for 2 h at room temperature while protected from light. The amount of human albumin present was measured at OD 420 nm using a microplate reader.

Results

Gene expression profiles that reflect the differentiation of HepaRG progenitor cells into hepatocyte-like cells

A protocol, which is described in the Materials and methods section (Fig. 1A), was used to induce the differentiation of human HepaRG progenitor cells in a synchronous fashion; the differentiation recapitulated the steps known to occur during hepatogenesis. Bipotent progenitor HepaRG cells, plated at 2.6×10^4 cells/cm² in the presence of insulin and corticosteroids, first gave rise to undifferentiated cells that actively proliferated for 7 days. These cells then committed into the hepatocyte and biliary differentiation pathways early after reaching confluence at day 14 (Fig. 1B, Panel [i]). The cells then organized themselves in either flat clear epithelial cells or cords of granular polygonal cells (Fig. 1B, Panel [ii]). At day 14, dimethyl sulfoxide (DMSO) was added into the culture medium, and maximum cell differentiation was reached after 2-week DMSO exposure (Fig. 1B, Panel [iii]). Hepatocyte-like cells that had exhibited a phenotype close to that of human hepatocytes, namely where periodic acid-Schiff (PAS) staining showed glycogen storage function, were identified as differentiated hepatocyte-like cells (Fig. 1B, Panels [iv], [v], and [vi], magenta staining shows presence of glycogen).

To determine whether our HepaRG-derived hepatocyte-like cells display the mature characteristics of a hepatic lineage, we examined in these cells the gene expression levels of various human hepatocyte markers, namely albumin, cytochrome P450 3A4 (CYP3A4), cytochrome P450 2B6 (CYP2B6) and cytokeratin

18 (KRT18) by reverse-transcription quantitative polymerase chain reaction (RT-qPCR); the results were normalized against undifferentiated HepaRG cells. As can be seen in Fig. 1C, all of these genes were expressed at significantly higher levels in the HepaRG-derived cells. To assess the functional status of HepaRG-derived hepatocyte-like cells, we determined the urea production and metabolic capacities of the differentiated cells. Urea production significantly increased during differentiation (Fig. 1D). In addition, cytochrome P450 enzyme 3A4 (CYP450) activity, which is one of the most important enzymes involved in the metabolism of xenobiotics in the liver [24], was also elevated during differentiation (Fig. 1E).

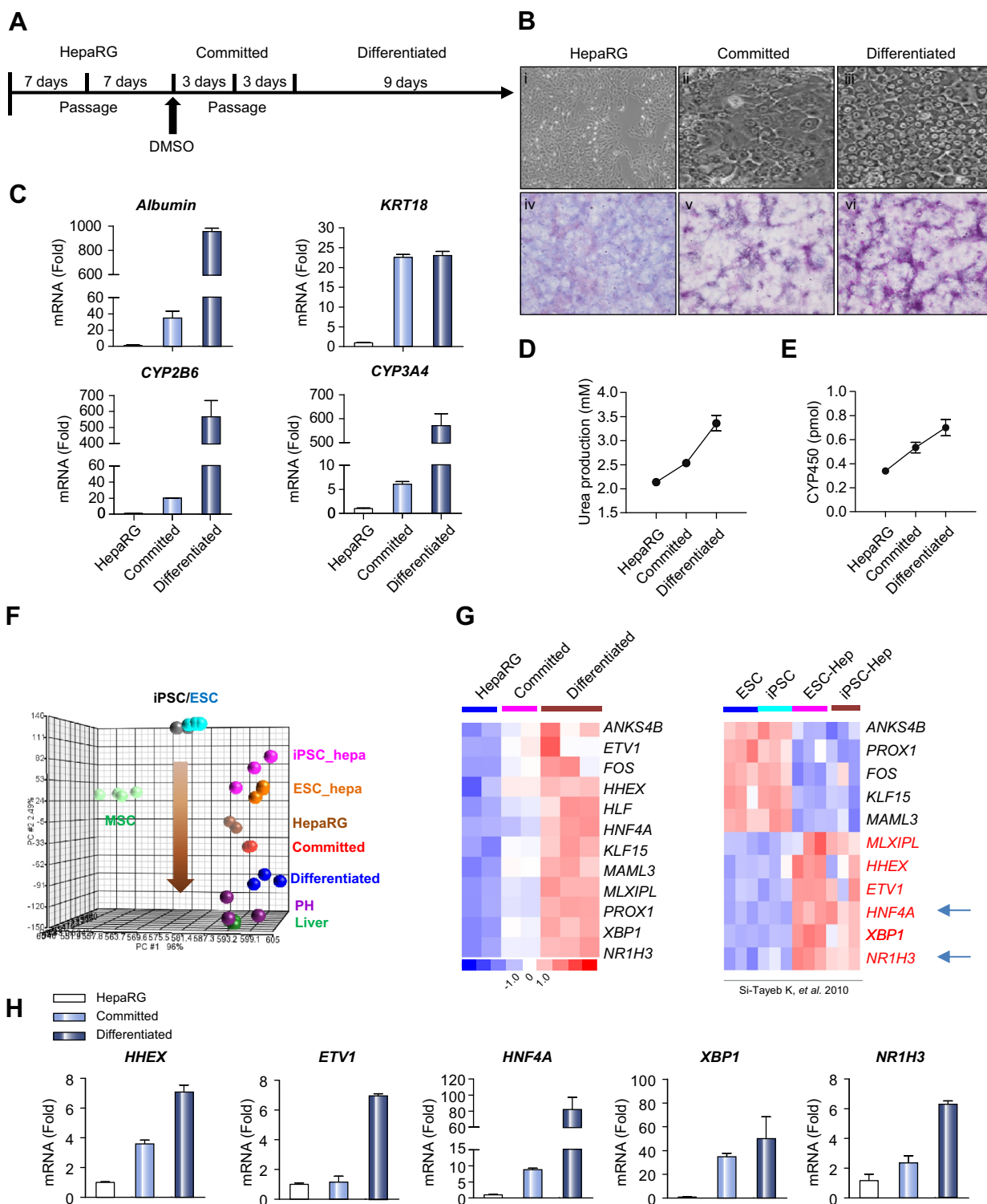
Gene expression microarray analysis of the hepatogenesis of HepaRG cells at 3 stages (undifferentiated, committed, and differentiated) was carried out. The principal component analysis (PCA) of the committed and differentiated HepaRG cells (red and blue spots, respectively), based on genes differentiating ESCs and primary hepatocytes (pFDR $q < 10^{-5}$) compared to the iPSC-derived or ESC-derived hepatic cells (magenta and orange spots, respectively), showed that the HepaRG-derived hepatocyte-like cells were distinct from the original HepaRG cells (brown spots) and were much closer to primary hepatocytes (PH) (purple spots) and liver tissue (green spots) (Fig. 1F). Our differentiated cells were found to be closest to primary hepatocytes. To ensure that the developmental signatures were not just specific to hepatocyte differentiation from HepaRG cells, genes that were differentially expressed between ESC and ESC-derived hepatocytes [25] were filtered and overlapped with the 1086 probe sets (Supplementary Fig. 1A). The results showed that a total of 895 probe sets were conserved between both HepaRG and ESC hepatogenesis (Supplementary Fig. 1B). These findings indicate that hepatocyte-like cells derived from HepaRG bipotent progenitor cells possess the largest number of primary hepatocyte features and functions and that studying the HepaRG differentiation process should help us to identify mechanisms that are critical to the hepatogenesis process.

NR1H3/LXR α is required for the hepatic differentiation of HepaRG cells

One of the major goals of this study was to identify hepatogenesis-determining factors. In this context, we favoured nuclear transcription factors (TFs) since TFs are critical cell fate determinants [26,27]. Six TFs, namely HHEX, ETV1, XBP-1, MLXIPL, NR1H3 and HNF4A, were found to be present at increased levels during hepatogenesis in both HepaRG progenitor cells and pluripotent stem cells (Fig. 1G). We confirmed that there was reliable expression of five of these TFs during the hepatic differentiation of HepaRG cells (Fig. 1H). The exception was MLXIPL where we failed to obtain the expression construct.

To evaluate the possible functions of these TFs, we determined the hepatogenesis-promoting ability of HepaRG cells overexpressing five of these TFs without DMSO treatment. The results revealed that the expression level of various hepatic genes were significantly higher in hepatocyte-like cells expressing NR1H3 and HNF4A (Fig. 2A and B); this was in contrast to the other three TFs, which showed no ability to promote the efficiency of hepatogenesis (Supplementary Fig. 1C).

NR1H3 (also known as liver X receptor α or LXR α) belongs to a hormone receptor superfamily of ligand-activated transcription factors [28] that regulate lipid metabolism [29,30] and the



inflammation response [31]. The involvement of NR1H3 in human ESC or iPSC hepatic differentiation is supported by the genomic data of Si-Tayeb *et al.* [25], which are presented in [Supplementary Fig. 2](#). HNF4A is known to directly control the expression of many hepatic genes and is also crucial for the maintenance of a network of transcription factors that is essential for normal hepatocyte function [32], serving as a positive control in this study.

We determined the functional status of NR1H3-derived hepatocyte cells, which exhibited higher glycogen storage ability than parental HepaRG cells and the level was similar to that of HNF4A-overexpressing cells ([Fig. 2C](#), magenta staining shows presence of glycogen). These cells also have a significantly higher urea production ([Fig. 2D](#)) and cytochrome P450 3A4 enzyme activity ([Fig. 2E](#)) compared to empty lentivirus vector controls. Furthermore, their activities were also increased during differentiation. These findings suggest that NR1H3, just like HNF4A, is able to improve the efficiency of HepaRG cells to differentiate into functional hepatocytes.

To further confirm the critical role of NR1H3 in the formation of hepatocyte-like cells, we generated shRNAs that were designed to target *NR1H3* or *HNF4A* using lentiviral transduction. In contrast to the differentiation of the control cells, the *NR1H3*- or *HNF4A*-depleted HepaRG cells were found to have severely diminished levels of the relevant hepatic mRNA ([Supplementary Fig. 3A and B](#)). Furthermore, glycogen storage, urea production, as well as cytochrome P450 enzyme activities were all significantly lower in HepaRG cells that lacked NR1H3 or HNF4A expression ([Supplementary Fig. 3C–E](#)); this supports the hypothesis that NR1H3 is a newly discovered crucial regulator involved in stem/progenitor cell hepatic differentiation.

Reciprocal regulation between NR1H3 and HNF4

To provide more mechanistic insights into how NR1H3 is able to regulate stem cell hepatogenesis, we performed oligonucleotide array analysis on HepaRG cells overexpressing NR1H3 and HNF4A; these were compared to empty lentiviral vector control cells after completing the first 7 days of differentiation without DMSO treatment ([Fig. 3A](#)). The aim was to provide an unbiased global transcriptome measurement that compared differentiated hepatocyte-like cells with NR1H3 and HNF4A overexpressing hepatocyte cells. The hepatic character was defined using a multidimensional scaling (MDS) plot and showed that NR1H3-overexpressing HepaRG cells were closer to differentiated hepatocyte-like cells than 7-day control differentiation and committed HepaRG cells ([Fig. 3B](#)). It should be noted that the transcriptome of NR1H3-overexpressing HepaRG cells showed

88.9% (965 probe sets of 1086 probe sets of the hepatogenesis in HepaRG cells at 3 stages) similarity to the transcriptome of HNF4A-overexpressing cells ([Fig. 3C](#)), indicating these two transcription factors have similar hepatogenesis-inducing functions. Genes activated or repressed by both NR1H3 and HNF4A are presented in [Supplementary Fig. 4A](#) using two heat maps.

Surprisingly, we found that overexpression or knockdown of *NR1H3* in HepaRG progenitor cells was able to induce the expression or suppression of the endogenous *HNF4A* gene, respectively ([Fig. 3D](#)). The *NR1H3* promoter is known to be occupied by HNF4A in both hepatocytes and pancreatic islets cells [14]. Furthermore, hepatocyte-like cells derived from *HNF4A*-knockdown ESCs expressed significantly less *NR1H3* mRNAs than control ESC-derived cells when *HNF4A* expression is targeted by shRNAs in human H9 pluripotent ESCs ([Supplementary Fig. 4B](#)). The latter finding was discovered by analysing the transcriptomes of both control ESCs and *HNF4A*-knockdown ESCs that had completed the hepatic differentiation protocol ([Supplementary Fig. 4B](#)) [15]. Based on the above findings, we performed reporter assays to investigate whether the *HNF4A* promoter can be targeted by NR1H3 as well as to identify any critical binding sites for NR1H3 within the *HNF4A* promoter. This was done by creating reporter constructs that contained different lengths of the *HNF4A* promoter ([Fig. 3E](#), left panel). An increase in *HNF4A* promoter activity was observed after transient transfection of the P2/-2200 reporter construct with NR1H3 ([Fig. 3E](#)). Deleting the region between -2200 and -1500 base pairs of the *HNF4A* promoter diminished *HNF4A* promoter activity by 17%, while further deletion of a region between -1000 to -500 base pairs totally abolished *HNF4A* promoter activity ([Fig. 3E](#), right panel). These results demonstrate that NR1H3 and HNF4A form a reciprocal regulation circuit that is involved in promoting stem cell hepatogenesis ([Fig. 3F](#)).

NR1H3-derived hepatocyte-like cells can rescue lethal fulminant hepatic failure

To assess the therapeutic potential of NR1H3-derived hepatocytes, a model of lethal fulminant hepatic failure caused by CCl₄ in NOD-SCID mice was used. A dose of CCl₄ at 0.35 ml/kg body weight resulted in lethality in all animals 2 weeks after administration of CCl₄. Transplantation of 2.5×10^7 viable HepaRG cell (empty lentivirus vector control) per kilogram body weight failed to rescue recipient animals from hepatic failure (0 of 8 mice survived). However, when the mice received NR1H3-derived hepatocytes, 87% of the recipient animals were rescued from lethal liver damaged (7 out of 8 mice survived) ([Fig. 4A](#)). Histopathologic analysis indicated the presence of massive

Fig. 1. Characterization and identification of mRNA involved in the differentiation of HepaRG cells into hepatocyte-like cells. (A) Schematic of the differentiation procedure. (B) Images showing the morphological changes in HepaRG cells that occur as they differentiate into hepatocyte-like cells. Panel (i): Morphology of undifferentiated HepaRG cells at 14 days after seeding at low density. Panel (ii): HepaRG cells cultured for 20 days without DMSO induction. Panel (iii): HepaRG cells cultured and maintained for 15 days in the presence of DMSO. Panels (iv–vi): Periodic acid-Schiff staining showing the glycogen storage in the differentiated HepaRG cells. (C) mRNA expression the hepatocyte markers albumin, cytochrome P450 3A4 (*CYP3A4*), cytochrome P450 2B6 (*CYP2B6*) and cytokeratin 18 (*KRT18*) after different times in culture; Gene expressions were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). (D) Differentiated HepaRG cells are able to secrete urea and (E) exhibit cytochrome P450 enzyme activity. (F) Principal component analysis showing transcriptome profile similarity between committed and differentiated HepaRG cells (red and blue spots, respectively), iPSC-derived or ESC-derived hepatic cells (magenta and orange spots, iPSC_hepa and ESC_hepa, respectively), original HepaRG cells (brown spots), primary hepatocyte cells (purple spots, PH) and liver tissue (green spots, Liver) (G) Two heat maps showing mRNA expression levels at different time points (red, high expression; blue, low expression). Six up-regulated transcription factors were identified during HepaRG cells differentiation. (H) Selected candidate transcription factors were analysed by qPCR.

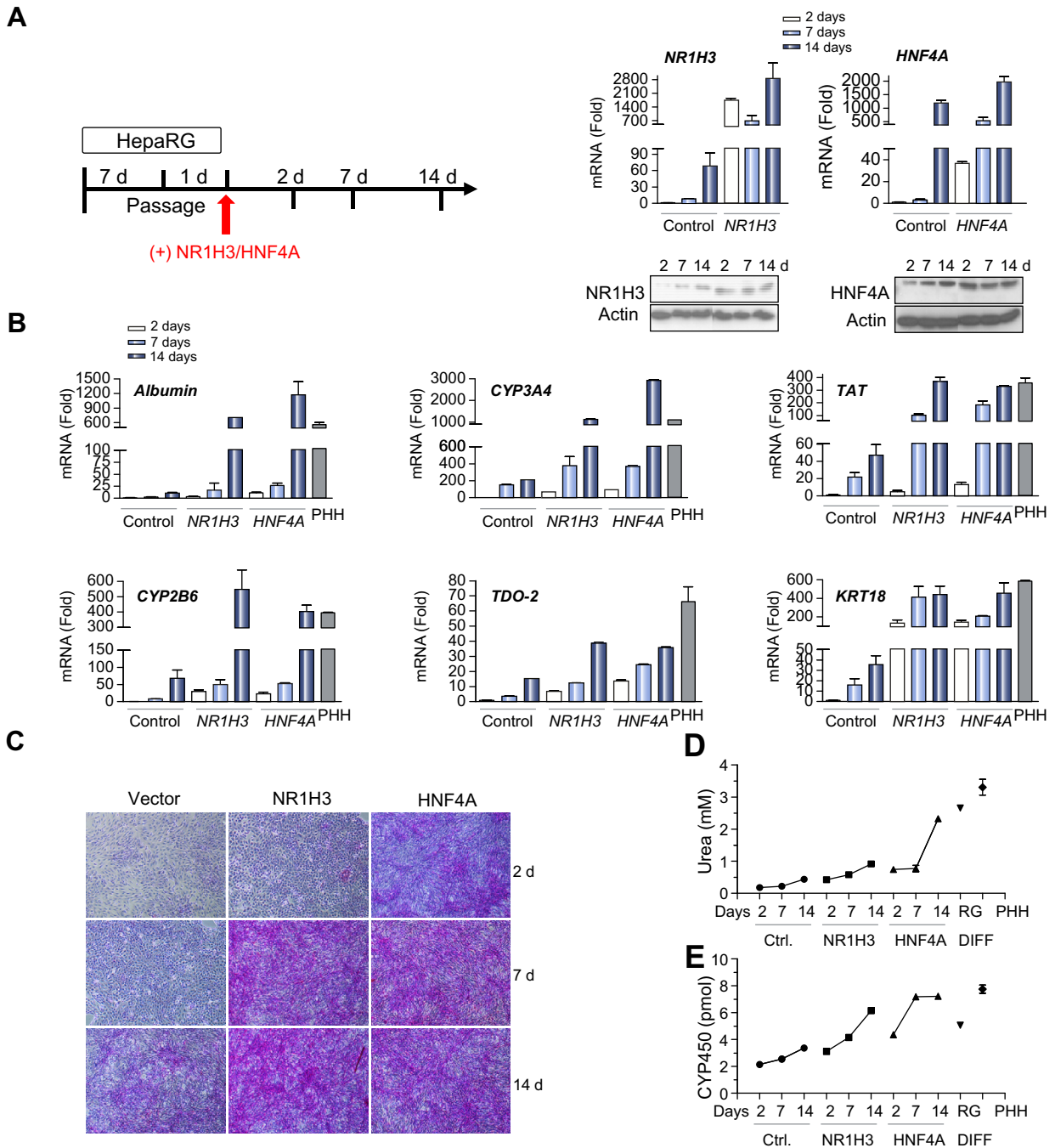


Fig. 2. NR1H3 is sufficient for differentiation of hepatocyte-like cells from HepaRG cells. (A) Schematic of the differentiation procedure by virus infection. Immunoblotting and RT-qPCR analysis of NR1H3 and HNF4A protein and mRNA levels during differentiation of HepaRG cells into hepatocyte-like cells. (B) RT-qPCR analysis shows high mRNA levels for mature hepatocyte markers throughout the differentiation when NR1H3 and HNF4A is overexpressed in HepaRG cells. After 14 days overexpression, the NR1H3-derived hepatocyte-like cells exhibited liver markers similar to primary human hepatocytes (PHH). (C) Periodic acid-Schiff staining shows the glycogen storage of NR1H3 and HNF4A-derived hepatocyte-like cells. (D) NR1H3 and HNF4A over-expression, increased urea production and (E) cytochrome P450 enzyme activity.

necrosis in the livers of the untreated control mice. This contrasted with the rescue of hepatic necrosis when the mice received NR1H3-derived hepatocytes; however, such rescue was absent when the mice received vector control HepaRG cells (Fig. 4B).

To investigate whether the transplanted cells were engrafted within the livers of the recipient mice, a human NR1H3 antibody was used to detect the presence of human NR1H3-derived hepatocytes in mouse liver. In addition, the hepatic functions of the

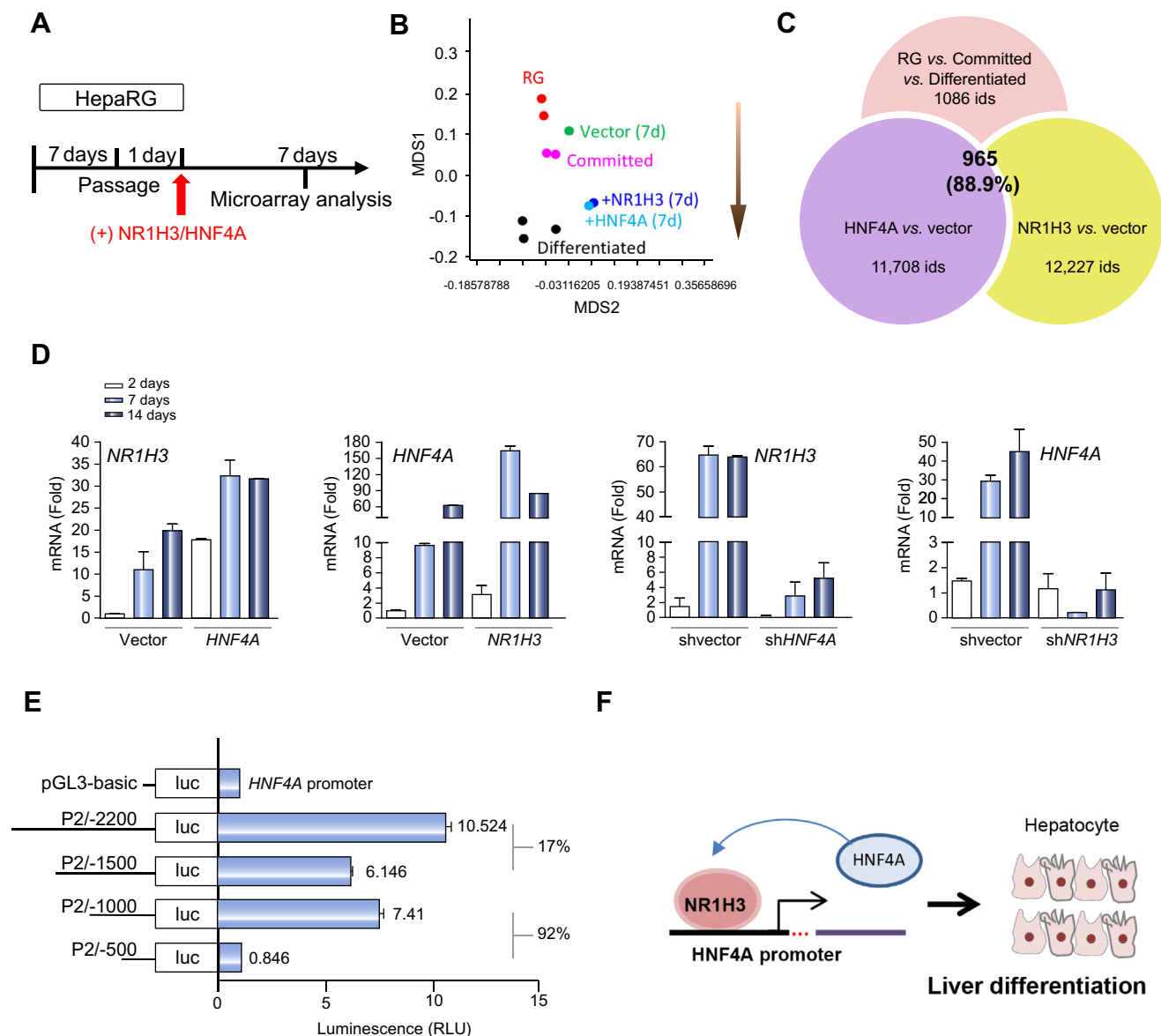


Fig. 3. NR1H3 promotes hepatic differentiation and regulates HNF4A expression. (A) Schematic overview of the virus infection time course. Gene expression analysis using microarray analysis was carried out on NR1H3 and HNF4A overexpressing HepaRG cells at day 7. (B) Multidimensional scaling plot showing the transcriptome profile similarity of the different groups; each spot represents an array sample. (C) Venn diagram showing the number of overlapping genes between NR1H3 overexpression induced hepatocyte differentiation, HNF4A overexpression induced hepatocyte differentiation and HepaRG differentiation. (D) mRNA expression levels of *NR1H3* and *HNF4A* examined by RT-qPCR during overexpression (left panel) or knockdown (right panel) of *HNF4A* or *NR1H3* in HepaRG cells, respectively. (E) *HNF4A* promoter reporter assays were performed by transfection of HepaRG cells with different promoter fragments inserted into a luciferase reporter plasmids. *NR1H3* regulates promoter activity by targeting different regions of the promoter, namely the -500 ~ -1000 bp. (F) Proposed model of hepatic differentiation regulated by NR1H3.

detected NR1H3-derived hepatocytes were identified by immunohistochemistry, namely the detection of human albumin expression in mouse livers (Fig. 4C and D). These findings show that human NR1H3-derived hepatocytes had become engrafted in the recipient's liver parenchyma and that these cells were able to successfully rescue the mice from hepatic failure. Consistently, biochemical tests showed the improved recovery of liver functions by transplantation of NR1H3-derived hepatocytes, which was similar to primary human hepatocytes (Supplementary Fig. 5 and Table 2).

Discussion

Since the HepaRG cell line was first described in 2002, many reports [33,34] have appeared that have described its unique features. These include the ability to differentiate towards both hepatocyte-like and biliary-like cells and the ability to stably express a range of important hepatic functions. In this study, we have demonstrated that the transcription factor NR1H3 contributes to the differentiation of functional hepatocytes from HepaRG cells and it does this by inducing HNF4A, a protein that

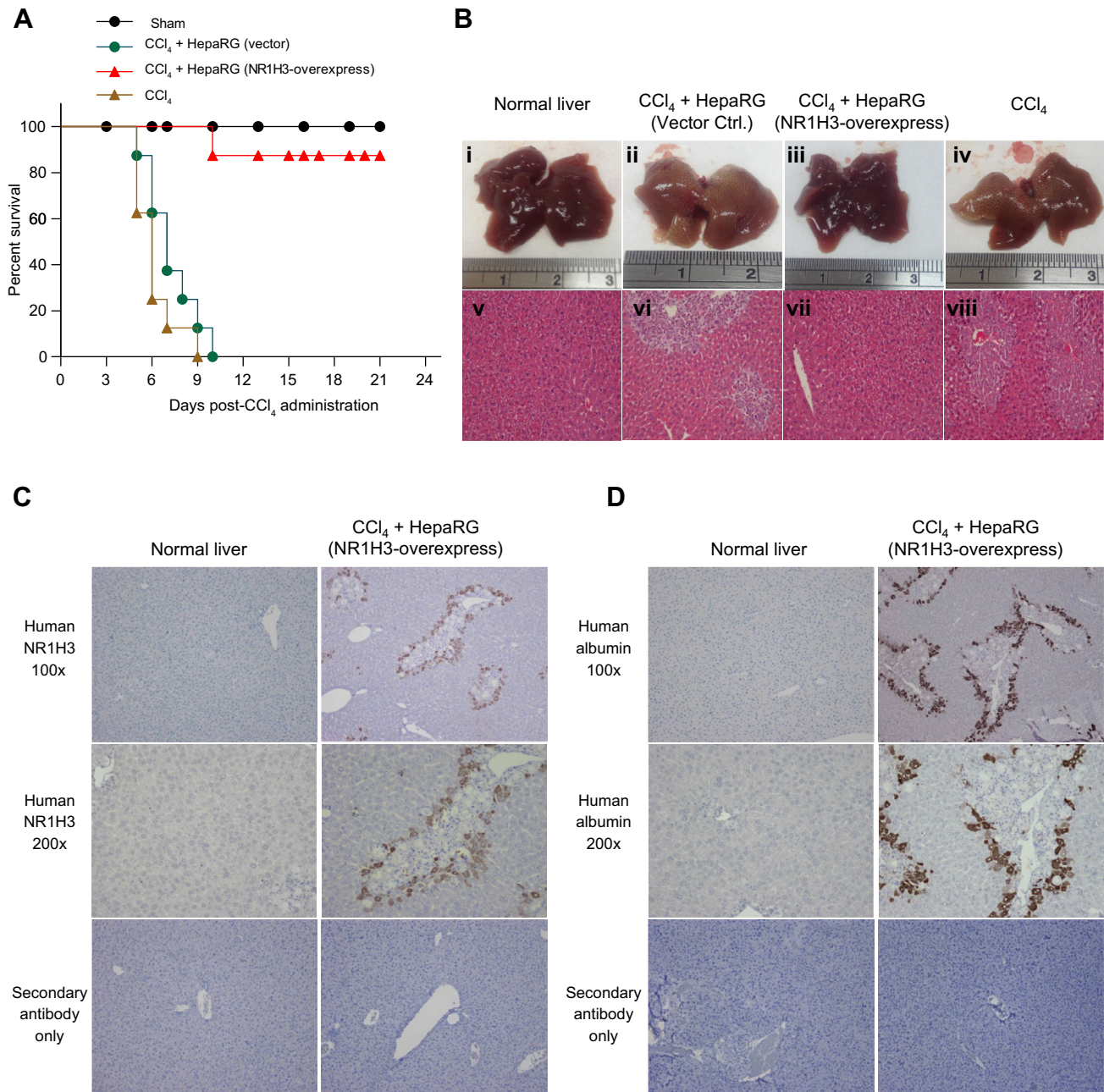


Fig. 4. NR1H3-derived hepatocyte-like cells can rescue CCl₄-induced lethal fulminant hepatic failure. (A) Survival curves of NOD-SCID mice (n = 8 in each group) that received intrasplenic cell transplantation with 2.5×10^7 NR1H3-derived hepatocytes per kilogram body weight. (B) Appearance and histopathology of livers showing organs that had or had not received cell transplantation with NR1H3-derived hepatocytes or vector only hepatocytes after administration of CCl₄. Panels (i) and (v), normal liver; Panels (iii) and (vii), NR1H3-derived hepatocytes transplanted after administration of CCl₄; Panels (ii) and (vi), vector control-hepatocytes transplanted after administration of CCl₄; Panels (iv) and (viii), intrasplenic saline injection after administration of CCl₄. (Original magnification, $\times 200$). (C, D) Samples were collected at 8 days after transplantation. Detection of human hepatocytes with anti-human NR1H3 and anti-human albumin antibodies by immunohistochemistry.

is well known for its hepatic fate-determining ability and for controlling the development of liver architecture. Overexpression of NR1H3 in HepaRG cells led to the efficient differentiation of HepaRG cells into hepatocyte-like cells that exhibit metabolic enzyme activity, mature hepatic markers, urea secretion and glycogen storage ability. Importantly, NR1H3-derived hepatocyte cells were found to rescue lethal fulminant hepatic failure. Our

results describe a novel mechanism that is able to successfully and efficiently generate functional hepatocyte-like cells that will be useful for studying liver development and regeneration.

The effect of NR1H3 on hepatocyte generation has not been identified previously. Other studies have focused on the cholesterol homeostasis and inflammation related activities of NR1H3, which is also known as liver X receptor α (LXR α), in the liver.

Table 2. Liver function tests of NOD-SCID mice that were administered 0.35 ml/kg CCl₄ and underwent transplantation with NR1H3-derived HepaRG cells, DMSO-induced differentiated HepaRG cells, vector control HepaRG cells or primary human hepatocytes.

	Serum glutamyl pyruvic transaminase (IU/L)	Human albumin/total albumin (%)
Normal NOD-SCID	20 ± 6.55	n.d.
Post-CCl₄ day 1	4088 ± 405.4	n.d.
Post-CCl₄ day 3		
IS-vector control	607 ± 31.28	3.2%
IS-PHH	107 ± 10.338	14.8%
IS-HepaRG-NR1H3	170 ± 15.79	11.1%
IS-HepaRG-DIFF	193 ± 17.017	12.07%
Post-CCl₄ day 5		
IS-vector control	572 ± 36	3.08%
IS-PHH	72 ± 7.17	17.1%
IS-HepaRG-NR1H3	120 ± 10.1	14.53%
IS-HepaRG-DIFF	121 ± 7.13	12.05%
Post-CCl₄ day 7		
Vector control	590 ± 44.43	2.5%
IS-PHH	56 ± 2.9	15.65%
IS-HepaRG-NR1H3	89 ± 8.85	12.59%
IS-HepaRG-DIFF	78 ± 6.28	10.95%
Post-CCl₄ day 14		
IS-vector control	n.d.	n.d.
IS-PHH	33 ± 1.5	13.72%
IS-HepaRG-NR1H3	55 ± 5.46	14.02%
IS-HepaRG-DIFF	70 ± 3.02	13.6%
Post-CCl₄ day 30		
IS-vector control	n.d.	n.d.
IS-PHH	29 ± 5.82	8.36%
IS-HepaRG-NR1H3	49 ± 2.01	7.78%
IS-HepaRG-DIFF	45 ± 5.32	6.19%

Data are presented as mean ± SD of 3 determinations.

IS, intrasplenic transplantation; ND, not done; DIFF, DMSO-induced differentiated HepaRG cells; PHH, primary human hepatocyte.

NR1H3 is critical for the peroxisome proliferator-activated receptor (PPAR) pathways, and there are clues that seem to support the hypothesis that the PPAR pathways play a role in hepatogenesis: PPAR-beta could facilitate the maturation of hepatic-like tissue derived from mouse embryonic stem cells and this was accompanied by mitochondriogenesis and membrane potential retention [35]. In addition, PPAR-alpha induced hepatic differentiation of rat oval cells, which act as bipotential progenitors for the two main hepatic lineages [36]. This is similar to the situation in HepaRG cells as rat oval cells are considered to be closely related to liver stem cells [36]. Inhibition of adult oval cell growth and viability has been noticed in the presence of a PPAR gamma agonist [37]. PPAR pathways as well as NR1H3, may have an important role in cellular energy metabolism during hepatogenesis.

Liver regeneration occurs both by replication of differentiated hepatocytes and through activation of a ductular reaction [38]. NR1H3-derived hepatocytes may enhance liver regeneration by several potential mechanisms: first, cytokines secreted by the implanted cells might improve liver regeneration via a paracrine

mechanism [39–41]. We found seven secreted factors are induced by NR1H3 in HepaRG cells, namely C5, CCL14/CCL15, CXCL2, IGF-1, MST-1, TNFSF10 and TSLP (Supplementary Fig. 6A). Among them, IGF-1 [42] and MST-1 [43] have been linked to liver regeneration and hepatic differentiation, and their increased levels in NR1H3-overexpressing HepaRG cells could be verified by RT-qPCR (Supplementary Fig. 6B). Secondly, transplanted cells may replace damaged tissues. We found that NR1H3 increases the expression levels of hepatic and proliferation-related factors such as HNF4A (Supplementary Figs. 8 and 9) [19], FOXA2 [44], PPAR gamma [45], HNF1, CEBP and Ki67 [46] (Supplementary Fig. 7). Together these factors may accelerate the proliferation of hepatocyte-like cells and help liver repair. Thirdly, since part of our NR1H3-derived cells express CK-19 (cholangiocytes marker) *in vivo* (Supplementary Fig. 10), a ductular reaction may also occur in response to acute severe and chronic liver injury [47,48].

HepaRG cells have been used in a variety of studies, including those involving liver toxicity [49,50], hepatitis viruses infection [51], human hepatic diseases and differentiation. The currently used differentiation procedure takes 4 weeks in DMSO-treated medium. In contrast, our results indicate that it is feasible to obtain functional hepatocyte-like cells within 7 days by over-expressing NR1H3 in HepaRG cells without DMSO treatment. Kotokorpi *et al.* [52] found that one LXR endogenous ligand, GW3965, is able to reduce the secretion of bile acids and increase lipid storage. Other synthetic agonists, including T0901317 and N,N-dimethyl-3β-hydroxycholeamide, have also been used to identify the functions of NR1H3 [53]. In such circumstances, since NR1H3 is involved in hepatic development, it is possible that the addition of NR1H3 agonists to the HepaRG culture medium might be an alternative approach to accelerate HepaRG cell hepatocyte differentiation *in vitro*.

Moreover, analysis of the gene expression profiles of ESC-derived and iPSC-derived hepatic progenies has shown that NR1H3, as well as HNF4A, are also highly expressed in hepatocyte-like cells derived from these pluripotent stem cells. This indicates that the activation of NR1H3 is universal in stem/progenitor cell hepatogenesis. The addition of NR1H3 agonists into differentiation cocktails might also be beneficial for the generation of functional hepatocyte-like cells from other stem cell resources and for further studies of hepatocyte transplantation in end-stage liver disease. Finally, it is also possible to accelerate liver regeneration *in vivo* by liver-specific target delivery of NR1H3 agonists, such as to package agonists into liver-specific vehicles [54,55].

In conclusion, we have identified a novel hepatogenesis regulator, NR1H3. This transcription factor activates and promotes hepatic differentiation by inducing *HNF4A* expression (Fig. 3F). We have also established the expression profiles that occur during HepaRG differentiation and these findings should help researchers to obtain a better understanding of the hepatic development. Furthermore, NR1H3-derived hepatocytes may serve as an alternative source of cells for further studies on liver development and regeneration.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

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