Hepatocyte Nuclear Factor 4 alpha (HNF4 α) Activation is Essential for Termination of Liver Regeneration

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Financial Support: These studies were supported by NIH-COBRE (P20 RR021940-03, P30 GM118247), NIEHS Toxicology Training Grant (T32ES007079-34) and NIH R01DK 0198414

Abstract

Hepatocyte Nuclear Factor 4 alpha (HNF4 α) is critical for hepatic differentiation. Recent studies have highlighted its role in inhibition of hepatocyte proliferation and tumor suppression.

However, the role of HNF4 α in liver regeneration is not known. We hypothesized that hepatocytes modulate HNF4 α activity when navigating between differentiated and proliferative states during liver regeneration. Western blot analysis revealed a rapid decline in nuclear and cytoplasmic HNF4 α protein levels accompanied with decreased target gene expression within 1 hour after 2/3 partial hepatectomy (post-PH) in C57BL/6J mice. HNF4 α protein expression did not recover to the pre-PH levels until day 3. Hepatocyte-specific deletion of HNF4 α (HNF4 α -KO) in mice resulted in 100% mortality post-PH despite increased proliferative marker expression throughout regeneration. Sustained loss of HNF4 α target gene expression throughout regeneration indicated HNF4 α -KO mice were unable to compensate for loss of HNF4 α transcriptional activity. Deletion of HNF4 α resulted in sustained proliferation accompanied by c-myc and cyclin D1 over expression and a complete deficiency of hepatocyte function after PH. Interestingly, overexpression of degradation-resistant HNF4 α in hepatocytes did not prevent initiation of regeneration after PH. Finally, AAV8-mediated reexpression of HNF4 α in hepatocytes of HNF4 α -KO mice post-PH restored HNF4 α protein levels, induced target gene expression and improved survival of HNF4 α -KO mice post-PH. In conclusion, these data indicate that HNF4 α reexpression following initial decrease is critical for hepatocytes to exit from cell cycle and resume function during the termination phase of liver regeneration. These results reveal the role of HNF4 α in liver regeneration and have implications for therapy of liver failure.

Introduction

HNF4 α is known as the master regulator of hepatocyte differentiation due its role in establishing hepatocyte-specific gene expression during embryonic development (1, 2), stabilizing the hepatic transcription factor network (3) and maintaining hepatocyte function (4). HNF4 α regulates genes involved in xenobiotic metabolism, carbohydrate metabolism, fatty acid metabolism, bile acid synthesis, blood coagulation, and ureagenesis (5). Expression of HNF4 α induces hepatocyte-like characteristics in induced pluripotent stem cells (6) and forced expression of HNF4 α can be used to differentiate and decrease cancer progression in hepatocellular carcinomas (HCC) and hepatoma cell lines (7, 8). Furthermore, decreased HNF4 α expression leads to loss of hepatic function and causes decompensation in cirrhotic rats (9).

Recent studies from our laboratory and others have revealed anti-proliferative properties of HNF4 α (10, 11). HNF4 α is anti-proliferative in kidney cells (12) and pancreatic β -cells (13). Furthermore, hepatocyte-specific deletion of HNF4 α (HNF4 α -KO) in mice results in spontaneous hepatocyte proliferation (10, 11) and promotes formation of diethylnitrosamine-induced HCCs (14).

During liver regeneration after 2/3 partial hepatectomy (PH), the most widely used model to study liver regeneration, multiple redundant mechanisms regulate initiation and termination of hepatocyte regeneration (15). Understanding the mechanisms that govern adult hepatocytes to navigate between quiescent and proliferative states could result in therapeutic targets for inducing hepatocyte proliferation during impaired regeneration or inhibiting excess proliferation

during carcinogenesis. Despite its role in maintaining hepatocyte differentiation and quiescence, little is known about the role of HNF4 α in hepatocyte regeneration or how decreased HNF4 α , a condition commonly found in diseased human livers (9, 16-18), would impact regeneration. In this study, we investigated the role of HNF4 α in regulation of liver regeneration after PH using wildtype (WT) and HNF4 α -KO mice. Our studies revealed that HNF4 α is indispensable for survival after PH and a critical component of termination of liver regeneration.

Materials and Methods

Animal Care and Surgeries.

Animals were housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care at the University of Kansas Medical Center under a standard 14-hr dark/10-hr light cycle with access to chow and water *ad libitum*. All studies were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. PH surgeries were performed and tissue samples obtained as previously described (19). Surgeries were performed between 9:00 am and 11:00 am. For liver regeneration in normal conditions, two-month-old male C57BL6/J mice were euthanized at 0, 1, 3, 6, 12, 24 and 48 hr, and 3, 5, 7, and 14 days after PH with 3 mice per time point. To study the effects of HNF4 α deletion on liver regeneration, seven days before surgery HNF4 α -floxed mice were injected intraperitoneally with AAV8-TBG-eGFP or AAV8-TBG-CRE resulting in WT and hepatocyte-specific HNF4 α -KO animals, respectively. These vectors were purchased from Penn Vector Core (Philadelphia, PA) and injected as previously described(20). WT and HNF4 α -KO mice were euthanized at 0, 1, 2, 5, 7 and 14 days after PH with 3-5 mice per group. Only two HNF4 α -KO mice survived to be included at the 7 day time point. HNF4 α reexpression was accomplished by tail vein injection of 2×10^{11} viral genomes/mouse of AAV8-CMV-HNF4 α generated in the laboratory of Dr. Holger Willenbring. Male HNF4 α -floxed mice were injected with AAV8-TBG-CRE to induce HNF4 α deletion and 7 days later were injected with AAV8-CMV-HNF4 α to restore HNF4 α expression. Liver and serum samples were collected seven days after AAV8-CMV-HNF4 α injection. Next, this system was used to test if HNF4 α reexpression could rescue HNF4 α -KO mice after partial hepatectomy. WT and HNF4 α -KO mice underwent PH 7 days after injection with AAV8-TBG-eGFP or AAV8-TBG-CRE with four mice per

treatment. Tail vein injection with reexpression vector or control saline occurred 2 days after surgery to allow for initiation of regeneration. Overexpression of HNF4 α was accomplished using Tet-On-HNF4 α transgenic mouse line developed at the KUMC Transgenic and Gene Targeting Institutional Facility. These mice contained a reverse tetracycline-controlled transcriptional activator which was constitutively expressed with a C/EBP β promoter. In the presence of the tetracycline-derivative Doxycycline (Dox), this transcription factor binds a tetracycline response element (TRE) upstream of an shRNA-resistant HNF4 α gene (kind gift from Dr. Stephen A. Duncan, Medical University of South Carolina). Tet-On-HNF4 α were heterozygous for both transgenes. Doxycycline was administered for two weeks prior to surgery by replacing drinking water with a 1 mg/mL Dox in 5% Sucrose solution. Untreated mice were given 5% sucrose solution only. Two mice were used for the untreated 6 hour time point and three to five mice used for all other treatments and time points.

Protein Isolation and Western blotting.

Nuclear and cytoplasmic lysates and RIPA extracts were prepared and used for Western blot analysis as reported (21). Primary antibodies are provided in Supplemental Table 1.

Densitometric analysis was performed using Image Studio Lite (LI-COR Biosciences).

Real Time PCR.

RNA isolation, conversion to cDNA and Real time PCR analysis was performed as previously described (22). RNA sequencing and bioinformatics analysis were performed as previously described(14). Genes of interest were identified based on RNA-Seq analysis comparing gene

expression in livers from WT and hepatocyte-specific HNF4 α -KO mice(14). Primer sequences are provided in Supplemental Table 2.

Staining Procedures.

Paraffin-embedded liver sections (4- μ m thick) were used for immunohistochemical staining of proliferating cell nuclear antigen (PCNA) as previously described(19). PCNA positive hepatocyte nuclei were quantified by counting three 40x fields per slide for each liver sample.

Serum Bilirubin

Total serum bilirubin was determined using the Total Bilirubin kit from Pointe Scientific (Cat #B7576) according to manufacturer's protocol.

Statistical Analysis

Results are expressed as mean $\pm$ standard error. One Way ANOVA and Student's *t* test was applied to all analyses with $p < 0.05$ being considered significant.

Results

Decreased HNF4 α Protein Expression and Transcriptional Activity During Initiation of Regeneration After Partial Hepatectomy.

First, we investigated the expression and activity of HNF4 α in liver regeneration after PH in C57BL/6J mice. We measured protein expression of HNF4 α adult isoforms using Western blotting in freshly prepared nuclear (Fig. 1A, 1C) and cytoplasmic (Fig. 1B) protein extracts. Nuclear HNF4 α levels started declining at 1 (hour) hr post-PH reaching the lowest level at 6 hr post-PH. Nuclear HNF4 α protein expression started rising at 12 hr, increased higher than the 0 hr levels peaking at 3 days post PH and then declined again to reach the 0 hr levels by 7 days post-PH (Fig. 1A, 1C). Cytoplasmic HNF4 α expression continually declined below 0 hr levels until 12h post-PH, then started to rise but did not return to 0 hr pre-PH levels till 14 days after PH. The expression of adult isoform of HNF4 α mRNA did not change over the entire regeneration time course (Fig. 1D).

We assessed HNF4 α transcriptional activity by qPCR analysis of its target genes at the 0, 6 and 12 hr time points when nuclear HNF4 α protein levels were at their lowest levels. We observed decreased expression of positive target genes (14) (Apoa2, ApoB, Ces3, Cldn1, Cyp2c37, Dio1, F12, Ugt2b1) at the 6 and 12-hr time points compared to 0 hr expression levels (Fig. 1E). We have previously identified novel negative targets of HNF4 α , which are suppressed by HNF4 α in the normal liver (14). qPCR analysis showed induction of HNF4 α negative target genes (Akr1b7, Ccnd1, Ect2, Myc) at the 6 and 12 hr time points (Fig. 1F). Together, these data indicate a decrease in HNF4 α activity at the onset of regeneration which is consistent with the changes we observed in HNF4 α protein expression (Fig. 1A-C).

HNF4 α Overexpression Delays But Does Not Prevent Hepatocyte Proliferation During Liver Regeneration

We used a Tet-On-HNF4 α transgenic mouse system (23) to overexpress HNF4 α in hepatocytes to test if the anti-proliferative effects of HNF4 α would prevent hepatocyte proliferation during the initiation of liver regeneration (Supp. Fig. 1). Western blot analysis confirmed increased HNF4 α in nuclear lysates from livers of doxycycline (Dox)-treated Tet-On-HNF4 α mice at 6 hours post-PH (Supp. Fig. 2A). This resulted in fewer PCNA-positive nuclei in Dox-treated mice 6 hours post-PH. However, no difference in PCNA staining was observed between untreated and Dox treated mice 48 hours post-PH (Supp. Fig. 2B). Interestingly, Dox treatment inhibited the occurrence of transient steatosis 48 hours post-PH (Supp. Fig 2C).

Hepatocyte-Specific HNF4 α -KO Mice Do Not Survive After PH.

Next, we investigated the effect of hepatocyte-specific HNF4 α deletion on liver regeneration after PH. WT and HNF4 α -KO mice underwent PH and were euthanized at 1, 2, 5, 7 and 14 days after surgery. HNF4 α protein remained undetectable in HNF4 α -KO mice throughout the time course after PH (Fig 2A). We observed 100% mortality in the HNF4 α -KO group by day 11 post-PH (Fig. 2B). Interestingly, the recovery of liver weight was similar between WT and HNF4 α -KO groups until 5 days post PH (Fig. 2C). Serum bilirubin was significantly elevated in HNF4 α -KO mice at days 1, 2 and 5 post-PH (Fig. 2D). While serum bilirubin in HNF4 α -KO animals did decrease over time, this assay was only performed on surviving mice and most animals in this group had died at later time points. We investigated changes in HNF4 α target gene expression in

WT and HNF4 α -KO mice at 7 days after PH, which revealed a significant decrease in expression of positive target genes including *Alas2*, *Apoa2*, *Apob*, *Cldn1*, *Cyp2c37*, *Dio1*, *F12* and *Ugt2b1* (Fig 3A). Similarly, the expression of the negative targets *Akr1b7*, *CcnD1*, *Cdkn3*, *Defb1*, *Ect2*, *Egr1*, *Myc* and *Slc34a2* was increased at 7 days post-PH (Fig. 3B).

Increased Hepatocyte Proliferation in HNF4 α -KO Livers Throughout Regeneration.

Western blot analysis indicated that protein expression of cyclin D1, the cyclin involved in driving G0 to G1 and G1 to S cell cycle transition, was significantly increased in HNF4 α -KO livers at all time points. The expression of CDK4, the cyclin dependent kinase interacting with cyclin D1 was lower in HNF4 α -KO liver at days 1 and 2 post PH but increased to levels comparable to WT mice by days 5 post PH. Cell proliferation assessed by Western blot and immunohistochemical analysis of PCNA revealed an elevated PCNA level in HNF4 α -KO mice at all time points (Fig. 4A). Immunohistochemical analysis of PCNA-positive nuclei per 40x field was significantly higher in HNF4 α -KO mice throughout the 7-day time course post PH (Fig. 4B-C).

Altered Proliferative Signaling in HNF4 α -KO Mice During Regeneration

We investigated the mechanism of sustained hepatocyte proliferation throughout regeneration in HNF4 α -KO mice by Western blot analysis of pathways commonly involved in hepatocyte proliferation. We focused on pathways downstream of primary hepatocyte mitogen signaling as well as those known to activate hepatocyte proliferation in the absence of HNF4 α (14, 24). Most interestingly, we observed complete loss of total EGFR and total c-MET protein expression in HNF4 α -KO animals at all time points throughout regeneration (Fig. 5A). Next, we examined

Wnt/ β -catenin pathway members (Fig. 5B). Total β -catenin levels were similar in WT and HNF4 α -KO mice before PH and did not change throughout the time course. However, inactive (Thr41/Ser45- phosphorylated) β -catenin was elevated in the WT animals at days 1 and 2 post-PH. Phosphorylated β -catenin was greater in HNF4 α -KO compared to WT mice 5 days post-PH. Densitometric analysis of inactive to total β -catenin ratio suggested activation of β -catenin was higher in HNF4 α -KO mice at 1 and 2 days post-PH. Total GSK-3 β levels did not change for both groups throughout the time course. Inactive (ser9-phospho) GSK-3 β was higher in both groups at days 1 and 5 post-PH, but no difference was observed between groups. Next, we investigated activation of several MAPK members including AKT, p38 and ERK1/2 (Fig. 5C). No difference in total AKT and total p38 protein levels was observed between groups throughout regeneration. Phosphorylation of AKT was inhibited in the HNF4 α -KO mice as compared to WT at days 0, 1 and 5 post-PH but was similar to WT at day 2 after PH. Phosphorylation (activation) of p38 was elevated in HNF4 α -KO mice at days 1 and 5 post-PH. Finally, Total ERK1/2 expression was elevated in HNF4 α -KO animals at all time points. Phosphorylation (activation) of ERK1 (upper band) was increased in HNF4 α -KO animals compared to WT at all time points. Phosphorylation (activation) of ERK2 (lower band) was inhibited in HNF4 α -KO group compared to WT at days 0 and 5 post-PH. ERK2 phosphorylation was elevated in HNF4 α -KO group compared to WT at day 7 post-PH. Finally, we measured expression of known regulator of hepatocyte proliferation and negative target of HNF4 α c-Myc (Fig. 5D). While c-Myc protein expression was similar between WT and HNF4 α -KO animals at day 0, c-Myc expression was elevated in HNF4 α -KO animals at all time points post-PH. Furthermore, c-Myc mRNA expression was elevated in HNF4 α -KO mice at all time points.

RNAseq revealed sustained increase in pro-proliferative and anti-differentiation signaling in HNF4 α -KO Mice Post-PH

To gain insights in the comprehensive signaling changes in HNF4 α -KO livers after PH, we performed RNA-Seq analysis at days 2 and 5 post-PH. These time points were selected to match the peak hepatocyte proliferation (day 2 post PH) and early termination phase of regeneration (day 5 post PH) in the liver regeneration process. Overall, a similar number of genes (~1,000) increased and decreased at day 2 and day 5 after PH in HNF4 α -KO mice as compared to WT mice (Supp. Table 3). Ingenuity Pathway Analysis (IPA) predicted activation and inhibition of transcription factors based on gene expression changes between genotypes. At 2 days post-PH (Table 1), IPA predicted activation of effectors of proinflammatory signaling (EGR1, IRF3, IRF6, IRF7) and proproliferative transcription factors (JUN, RB1, P300, TCF3) in HNF4 α -KO liver. Interestingly, HNF4 α -KO also showed activation of TGF- β signaling as indicated by activation of SMAD2 and SMAD4 as well as inhibition of SMAD7 (25). The transcription factors predicted to be inhibited 2 days post-PH included HNF4 α and its coactivators HNF1A and PPARGC1A (PGC1A). Furthermore, Estrogen Receptor α (Esrra), which is known to regulate gene expression in coordination with HNF4 α and PGC1A (26), was also predicted to be inhibited. TAF4, a known HNF4 α cofactor which is required for HNF4 α transcriptional activity (27), was also inhibited. MED1, which is essential for PPAR α activity and required for survival after PH (28), was inhibited in HNF4 α -KO 2 days post-PH. ZBTB20, a known suppressor of hepatocyte proliferation and repressor of alpha-fetoprotein (29, 30), was predicted to be inhibited.

At day 5 post-PH (Table 2), the analysis predicted activation of transcription factor proinflammatory damage associated molecular pattern (DAMP) protein HMGB1 in HNF4 α -KO mice indicating sustained inflammatory signaling in HNF4 α -KO mice throughout regeneration. The TGF β effector SMAD2 was also activated at day 5 post-PH. Proliferative marker EP300 was activated at day 5 post-PH. The HNF4 α negative target gene and proliferative marker CCND1 was activated in the HNF4 α -KO group at day 5 post-PH indicated sustained proliferative signaling compared to WT mice. Activation of SNAI2 was predicted in the HNF4 α -KO group at 5 days post-PH. SNAI2 is repressed by HNF4 α and is known to promote EMT (31). PLAG1 is a fetal gene overexpressed in hepatoblastomas (32) and was predicted to be activated in the HNF4 α -KO group at day 5. Activation of TRIM24, a transcription factor with oncogenic activity (33), was predicted at day 5 post PH in the HNF4 α -KO mice. Transcription factors including HNF4A, HNF1A, PPARGC1A, and Esrra were inhibited at day 2 post-PH and continued to be inhibited at day 5 post-PH. Inhibition of SIRT2 was observed at 5 days post-PH in HNF4 α -KO mice consistent with its known role in sharing many of the same target genes as HNF4 α (34). PPARGC1B is a known coactivator of PPARGC1A and was inhibited. NCOA2 and EBF1, known tumor suppressors(35, 36), were inhibited in HNF4 α -KO mice day 5 post-PH. NFIX, a transcription factor known to inhibit development of HCC(37), was inhibited. Finally, the master regulators of sterol and lipid metabolism, SREBF1 and SREBF2, were inhibited 5 days post-PH.

IPA analysis also predicted activity of diseases and organ function based on gene expression differences between WT and HNF4 α -KO mice at 2 and 5 days post-PH. The comparison at day 2 post-PH (Supp. Table 4) predicted HNF4 α -KO mice would exhibit activation of pathways

involving inflammation, tumorigenesis and wound healing. Inhibited functions included numerous basic liver processes such as transport, metabolism and synthesis of cholesterol, lipids, bile acids and xenobiotics. This pattern continued at day 5 post-PH (Supp. Table 5). Activated functions continued to be related to cell proliferation, tumorigenesis and inflammation. Additionally, functions related to embryonic organ tissue development were activated in the HNF4 α -KO group. Functions related to transport and metabolism of lipids, cholesterol and vitamins remained inhibited in HNF4 α -KO mice 5 days post-PH.

Reexpression of HNF4 α Restores Hepatocyte Quiescence and Gene Expression and Extends Survival of HNF4 α -KO Animals Post-PH

Next we tested if reexpression of HNF4 α in hepatocytes by intravenous injection of AAV8-CMV-HNF4 α could rescue HNF4 α -KO mice after PH by restoring HNF4 α transcriptional activity after cell division. First we reexpressed HNF4 α in HNF4 α -KO mice and measured HNF4 α target gene expression. HNF4 α reexpression increased total c-MET and total EGFR protein expression to WT levels and reduced cyclin D1 expression to WT levels (Fig. 6A). Hepatocyte proliferation was assessed by immunohistochemical analysis of PCNA positive nuclei. High levels of hepatocyte proliferation were observed in the HNF4 α -KO group. HNF4 α reexpression resulted in decreased hepatocyte proliferation compared to HNF4 α -KO mice (Fig. 6B,C). Finally, we reexpressed HNF4 α in HNF4 α -KO mice 2 days post-PH. This treatment improved survival compared to HNF4 α -KO mice (Fig. 6D).

Discussion

HNF4 α is the master regulator of hepatocyte differentiation because it is required for hepatocyte differentiation during embryonic liver development and it maintains hepatocyte specific gene expression in adult hepatocytes (3-5). HNF4 α also exerts strong anti-proliferative effects on hepatocytes and decreased HNF4 α expression and activity results in loss of quiescence, which can lead to HCC (14, 17, 18). Despite being recognized as one of the strongest anti-proliferative proteins in the liver, the role of HNF4 α during liver regeneration is not known. We investigated the role of HNF4 α in liver regeneration after PH, where adult hepatocytes exit the physiological quiescent state and enter the cell cycle before returning to the quiescent state once liver regeneration has been completed. We hypothesized that decreased HNF4 α expression and function would occur during the initiation of regeneration to alleviate the antiproliferative effects of HNF4 α as quiescent hepatocytes enter the cell cycle during the initiation of liver regeneration. Indeed, we observed decreased nuclear and cytoplasmic levels of HNF4 α occurring within hours after surgery. This decreased protein expression was not due to changes in transcription of the HNF4 α gene because there were no changes in HNF4 α mRNA post-PH. Decreased periportal HNF4 α staining after PH has been previously reported (38). However, HNF4 α expression during liver regeneration has never been studied in a time course at this level of resolution until now. Posttranslational modifications (PTMs) of HNF4 α which result in decreased nuclear localization are known (39, 40), and some PTMs can lead to proteosomal degradation of HNF4 α (41). Future studies are needed to investigate whether these posttranslational modifications, especially phosphorylation of HNF4 α , is the mechanism behind the decrease in HNF4 α protein levels during the initiation of liver regeneration. Decrease in expression of HNF4 α protein after

PH resulted in decrease in its transcriptional activity as measured by its target genes, which play a critical role in liver physiology (14). Our observations independently reproduce a microarray study describing decreased HNF4 α target gene expression 4 hours post-PH (42). This highlights functional differences between quiescent and proliferating hepatocytes and suggests restoring HNF4 α in hepatocytes responding to chronic injury could successfully restore hepatic function (9, 43).

Next, we hypothesized that overexpressing HNF4 α in hepatocytes at time points after PH when HNF4 α levels were decreased would prevent hepatocytes from proliferating and halt or delay initiation of liver regeneration. Interestingly, our experiments with Tet-On driven HNF4 α expression revealed no significant effect on overall hepatocyte proliferation after PH. These data suggest that rapid down regulation of HNF4 α after PH may accelerate hepatocyte cell cycle entry, but is not absolutely necessary for initiation of cell cycle progression after PH. It is also possible that HNF4 α overexpression resulted in delayed initiation because the mechanism responsible for HNF4 α degradation was still active and more time was needed to degrade the overexpressed protein. Inhibiting the mechanism responsible for HNF4 α degradation could be a better strategy to study the effects of increased HNF4 α expression during initiation of liver regeneration once those mechanisms are established. One interesting observation from the HNF4 α overexpression study was the lack of steatosis in HNF4 α overexpressing mice, which is known to occur after PH (44). These data indicate that the initial loss of HNF4 α could be mechanistically involved in the transient steatosis observed following PH and those mechanisms may be independent of initiation of cell proliferation.

The most striking observation from our studies is 100% mortality in HNF4 α -KO mice following PH within 11 days secondary to loss of hepatic function. Several HNF4 α -KO mice exhibited signs of hepatic encephalopathy such as lack of righting reflex prior to death (data not shown). Decreased hepatic function further manifested as elevated bilirubin levels in HNF4 α -KO mice after PH. The fact that HNF4 α -KO hepatocytes were not able to compensate for loss of HNF4 α function (as measured by target gene expression) further support the role of HNF4 α as a master regulator of hepatic differentiation. Consistent with our previous findings that HNF4 α is anti-proliferative in hepatocytes, the HNF4 α -KO mice showed a consistently higher cell proliferation, which never declined after PH. But the lack of HNF4 α resulted in failure to redifferentiate the newly divided cells resulting in significant decline in hepatic function leading to death. This was evident by the complete lack of hepatocyte differentiation genes at 7 days post PH in HNF4 α -KO mice as compared to the WT mice. This was consistent with the significant increase in nuclear HNF4 α expression observed in the control mice (Fig. 1) at days 3 and 5 after PH, the time points when the cell proliferation decreases and redifferentiation starts. Taken together, these data clearly demonstrate that HNF4 α -mediated differentiation of the newly divided hepatocytes is an essential part of the termination of regeneration and failure to redifferentiate can lead to cell and animal mortality.

Another important observation in our studies was the complete lack of MET and EGFR expression in HNF4 α -KO mice. It is known that signaling induced by primary hepatic mitogens HGF and EGF family members (EGF, TGF α etc.) via their cognate receptors c-MET and EGFR is essential for liver regeneration after PH (24). However, HNF4 α -KO mice seem to be an exception to this rule. We observed complete loss of c-MET and EGFR expression in HNF4 α -

KO mice, yet hepatocyte proliferation occurred in these mice at high levels before and throughout liver regeneration. To address the alternate mechanisms at play, we investigated several known pathways including Wnt/ β -catenin pathway and MAPK signaling. Whereas, significantly higher β -catenin signaling was observed in HNF4 α -KO mice as evident by decrease in phosphorylated (inactive) β -catenin, this was observed only during the early time points. Similarly, the AKT, p38 and ERK-1/2 pathways also revealed only moderate differences between WT and HNF4 α -KO mice after PH, which can not explain the sustained proliferation of hepatocytes in HNF4 α -KO mice. However, we observed a significant activation of c-myc in HNF4 α -KO mice consistent with the previous studies (14). Also, the role of myc in liver regeneration and cancer is well documented. Our studies show that HNF4 α deleted hepatocytes lose two of the primary mitogenic pathways, namely MET and EGFR, but proliferation continues mainly via myc overexpression.

The RNAseq analysis further revealed mechanisms that drive continued proliferation and also the mechanisms that lead to liver failure and death of the HNF4 α -KO mice after PH. We investigated days 2 post-PH, the time points with peak proliferation, and 5 days post-PH the time point when termination of regeneration begins. At both time points, the HNF4 α -KO livers exhibited a proinflammatory and profibrotic transcriptional profile. Elevated inflammation in HNF4 α -KO mice was not caused by hepatocellular injury, as there was no difference in ALT levels or histopathological changes between groups (data not shown). However, previous studies have reported elevated bile acid levels in HNF4 α -KO livers, which could cause increased inflammatory signaling (45). Furthermore, many HNF4 α negative target genes such as EGR1 are pro-inflammatory. IPA analysis also supported our findings of dedifferentiation and loss of

hepatocyte function in HNF4 α -KO mice. IPA predicted consistent inhibition of several prominent hepatocyte functions in HNF4 α -KO at day 2 and day 5 post-PH. However, we observed a shift in the characteristics of activated functions between the two time points. Most of the activated functions in HNF4 α -KO mice at day 2 included those involved in inflammation. However, the activated functions in HNF4 α -KO mice at day 5 included many more functions involved in proliferation, tumorigenesis and a developmentally immature phenotype in addition to inflammation. Furthermore, HNF4 α -KO mice at day 5 were predicted to express higher levels of known oncogenes (TRIM24, SNAI2, PLAG1) which were not activated at day 2. This transformation from an abnormal regenerative response to the beginnings of a pathological condition raise the question of how hepatocytes with poor expression of HNF4 α may respond to chronic or intermittent low level liver injury. The decreased expression of HNF4 α expressed in many liver diseases and HCC may be an example of the innate regenerative capability of the liver contributing to the progression of liver disease.

We observed that reexpression of HNF4 α in HNF4 α -KO mice using the AAV8 resulted in restoration of majority of the WT gene expression pattern. However, when HNF4 α construct was introduced after PH, we did not observe complete prevention of death. This may be due to several reasons including the timing of HNF4 α reexpression (two days after PH may be too early), the possibility that dividing cells may have decreased sensitivity to AAV8 and that the dividing cells may reject AAV8-mediated introduction of exogenous material. Nevertheless, we did observe a significant increase in survival following HNF4 α reintroduction in HNF4 α -KO mice.

In summary, our studies are the first to examine and manipulate HNF4 α expression over multiple time points throughout the initiation, progression and termination of liver regeneration after partial hepatectomy. Our findings suggest downregulation of HNF4 α may contribute to hepatocyte cell cycle entry during initiation of regeneration. More importantly, reestablishment of HNF4 α activity during termination of regeneration is absolutely required for termination of regeneration and resumption of hepatocyte function. This study uncovers new evidence of HNF4 α mediated expression of EGFR and c-MET and demonstrate that HNF4 α -KO mice are capable of mounting a regenerative response despite lacking these receptors. Furthermore, regeneration in the absence of HNF4 α results in a dedifferentiated, pro-carcinogenic hepatocyte phenotype. These results confirm the role of HNF4 α as a major player in hepatocyte proliferation and differentiation during liver regeneration.

Figure Legends

Figure 1. Decreased HNF4 α protein expression and transcriptional activity during initiation of regeneration after partial hepatectomy. Western blot analysis of HNF4 α adult isoform in (A) nuclear and (B) cytoplasmic lysates from mouse liver over a time course of 0 – 14 days after 2/3 PH. (C) Densitometric analysis of nuclear HNF4 α blot. (D) qPCR analysis of HNF4 α adult isoform mRNA over a time course of 0 – 14 days after 2/3 PH. Fold change calculated by comparison to 0 hr time point. qPCR analysis of positively regulated (E) and negatively regulated (F) HNF4 α target genes at 0, 6 and 12 hr post-PH. Fold change calculated by comparison to 0 hr time point. *indicate significant difference at $P \leq 0.05$.

Figure 2. Complete mortality of HNF4 α -KO mice following PH. (A) Western blot of HNF4 α confirming efficient KO of HNF4 α in pooled liver lysates at all time points post-PH. (B) Kaplan-Meier survival analysis of WT and HNF4 α -KO groups after PH. (C) Liver weight to body weight ratios and (D) serum bilirubin levels in WT and HNF4 α -KO mice after PH. *indicate significant difference at $P \leq 0.05$ between WT and HNF4 α -KO.

Figure 3: Sustained Loss of HNF4 α Transcriptional Activity In HNF4 α -KO Mice 7 Days Post-PH. qPCR analysis of mRNA isolated from frozen liver in WT and HNF4 α -KO mice 7 days post-PH. (A) Decreased expression of positive targets of HNF4 α and (B) increased expression of negative targets of HNF4 α . *indicate significant difference at $P \leq 0.05$ between WT and HNF4 α -KO.

Figure 4. Increased hepatocyte proliferation in HNF4 α -KO livers throughout regeneration. (A) Western blot analysis of Cyclin D1, CDK4, and PCNA over a time course of 0 to 7 days post-PH. (B) Quantification of immunohistochemical analysis for PCNA positive nuclei in liver sections of WT and HNF4 α -KO mice throughout time course post-PH. (C) Representative photomicrographs (400x) of PCNA-stained liver sections from WT and HNF4 α -KO mice throughout time course post-PH. *indicate significant difference at $P \leq 0.05$ between WT and HNF4 α -KO.

Figure 5. Altered proliferative signaling in HNF4 α -KO mice during regeneration. Western blot analysis of (A) total EGFR and c-Met, (B) total and phosphorylated β -catenin and total and phosphorylated GSK3 β , (C) line graphs showing densitometric signal of inactive to total β -catenin, (D) Western blots for Total AKT, Phospho-AKT, Total p38, Phospho-p38, ERK1/2, Phospho-ERK1/2, (E) qPCR analysis of c-Myc mRNA from WT and HNF4 α -KO livers over time course post-PH, (F) Western blot analysis of c-Myc, (G) and densitometric analysis of c-Myc. *indicate significant difference at $P \leq 0.05$ between WT and HNF4 α -KO.

Figure 6. Reexpression of HNF4 α restores hepatocyte quiescence and gene expression and extends survival of HNF4 α -KO animals post-PH. (A) Western blot analysis of HNF4 α , Cyclin D1, c-Met and EGFR in WT, HNF4 α -KO and HNF4 α -Reexp. mice, (B) Immunohistochemical analysis of PCNA positive nuclei in liver sections from WT, HNF4 α -KO and HNF4 α -Reexp. mice, (C) Representative photomicrographs (40x) of PCNA-stained liver sections, (D) Kaplan-

Meier survival analysis of WT and HNF4 α -Reexp mice after PH. *indicate significant difference at $P \leq 0.05$.

*Table 1: Predicted Activity of Transcription Factors in HNF4 α -KO Mice Compared to WT Mice
2 Days Post-PH.*

Day 2 Post-PH			
Activated Pathways	Activation z-score	Inhibited Pathways	Activation z-score
JUN	3.31	HNF4A	-6.498
EGR1	2.814	HNF1A	-4.428
RB1	2.497	Esrra	-3.357
IRF3	2.42	PPARGC1A	-3.228
FOXO4	2.377	NFIX	-2.433
EP300	2.305	ZBTB20	-2.294
SPI1	2.248	MED1	-2.272
THRAP3	2.219	CLOCK	-2.219
IRF6	2.215	NFIC	-2.2
SMAD4	2.201	GATA2	-2.059
MTA1	2.194	NKX2-3	-2.04
Gm21596/Hmgb1	2.169	E2F1	-2.027
IRF7	2.163	SMAD7	-2.027
SMAD2	2.068	TAF4	-2.008
TCF3	2.022		

*Table 2: Predicted Activity of Transcription Factors in HNF4 α -KO Mice Compared to WT Mice
5 Days Post-PH.*

Day 5 Post-PH

Activated Pathways	Activation z-score	Inhibited Pathways	Activation z-score
Gm21596/Hmgbl	2.767	HNF4A	-6.471
EP300	2.565	HNF1A	-4.641
TRIM24	2.554	SREBF2	-4.603
SMAD2	2.401	SREBF1	-3.768
CCND1	2.307	PPARGC1A	-3.045
SNAI2	2.1	Esrra	-2.887
ANKRD42	2	IRF7	-2.476
PLAG1	2	SIRT2	-2.449
		NFIX	-2.433
		PPARGC1B	-2.424
		BCL6	-2.229
		EBF1	-2.177
		ZEB1	-2.077
		NCOA2	-2.028

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Figure 1

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