

ROLE OF HEPATOCYTE NUCLEAR FACTOR 4 α IN HEPATOCYTE
PROLIFERATION

BY

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Chairperson: Udayan Apte, PhD, DABT

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Abstract

Hepatocyte Nuclear Factor 4 alpha (HNF4 α) is the master regulator of hepatocyte differentiation. It is involved in the up-regulation of genes involved in many classic hepatic functions including: bile acid metabolism, xenobiotic metabolism, glucose homeostasis, lipid metabolism, coagulation factor synthesis, etc. However, the role of HNF4 α in regulation of hepatocyte proliferation was not known. The primary goal of this dissertation was to investigate the role of HNF4 α in the regulation of hepatocyte proliferation. In these studies we utilized two novel inducible, hepatocyte specific, HNF4 α knockdown mouse models. Past models of HNF4 α deficiency result in deletion within the first few weeks of birth and lead to lethality within 6-8 weeks. This makes it difficult to address a role for HNF4 α in hepatocyte proliferation because the liver is still growing and differentiating within this time frame. Hepatocyte-specific deletion of HNF4 α in adult mice resulted in fat accumulation (steatosis), glycogen depletion, and increased hepatocyte proliferation with a significant increase in liver/body weight ratio without complimentary liver regeneration due to hepatocyte cell death. Global gene expression analysis (microarray and RNA-Seq) revealed that a significant number of the 300+ down-regulated genes are involved in hepatic differentiation, many of which are known HNF4 α targets. Interestingly, a significant number of the 500+ up-regulated genes are associated with cell proliferation and cancer. Further, a combined bioinformatics analysis of ChIP-sequencing and RNA-sequencing data indicated that a substantial number of up-regulated genes are putative HNF4 α targets. We have used chromatin immunoprecipitation (ChIP) to

confirm three of these targets: *Ect2*, *Osgin1*, and *Hjurp*. Ingenuity Pathway Analysis (IPA)-mediated functional analysis revealed the most significantly activated gene network after HNF4 α deletion is regulated by c-Myc.

To determine the role of HNF4 α in pathogenesis of hepatocellular carcinoma (HCC), we performed the classic initiation-promotion experiment using diethylnitrosamine (DEN). Deletion of HNF4 α resulted in extensive promotion of DEN-induced hepatic tumors, which were highly proliferative and less differentiated. Further, the HCC observed in HNF4 α -deleted mice exhibited significant up-regulation of c-Myc and its target genes.

We hypothesized that HNF4 α inhibits hepatocyte proliferation by repression of target genes. One possible mechanism is that HNF4 α influences the epigenetic state of a given gene to promote or inhibit gene expression. We studied various epigenetic modifications associated with gene activation and repression on a 9 kb segment of the promoter of *Ect2*, a validated HNF4 α negative target gene also known to activate hepatocyte proliferation. ChIP analysis performed on chromatin isolated from control and HNF4 α KO livers indicated that a deletion of HNF4 α resulted in an epigenetic switch in histone modifications at the *Ect2* promoter from an inhibited to an active state; primarily affecting acetylation of histone H3K9.

These data indicate that HNF4 α inhibits hepatocyte proliferation, is a potential tumor suppressor in the liver, and plays a critical role in chemical carcinogenesis. We also provide data that support the hypothesis that HNF4 α may be functioning through an influence on the epigenetic state of select genes.

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1. Introduction

1.1 Hepatocyte Nuclear Factors

The liver performs essential functions in the body by expressing hepatocyte-specific genes which encode for plasma proteins, clotting factors, enzymes involved in detoxification, gluconeogenesis, glycogen synthesis, and glucose, fat, and cholesterol metabolism (Jungermann and Katz 1989). Functional analysis of numerous hepatocyte-specific DNA regulatory regions have shown that they are composed of multiple cis-acting elements that bind different families of liver-enriched transcription factors termed hepatocyte nuclear factors (HNFs) (Costa, Kalinichenko et al. 2003). Early studies involved transient transfections of hepatoma cell lines utilizing DNA regulatory regions containing targeted mutations in transcription factor binding sites fused to a reporter gene. Expression levels of the reporter gene were then used to monitor promoter activity (Cereghini, Raymondjean et al. 1987; Courtois, Morgan et al. 1987; Courtois, Baumhueter et al. 1988; Monaci, Nicosia et al. 1988; Costa, Grayson et al. 1989; Costa and Grayson 1991; DiPersio, Jackson et al. 1991; Pani, Quian et al. 1992; Lu, Huang et al. 1999; Vorachek, Stepan et al. 2000). The results of these experiments suggested that hepatocyte-specific gene transcription requires binding of multiple HNFs in order to provide synergistic transcriptional activation. The requirement of multiple factors highlights an important mechanism in tissue-specific gene expression. Analysis of liver gene expression profiles in HNF-deficient mice revealed distinct phenotypes because of the role that each HNF plays in the transcription of distinct sets of hepatocyte-specific genes (Costa, Kalinichenko et al. 2003).

1.2 Hepatocyte Nuclear Factor 4 alpha

One such HNF is hepatocyte nuclear factor 4 alpha (*HNF4α*, *NR2A1*). HNF4α is a highly conserved member of the nuclear receptor superfamily. It was first discovered bound to sites required for the transcription of transthyretin (*TTR*) and apolipoprotein CIII (*APOCIII*) within rat liver extracts (Sladek, Zhong et al. 1990). HNF4α is expressed at high levels in the liver and kidney, and to a lesser degree (<50%) in the small intestine, colon, and pancreatic β cells (Drewes, Senkel et al. 1996; Jiang, Tanaka et al. 2003). In mouse liver development, HNF4α is expressed in the primary and extraembryonic visceral endoderm prior to gastrulation and in epithelial cells at the onset of liver, pancreas, and intestine formation (Duncan, Manova et al. 1994). HNF4α-null embryos exhibit severe visceral endoderm defects preventing gastrulation and causing failure to develop past 6.5 dpc (Chen, Manova et al. 1994).

The functional domains of HNF4α consist of an N-terminal activation domain (AF-1, also referred to as A/B domain), a zinc finger domain that serves as the DNA binding domain (C domain), a putative ligand binding domain (E domain), and a C-terminal domain that contains a region involved in homodimerization, activation function (AF-2), and a short repressor region (Ryffel 2001) (Fig. 1.2.1). The HNF4α gene consists of 13 exons spanning over 70 kb. It has multiple alternatively spliced variants. Multiple isoforms are proposed to exist in mammals and are thought to have different physiological roles in development and transcriptional regulation of target genes

Figure 1.2.1



Schematic of HNF4α protein structure. The HNF4α protein structure consists of an activation domain (A/B), zinc finger DNA binding domain (C), a hinge domain (D), a putative ligand binding domain (E), and a second activation domain (F) that is thought to be responsible for homodimerization and contain a short repressor domain.

(Jiang, Tanaka et al. 2003). Further, HNF4 α has two, differentially utilized promoters. During early liver development HNF4 α initiates from the P2 promoter, but as the liver differentiates, transcription of the HNF4 α gene begins to favor the P1 promoter. P2 isoforms appear to activate genes involved in early liver development, such as α -fetoprotein and transthyretin, while P1 isoforms appear to activate genes involved in later hepatic differentiation, such as *apoCIII* (Drewes, Senkel et al. 1996; Torres-Padilla, Fougere-Deschatrette et al. 2001).

Recent studies have highlighted the importance of the DNA-binding domain and the ligand-binding domain for proper binding of HNF4 α to its response elements. A lack of the ligand-binding domain can reduce the affinity of HNF4 α for its response elements by 75-fold (Chandra, Huang et al. 2013). HNF4 α 's ligand-binding domain interacts with endogenous fatty acids, such as linoleic acid. The presence of the fatty acid is thought to lend structural integrity to the protein, but the presence of linoleic acid has not been shown to confer significant transcriptional activation (Yuan, Ta et al. 2009). It is likely that the fatty acid is needed for stabilization of the protein instead of switching it off and on.

HNF4 α functions by binding as a homodimer to its DNA recognition site, a direct repeat element (AGGTCA) with either a one or two nucleotide spacer (AGGTCA n AGGTCA or AGGTCA nn AGGTCA; designated DR1 or DR2, respectively). After binding to DNA, HNF4 α has traditionally been thought to recruit transcriptional co-activators and accessory proteins to positively regulate the expression of its target genes. It is linked to important hepatic functions such as glycolysis, gluconeogenesis, ureagenesis, fatty acid metabolism, bile acid

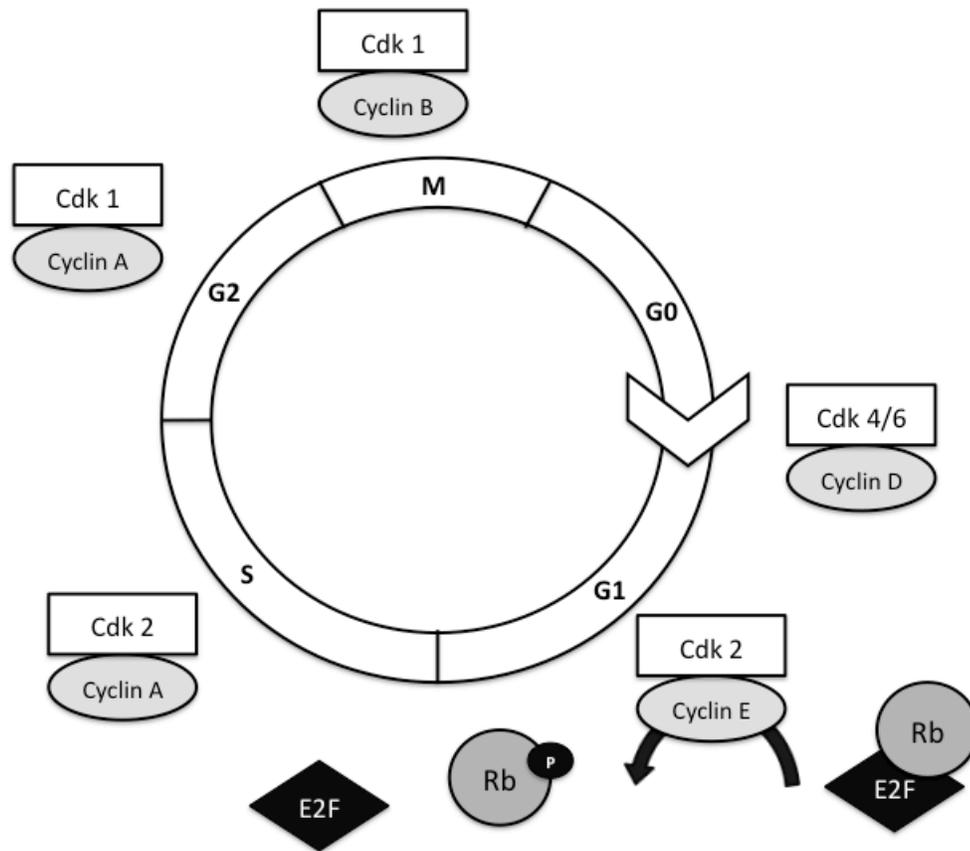
synthesis, drug metabolism, apolipoprotein synthesis, and blood coagulation by regulating the transcription of many of the genes involved in each of these functions (Hayhurst, Lee et al. 2001; Inoue, Yu et al. 2004; Inoue, Peters et al. 2006; Inoue, Yu et al. 2006; Gonzalez 2008). Because of its involvement in a wide array of hepatic functions, HNF4 α is known throughout the literature as the master regulator of hepatic differentiation.

Postnatal hepatocyte-specific deletion of HNF4 α using an HNF4 α ^{F1/F1} allele utilizing an enhancer driven Cre recombinase under the albumin promoter (Alb-Cre) showed that a loss of HNF4 α results in accumulation of lipid, reduced serum cholesterol and triglyceride levels, and increased serum bile acids (Hayhurst, Lee et al. 2001). Further, HNF4 α -null livers exhibited a decrease in classic hepatocyte gene expression such as apolipoprotein B, microsomal triglyceride transfer protein, liver fatty acid binding protein, and bile acid transport proteins sodium taurocholate cotransporting polypeptide (*NTCP*) and organic anion transporting polypeptide 1A1 (*OATP1A1*) (Hayhurst, Lee et al. 2001). The absence of HNF4 α in this model results in metabolic disruption and increased mortality (Hayhurst, Lee et al. 2001; Inoue, Yu et al. 2004; Inoue, Peters et al. 2006; Inoue, Yu et al. 2006).

1.3 Cell Cycle Regulation

Cell proliferation is a tightly regulated process with multiple transition checkpoints including G1/S (DNA replication) and G2/M (mitosis) (Fig. 1.3.1). Temporal activation of multiple cyclin-dependent kinases (Cdk), in complex with

Figure 1.3.1



Schematic of the cell cycle. Quiescent cells (G0) transition into the cell cycle following activation of cyclin-dependent kinase (cdk) 4/6 by cyclin D. Cdk2 in complex with cyclin E or cyclin A cooperates with active cdk4/6 to phosphorylate retinoblastoma (Rb) resulting in a release in its inhibition of the transcription factor E2F allowing for it to activate transcription for genes involved in the S phase. The cell cycle progresses through G2 following the activation of cdk1 by cyclin A. Final activation of cdk1 by cyclin B results in M-phase progression.

their corresponding cyclin regulatory subunits, control transitioning from one phase of the cell cycle to the next. Stimulation of Cdk2-cyclin E/A or Cdk1-cyclin B is essential for progression throughout the cell cycle G1/S and G2/M transitions, respectively. Transitioning from a quiescent state (G0) into the first phase of the cell cycle (G1) requires activation of Cdk4/6 by cyclin D. It is well established that Cdk2 in complex with either cyclin E or cyclin A cooperates with cyclin D-Cdk4/6 to phosphorylate the retinoblastoma protein, which releases bound E2F transcription factor and allows it to stimulate the expression of genes required for S-phase (Harbour and Dean 2000; Ishida, Huang et al. 2001). The Cdk1-cyclin B complex provides signaling for M-phase progression through phosphorylation of protein substrates essential for chromosome segregation, breakdown of the nuclear envelope, and cytokinesis (Ookata, Hisanaga et al. 1993; Blangy, Lane et al. 1995; Kimura, Hirano et al. 1998; Ohi and Gould 1999; Sutani, Yuasa et al. 1999; Nigg 2001).

1.4 Regulation of Hepatocyte Proliferation

The mammalian liver has a huge capacity for regeneration (Michalopoulos 2007). The liver evolved the capacity to regenerate because it is exposed to high levels of endogenous and exogenous compounds that result in liver injury and death of tissue. Liver is capable of regenerating to its original size in response to cellular injury due to toxins, viral hepatitis, or surgical resection in order to maintain a given liver-to-body weight ratio (Fausto, Laird et al. 1995; Taub 1996;

Michalopoulos 2007). A major model used for studying hepatocyte proliferation/liver regeneration is the two-thirds partial hepatectomy (PHx). PHx results in the synchronized induction of (S-phase) and mitosis throughout the remaining liver. The proliferative response is initiated by complement activation (C3 and C5), which results in the downstream release of cytokines such as tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6) (Strey, Markiewski et al. 2003). This helps to facilitate signaling to stimulate terminally differentiated hepatocytes to transition into the cell cycle and is followed by the release of growth factors such as tumor growth factor α (TGF- α), hepatocyte growth factor (HGF), epidermal growth factor (EGF) all of which are thought to aid in the progression of hepatocytes through the cell cycle. Hepatocyte proliferation in response to liver regeneration coincides with a potent activation of transcription factors such as c-Jun, c-Fos, c-Myc, NF- κ β , Stat-3, Egr-1, and C/EBP β (Peng, Du et al. 1999; Taub, Greenbaum et al. 1999; Diehl 2000; Michalopoulos 2007; Fausto, Campbell et al. 2012).

1.5 HNF4 α and Hepatocyte Proliferation

Early evidence in other organ systems. Early evidence suggesting that HNF4 α is involved in cell proliferation came from the observation that cancers of multiple organs expressing HNF4 α show a decrease in HNF4 α expression. Analysis of human renal cell carcinoma showed a down-regulation of HNF4 α mRNA and protein expression along with suppression in HNF4 α DNA binding activity (Sel, Ebert et al. 1996; Lenburg, Liou et al. 2003). As a follow up to these studies,

Lucas, et al. showed that expression of HNF4 α in HEK293 cells caused an inhibition in cell proliferation and characterized gene changes that correlate with the changes seen in renal cell carcinomas (Lucas, Grigo et al. 2005). Grigo, et al. narrowed the list of genes thought to be responsible for HNF4 α 's effect on renal cell carcinoma to 14 genes including: *CDKN1A* (p21), *TGFA*, *MME* (*NEP*), *ADAMTS1*, *SEPP1*, *THEM2*, *BPHL*, *DSC2*, *ANK3*, *ALDH6A1*, *EPHX2*, *NELL2*, *EFHD1*, and *PROS1* (Grigo, Wirsing et al. 2008).

Chiba, et al. developed an F9 murine embryonal cell line with an inducible form of HNF4 α . In this model they provide evidence that expression of HNF4 α inhibits cell proliferation. They showed that F9 cells, which express HNF4 α , become arrested in the G0/G1 phase of the cell cycle due to an up-regulation of *CDKN1A* (p21) in a p53-independent manner. They further confirmed their findings in rat lung endothelial cells (Chiba, Itoh et al. 2005).

Erdmann, et al. investigated whether the presence of HNF4 α could inhibit proliferation of pancreatic β -cells by over-expressing HNF4 α in rat INS-1 cells, a rat insulinoma cell line. They found that over-expression of the HNF4 α isoform 2 led to pronounced morphological changes and a decrease in cell proliferation (Erdmann, Senkel et al. 2007).

Early evidence in the liver. It is well established that HNF4 α is essential for normal hepatic development and maintenance of a differentiated phenotype. Mice, which lack HNF4 α , fail to develop functional hepatic tissue marked by a lack of hepatic gene expression (Duncan, Nagy et al. 1997; Li, Ning et al. 2000).

A study performed by Mizuguchi, et al. (Mizuguchi, Mitaka et al. 1998), suggested that the maintenance of a differentiated phenotype might inhibit hepatocyte proliferation in an *in vitro* system. Primary rat hepatocytes treated with 2% DMSO maintained a more normal expression of hepatic markers including higher HNF4 α expression and exhibited decreased DNA synthesis (BrdU incorporation assay). Späth and Weiss expressed HNF4 α in the rat hepatoma cell line, H5, and showed that following HNF4 α expression the cells took on a more hepatocyte-like morphology along with a reactivation of hepatocyte-specific genes (Spath and Weiss 1998). Also, they observed a decrease in cell proliferation; however, many of their observations were made only in the presence of dexamethasone.

Later studies by Hayhurst, et al. using an HNF4 α hepatocyte-specific KO utilizing the Cre-Lox system under the albumin promoter (AlbCre) shed some light on post-natal liver development and a possible role in the inhibition of hepatocyte proliferation. HNF4 α KO in livers showed a significant increase in liver/body weight ratio (4.0 ± 0.3 to 7.3 ± 0.9) (Hayhurst, Lee et al. 2001). This observed increase was not further studied in this report, but it is some of the first evidence that HNF4 α may inhibit hepatocyte proliferation and maintain a normal liver/body weight ratio.

Lazarevich, et al. first described a link between HNF4 α 's known functions in promoting differentiation and how this may be inhibited in times of increased proliferation (Lazarevich, Cheremnova et al. 2004). It is well accepted that there is a link between a cell's state of differentiation and its propensity for proliferation.

Generally, a cell that is well differentiated does not have a propensity to proliferate (Holland 2000). In the Lazarevich study they show that a slow-growing HCC can progress to a fast-growing, de-differentiated HCC; this correlates with a repression of HNF4 α . They found that a forced expression of HNF4 α in the fast-growing HCC can cause re-differentiation of the tumor cells towards a more hepatocyte-like phenotype. The observed change occurs rapidly suggesting that the progression of the tumor was due to a limited number of gene changes. Further, they observe that the forced expression of HNF4 α suppressed the proliferation of the fast-growing HCC cells suggesting that HNF4 α may have a tumor suppressing effect. The authors conclude that a loss of HNF4 α may be a critical event in HCC progression and mediates a de-differentiation, loss of cell adhesion, and an increase in cell proliferation and invasiveness.

Lazarevich, et al. conducted a more recent study to investigate HNF4 α as a marker for epithelial tumor progression (Lazarevich, Shavochkina et al. 2010). In these studies, tumor progression to a dedifferentiated phenotype correlated with a reduction in HNF4 α in mice. They also observed that a decrease in HNF4 α gene expression correlated with an “unfavorable” prognosis of HCC in humans and that a repression of HNF4 α in human pancreatic adenocarcinomas correlated with more dedifferentiated tumors.

1.6 Purpose and Aims

Currently a lot is known about the mechanisms by which hepatocyte proliferation is initiated in terms of liver regeneration, but not much is known about the

mechanisms by which hepatocyte proliferation is terminated and inhibited. We believe that elucidating these mechanisms will help us understand if they are involved in the pathogenesis of cancers within the liver, such as HCC. Recent studies have suggested a potential role for HNF4 α in the inhibition of hepatocyte proliferation. The objective of this dissertation was to determine a role for HNF4 α in normal hepatocyte proliferation and begin to investigate the mechanisms by which HNF4 α is regulating it.

Specific Aim #1: Determine the role of HNF4 α in Hepatocyte Proliferation

- Develop novel mouse models to determine the effect of HNF4 α deletion on hepatocyte proliferation in the adult mouse liver
- Determine global gene expression changes following HNF4 α deletion.
- Determine if an over-expression of HNF4 α in mouse hepatoma cells will result in inhibition of the cell cycle.

We hypothesized that a deletion of HNF4 α will result in increased hepatocyte proliferation. Because HNF4 α is a transcription factor, we also hypothesized that HNF4 α is functioning to inhibit genes involved in mitogenesis. Further, we predicted that an over-expression of HNF4 α in mouse hepatoma cells will result in an inhibition of the cell cycle due to inhibition of pro-mitogenic genes.

Specific Aim #2: Determine a role for HNF4 α in the pathogenesis of HCC

- Determine the effect of HNF4 α deletion on diethylnitrosamine (DEN)-induced hepatocellular carcinoma
- Determine the mechanism by which HNF4 α is functioning as a tumor suppressor.

We hypothesized that a deletion of HNF4 α in combination with the known hepatic carcinogen, diethylnitrosamine (DEN), will result in increased tumorigenesis. Also, we hypothesized that HNF4 α is functioning as a tumor suppressor by inhibiting the expression of pro-mitogenic genes in normal liver tissue versus transformed liver tissue.

Specific Aim #3: Determine the mechanism by which HNF4 α inhibits pro-mitogenic gene expression

- Determine to what extent HNF4 α inhibits select pro-mitogenic genes (*Ect2*) through epigenetic modifications, such as histone modifications.
- Characterize a switch in the epigenetic signature of select genes (*Ect2*) following a deletion of HNF4 α in the mature mouse liver from a repressed to an active state.

We hypothesized that HNF4 α represses transcription of select pro-mitogenic genes through influencing the epigenetic state of the gene's promoter region.

Further, we hypothesized that a deletion of HNF4 α will result in a switch of the epigenetic signature at the select promoters from a repressed to an active state of transcription.

Chapter 2

Liver-specific Deletion of Hepatocyte Nuclear Factor-4 α in Adult Mice Results in Increased Hepatocyte Proliferation

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2.1 Abstract

HNF4 α is known as the master regulator of hepatocyte differentiation. Recent studies indicate that HNF4 α may inhibit hepatocyte proliferation via yet to be identified mechanisms. We investigated the role of HNF4 α in the regulation of hepatocyte proliferation using two novel HNF4 α knockdown mouse model based on inducible Cre recombinase. Our first model utilized delivery of the Cre recombinase using AAV8 virus, while the second model utilized a tamoxifen-inducible Cre recombinase. Hepatocyte-specific deletion of HNF4 α resulted in increased hepatocyte proliferation. Global gene expression analysis showed that a majority of the down-regulated genes were previously known HNF4 α target genes involved in hepatic differentiation. Interestingly, many of the 500+ up-regulated genes were associated with cell proliferation and cancer. Further, we identified potential negative target genes of HNF4 α , many of which are involved in the stimulation of cell proliferation. We confirmed binding of HNF4 α at three of these genes using ChIP analysis. Furthermore, *in vitro* over-expression of HNF4 α in mouse HCC cells resulted in a decrease in pro-mitogenic gene expression and cell cycle arrest. Taken together, these data indicate that, apart from its role in hepatocyte differentiation, HNF4 α actively inhibits hepatocyte proliferation by repression of specific pro-mitogenic genes.

2.2 Introduction

Because of the importance of HNF4 α in liver development, a whole body HNF4 α KO results in embryonic lethality. Therefore, the Cre-Lox system under the albumin promoter was utilized to create a liver-specific inducible KO of HNF4 α (Hayhurst, Lee et al. 2001). This model provided some of the first evidence that a lack of HNF4 α results in increased hepatocyte proliferation supported by an increase in liver/body weight ratio present in these mice.

One problem with using this model to study the role of HNF4 α in hepatocyte proliferation is that the mice display severe hepatic metabolic disruption and death by 6-8 weeks of age. Another problem with using this model to study hepatocyte proliferation is that the deletion of HNF4 α happens early in life, when the liver is still growing and differentiating. Many gene changes are occurring throughout this time period, which would make the elucidation of the mechanism extremely difficult.

To overcome the lack of suitable model to study the role of HNF4 α in hepatocyte proliferation, we developed two novel models of HNF4 α deletion using the Cre recombinase system. Our first model utilizes adeno-associated virus 8 (AAV8) in order to deliver the Cre recombinase to HNF4 α -floxed mice, which resulted in HNF4 α deletion within one week. In our second model, we crossed the HNF4 α ^{F1/F1} mice with mice containing Cre recombinase governed by a tamoxifen (TAM)-inducible albumin promoter (AlbERT2Cre). Liver-specific HNF4 α deletion was achieved in these mice by activation of Cre using TAM. Our aim was to utilize these models to acutely delete HNF4 α after the liver has fully

matured in order to determine a role for HNF4 α in controlling hepatocyte proliferation and further use them to elucidate the mechanisms by which HNF4 α inhibits hepatocyte proliferation. This model will allow us to more accurately detect any changes in hepatocyte proliferation that would be due to a direct loss of HNF4 α versus hepatocyte proliferation associated with normal growth and maturation of the liver that occurs within the first 3 months of life.

As mentioned previously, HNF4 α is a transcription factor. Therefore, the mechanism by which HNF4 α inhibits hepatocyte proliferation may lie in its influence on gene expression within the liver. HNF4 α has been shown to inhibit pro-mitogenic gene expression in multiple models (Chiba, Itoh et al. 2005; Lucas, Grigo et al. 2005; Grigo, Wirsing et al. 2008). We believe that utilizing this model will allow us to more accurately investigate the mechanism by which HNF4 α may be inhibiting hepatocyte proliferation by its influence on pro-mitogenic gene expression. The fully mature liver will have less gene expression activity associated with growth and proliferation that would be observed in a liver that is still growing and maturing.

Following generation of the two HNF4 α KO models, we observed a significant decrease of HNF4 α protein level within one week after introduction/activation of the Cre recombinase. The knockdown of HNF4 α protein correlated with a significant increase in liver/body weight ratio due to an increase in hepatocyte proliferation. We also observed an increase in pro-mitogenic gene expression in the HNF4 α KO mice. We performed ChIP analysis on select genes

in order to show that HNF4 α not only activates genes involved in hepatocyte differentiation but to also inhibit genes involved with hepatocyte proliferation.

In order to corroborate these results, we performed an *in vitro* over expression of HNF4 α in mouse hepatoma cells. We observed a decrease in proliferation that is associated with a decrease in pro-mitogenic gene expression in the mouse hepatoma cells. HNF4 α over expression resulted in cell cycle arrest within 24 hrs. Therefore, we were able to conclude that HNF4 α has a dual role in the liver: promoting differentiation and inhibiting proliferation.

2.3 Materials and Methods

2.3.1

Animals, Treatments, and Tissue Collection. The HNF4 α ^{F1/F1} mice used in these studies were previously described (Hayhurst, Lee et al. 2001). All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities at the University of Kansas Medical Center under a standard 12-h light/dark cycle with access to chow and water ad libitum. All studies were approved by the Institutional Animal Care and Use Committee of KUMC.

Mice were euthanized by cervical dislocation under isoflurane anesthesia, and livers were collected. Pieces of liver were fixed in 10% neutral buffered formalin for 48 h and further processed to obtain paraffin blocks, and 4- μ m-thick sections were obtained. A piece of liver was frozen in optimum cutting temperature (OCT) medium and used to obtain fresh frozen sections. A part of the liver tissue was used to prepare fresh nuclear and cytoplasmic protein extracts using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA). The remaining liver tissue was frozen in liquid N₂ and stored at -80°C until used to prepare RIPA extracts.

2.3.2

Generation of the AAV8-dependent HNF4 α KO Model. Three-month-old HNF4 α ^{F1/F1} mice (provided by Dr. Frank Gonzalez of NCI-NIH) were treated with an adeno-associated virus 8 (AAV8) containing the gene for Cre recombinase under the major urinary protein promoter (MUP-iCre-AAV8), or control AAV8

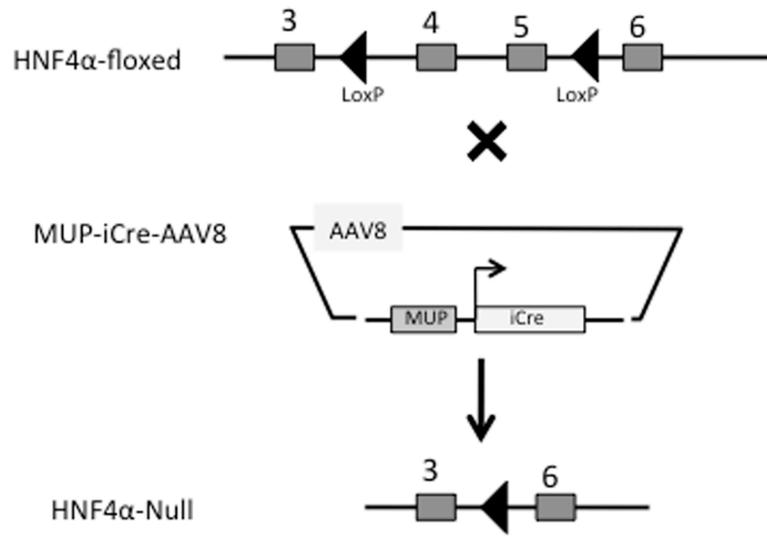
containing the gene for green fluorescence protein (MUP-EGFP-AAV8) (Fig. 2.3.2.1). The mice were injected with a single injection of MUP-iCre-AAV8 or MUP-EGFP-AAV8 at the dose of 400 μ L virus/mouse (n=7), intraperitoneally in three separate experiments. Only male mice were used because the MUP promoter has an ~5-fold higher expression in male mice as compared to female mice (Hastie, Held et al. 1979). Mice were sacrificed and tissue was harvested 7 days after infection with AAV8.

2.3.3

Generation of the AlbERT2Cre HNF4 α KO Model. The HNF4 α ^{F1/F1} mice and the TAM-inducible Albumin cre mice (AlbERT2Cre⁺, provided by Dr. Pierre Chambon, IGBMC-France) used in these studies have been previously described (Hayhurst, Lee et al. 2001). The HNF4 α ^{F1/F1}, AlbERT2Cre⁺ mice were produced by standard animal breeding and identified using PCR-based genotyping of DNA isolated from tail biopsies (Fig. 2.3.3.1).

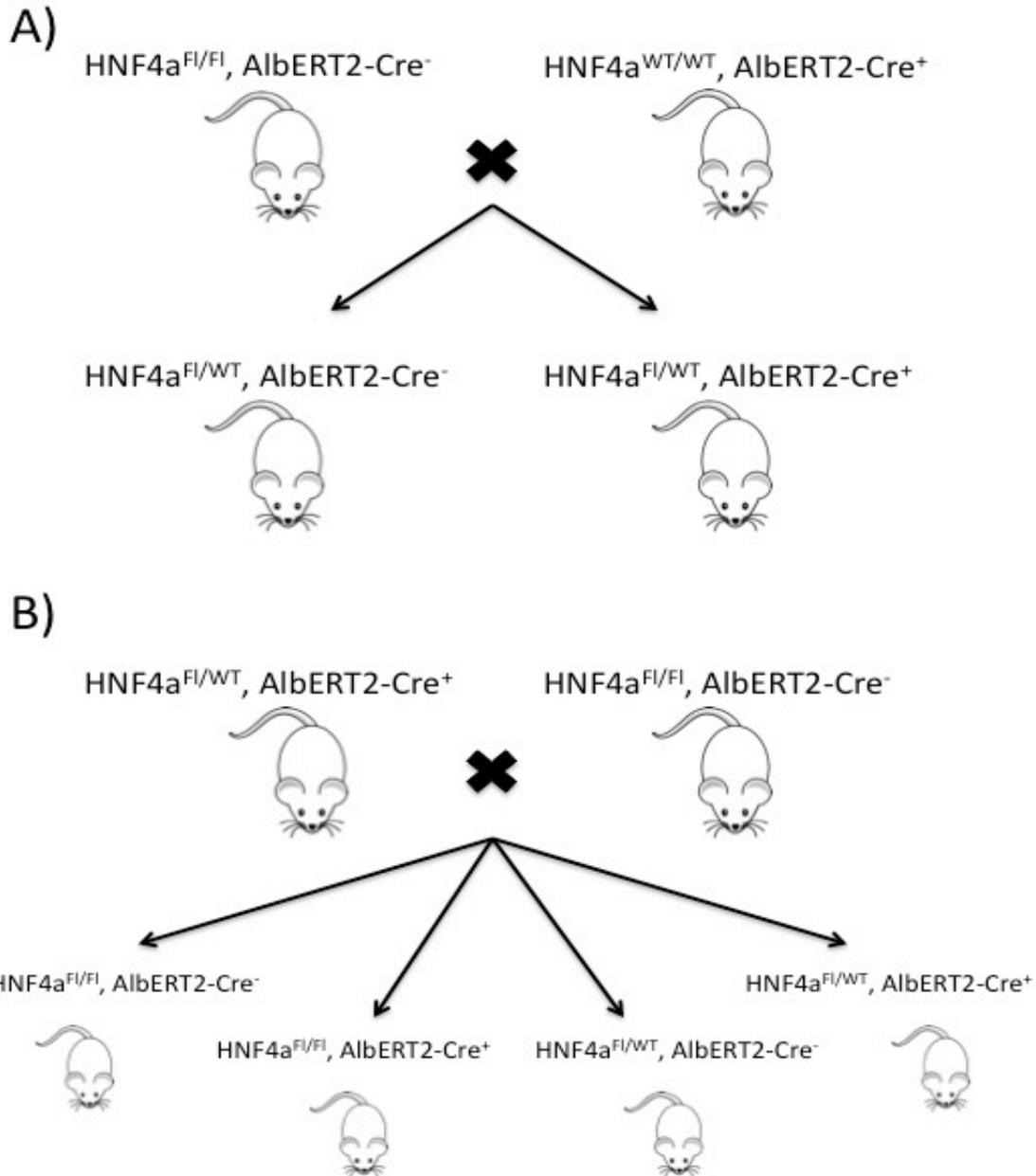
Three-month-old male, HNF4 α ^{F1/F1}, AlbERT2Cre⁺ mice were treated with TAM (6 μ g/mouse, IP, referred to as HNF4 α -KO), or with vehicle alone (corn oil, IP, referred to as Control), subcutaneously. To account for changes induced by TAM, three-month-old male, HNF4 α ^{F1/F1}, AlbERT2Cre⁻ mice were treated with TAM (6 μ g/mouse, IP, referred as TAM Control). Mice were euthanized and tissue was harvested 7 days post-injection (Fig. 2.3.3.2).

Figure 2.3.2.1



Novel model of HNF4 α deletion in adult liver. Scheme of HNF4 α deletion in the HNF4 $\alpha^{fl/fl}$ mice using major urinary protein promoter driven Cre recombinase delivered by adeno-associated virus 8

Figure 2.3.3.1

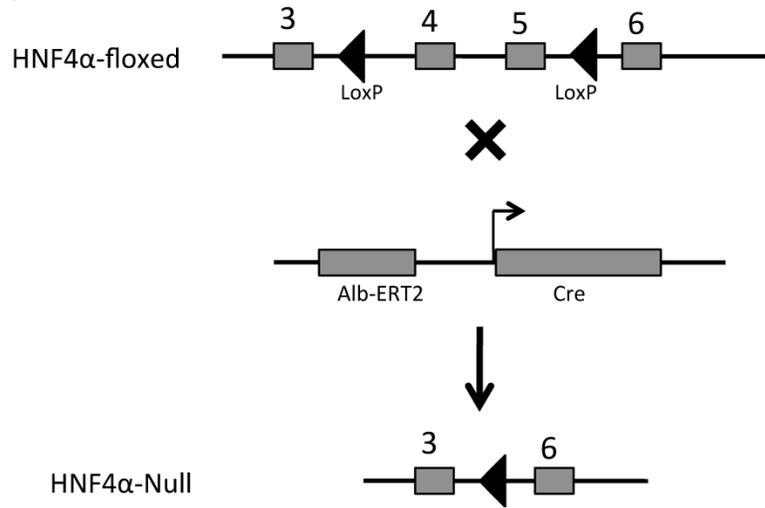


Generation of AlbERT2Cre HNF4 α KO Mice. (A) First breeding pair used to generate $\text{HNF4}\alpha^{\text{FI/WT}}, \text{AlbERT2-Cre}^+$ and $\text{HNF4}\alpha^{\text{FI/WT}}, \text{AlbERT2-Cre}^-$ mice. (B) Second breeding pair used to generate $\text{HNF4}\alpha^{\text{FI/FI}}, \text{AlbERT2-Cre}^-$ and $\text{HNF4}\alpha^{\text{FI/FI}}, \text{AlbERT2-Cre}^+$ mice. The $\text{HNF4}\alpha^{\text{FI/FI}}, \text{AlbERT2-Cre}^+$ mice are used for KO of HNF4 α and for the vehicle control while the $\text{HNF4}\alpha^{\text{FI/FI}}, \text{AlbERT2-Cre}^-$ mice were

used for the TAM-control.

Figure 2.3.3.2

(A)



Deletion of HNF4 α using TAM (TAM) driven albumin cre mice. (A) Scheme of HNF4 α deletion using the HNF4 α ^{F1/F1}, AlbERT2-Cre⁺ mice.

2.3.4

Western Blotting. RIPA extracts obtained from whole liver tissues were used for Western blot analysis. Total protein was isolated using 1% sodium dodecyl sulfate (SDS) in RIPA buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1% Triton X-100, 0.25% sodium deoxycholate, 0.6-2 µg /mL aprotinin, 10 µM pepstatin). Protein concentrations were determined using the bicinchoninic acid protein assay reagents (BCA method) (Pierce Chemical; Rockford, IL.).

Total cell lysates (50 µg) were separated by SDS-polyacrylamide gel electrophoresis in 4% to 12% NuPage Bis-Tris gradient gels with MOPS buffer (Invitrogen; Carlsbad, CA), then transferred to Immobilon-P membranes (Millipore; Bedford, MA) in NuPage transfer buffer containing 20% methanol. Membranes were stained using Ponceau S to analyze loading and transfer efficiency.

Membranes were probed using antibodies for HNF4α (1:1000; R&D Systems; Minneapolis, MN), and β-Actin (1:1000; Cell Signaling; Danvers, MA). Briefly, membranes were blocked using Blotto (2.5 M NaCl, 1 M Tris pH 7.6, 0.1% Tween-20) containing 5% nonfat milk. Membranes were then probed using primary and secondary antibodies in Blotto containing 1% nonfat milk, and developed using SuperSignal West Pico chemiluminescence substrate (Thermo Fisher Scientific; Rockford, IL) and exposed to x-ray film (MidSci; St. Louis, MO).

2.3.5

Immunohistochemistry. Paraffin-embedded liver sections (4 µm thick) were used for immunohistochemical staining. Slides were stained with hematoxylin and

eosin (H&E) using a Leica CV 5030 autostainer (Leica Microsystems; Buffalo Grove, IL). Paraffin-embedded sections were also stained with periodic acid-Schiff (PAS) stain to detect glycogen accumulation. Briefly, after deparaffinization sections were oxidized in 0.5% periodic acid solution (0.5 g in ddH₂O) for 5 min and washed in ddH₂O for 5 min. Sections were then placed in Schiff reagent for 15 min, washed in tap water for 5 min, and counterstained in hematoxylin for 1 min. This was followed by washing with tap water for 5 min, dehydration, and mounting.

Cell proliferation was detected by immunostaining the paraffin-sections for proliferating cell nuclear antigen (PCNA) as previously described (Yamagata, Furuta et al. 1996; Xu, Hui et al. 2001). Fresh frozen sections (5 µm thick) were used to detect Ki-67 immunofluorescence, as described previously (Borude, Edwards et al. 2012; Walesky, Gunewardena et al. 2012). Ki-67 is a marker for actively proliferating cells, much like PCNA.

Fresh frozen sections (5 µm thick) were used to detect lipid accumulation by staining with Oil Red O. Briefly, Oil Red O stock solution was made by combining 2 g of Oil Red O (Sigma-Aldrich; St. Louis, MO) with 400 mL isopropanol. Oil Red O working solution was made fresh the day of staining by combining 240 mL of working solution with 160 mL of ddH₂O. The working solution was then filtered using a TPP bottle top 0.2 µm filter (MidSci; St. Louis, MO). Fresh frozen sections were air-dried at room temp for 2 min and fixed with 10% formalin for 5 min. Sections were washed with running tap water for 10 min followed by a rinse in 60% isopropanol to get rid of excess water. Freshly

prepared Oil Red O working solution was then used to stain the sections for 20 min followed by a rinse in 60% isopropanol to get rid of excess Oil Red O. Nuclei were stained with hematoxylin for 2 min followed by rinsing with tap water.

Apoptosis was measured using the *In Situ* Cell Death Detection Kit, TMR red (Roche Applied Science; Indianapolis, IN; Cat # 12156792910) according to the manufacturer's protocol.

2.3.6

Microarray Analysis. Gene expression analysis for determining the global changes in gene expression following HNF4 α KO in mature mouse livers (AAV8 model) was carried out using the Affymetrix Mouse430_2.0 gene chip. Livers were pooled (100 mg per mouse) from 3 individual mice each for HNF4 α ^{F1/F1} mice treated with MUP-iCre-AAV8 vector and HNF4 α ^{F1/F1} mice treated with MUP-EGFP-AAV8 control vector for RNA isolation using the TRIzol® Reagent with Phase Lock Gel Heavy protocol. The microarrays were background corrected, normalized and gene-level summarized using the Robust Multichip Average (RMA) procedure (Irizarry, Bolstad et al. 2003). The resulting log (base 2) transformed signal intensities were used for ascertaining differentially expressed genes. Fold changes were calculated by transforming to the linear scale the difference in log intensity values between control and HNF4 α KO. All computations were performed in Matlab (R2009b, The MathWorks Inc, Natick, MA) and the Partek Genomic suite (v 6.5, Partek Inc., St. Louis, MO).

2.3.7

RNA-Sequencing. Total RNA was isolated from liver tissue using the phenol/chloroform extraction protocol. Integrity of RNA was analyzed by the Microarray Core Facility at KUMC (Kansas City, KS) using an Agilent Bioanalyzer 2100 (Agilent Technologies; Santa Clara, CA).

We performed two separate and independent RNA-Seq experiments for the same treatment conditions, Cre+ \backslash Tamoxifen, Cre- \backslash Tamoxifen and Cre+ \backslash Corn Oil. In the first instance (Run1), the total processed RNA extracted from pooled mouse liver samples (3 mice per group) treated with Cre+ \backslash Tamoxifen, Cre- \backslash Tamoxifen and Cre+ \backslash Corn Oil was sequenced in an Illumina HiSeq 2000 sequencing machine (Illumina, San Diego, CA). The initial library of 10nM concentration for each of the three samples was split into two diluted concentrations of 5pM and 3pM and sequenced separately at a 2 \times 100 bp paired-end resolution and the output of the sequencing runs combined for downstream analysis. In order to complement the initial RNA-Seq analysis, we ran a second RNA-seq experiment (Run2) on biological replicate samples (n=2) of mouse liver treated with Cre+ \backslash Tamoxifen, Cre- \backslash Tamoxifen and Cre+ \backslash Corn Oil. These samples were sequenced at a 50bp single-end resolution. The RNA-Seq data obtained from both experiments was used for the bioinformatics analysis. Reads from both experiments were mapped to the mouse reference genome (NCBI37/mm9) using TopHat v1.4.1 (Trapnell et al.). TopHat was run with default parameters and for Run1 with paired reads, a mate inner distance of -40 was set to accommodate the 260bp average fragment length. The binary output files generated by TopHat were passed to CuffDiff (Trapnell, Williams et al. 2010) for

calculating differential gene expression. For samples from both runs, differential gene expression was first calculated for Cre+\Tamoxifen against Cre+\Corn Oil and Cre+\Tamoxifen against Cre-\ Tamoxifen. For each run, genes that were significantly differentially expressed in both these measures based on an absolute fold change of at least 1.5 and a q-value (the p-value adjusted for multiple hypothesis correction using the Benjamin-Hochberg procedure) less than or equal to 0.05 were selected. This data filtering resulted in 1096 genes from Run1 and 3436 genes from Run2. Of these, 877 genes were common to both runs (right tailed Fisher's exact test significance p-value <1E-100). The RNA-seq data have been submitted to the Sequence Read Archive (SRA) of NCBI.

2.3.8

Chromatin Immunoprecipitation-Sequencing (ChIP-Seq) Data Analysis.

Previously published ChIP-Seq data were downloaded from the NCBI Short Read Archive (HNF4 α : SRR014519.fastq,SRR014520.fastq; INPUT: SRR043782.fastq,SRR043783.fastq,SRR043784.fastq) (Hoffman, Robertson et al. 2010). Raw sequencing reads were aligned to the mouse genome (mm9) using bowtie-0.12.3 (Langmead, Trapnell et al. 2009). Determination of binding sites was performed using MACS with default settings (Zhang, Liu et al. 2008). Binding sites were annotated by PeakAnalyzer (Salmon-Divon, Dvinge et al. 2010) using the Nearest TSS option, filtered to contain only the binding sites within 10 kb of a transcriptional start site, and joined with expression array data using custom scripts.

2.3.9

RNA-Seq/ChIP-Seq Comparative Analysis. We analyzed publically available ChIP-Seq data (SRA008281) from Hoffmen et al. (Hoffman, Robertson et al. 2010) to obtain an unbiased whole genome mapping of Hnf4 α binding sites in mouse. Sequences were aligned using Bowtie2 (ver 2.0.2) to the latest mouse reference genome (GRCm38/mm10) using default parameters (Langmead and Salzberg 2012). Peak detection was performed using the Model-based Analysis of ChIP-Seq (MACS) algorithm with the peak detection p-value cutoff set at 1e-5 (default) (Zhang, Liu et al. 2008). This resulted in a set of 9281 significant (FDR less than 1 in a 100) Hnf4 α binding sites. We searched for the Hnf4 α consensus sequence within a 250 bp region from either side of the called peaks using a weight-matrix match with at least 80% similarity. The Hnf4 α weight matrix obtained from the JASPAR database (Sandelin, Alkema et al. 2004) was used as a surrogate to model Hnf4 α binding sites. A substantial proportion (92%) of the highly enriched Hnf4 α binding sites consisted of at least one Hnf4 α consensus site. All identified Hnf4 α binding sites were annotated with their closest up-stream, down-stream and overlapping genes using Ensembl gene annotations.

We looked at how many of the 877 putative Hnf4 α perturbed genes identified in our experiment were among the putative Hnf4 α target genes identified by the ChIP-Seq experiment. The significance of the overlap of genes between the two studies was calculated using the right tailed Fisher's exact test. The statistic was calculated separately for genes with up-stream, down-stream, and overlapping Hnf4 α targets and in combination.

2.3.10

Real-Time PCR. Gene expression was estimated by comparing mRNA levels from HNF4 α ^{F1/F1} mice treated with MUP-iCre-AAV8 vector and HNF4 α ^{F1/F1} mice treated with MUP-EGFP-AAV8 control vector by real-time PCR (RT-PCR). RNA concentration was estimated by spectrophotometry and samples were diluted to a concentration of 0.05 $\mu\text{g}/\mu\text{L}$. cDNA was made using 1 μg of RNA per sample in combination with cDNA master mix reagents (4.03 μL sterile water, 2.5 μL 5X RT buffer, 0.5 μL dNTPs, 0.16 μL random primers, 0.16 μL rRNasin, 0.16 μL MMLV reverse transcriptase) (Promega; Madison, WI). The RNA/Master Mix solution was then incubated at 42°C for 15 min followed by 95°C for 5 min. Analysis of the cDNA was done by adding 5 μL of diluted cDNA (1:10 in DEPC water) combined with 20 μL of primer pair/SyBr Green mix (3 μL of 5 μM primer pairs, 4.5 μL sterile water, 12.5 μL SyBr Green) (Applied Biosystems; Carlsbad, CA). Primers for RT-PCR include: cyclin D1, cyclin B1, cyclin B2, cyclin A2, cdca3, cdc20, aurora kinase A, ect2, egr1, polo-like kinase 1, eid1, and birc5. RT-PCR was then conducted using the 7300 Real-Time PCR System (Applied Biosystems; Foster City, CA).

2.3.11

Chromatin Immunoprecipitation. ChIP was performed using whole liver tissue from HNF4 α ^{F1/F1} mice treated with MUP-iCre-AAV8 vector and HNF4 α ^{F1/F1} mice treated with MUP-EGFP-AAV8 control vector using a protocol adapted from a previously published protocol by Bucholz, et al (Bucholz 2007). Approximately 200 mg of frozen liver tissue was used to for chromatin isolation from 3 mice of

each group. Isolated chromatin was then incubated with mouse IgG (1 µg; Millipore; Billerica, MA), DNA polymerase II (1 µg; Millipore; Billerica, MA), and HNF4α (10 µg; R&D Systems; Minneapolis, MN) antibodies for immunoprecipitation. Analysis was performed by RT-PCR using primers for *Ect2*, *Osgin1*, and *Hjurp*.

2.3.12

Over-expression of HNF4α in Hepa1C1C cells. HNF4α was ectopically over expressed by transfecting mouse HCC cells (Hepa1C1C) with an HNF4α over expression plasmid using the X-treme Gene 9 transfection reagent (Roche; Indianapolis, IN). The HNF4α over expression plasmid (Supplemental Figure 3) was a gift from Aaron Bell, PhD (University of Pittsburgh). Cells were plated at 1.5×10^6 /plate in a 100 mm plate. Cells were allowed to grow for 24 hrs before transfection with either HNF4α over-expression plasmid or empty vector. Other controls included cells treated with transfection reagent alone or with DMEM alone. Cells were then allowed to grow for 24 hrs before they were used for further analysis.

Over-expression of HNF4α was measured at the protein level by Western blot. Total protein was harvested and concentrations were estimated for all groups using RIPA buffer and the BCA protocol, as described previously. Western blotting was done using 50 µg of protein per sample and incubating with the HNF4α antibody (1:1000), using Gapdh (1:2000) as a loading control.

2.3.13

Fluorescence Activated Cell Sorting (FACS). In order to select positively transfected cells, FACS was performed utilizing GFP expressed in the presence of the HNF4 α over-expression plasmid. FACS was performed by the Flow Cytometry Core Facility at KUMC (Kansas City, KS).

Cells were plated at 1.5×10^6 cells/plate in 100 mm plates using the same groups as previously discussed. Cells were transfected 24 hrs after plating and were collected 24 hrs post-transfection for sorting based upon their expression of GFP using the BD FACS Aria system (BD Biosciences; San Jose, CA). After sorting, total protein (RIPA) and total RNA (TRIzol) was collected for further analysis.

Over-expression of HNF4 α was measured at the protein level via Western Blot. Pro-mitogenic gene expression was measured using RT-PCR and previously described primer sets.

2.3.14

Cell Cycle Analysis. Cell cycle analysis was performed on cells transfected with the HNF4 α over-expression plasmid, or the control plasmid, both of which express GFP for selection of positively transfected cells. Cells were plated at 1.5×10^6 cells/plate in 100 mm plates. Cells were transfected 24 hrs after plating and were collected for cell cycle analysis 24 hrs post-transfection. Collected cells were fixed with paraformaldehyde (2% w/v) for 1 hr at 4°C. Cells were then permeabilized with cold 70% ethanol overnight at 4°C. The following day cells were stained with propidium iodide (40 μ g/mL in sterile PBS) for 30 min at 37°C. Cell cycle was then analyzed by the Flow Cytometry Core Facility (KUMC;

Kansas City, KS) using the BD LSRII (BD Biosciences; San Jose, CA) and Flowjo flow cytometry analysis software.

2.3.15

Statistical Analysis. For all experiments not associated with RNA sequencing, such as ALT measurements, results are expressed as mean \pm standard deviation. Student's T-test was applied to all analyses, except RNA sequencing, with a *p-value* <0.05 being considered significant.

2.4 Results

2.4.1 Deletion of HNF4 α :

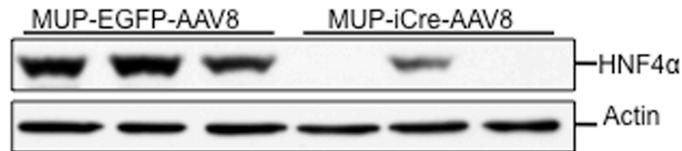
Treatment of HNF4 α ^{F1/F1} mice with MUP-iCre-AAV8 resulted in deletion of HNF4 α as demonstrated by Western blot analysis (Fig. 2.4.1.1A). Data show 70-100% decrease in HNF4 α protein level following treatment with MUP-iCre-AAV8, as compared to MUP-EGFP-AAV8. In 2 of the 7 mice studied we observed partial deletion of HNF4 α whereas, in the rest of the mice, a complete deletion of HNF4 α was observed (Western blots show 3 representative mice). The mice with complete deletion of HNF4 α were used for all further analysis.

Treatment of HNF4 α ^{F1/F1}, AlbERT2Cre⁺ mice with TAM resulted in deletion of HNF4 α as demonstrated by Western blot analysis (Fig. 2.4.1.1B). Data show ~80-90% decrease in HNF4 α protein level in the KO, as compared to controls HNF4 α ^{F1/F1} AlbERT2Cre⁺ treated with corn oil and HNF4 α ^{F1/F1} AlbERT2Cre⁻ treated with TAM, 7 days after treatment. Deletion of HNF4 α did not result in significant liver injury as indicated by serum ALT and glucose concentrations (Fig. 2.4.1.2).

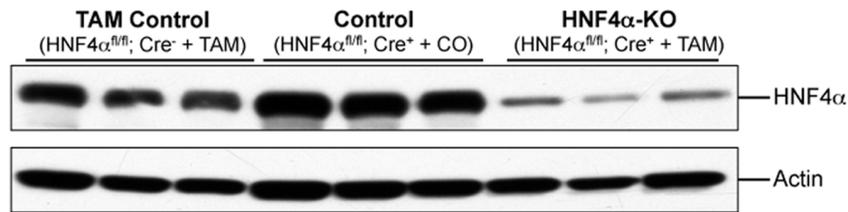
2.4.2 Histological changes following HNF4 α deletion: H&E staining of liver sections indicated that there was no histopathological liver injury following deletion of HNF4 α in either HNF4 α deletion model (Fig. 2.4.2.1 and 2.4.2.2, H&E). There was no apparent apoptosis, necrosis, or infiltration of immune cells (hallmarks of injury). However, the hepatocytes exhibited extensive

Figure 2.4.1.1

A)



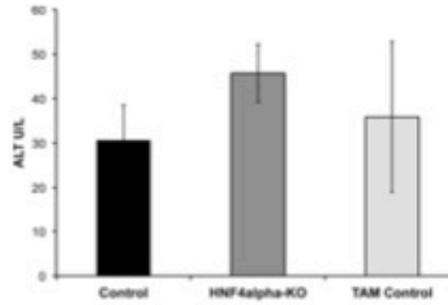
B)



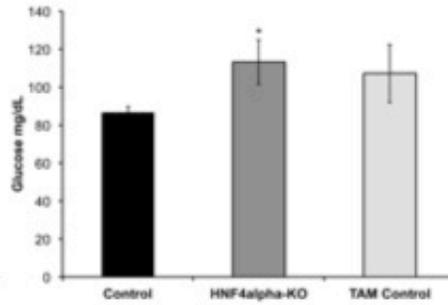
Western Blot Analysis of HNF4 α Deletion in Both Models. (A) Western blot of HNF4 α protein expression in MUP-EGFP-AAV8 (Control) and MUP-iCre-AAV8 (HNF4 α KO). Three representative samples are shown for each group. (B) Western blot of HNF4 α protein expression in HNF4 α ^{F1/F1}, Cre⁻ + TAM (TAM Control), HNF4 α ^{F1/F1}, Cre⁺ + CO (Control), and HNF4 α ^{F1/F1}, Cre⁺ + TAM (HNF4 α KO). Three representative samples are shown for each group. Actin is shown as a loading control.

Figure 2.4.1.2

A)

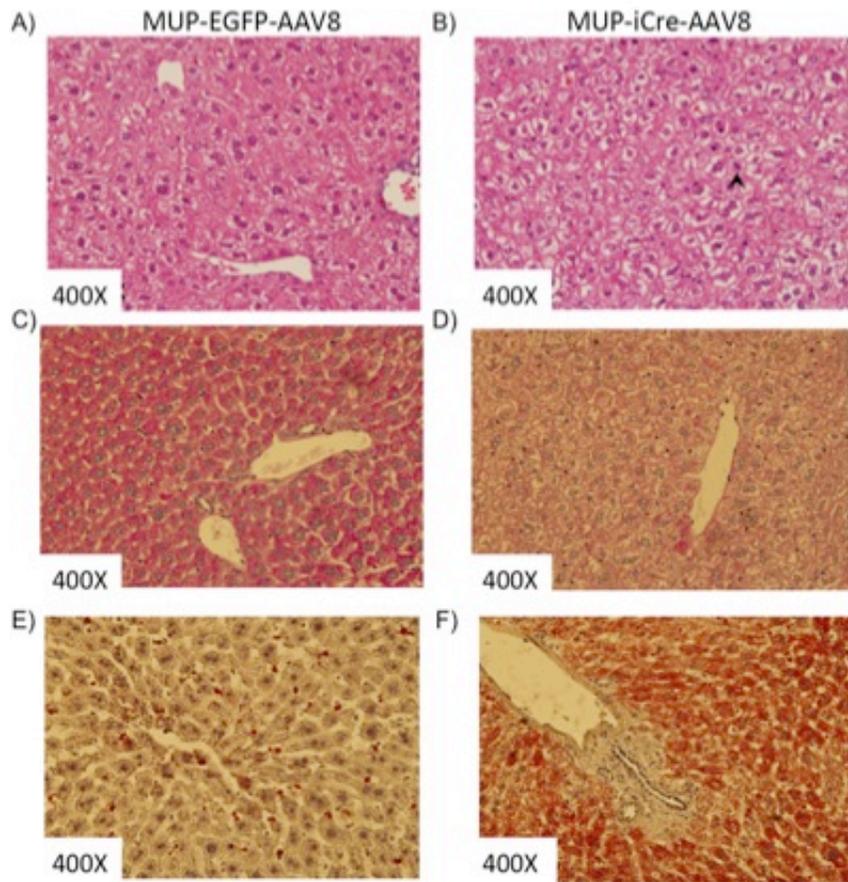


B)



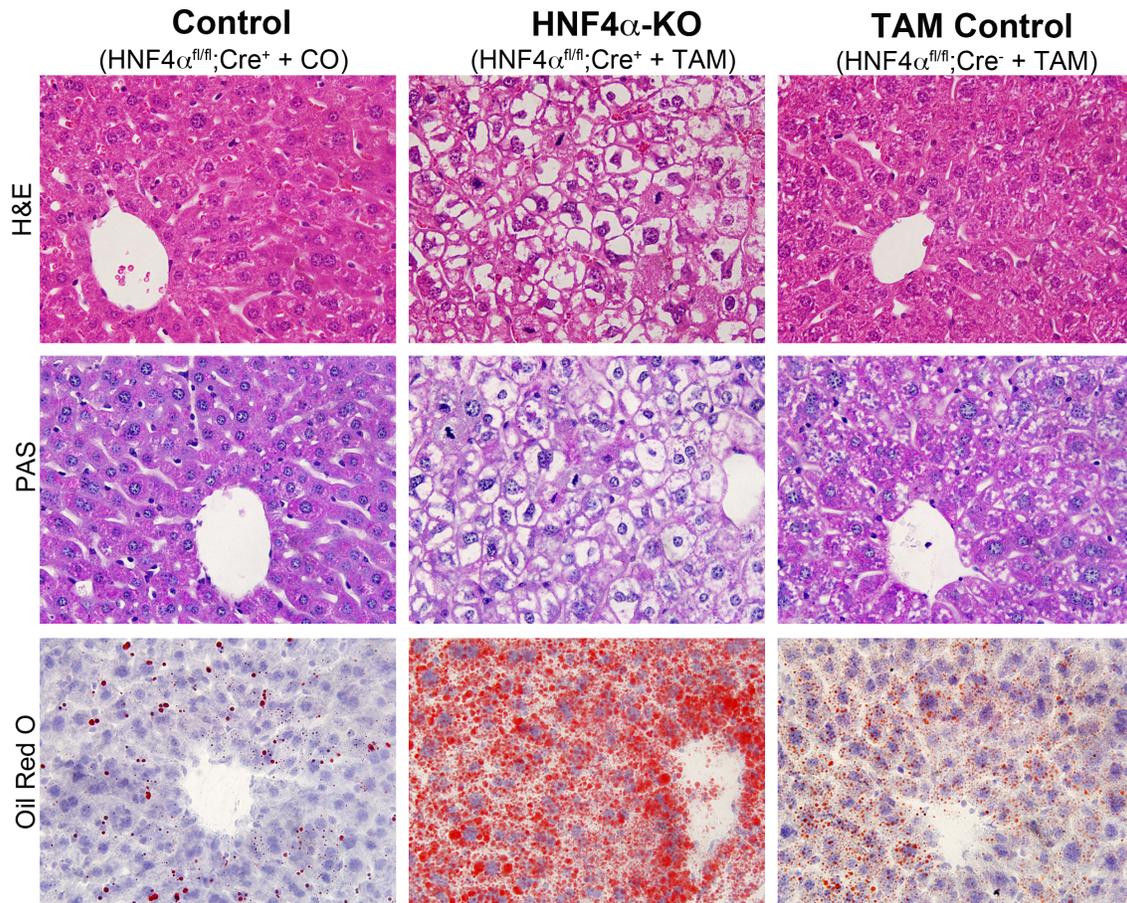
Deletion of HNF4 α did not result in liver injury. Liver injury was measured at the level of (A) serum alanine aminotransferase (ALT) and (B) serum glucose in the AlbERT2Cre HNF4 α KO mice. We did not observe a significant change in either ALT or glucose.

Figure 2.4.2.1



Histopathological Analysis of MUP-iCre-AAV8 HNF4 α KO Livers. Left pane shows representative photomicrographs of liver sections of MUP-EGFP-AAV8-treated HNF4 $\alpha^{fl/fl}$ mice (Control) stained for H&E (A), PAS (C), and Oil Red O (E). Right panel shows representative photomicrographs of liver sections of MUP-iCre-AAV8-treated HNF4 $\alpha^{fl/fl}$ mice (HNF4 α KO) stained for H&E (B), PAS (D), and Oil Red O. All images 400x magnification.

Figure 2.4.2.2

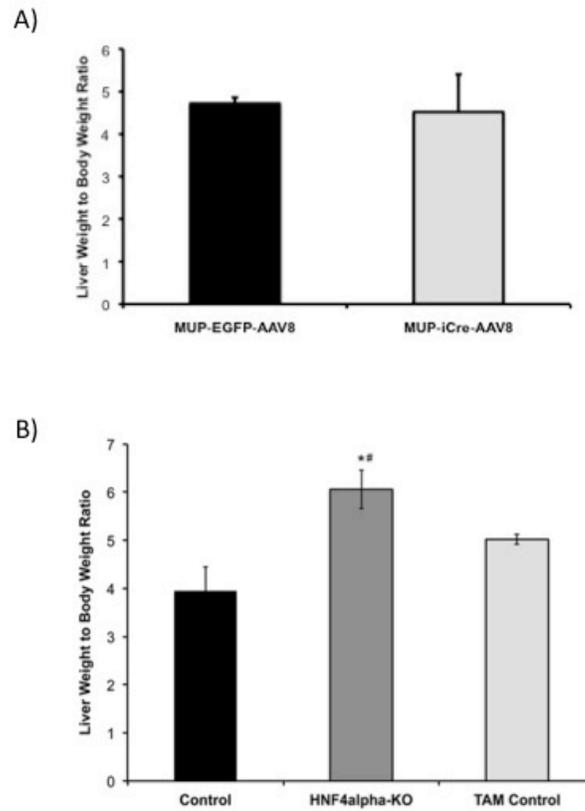


Histopathological Analysis of AlbERT2Cre HNF4 α KO livers. Representative photographs of paraffin embedded liver sections from the livers of Control, HNF4 α -KO, and TAM Control livers seven days after either TAM or corn oil treatment. Sections were stained using H&E (upper panel), and PAS for glycogen staining (middle panel). Frozen sections from the same samples were used for Oil Red O staining (lower panel). All images are 600x.

vacuolization giving them an 'empty' appearance. Previously published studies using the original, non-inducible AlbCre HNF4 α KO mice suggested that these open spaces might be due to an alteration of fat and glycogen accumulation (Hayhurst, Lee et al. 2001). Therefore, we decided to stain for fat (Oil Red O) and glycogen (PAS) deposits. PAS staining (magenta color) revealed decreased hepatic glycogen content following deletion of HNF4 α (Fig. 2.4.2.1D and 2.4.2.2, PAS). A concomitant increase in lipid accumulation after HNF4 α deletion was demonstrated by Oil Red O staining (Fig. 2.4.2.1F and 2.4.2.2, Oil Red O).

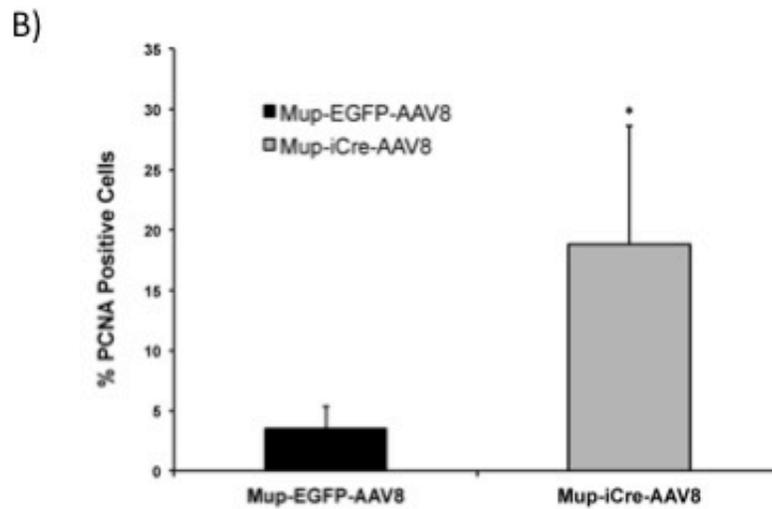
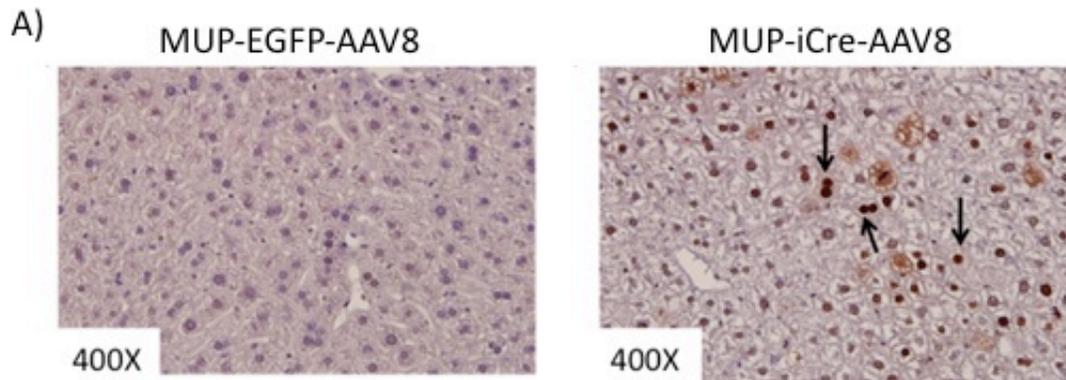
2.4.3 Deletion of HNF4 α results in Hepatomegaly due to Hyperplasia. HNF4 α deletion resulted in a significant increase in liver/body weight ratio in the AlbERT2Cre HNF4 α KO mice (Fig. 2.4.3.1B). The MUP-iCre-AAV8 HNF4 α KO mice did not show a statistically significant change in liver/body weight ratio (Fig. 2.4.3.1A). We investigated whether the increase in liver size was due to hyperplasia within the liver by staining for markers of actively proliferating cells, PCNA and Ki-67. HNF4 α deletion resulted in a significant increase in hepatocyte proliferation as demonstrated by PCNA analysis in both models of HNF4 α deletion (Fig. 2.4.3.2A and 2.4.3.3A). HNF4 α deletion resulted in an ~15% increase in actively proliferating cells as compared to control in the MUP-iCre-AAV8 HNF4 α KO model (Fig. 2.4.3.2B) and an ~20% increase in actively proliferating cells in the AlbERT2Cre HNF4 α KO model (Fig. 2.4.3.3B). The PCNA data were further confirmed by Ki-67 immunofluorescence in the AlbERT2Cre HNF4 α KO model (Fig. 2.4.3.4).

Figure 2.4.3.1



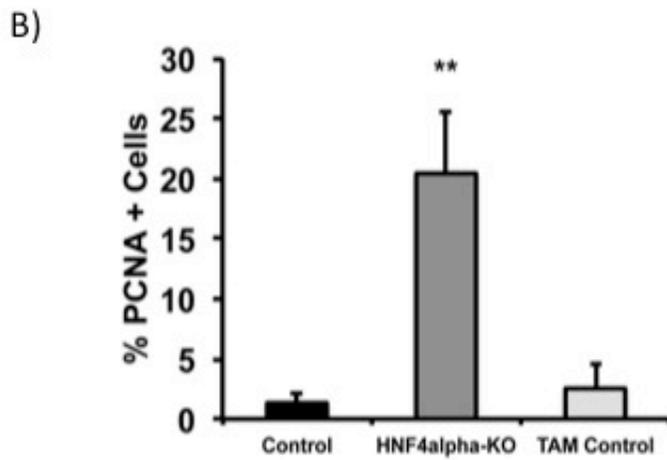
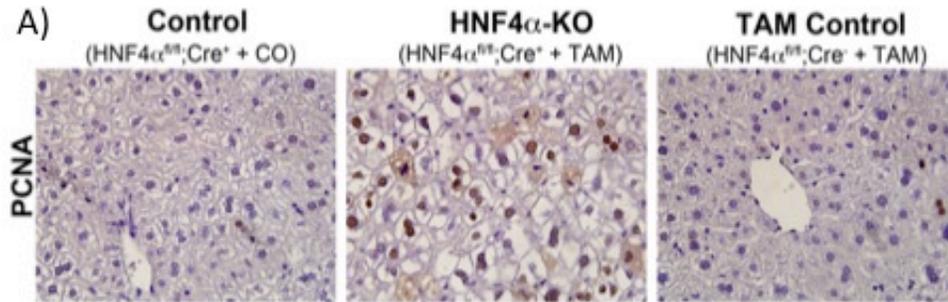
Increase in Liver Weight to Body Weight Ratio in AlbERT2Cre HNF4 α KO Mice. Deletion of HNF4 α did not cause a change in liver weight to body weight ratio in the MUP-iCre-AAV8 HNF4 α KO mice (A); however, a statistically significant increase was observed in the AlbERT2Cre HNF4 α KO mice versus both the Control and the TAM Control mice (B).

Figure 2.4.3.2



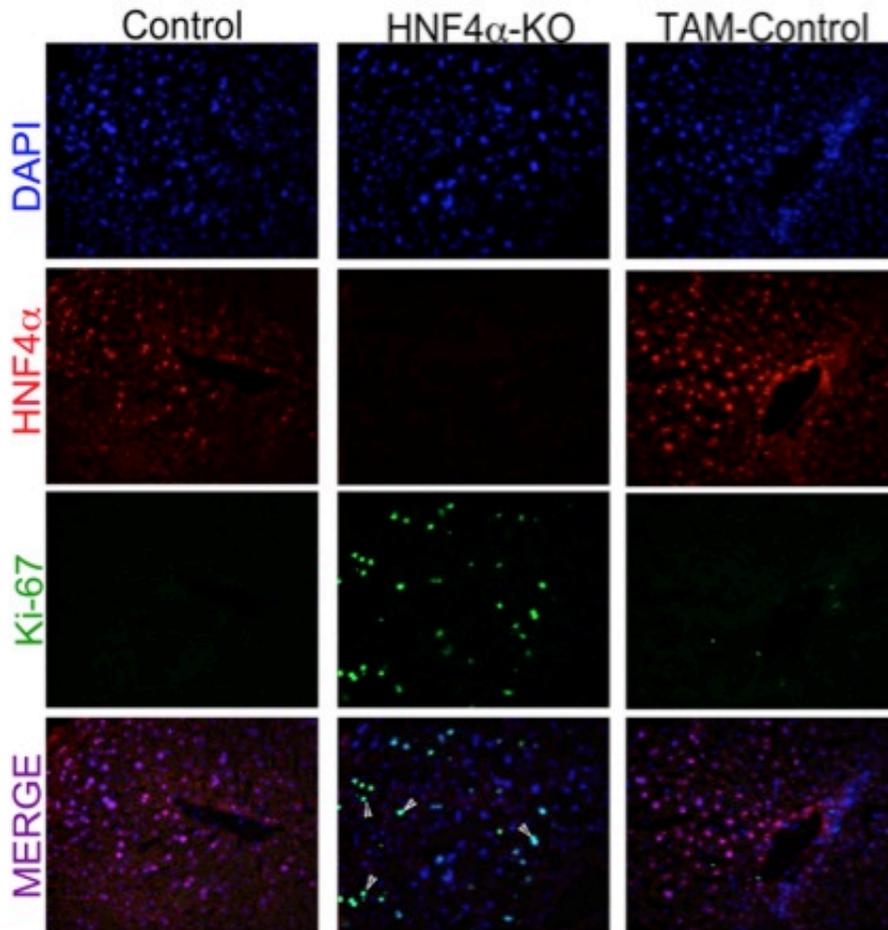
*Deletion of HNF4 α Results in Increased Cell Proliferation in the MUP-iCre-AAV8 HNF4 α KO Mice. A) Increased cell proliferation as shown by staining for PCNA. Arrows display cells in mitosis. All sections are at 400X magnification. B) Deletion of HNF4 α results in an ~15% increase in PCNA-positive cells as compared to control. * denotes a *p-value* < 0.05.*

Figure 2.4.3.3



*Deletion of HNF4 α Results in Increased Cell Proliferation in the AlbERT2Cre HNF4 α KO Mice. A) Increased cell proliferation as shown by staining for PCNA. All sections are at 400X magnification. B) Deletion of HNF4 α results in an ~20% increase in PCNA-positive cells as compared to controls. * denotes a *p*-value < 0.05.*

Figure 2.4.3.4



Deletion of HNF4 α Results in Increased Cell Proliferation in the AlbERT2Cre HNF4 α KO Mice. Confirmation of increased cell proliferation in the AlbERT2Cre HNF4 α KO mice by staining for cell proliferation marker, Ki-67. HNF4 α KO samples show a lack of staining for HNF4 α with increased staining for Ki-67 (arrowheads). All sections are at 400X magnification.

2.4.4 Increased Pro-Mitogenic Gene Expression following Deletion of HNF4 α

To determine the mechanisms of increased cell proliferation after HNF4 α deletion, we performed global gene expression analysis on each of our models. We performed cDNA microarray on total RNA isolated from liver tissues of MUP-EGFP-AAV8 and MUP-iCre-AAV8 treated mice. Deletion of HNF4 α in the MUP-iCre-AAV8 HNF4 α KO model resulted in changes in a high number of genes (Table 2.4.4.1). Further, we performed RNA-sequencing analysis on total RNA isolated from the livers of HNF4 α ^{F1/F1}, AlbERT2Cre⁺ mice treated with TAM (HNF4 α -KO), vehicle alone (Control), and AlbERT2Cre⁻ mice treated with TAM (TAM Control). High-throughput sequencing generated 117, 179, and 136 million reads for the Cre⁺\TAM, Cre⁻\TAM and Cre⁺\Corn Oil samples respectively. Of these, TopHat was able to map 103, 163 and 121 million reads to the mouse reference genome respectively. Deletion of HNF4 α in the AlbERT2Cre HNF4 α KO model further confirmed that a large number of genes are affected by a loss of HNF4 α (2.4.4.1).

Many of the down-regulated genes are previously identified HNF4 α target genes involved in classic hepatocyte function (Pineda Torra, Jamshidi et al. 2002; Battle, Konopka et al. 2006; Garrison, Battle et al. 2006; Bolotin, Liao et al. 2010) (Table 2.4.4.2). Interestingly, a significant number of genes that are up-regulated following HNF4 α deletion are pro-mitogenic with a large number directly associated with the cell cycle (Table 2.4.4.3). Gene changes were confirmed

Table 2.4.4.1

	MUP-iCre-AAV8	AlbERT2Cre
Up-regulated	1,196	515
Down-regulated	955	362

Number of Genes Changed Following Deletion of HNF4 α in Both HNF4 α KO Models

Table 2.4.4.2

Gene Name	Gene Symbol	Fold Change in MUP-iCre-AAV8	Fold Change in AlbERT2Cre
UDP Glucuronosyltransferase 2 Family, Polypeptide B4	<i>Ugt2b1</i>	-6.38	-159.92
Apolipoprotein A-II	<i>Apoa2</i>	-3.49	-54.52
Coagulation Factor XII	<i>F12</i>	-16.89	-22.06
Cytochrome P450, Family 8, Subfamily B, Polypeptide 1	<i>Cyp8b1</i>	-12.08	-25.47
Apolipoprotein A-IV	<i>Apoa4</i>	-35.81	-3.93
Sulfotransferase Family, Cytosolic, 1A, Phenol-Preferring, Member 1	<i>Sult1a1</i>	-24.67	-11.72
Claudin 2	<i>Cldn2</i>	-18.55	-4.18
Apolipoprotein B	<i>Apob</i>	-7.77	-7.13
Claudin 1	<i>Cldn1</i>	-5.82	-4.79
Cytochrome P450, Family 7, Subfamily B, Polypeptide 1	<i>Cyp7b1</i>	-2.93	-13.68

Down-regulated genes following deletion of HNF4α. Global gene expression changes following deletion of HNF4α were analyzed using cDNA microarray analysis. A significant number of down-regulated genes were observed to be known targets of hepatic differentiation.

Table 2.4.2.3

Gene Name	Gene Symbol	Fold Change in MUP-iCre-AAV8	Fold Change in AlbERT2Cre
Cyclin B1	<i>Ccnb1</i>	9.51	58.75
Cell Division Cycle Homolog 25 C	<i>Cdc25c</i>	1.44	21.07
Cyclin-Dependent Kinase Inhibitor 3	<i>Cdkn3</i>	3.33	144.66
Cell Division Cycle 20 Homolog	<i>Cdc20</i>	1.08	74.59
Antigen Identified by Antibody Ki-67	<i>Ki-67</i>	N/A	23.71
Polo-Like Kinase 1	<i>Plk1</i>	2.58	12.90
Cyclin B2	<i>Ccnb2</i>	7.19	21.79
Cyclin A2	<i>Ccna2</i>	6.41	20.89
Epithelial Cell Transforming Sequence 2	<i>Ect2</i>	11.8	20.53
Cell Division Cycle Associated 3	<i>Cdca3</i>	4.75	13.16
Cell Division Cycle Associated 8	<i>Cdca8</i>	3.42	8.02
Early Growth Response 1	<i>Egr1</i>	33.43	5.44
Myelocytomatosis Viral Oncogene Homolog	<i>Myc</i>	N/A	3.84

Up-regulated genes following deletion of HNF4 α . Global gene expression changes following deletion of HNF4 α were analyzed using cDNA microarray analysis. A significant number of up-regulated genes are known targets of cell proliferation and cancer.

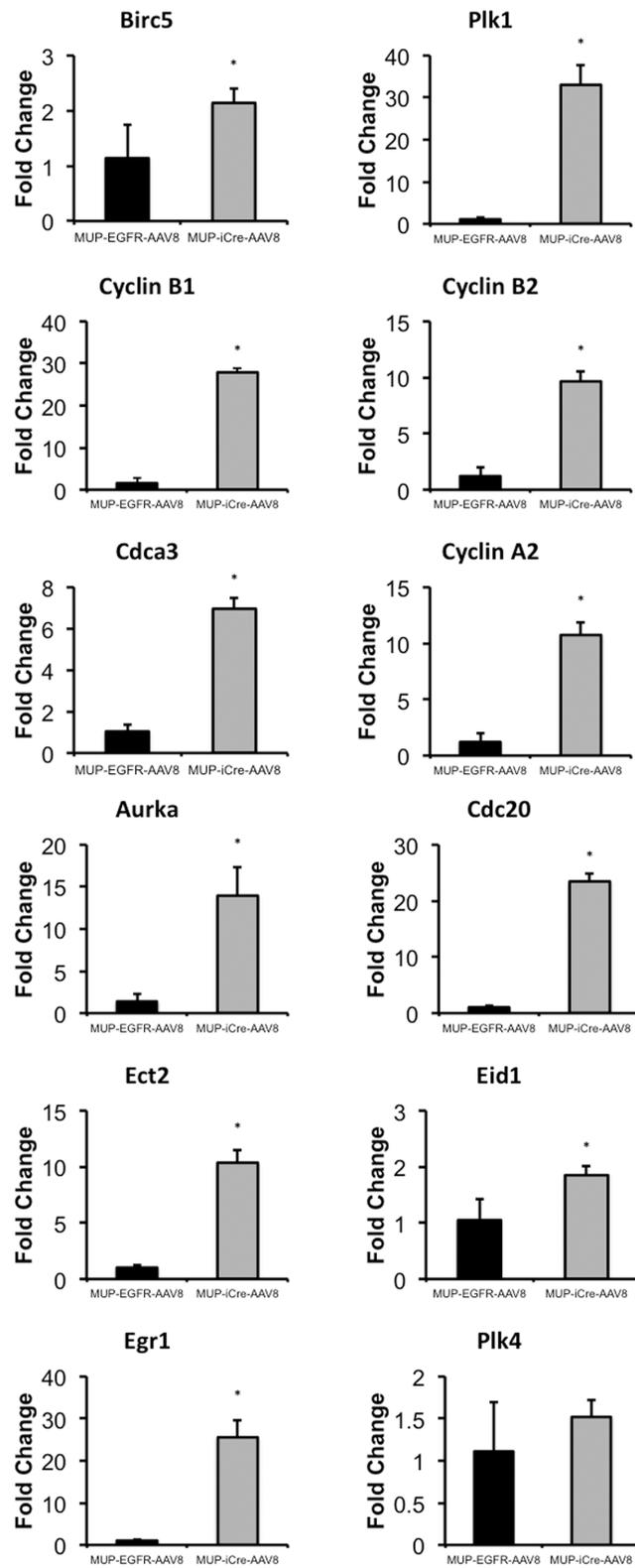
by RT-PCR (Fig. 2.4.4.4) for 12 of the genes found to be significantly changed in the MUP-iCre-AAV8 HNF4 α KO mice. These data indicate that loss of HNF4 α result in activation of pro-mitogenic genes including: *Egr1*, *Cdc20*, *Ect2*, *Birc5*, *Ccnb1*, *Ccnb2*, *Ccna2*, *Cdca3*, *Eid1*, *Aurka*, *Plk1*, and *Plk4*.

2.4.5 Identification of novel ‘negative’ target genes of HNF4 α

Because HNF4 α is a transcription factor and regulates gene expression via direct promoter binding, we further determined how many of the genes changed after HNF4 α deletion were direct HNF4 α targets. For this, we compared our cDNA microarray data to published chromatin immunoprecipitation-sequencing (ChIP-Seq) data (Hoffman, Robertson et al. 2010). We identified 29 up-regulated genes following HNF4 α deletion that contained a putative HNF4 α binding site within 10 kb of the transcriptional start site (TSS) (Table 2.4.5.1).

In order to corroborate the observation we made in the MUP-iCre-AAV8 HNF4 α KO model, we compared RNA-Seq data from the AlbERT2Cre HNF4 α KO model with the same ChIP-Seq data used to perform the comparative analysis in the MUP-iCre-AAV8 model (Hoffman, Robertson et al. 2010). RNA-Seq/ChIP-Seq comparative analysis revealed that of the total gene changes observed following deletion of HNF4 α (877), ~53% of these (462) contained a putative HNF4 α binding site within 50 kb of the transcriptional start site (TSS). Further, ~45% (395) contained a putative HNF4 α binding site within 10 kb of the TSS.

Figure 2.4.4.4



HNF4 α deletion results in increased pro-mitogenic gene expression. Real Time PCR analysis of various pro-mitogenic genes in livers of HNF4 α ^{fl/fl} mice treated with either MUP-EGFP-AAV8 or MUP-iCre-AAV8 for one week. *indicates statistically significant difference at P<0.05.

Table 2.4.5.1

Number	Gene	Chromosome	Distance to TSS
1	<i>Acot1</i>	12	873
2	<i>Acot3</i>	12	-1292
3	<i>Atp4a</i>	7	7582
4	<i>Birc5</i>	11	-3471
5	<i>Cry1</i>	10	-5224
6	<i>Cyp39a1</i>	17	1236
7	<i>Ect2</i>	3	-2512
8	<i>Flot2</i>	11	7035
9	<i>Hjurp</i>	1	9111
10	<i>Kynu</i>	2	710
11	<i>Lgals4</i>	7	3058
12	<i>Mki67</i>	7	-7580
13	<i>Mmp14</i>	14	-1181
14	<i>Nfe2l2</i>	2	-666
15	<i>Nln</i>	13	-1325
16	<i>Osgin1</i>	8	3728

17	<i>Pcp4l1</i>	1	-8176
18	<i>Rhpn2</i>	7	797
19	<i>Rrm2</i>	12	-2502
20	<i>Serpina6</i>	12	-3076
21	<i>Shroom1</i>	11	246
22	<i>Slc16a6</i>	11	-4619
23	<i>Slc1a4</i>	11	-503
24	<i>Synpo</i>	18	3786
25	<i>Tifa</i>	3	3244
26	<i>Tkt</i>	14	-1021
27	<i>Tm4sf4</i>	3	9948
28	<i>Tsku</i>	7	9502
29	<i>Zfp62</i>	11	-7894

Up-regulated Genes in MUP-iCre-AAV8 HNF4 α KO Mice with a Putative HNF4 α -binding Site within 10 kb of the Transcriptional Start Site (TSS)

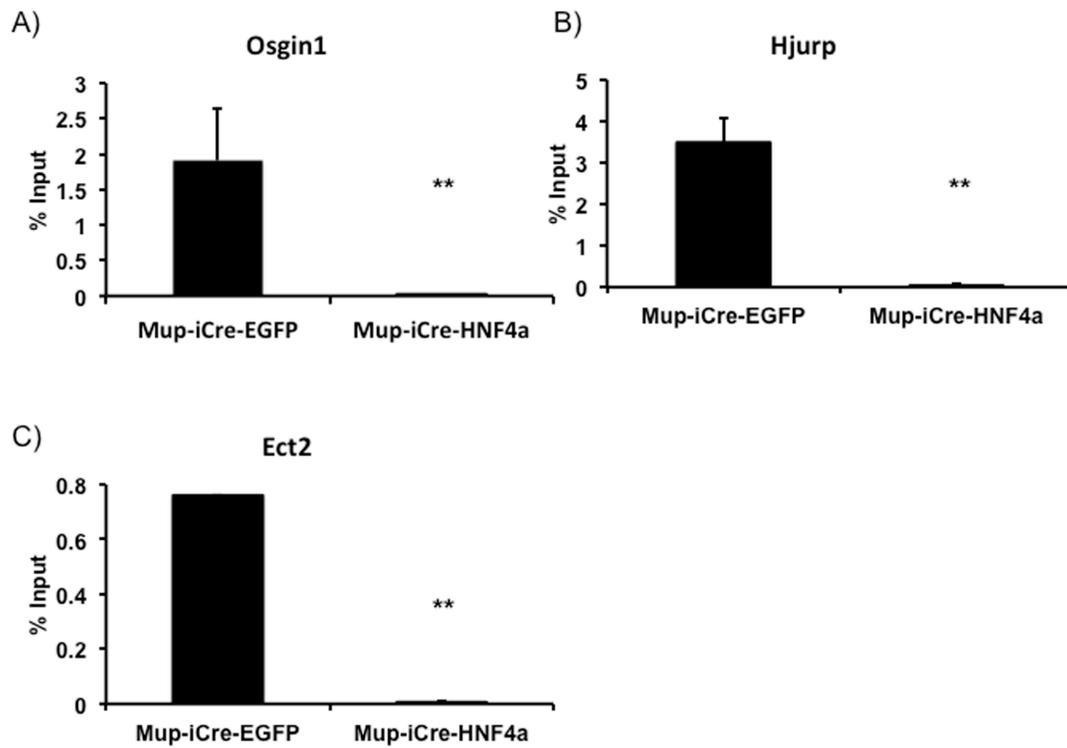
2.4.6 Direct Interaction of HNF4 α with the Promoter Region of Pro-Mitogenic Genes *Ect2*, *Hjurp*, and *Osgin1*

We further confirmed the HNF4 α binding sites on three of the pro-mitogenic genes that our comparative analyses identified to be putative HNF4 α target genes: *Ect2* oncogene, *Hjurp*, and *Osgin1*, which are known to be involved in hepatocyte proliferation, using ChIP assay (Ong, Ng et al. 2004; Kato, Sato et al. 2007; Morin, Flors et al. 2009). Our results confirmed the putative HNF4 α binding sites identified in the comparative analyses for *Ect2*, *Hjurp*, and *Osgin1* (Fig. 2.4.6.1). HNF4 α showed a high degree of binding when HNF4 α is present (MUP-EGFP-AAV8), but showed no enrichment of signal when HNF4 α was deleted (MUP-iCre-AAV8).

2.4.7 In Vitro Over-Expression of HNF4 α Results in Decreased Hepatocyte Proliferation and a Down Regulation of Pro-Mitogenic Gene Expression

To determine if HNF4 α over-expression results in decreased cell proliferation, we over expressed HNF4 α in mouse HCC cells (Hepa1C1C). The HNF4 α over-expressing cells were selected by flow cytometry analysis using GFP as a marker. The HNF4 α over-expression plasmid used in these studies also expresses GFP under a separate promoter allowing selection of transfected cells. HNF4 α over-expression was confirmed at the protein level by Western blot analysis (Fig. 2.4.7.1A) using both unselected and GFP-selected cells. Next we studied the effect of HNF4 α over-expression on cell cycle using propidium iodide-aided flow cytometric analysis. The over-expression of HNF4 α resulted in an

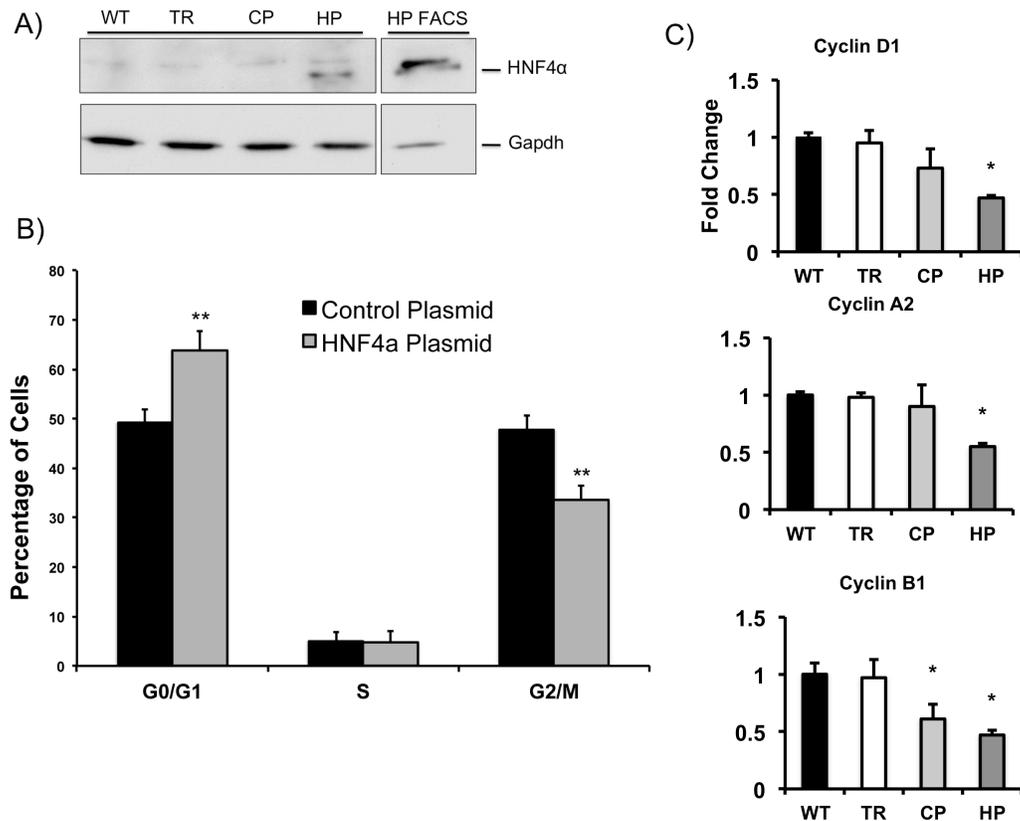
Figure 2.4.6.1



ChIP-qPCR Confirmation of HNF4 α binding sites on pro-mitogenic genes. Bar graphs showing ChIP-qPCR of HNF4 α binding on *Osgin1* (A), *Hjurp* (B) and *Ect2* (C) in MUP-EGFP-AAV8 (Control, normal HNF4 α level). and lack of HNF4 α binding in MUP-iCre-AAV8 (HNF4 α KO). **indicates statistically significant difference at P<0.01.

increase in the number of cells in G0/G1 phase and a decreased number of cells in G2/M phase of the cell cycle (Fig. 2.4.7.1B). RT-PCR analysis performed on GFP-selected cells indicated that over-expression of HNF4 α resulted in decreased expression of cell cycle genes such as Cyclin D1, B1, and A2 (Fig. 2.4.7.1C). These data indicate that over-expression of HNF4 α results in cell cycle arrest mainly in the G0/G1 phase of cell cycle.

Figure 2.4.7.1



HNF4α over-expression results in cell cycle arrest. (A) Western blot analysis of HNF4α of Hepa1C1C cells non-transfected (WT), treated with transfection reagent alone (TR), Control plasmid (CP), HNF4α over-expression construct (HP) and GFP sorted cells after transfection with HNF4α over-expression construct. (B) PI-based flow cytometric cell cycle analysis of GFP-positive cells transfected with either the Control plasmid or HNF4α over-expression construct. ** indicates statistically significant difference at $P < 0.01$ (C) Real time PCR analysis of cell cycle genes in GFP-sorted cells transfected with either the Control plasmid or HNF4α over-expression construct. * indicates statistically significant difference at $P < 0.05$

2.5 Discussion

HNF4 α regulates a majority of hepatocyte-specific genes and its role in hepatic differentiation is well-recognized (Hayhurst, Lee et al. 2001; Inoue, Hayhurst et al. 2002; Inoue, Yu et al. 2004; Inoue, Peters et al. 2006; Inoue, Yu et al. 2006; Gonzalez 2008). Recent studies have suggested a novel role of HNF4 α in the regulation of hepatocyte proliferation (Flodby, Liao et al. 1995; Kalkuhl, Kaestner et al. 1996; Hayhurst, Lee et al. 2001; Xu, Hui et al. 2001; Lazarevich, Cheremnova et al. 2004; Erdmann, Senkel et al. 2007; Gonzalez 2008; Grigo, Wirsing et al. 2008; Ning, Ding et al. 2010). However, the mechanisms of HNF4 α -mediated inhibition of hepatocyte proliferation remain unknown. In this study, we elucidated a mechanism by which HNF4 α has an anti-proliferative effect on hepatocytes within the mature mouse liver. We have demonstrated that a loss of HNF4 α leads to an increase in hepatocyte proliferation in correlation with an increase in pro-mitogenic gene expression.

Previous models of HNF4 α deletion resulted in a loss of HNF4 α relatively early in the life, when the liver is still growing and maturing (Hayhurst, Lee et al. 2001; Parviz, Matullo et al. 2003). This may limit the interpretation of data. Further, none of those studies have specifically investigated the role of HNF4 α in cell proliferation. We developed two novel models of HNF4 α deletion in adult mice where liver maturation has completed and the loss of HNF4 α would not interfere in postnatal liver development. Our models utilize the Cre-lox method of deletion. The first model utilizes delivery of the Cre recombinase using an AAV8 viral vector under a liver-specific promoter (MUP) to HNF4 α ^{F1/F1} mice (MUP-iCre-

AAV8). Our second model utilizes a tamoxifen-inducible Cre recombinase under the liver-specific albumin promoter (AlbERT2Cre).

Following deletion of HNF4 α *in vivo*, we observed no significant injury within the liver. We did, however, observe an increase in lipid accumulation and a decrease in glycogen accumulation. Hepatocytes in normal liver store a significant amount of glycogen, but hepatocytes in HNF4 α -KO mice exhibited a decrease in glycogen and a substantial increase in hepatic fat content. These data are reflective of the metabolic changes induced in the liver due to a lack of HNF4 α , which regulates many of the genes involved in glycogen synthesis (*Gys2*) (Ladias, Hadzopoulou-Cladaras et al. 1992) and lipid transport (*Apoa2*, *Apoa4*, *Apob*, *Apoc2*, *Apoc3*, and *MTP*) (Bonzo, Ferry et al. 2012).

Interestingly, we observed an increase in liver/body weight ratio within the HNF4 α KO mice. We investigated whether the increased liver size was due to hyperplasia within the liver. We observed a dramatic increase in hepatocyte proliferation in both HNF4 α deletion models, which explains the increased liver size.

Because HNF4 α is a transcription factor, we investigated whether the increased hepatocyte proliferation was due to global gene expression changes in pro-mitogenic genes. Following deletion of HNF4 α , we observed a down-regulation of classic genes involved in hepatocyte differentiation, many of which are previously characterized targets of HNF4 α . A significant number of the up-regulated genes are genes involved in cell proliferation and the cell cycle.

As a proof-of-principle experiment, we performed an *in vitro* over-expression of HNF4 α . Over-expression of HNF4 α *in vitro* yielded a decrease in pro-mitogenic gene expression with an arrest in the cell cycle at the G2/M phase. We hypothesize that HNF4 α , which is typically thought of as a positive transcriptional regulator, may be functioning as a negative transcriptional regulator for select genes involved in the inhibition of hepatocyte proliferation. Thus, HNF4 α may play a dual role in the mature mouse liver, acting as a transcriptional activator for genes involved in differentiation and as a transcriptional repressor for genes involved in hepatocyte proliferation. A disruption in normal HNF4 α function could lead not only to decreased hepatic differentiation but increased proliferation due to loss of inhibition of strong mitogenic genes.

We have identified a number of genes that are up-regulated following HNF4 α deletion, indicating that they are negatively regulated by HNF4 α . Our data indicate that HNF4 α may be negatively regulating these genes by direct binding to specific sites on their promoters. We have confirmed the ability of HNF4 α to bind to the promoter regions of some of these genes including *Ect2*, *Hjurp*, and *Osgin1*. Further studies, included in chapter 4, indicate that the mechanism of this inhibition is epigenetic regulation via promoter acetylation.

A vast number of the up-regulated genes do not have an HNF4 α binding site therefore direct regulation of these genes at the level of transcription is unlikely. It is possible that HNF4 α may regulate these genes indirectly via an intermediary pathway, or via miRNAs as shown by Hatziapostolou, et al.

(Hatziapostolou, Polytarchou et al. 2011). They provide evidence of an “HNF4 α circuit” involving miR-124, *IL6R*, *STAT3*, and miR-24/miR-629 in the regulation of hepatocarcinogenesis. They show a correlation between the down-regulation of HNF4 α and miR-24 and an up-regulation of *IL6R* and *STAT3* associated with the progression of HCC. We cannot comment on the expression of miRs in our model at this time, but we do not observe an increase in *IL6R* or *STAT3*. This is likely due to a lack of inflammatory responses within our model, which may be a mediating event in the activation of the “HNF4 α circuit”. With this said, it is still very much a possibility that HNF4 α is regulating many of the gene expression changes that we observe by an indirect mechanism involving miRNAs.

One potential mechanism of HNF4 α -induced gene silencing is the influence of epigenetic modifications (histone modifications and DNA methylation). Ungaro, et al. (2010) provided some of the first evidence that HNF4 α is a negative transcriptional regulator (Ungaro, Teperino et al. 2010). Their data supports the hypothesis that HNF4 α negatively regulates the expression of the *PED* gene in the liver. They also provide evidence that HNF4 α silences the *PED* gene by influencing chromatin remodeling through epigenetic modifications at the *PED* promoter. Based on these studies, and our data, we hypothesize that the inhibition of hepatocyte proliferation associated with the presence of HNF4 α is due to repression of pro-mitogenic genes by HNF4 α -induced histone modifications, such as a decrease in H3K9/K14 acetylation and H3K4 dimethylation. We further hypothesize that HNF4 α is recruiting select co-factors at pro-mitogenic genes in order to influence these histone modifications.

In summary, we have developed two novel models of HNF4 α deletion in adult mouse liver and demonstrated that loss of HNF4 α results in activation of hepatocyte proliferation in mature liver. Our data suggests a novel role of HNF4 α in the mature mouse liver in the inhibition of hepatocyte proliferation. Elucidating the mechanism by which HNF4 α influences hepatocyte proliferation may highlight its significance in complex liver pathologies, such as HCC, leading to its utilization as a prognostic marker and/or therapeutic target.

Chapter 3

Hepatocyte Nuclear Factor 4 alpha Deletion Promotes Diethylnitrosamine-induced Hepatocellular Carcinoma in Rodents

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3.1 Abstract

Our studies reported in Chapter 2, indicate that HNF4 α , the master regulator of hepatocyte differentiation, can also inhibit hepatocyte proliferation in mature liver. To further investigate the mechanisms of HNF4 α -induced inhibition of hepatocyte proliferation, we developed two models of HNF4 α deletion in mature mouse liver. Hepatocyte-specific deletion of HNF4 α in adult mice resulted in increased hepatocyte proliferation with a significant increase in liver/body weight ratio. To further determine the mechanisms of increased cell proliferation after HNF4 α deletion, we performed global gene expression changes using Illumina HiSeq-based RNA sequencing. RNA-Seq studies revealed that, a significant number of up-regulated genes following deletion of HNF4 α were associated with cancer pathogenesis, cell cycle control, and cell proliferation. Next, we performed Ingenuity Pathway Analysis to determine major pathways changed after HNF4 α deletion. The pathway analysis revealed that c-Myc-regulated gene expression network was highly activated following HNF4 α deletion. Finally, to determine whether deletion of HNF4 α affects cancer pathogenesis, HNF4 α knockdown was induced in mice treated with the known hepatic carcinogen diethylnitrosamine (DEN). Deletion of HNF4 α significantly increased the number and size of DEN-induced hepatic tumors. Pathological analysis revealed that tumors in HNF4 α deleted mice were well-differentiated hepatocellular carcinoma (HCC) and mixed HCC-cholangiocarcinoma. Analysis of tumors and surrounding normal liver tissue in DEN-treated HNF4 α knockout mice showed significant induction in c-Myc expression. Taken together, deletion of HNF4 α in adult hepatocytes results in

increased hepatocyte proliferation and promotion of DEN-induced hepatic tumors secondary to aberrant c-Myc activation.

3.2 Introduction

Hepatocyte nuclear factor 4 alpha (*HNF4α*, *NR2A1*) is the master regulator of hepatocyte differentiation (Sladek, Zhong et al. 1990; Sladek 1993). In the previous chapter we provided evidence that HNF4α plays a dual role in the liver by promoting differentiation and inhibiting proliferation. We developed two novel models of HNF4α deletion (MUP-iCre-AAV8 and AlbERT2Cre) in order to knockout HNF4α after the liver has fully matured and observe its effect on proliferation. Because HNF4α plays a role in inhibiting hepatocyte proliferation, it is highly likely that HNF4α may be involved in the pathogenesis of cancers within the liver, such as hepatocellular carcinoma (HCC).

HCC is the fifth-most common cancer and the third most frequent cause of cancer death globally (Llovet, Burroughs et al. 2003). Recent studies revealed that HNF4α is suppressed in human HCC tissues compared with adjacent, non-cancerous tissues (Lazarevich, Cheremnova et al. 2004). Lazarevich, et al. first described a link between HNF4α's known functions in promoting differentiation and how this is inhibited in times of increased proliferation. It is well accepted that there is a link between a cell's state of differentiation and its propensity for proliferation. Generally, a cell that is well differentiated does not have a propensity to proliferate. In their study, Lazarevich and colleagues show that a slow-growing HCC can progress to a fast-growing, de-differentiated HCC and this correlates with a repression in HNF4α. They found that forced expression of HNF4α in the fast-growing HCC induces re-differentiation of the tumor cells towards a more hepatocyte-like phenotype. The observed change occurs rapidly

suggesting that the progression of the tumor was due to a limited number of gene changes. Further, they observe that the forced expression of HNF4 α suppressed the proliferation of the fast-growing HCC cells suggesting that HNF4 α may have a tumor-suppressing effect. They conclude that a loss of HNF4 α may be a critical event in HCC progression that mediates hepatocyte de-differentiation, loss of cell adhesion, and an increase in cell proliferation and invasiveness.

Lazarevich, et al. conducted a more recent study to investigate HNF4 α as a marker for epithelial tumor progression (Lazarevich, Shavochkina et al. 2010). In these studies, Lazarevich and colleagues found that tumor progression to a dedifferentiated phenotype was associated with a reduction in HNF4 α in mice. Further, they found that de-differentiated human HCCs exhibited the same phenomenon.

The study in the previous chapter focused on a deletion of HNF4 α at a one-week time point. In that study, we performed global gene expression analysis in order to determine if a loss of HNF4 α correlates with gene changes that promote hepatocyte proliferation giving strength to our overall hypothesis that HNF4 α inhibits hepatocyte proliferation. In the current study, we expanded upon this focus by conducting Ingenuity Pathway Analysis (IPA) on the RNA-Seq data obtained from the AlbERT2Cre HNF4 α KO mice. IPA revealed significant changes in gene networks associated with cell cycle control, cell proliferation, and cancer. It also revealed activation of the oncogene, c-Myc. c-Myc is commonly up-regulated in human HCCs. Further, transgenic mice that over

express c-Myc result in chronic hepatic proliferation and increased liver cancer (Calvisi and Thorgeirsson 2005).

Because of the information we received from IPA, we decided to investigate a potential role for HNF4 α in the pathogenesis of HCC. It has been speculated that deletion of HNF4 α will result in rapid liver failure making it difficult to directly study its role in the pathogenesis of HCC (Bonzo, Ferry et al. 2012). Whether HNF4 α deletion itself can result in hepatic tumorigenesis is not known and may be difficult to study due to limitations of the model system; therefore, we decided to investigate whether HNF4 α deletion can promote existing tumors in the liver; this can be tested using the two-stage diethylnitrosamine (DEN)-induced chemical carcinogenesis model.

The results presented in the remainder of this chapter indicate that HNF4 α deletion during the late stage of HCC progression substantially promotes DEN-induced hepatic tumor formation. Our results show that deletion of HNF4 α in mice treated with DEN results in a vast expansion in tumor size and tumor number. Furthermore, the tumors, as well as surrounding tumor stroma, in HNF4 α -KO mice showed extensive up regulation of c-Myc and Cyclin D1. We believe that the data presented in this chapter helps to strengthen the hypothesis that HNF4 α is a tumor suppressor and could potentially be utilized in the future as a target for therapeutics or as a prognostic marker for HCC.

3.3 Materials and Methods

3.3.1

Animals, Treatments, and Tissue Collection. The HNF4 $\alpha^{F1/F1}$, AlbCreERT2⁺ mice were produced by standard animal breeding and identified using PCR based genotyping of genomic DNA isolated from tail biopsies (Fig. 2.3.3.1). All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities at the University of Kansas Medical Center under a standard 12-h light/dark cycle with access to chow and water *ad libitum*. The Institutional Animal Care and Use Committee approved all of the studies. Mice were euthanized by cervical dislocation under isoflurane anesthesia and livers and serum were collected.

3.3.2

Diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC): For the DEN-induced HCC protocol, male HNF4 $\alpha^{F1/F1}$, AlbERT2-Cre⁺ mice were injected, subcutaneously, with 15 μ g/g DEN (in 0.9% saline) at postnatal day 12-15. At 8 months, these mice were divided into 2 groups and treated with either TAM (6 μ g/mouse) or corn oil, and sacrificed two months later at 10 months of age. A separate group of age-matched HNF4 $\alpha^{F1/F1}$, AlbERT2Cre⁻ mice was also treated with 15 μ g/g DEN at PND 12-15 and then injected with TAM ((6 μ g/mouse) at 8 months of age. Liver and serum samples were obtained and processed as described before (Walesky, Gunewardena et al. 2012). Liver and body weights of mice were noted at the time of sacrifice and used to determine liver/body weight ratios. Liver injury and function were determined by serum alanine

aminotransferase (ALT), serum bilirubin, and serum glucose levels measured using the Infinity™ ALT (GPT) and the Infinity™ Glucose kit (Thermo Scientific; Middletown, VA) according to the manufacturer's protocol.

3.3.3

Western Blotting. RIPA extracts obtained from whole liver tissues were used for Western blot analysis; Western blots were performed using the previously described protocol (2.3.4). The antibodies used in this study are as follows: *HNF4 α* , (1:1000; R&D Systems; Minneapolis, MN; Cat. # PP-H1415-00), Cyclin D1 (Cat. # 2978), c-Myc (Cat. # 5605), and β -Actin (Cat. # 4970) (1:1000; Cell Signaling; Danvers, MA).

3.3.4

Staining Procedures. Paraffin-embedded liver sections (4 μ m thick) were used for H&E and immunohistochemical staining of PCNA as described previously (2.3.5).

3.3.5

RNA-Seq and Functional Analysis. Total RNA was isolated from liver tissue using the phenol/chloroform extraction protocol. Integrity of RNA was analyzed by the Microarray Core Facility at KUMC (Kansas City, KS) using an Agilent Bioanalyzer 2100 (Agilent Technologies; Santa Clara, CA).

We performed two separate and independent RNA-Seq experiments for the same treatment conditions, Cre+\Tamoxifen, Cre-\ Tamoxifen and Cre+\Corn Oil all at a 7 day time point. . In the first instance (Run1), the total processed RNA extracted from pooled mouse liver samples (3 mice per group) treated with Cre+\Tamoxifen, Cre-\ Tamoxifen and Cre+\Corn Oil was sequenced in an

Illumina HiSeq 2000 sequencing machine (Illumina, San Diego, CA). The initial library of 10nM concentration for each of the three samples was split into two diluted concentrations of 5pM and 3pM and sequenced separately at a 2×100 bp paired-end resolution and the output of the sequencing runs combined for downstream analysis. In order to complement the initial RNA-Seq analysis, we ran a second RNA-seq experiment (Run2) on biological replicate samples (n=2) of mouse liver treated with Cre+Tamoxifen, Cre- Tamoxifen and Cre+Corn Oil. These samples were sequenced at a 50bp single-end resolution. The RNA-seq data obtained from both experiments was used for the bioinformatics analysis. RNA-seq data from both experiments were mapped to the mouse reference genome (NCBI37/mm9) using TopHat v1.4.1 (Trapnell et al.). TopHat was run with default parameters and for Run1 with paired reads, a mate inner distance of -40 was set to accommodate the 260bp average fragment length. The binary output files generated by TopHat were passed to CuffDiff (Trapnell, Williams et al. 2010) for calculating differential gene expression. For samples from both runs, differential gene expression was first calculated for Cre+Tamoxifen against Cre+Corn Oil and Cre+Tamoxifen against Cre- Tamoxifen. For each run, genes that were differentially expressed in both these measures based on an absolute fold change of at least 1.5 and a q-value (the p-value adjusted for multiple hypothesis correction using the Benjamini-Hochberg procedure) less than or equal to 0.05 were selected. This data filtering resulted in 1096 genes from Run1 and 3436 genes from Run2. Of these 877 genes were common to both runs (right tailed Fisher's exact test significance p-value <1E-100). These genes were

uploaded to Ingenuity Pathways Analysis (IPA, Ingenuity Systems, version 7.6 (www.ingenuity.com)) for gene set enrichment analysis. Out of the 877 genes uploaded, 864 genes qualified for analysis in IPA. The analysis was performed in IPA with default parameters. The RNA-seq data were submitted to the Sequence Read Archive (SRA) of NCBI.

Further, we performed RNA-Seq using a similar protocol on three separate tumors from the male HNF4 α ^{F1/F1}; AlbERT2Cre⁺ mice injected, subcutaneously, with 15 μ g/g DEN along with three separate tumors from control mice treated with DEN alone. Reads were compared using the previously described protocol. These samples were sequenced at a 50 bp single-end resolution. The RNA-seq data was used for the bioinformatics analysis. Reads were mapped to the mouse reference genome (NCBI37/mm9) using TopHat v1.4.1 (Trapnell et al.). TopHat was run with default parameters. The binary output files generated by TopHat were passed to CuffDiff (Trapnell, Williams et al. 2010) for calculating differential gene expression. Genes that were differentially expressed based on an absolute fold change of at least 1.5 and a q-value (the p-value adjusted for multiple hypothesis correction using the Benjamini-Hochberg procedure) less than or equal to 0.05 were selected. This data filtering resulted in 2,168 genes.

3.3.6

Statistical Analysis. For all experiments not associated with RNA sequencing are expressed as mean \pm standard deviation. Student's T-test was applied to all analyses with a *p-value* <0.05 being considered significant.

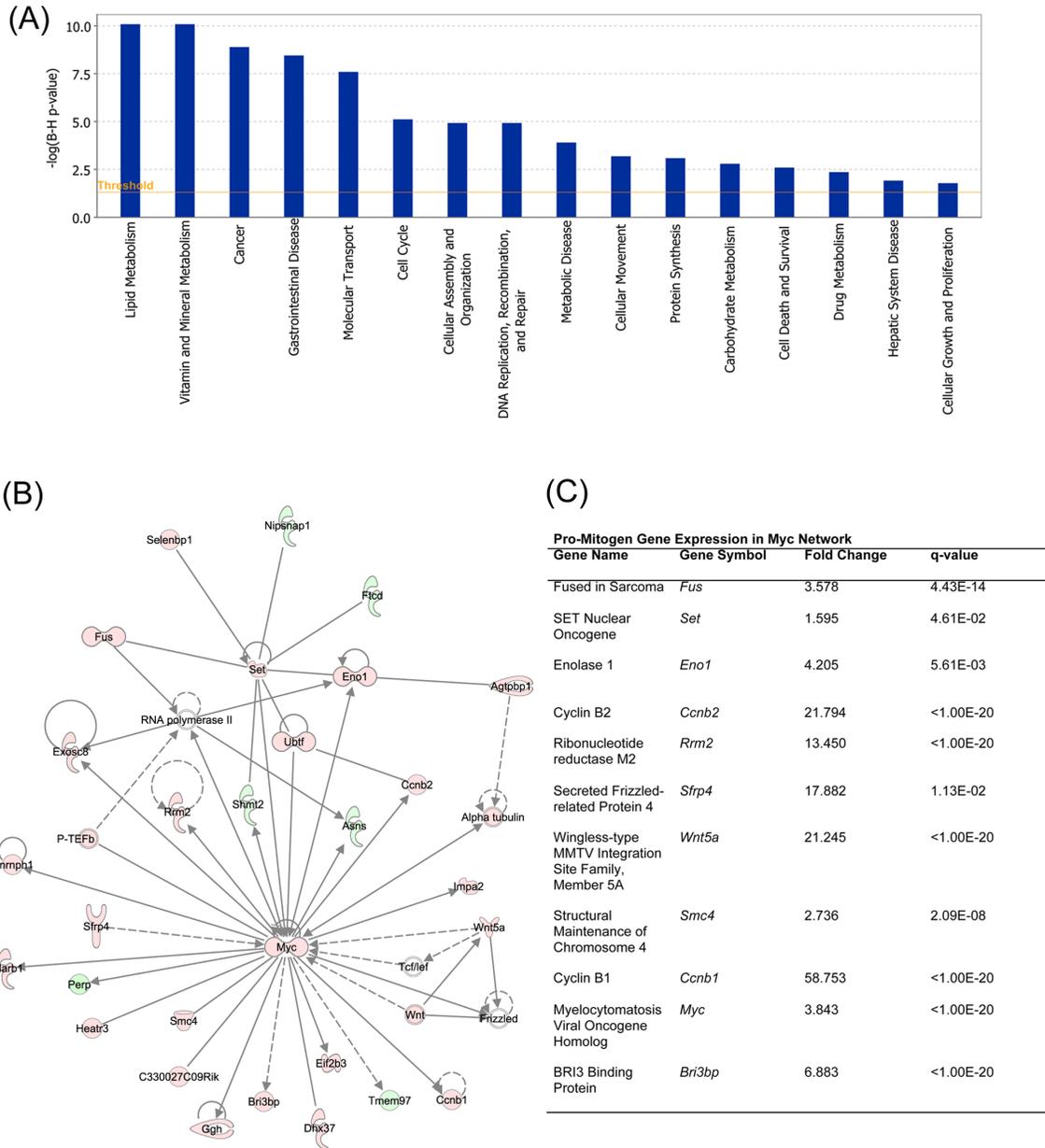
3.4 Results

3.4.1 HNF4 α Deletion Results in Increased Expression of Gene Networks involved in Cell Cycle Control, Cell Proliferation, and Cancer

In the previous chapter, we provided evidence that deletion of HNF4 α resulted in the down-regulation of many genes involved in hepatocyte function, such as xenobiotic metabolism, cholesterol metabolism, coagulation, bile acid synthesis, etc. (Fig. 2.4.2.2). Interestingly, many of the up-regulated genes are involved in the cell cycle and cancer (Table 2.4.2.3).

To identify patterns in gene expression changes, we utilized Ingenuity Pathway Analysis (IPA, Ingenuity[®] Systems, www.ingenuity.com). Functional analysis of gene expression changes revealed that genes involved with cancer pathogenesis are one of the most significant groups of genes changed (Fig. 3.4.1.1A). Other groups of significantly changed genes include genes involved in cell cycle and cellular growth and proliferation (Fig. 3.4.1.1A). IPA further revealed changes in major transcription factor activity following HNF4 α deletion. The c-Myc-regulated gene expression network showed the most significant changes in gene expression that correlate with activation of c-Myc following HNF4 α deletion (Fig. 3.4.1.1B and 3.4.1.1C). This includes genes involved in cell proliferation including *ccnb1*, *ccnb2*, *fus*, and *set* oncogene. Also, many other transcription factor networks involved in cell proliferation and cancer were significantly activated. As expected, gene network associated with HNF4 α was inhibited (regulation z-score -5.0). Other factors inhibited include Cdkn1A

Figure 3.4.1.1



Gene expression changes in livers following HNF4 α deletion. Global gene expression analysis was conducted using Illumina-based RNA sequencing as described in Methods. The data were used for Ingenuity Pathway Analysis. (A) Bar graph showing top gene categories altered following HNF4 α deletion. (B) c-Myc Gene network showing various genes either up-regulated (red) or down-regulated (green) in HNF4 α -KO mice as compared to Control. (C) Table showing up-regulated pro-mitogenic genes within the c-Myc-regulated gene network.

(p21), Smarcb1, Tob1, and Cdkn2A (p16), all of which are associated with cancer pathogenesis.

3.4.2 HNF4 α Deletion Results in Increased Promotion of DEN-induced Hepatocellular Carcinoma

To determine the effect of HNF4 α deletion on hepatic tumor progression we utilized a DEN-induced HCC model. HNF4 α ^{F^l/F^l}, AlbERT2Cre⁺ mice were treated with known hepatic carcinogen, DEN, at post-natal day 15 and then treated with TAM (HNF4 α -KO) or corn oil (control) at 8 months of age followed by tissue collection 2 months later at 10 months of age (Fig. 3.4.2.1A). Deletion of HNF4 α for only a 2 month period resulted in increased HCC progression demonstrated by an increase in tumor number and size (Fig. 3.4.2.1B, arrows), along with a 2-fold increase in liver/body weight ratio (Fig. 3.4.2.1C). HNF4 α -KO livers display advanced tumor morphology and significantly increased proliferation when compared to control livers via H&E (Fig. 3.4.2.1D) and PCNA staining, respectively (Fig. 3.4.2.1E). The control mice treated with DEN exhibited mainly hyperproliferative nodules and a few high-grade dysplastic nodules with few early-stage HCCs. In contrast, the HNF4 α -KO mice treated with DEN exhibited extensive dysplastic nodules, HCCs (Fig. 3.4.2.1D-ii and iv), and tumors with mixed HCC-cholangiocarcinoma morphology (Fig. 3.4.2.1D-iii and v). The tumors in HNF4 α -KO mice exhibited distinct histological features including expansion of oval cell population (Fig. 3.4.2.1D-ii, 3.4.2.1D-iv and 3.4.2.1E-iii) and presence of inflammatory cell foci (Fig. 3.4.2.1D-vi).

HNF4 α deletion promotes DEN-induced hepatic tumors in mice. (A) Scheme showing protocol of DEN-induced hepatic tumor induction. (B) Representative micrographs of livers of Control mice (left) and HNF4 α -KO mice (right) treated with DEN. (C) Liver to body weight ratios of Control and HNF4 α -KO mice treated with DEN. (D) Representative photomicrographs of paraffin embedded liver sections stained for H&E from Control (i) and HNF4 α -KO mice (ii to vi). Large arrowheads show oval cell-like cells in (ii) and (iv). Arrows point to biliary duct proliferation in the mixed type tumors in (iii) and (v). Small arrowheads indicate inflammatory cell focus in (vi). (E) Representative photomicrographs of PCNA immunohistochemistry on paraffin section from control (i) and HNF4 α -KO mice (ii and iii). Arrowheads point to proliferating biliary duct cells.

3.4.4 HNF4 α Deletion Results in Increased Pro-Mitogenic Signaling in DEN-induced Hepatocellular Carcinoma

We hypothesized that increased progression of HCC in HNF4 α -KO mice treated with DEN increased pro-mitogenic signaling. We performed RNA-Seq analysis on three separate tumors from HNF4 α ^{F1/F1}, AlbERT2-Cre⁺ mice injected with 15 μ g/g DEN along with three separate tumors from control mice treated with DEN alone in hope to characterize gene changes that are unique to a deletion of HNF4 α . These unique gene signatures may partially explain why the tumors, which lack HNF4 α , have increased progression.

RNA-Seq revealed a significant change in global gene expression in tumors that lack HNF4 α . A total of 2,168 genes (1,244 up-regulated and 924 down-regulated) were changed following a deletion of HNF4 α . Further, a significant number of the down-regulated genes are genes associated with classic hepatic functions (Fig. 3.4.4.1). A significant number of the genes that are up-regulated are involved in cell proliferation and cancer (Fig. 3.4.4.2).

RNA-Seq showed a significant increase in Cyclin D1 expression but no change in c-Myc. Because data from IPA analysis suggested activation of the c-Myc gene network, we decided to look at c-Myc protein level. Western blot analysis of normal liver tissue (control), control+DEN (normal tissue), tumor tissues and surrounding normal tissues from HNF4 α -KO mice treated with DEN indicated increased Cyclin D1 and c-Myc protein expression only in normal liver tissue surrounding the tumors and in the tumors observed in HNF4 α -KO mice treated with DEN (Fig. 3.4.4.3A).

Figure 3.4.4.1

Gene Name	Gene Symbol	Fold Change
UDP Glucuronosyltransferase 2 Family, Polypeptide B4	<i>Ugt2b1</i>	-5.86
Apolipoprotein A-II	<i>Apoa2</i>	-3.84
Coagulation Factor XII	<i>F12</i>	-3.03
Cytochrome P450, Family 8, Subfamily B, Polypeptide 1	<i>Cyp8b1</i>	-4.41
Apolipoprotein A-IV	<i>Apoa4</i>	-2.17
Sulfotransferase Family, Cytosolic, 1A, Phenol- Preferring, Member 1	<i>Sult1a1</i>	-1.42
Claudin 2	<i>Cldn2</i>	-3.55
Apolipoprotein B	<i>Apob</i>	-3.13
Claudin 1	<i>Cldn1</i>	-3.32
Cytochrome P450, Family 7, Subfamily B, Polypeptide 1	<i>Cyp7b1</i>	-3.91
Cyclin F	<i>Ccnf</i>	-2.68

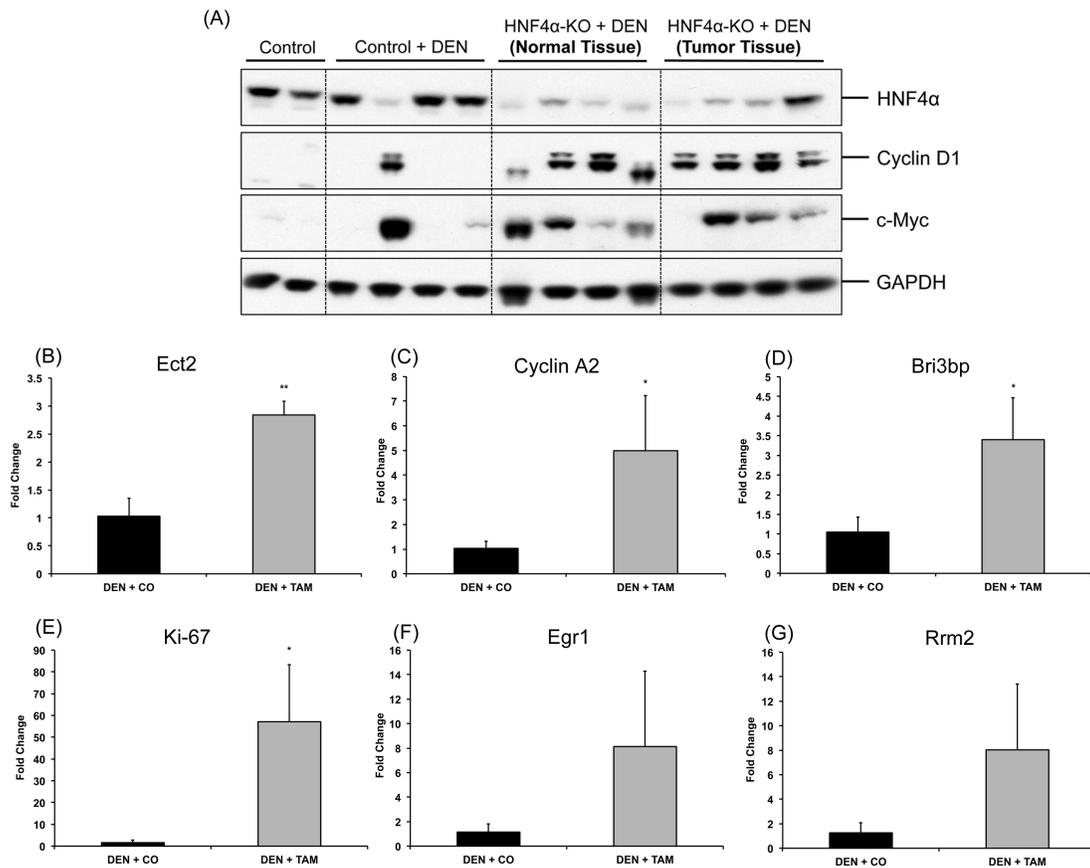
Down-regulated genes in HNF4 α KO Tumors. Global gene expression changes following deletion of HNF4 α in DEN-treated mice were analyzed using RNA-Seq analysis. A significant number of down-regulated genes are known targets of hepatic differentiation.

Figure 3.4.4.2

Gene Name	Gene Symbol	Fold Change
Cyclin D1	<i>Ccnd1</i>	3.59
Cyclin B1	<i>Ccnb1</i>	2.37
Antigen Identified by Monoclonal Antibody Ki- 67	<i>Mki-67</i>	1.61
Early Growth Response 1	<i>Egr1</i>	1.93
Ribonucleotide Reductase M2	<i>Rrm2</i>	2.21

Up-regulated genes in HNF4 α KO Tumors. Global gene expression changes following deletion of HNF4 α in DEN-treated mice were analyzed using RNA-Seq analysis. A significant number of up-regulated genes are involved in cell proliferation and cancer pathogenesis.

Figure 3.4.4.3



Increased pro-mitogenic gene expression in HNF4 α -KO tumors. (A) Western blot analysis of HNF4 α , Cyclin D1, and c-Myc using either nuclear proteins (HNF4 α) or total liver extracts (all other) of Control mice, Control mice treated with DEN, normal liver tissue of HNF4 α -KO treated with DEN and tumor tissue of HNF4 α -KO treated with DEN. (B) to (G) Real time PCR analysis of putative negative targets of HNF4 α identified by combined RNA-seq-ChIP-seq bioinformatics analysis.

Real time PCR analysis confirmed that expression of several genes up regulated following HNF4 α deletion were increased in the tumors observed in HNF4 α -KO mice (Fig. 3.4.4.3B to 3.4.4.3G).

3.5 Discussion

HNF4 α is the master regulator of hepatocyte differentiation because it regulates many hepatocyte-specific genes involved in bile acid, drug, and lipid metabolism as well as, blood coagulation. Our studies presented in Chapter 2 revealed a novel function of HNF4 α in the regulation of hepatocyte proliferation and indicate that HNF4 α may actively inhibit hepatocyte proliferation. Furthermore, the exact role of HNF4 α in the pathogenesis of liver cancers is not known. In this study, we established a role for HNF4 α in cancer pathogenesis using our AlbERT2Cre HNF4 α KO mouse model combined with the known hepatic carcinogen, DEN, in a classic initiation/promotion experiment.

In the previous chapter we performed a global gene expression study using Illumina HiSeq2000-based RNA sequencing in order to investigate the mechanism by which HNF4 α is inhibiting hepatocyte proliferation. We found that a KO of HNF4 α causes an up-regulation of many pro-mitogenic genes. In the current study we performed bioinformatic analysis of our RNA-Seq data. IPA-mediated functional analysis revealed that the major classes of genes changed following HNF4 α deletion are in cell cycle control, cancer, and cell proliferation categories. The up-regulation of pro-mitogenic genes explains the significant increase in proliferation within the livers of HNF4 α -KO mice.

Our analysis also revealed that a large number of the genes up-regulated after HNF4 α deletion are regulated by c-Myc. The RNA-seq data showed a 3.8-fold increase in c-Myc gene expression after deletion of HNF4 α for one week, corroborating these results. Previous studies have indicated that HNF4 α

competes with c-Myc for binding on the promoter of cell cycle inhibitor *p21/WAF1* (Hwang-Verslues and Sladek 2008). Further analysis revealed that several genes up-regulated in the c-Myc gene network are involved in stimulation of cell proliferation and cancer pathogenesis including the *set* oncoprotein, *fus*, *ccnb1*, and *ccnb2*. These data indicate that HNF4 α may indirectly down-regulate these genes via suppressing c-Myc activation in normal adult hepatocytes.

It has been speculated that deletion of HNF4 α will result in rapid liver failure making it difficult to directly study its role in the pathogenesis of HCC (Bonzo, Ferry et al. 2012). Whether HNF4 α deletion itself can result in hepatocarcinogenesis is not known and may be difficult to study due to limitations of the model system. Therefore, we decided to investigate whether HNF4 α deletion can promote existing tumors in the liver using the two-stage DEN-induced chemical carcinogenesis model. Our studies indicated that HNF4 α deletion during the late stage of HCC progression can substantially promote DEN-induced hepatic tumor formation. Our results show that deletion of HNF4 α in mice treated with DEN results in a vast expansion in tumor size and tumor number.

In order to investigate the mechanism by which a deletion of HNF4 α causes an increase in the tumorigenicity of DEN-initiated tumors, we performed RNA-Seq analysis on tumors from DEN-treated mice and DEN-treated mice combined with a deletion of HNF4 α . RNA-Seq analysis revealed a large number of gene expression changes unique to the mice treated with DEN combined with a deletion of HNF4 α . Many of the down-regulated genes are involved in hepatic

differentiation. We expected to see these gene changes since we observed many of them in our HNF4 α deletion studies in Chapter 2. These results gave us confidence that our HNF4 α deletion was effective since they were similar to what we had previously observed. We observed an up-regulation of pro-mitogenic genes such as *ccnd1*, *ccnb1*, *Ki-67*, *Egr-1* and *Rrm2*. We did not observe changes in many of the pro-mitogenic genes characterized in Chapter 2. It is possible that these genes are up-regulated in both the DEN-treated mice and the DEN-treated mice combined with a deletion of HNF4 α . The five genes listed previously may be able to partially explain why the HNF4 α KO tumors grow faster and are more in number.

Our bioinformatic analysis pointed toward the importance of c-Myc activation in our initial studies at a one-week time point. Because c-Myc is an oncogene, we hypothesized that it may be important in the mechanism(s) responsible for the increased tumorigenicity we observed in the DEN-treated HNF4 α KO mice. We did not observe an up-regulation of c-Myc at the RNA level; however, the tumors, as well as surrounding tissues, in HNF4 α KO mice treated with DEN showed extensive up-regulation of c-Myc and Cyclin D1 at the protein level. These data further support the hypothesis that HNF4 α inhibits hepatocyte proliferation by inhibiting the c-Myc gene network.

We saw a reduction in Cyclin F expression. Cyclin F is expressed during S phase and peaks during the G2 phase of the cell cycle. Cyclin F interacts with ribonucleotide reductase family member 2, RRM2, and is thought to promote its ubiquitylation and degradation (D'Angiolella, Donato et al. 2012). Ribonucleotide

reducases catalyze the conversion of ribonucleotides to dNTPs for synthesis of DNA during replication and repair. Cyclin F-mediated degradation of RRM2 is essential to maintain balanced levels of dNTPs. Failure to regulate dNTP levels causes genome instability and a phenotype associated with hypermutation (D'Angiolella, Esencay et al. 2013). Cells in which cyclin F expression is silenced accumulate high levels of dNTPs and display an increase in mutation frequency compared with control cells (D'Angiolella, Donato et al. 2012). HNF4 α KO tumors express lower levels of cyclin F and increased expression of RRM2. This outlines a potential mechanism by which HNF4 α KO may promote tumorigenesis, and needs further investigation.

Taken together, our data indicates that HNF4 α is not only an important factor in the regulation of hepatocyte differentiation, but also as a critical player in the inhibition of hepatic proliferation in association with increased tumorigenesis. Our study sheds light on the mechanism of HNF4 α -mediated inhibition of cell proliferation in tumors and indicates that HNF4 α inhibits hepatocyte proliferation by down-regulation of pro-mitogenic genes such as c-Myc. These data suggest a novel role as a tumor suppressor and highlight HNF4 α as a potential therapeutic target, as well as a prognostic marker, for liver cancers.

Chapter 4

Role of HNF4 α in the Transcriptional Control of the Ect2 Oncogene by Influencing Histone Deacetylation and Chromatin Condensation

4.1 Abstract

HNF4 α is the master regulator of hepatocyte differentiation. Recent evidence has highlighted a role of HNF4 α in the regulation of hepatocyte proliferation and as a tumor suppressor. Evidence suggests the mechanism by which HNF4 α inhibits hepatocyte proliferation is through the down-regulation of select pro-mitogenic genes. In this study, we investigated a role for HNF4 α in the inhibition of select pro-mitogenic genes by influencing histone modifications. We have previously shown that the oncogene, Ect2, is a direct target of HNF4 α and is up-regulated following deletion of HNF4 α . We investigated four common histone modifications and found that HNF4 α has a large influence on the acetylation state of the Ect2 promoter region. We also found that HNF4 α interacts with the histone deacetylase recruiter, SMRT, in our model and they are both found at the Ect2 promoter when it has a low acetylation state. In conclusion, we have provided evidence that HNF4 α can affect the acetylation state of the Ect2 oncogene and believe it is doing so through the recruitment of a histone deacetylase complex via SMRT.

4.2 Introduction

Recent studies highlighted a novel role of HNF4 α in the inhibition of cell proliferation within multiple tissue types, including the liver (Flodby, Liao et al. 1995; Kalkuhl, Kaestner et al. 1996; Hayhurst, Lee et al. 2001; Xu, Hui et al. 2001; Lazarevich, Cheremnova et al. 2004; Erdmann, Senkel et al. 2007; Gonzalez 2008; Grigo, Wirsing et al. 2008; Ning, Ding et al. 2010; Bonzo, Ferry et al. 2012; Walesky, Edwards et al. 2013; Walesky, Gunewardena et al. 2013). Further, studies by our lab and Bonzo, et al. have shed light on a potential mechanism by which HNF4 α inhibits cell proliferation (Bonzo, Ferry et al. 2012; Walesky, Edwards et al. 2013; Walesky, Gunewardena et al. 2013). Because HNF4 α is a transcription factor, we investigated gene expression changes following an acute deletion of HNF4 α within the mature mouse liver. A large number of genes down-regulated by a deletion of HNF4 α are involved in hepatocyte differentiation, many of which are previously characterized targets of HNF4 α . Interestingly, we found that many of the genes that are up-regulated are involved in cell cycle progression, cell proliferation, and cancer.

One gene that we continually found up-regulated after HNF4 α deletion is epithelial cell transforming sequence 2 oncogene (*Ect2*), a member of the Dbl family of guanine nucleotide exchange factors (Fields and Justilien 2010). It is important in cell division by influencing cytokinesis, mitotic spindle assembly, and cell polarity (Tatsumoto, Xie et al. 1999; Joberty, Petersen et al. 2000; Tatsumoto, Sakata et al. 2003; Liu, Ishida et al. 2004; Kim, Billadeau et al. 2005; Ocegüera-Yanez, Kimura et al. 2005). *Ect2* is up-regulated in regenerating livers

of mice (Sakata, Rubin et al. 2000) and is up-regulated in patients susceptible to HCC recurrence (Wang, Ooi et al. 2011). *Ect2* is up-regulated over 20-fold following an acute deletion of HNF4 α and ~3-fold in HNF4 α -KO mouse liver tumors induced by the known hepatic carcinogen diethylnitrosamine, DEN (Walesky, Edwards et al. 2013). We have also characterized *Ect2* as a novel, direct target of HNF4 α by performing chromatin immunoprecipitation (ChIP) assays (Walesky, Gunewardena et al. 2013).

Gene regulation through the alteration of epigenetic modifications, in particular histone modifications, is an area of increased interest throughout the scientific community. Histone modifications are a major mechanism regulating the dynamic structure of the nucleosome influencing chromatin accessibility and gene regulation (Patel and Wang 2013). Histone acetylation facilitates chromatin remodeling to a more accessible form of DNA allowing for the binding of protein complexes, such as the RNA polymerase machinery (Zentner and Henikoff 2013).

Following the discovery of *Ect2* being a direct target of HNF4 α , we wanted to investigate potential mechanisms by which HNF4 α inhibits *Ect2* expression in normal, non-proliferating hepatocytes. One potential mechanism is that HNF4 α can bind directly to the *Ect2* promoter and facilitate the formation of a complex that influences epigenetic modifications resulting in silencing of the gene. In this study, we decided to investigate whether or not the presence of HNF4 α can affect which histone modifications are present at the promoter of *Ect2*. We found that following deletion of HNF4 α there was a switch in histone modifications from

a transcriptionally inactive to an active state with the most dramatic changes in the acetylation state of the *Ect2* promoter region. Further, we found that HNF4 α can directly interact with the histone deacetylase recruiter, SMRT, and they are both present at the *Ect2* promoter when *Ect2* expression is reduced. The current study highlights a potential mechanism of HNF4 α -dependent silencing of pro-mitogenic genes in controlling hepatocyte proliferation within the mature mouse liver.

4.3 Materials and Methods

4.3.1

Animals, Treatment, and Tissue Collection. The HNF4 $\alpha^{F1/F1}$, AlbCreERT2⁺ mice used in this study have been previously described in Chapter 2 (Walesky, Edwards et al. 2013). All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities at the University of Kansas Medical Center under a standard 12-hour light/dark cycle with access to chow and water *ad libitum*. The Institutional Animal Care and Use Committee approved all of the studies.

Three-month-old male, HNF4 $\alpha^{F1/F1}$, AlbERT2-Cre⁺ mice were treated with TAM (6 ug/mouse, intraperitoneally, referred to as HNF4 α -KO), or with vehicle alone (corn oil, intraperitoneally, referred to as Control) (Fig. 2.3.3.2). To account for changes induced by TAM, 3-month-old, HNF4 $\alpha^{F1/F1}$, AlbERT2-Cre⁻ mice were treated with TAM (6 ug/mouse, intraperitoneally, referred to as TAM Control). Mice were euthanized by cervical dislocation under isoflurane anesthesia; livers and blood were collected 7 days postinjection.

4.3.2

Western Blotting. RIPA extracts obtained from whole liver tissue were used for Western blot analysis using the described protocol (Borude, Edwards et al. 2012). The antibodies used for Western blotting include HNF4 α (1:1000; R&D Systems, Minneapolis, MN; Cat. No. PP-H1415-00) and Gapdh (1:1000; Cell Signaling, Danvers, MA; Cat. No. 2118).

4.3.3

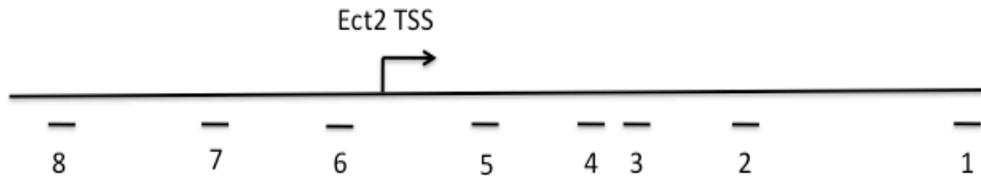
Chromatin Immunoprecipitation, ChIP. We performed ChIP in order to determine the presence of specific histone modifications within a 9 kb region surrounding the Ect2 transcriptional start site (Chr 3: 27,046,776 – 27,055,776). The ChIP protocol used was previously described in chapter 2 (Walesky, Gunewardena et al. 2013). Antibodies used for ChIP are as follows: H3K9-acetyl (10 ug; Abcam, Cambridge, MA; Cat. No. ab12179), H3K9-dimethyl (10 ug; Abcam, Cambridge, MA; Cat. No. ab 1220), H3K4-trimethyl (10 ug; Abcam, Cambridge, MA; Cat. No. ab1012), H3K27-trimethyl (10 ug; Abcam, Cambridge, MA; Cat. No. ab 6002). Primers were designed in order to detect the entire region (Fig. 4.3.3.1 and Table 4.3.3.2).

ChIP was also used in order to confirm the presence of HNF4 α and SMRT at the Ect2 promoter. Antibodies used for this ChIP experiment include HNF4 α (10 ug; R&D Systems, Minneapolis, MN; Cat. No. PP-H1415-00) and SMRT, or NCOR2 (10 ug; Abcam, Cambridge, MA; Cat. No. ab2781). Primers were designed for three putative HNF4 α binding sites within 16 kb of the Ect2 transcriptional start site estimated by the HNF4 α motif finder designed and maintained by the laboratory of Frances Sladek, PhD (Fig. 4.3.3.3 and Table 4.3.3.4). The HNF4 α motif finder can be found at <http://nrmotif.ucr.edu/NRBSScan/H4sbm.htm>.

4.3.4

Co-Immunoprecipitation. We performed a protein-protein immunoprecipitation assay in order to confirm a direct interaction between HNF4 α and the HDAC

Figure 4.3.3.1



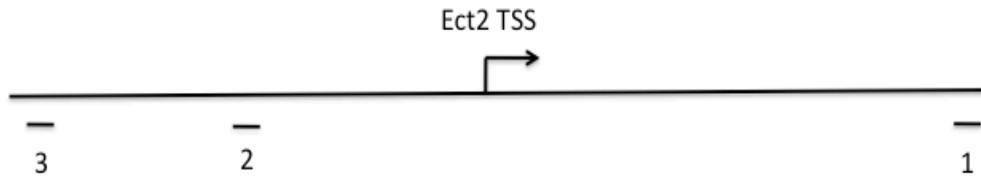
Strategy for detecting histone modifications across the Ect2 promoter region. Primers pairs were designed in order to detect specific histone modifications throughout a 9 kb region surrounding the transcriptional start site for Ect2.

Table 4.3.3.2

Site	Sequence	Product Length	Distance from TSS
1	5'-ATGTAATGGAAGGTCAGGGC-3' (Sense) 5'-TTCGTGCTATTCTCCAGTGTG-3' (Antisense)	138 bp	+5.3 kb
2	5'-AGGTAGGCTAGATGGTCAGTC-3' (Sense) 5'-TGGAGGTTTTGAGACAGGC-3' (Antisense)	103 bp	+3.3 kb
3	5'-GTTTGTCTTATCCACGGCAATC-3' (Sense) 5'-GAGGGCTGTATGAAGTTGGTC-3' (Antisense)	96 bp	+2.1 kb
4	5'-AGCTTGAACCTGTGAGATGAG-3' (Sense) 5'-TCTCCCTCTTCCCTCTCC-3' (Antisense)	73 bp	+1.8 kb
5	5'-GTAATCCGAGCACCCAGAAG-3' (Sense) 5'-ACCCCAGCATAGACTTGAATTG-3' (Antisense)	85 bp	+0.9 kb
6	5'-AGGTGTTTCTGTGGTTACCG-3' (Sense) 5'-TCTTGAAGTTGGAGGTGATGG-3' (Antisense)	88 bp	-0.4 kb
7	5'-TGAGGAAATGCTGACAGGAAG-3' (Sense) 5'- CGGAGTAGGGATTCAAAGATAGAG-3' (Antisense)	127 bp	-1.9 kb
8	5'-GCCCATACTAATCCAGACACC-3' (Sense) 5'-CACGGTCCAGTCTTGATCTTC-3' (Antisense)	75 bp	-2.8 kb

Primer sets used for detecting histone modifications across the Ect2 promoter region. Primer sequences used for chromatin immunoprecipitation of the Ect2 promoter region.

Figure 4.3.3.3



Strategy for detecting HNF4 α and SMRT binding at the Ect2 promoter region. (A) Primers pairs were designed in order to detect HNF4 α and SMRT binding at the Ect2 promoter.

Table 4.3.3.4

Site	Sequence	Product Size	Distance from TSS
HNF4 α Site 1	5'-ATGTAATGGAAGGTCAGGGC- 3' (Sense) 5'- TTCGTGCTATTCTCCAGTGTG-3' (Antisense)	138 bp	+5.5 kb
HNF4 α Site 2	5'- TCTCTCTTCCTCTCCTTCTCTTC- 3' (Sense) 5'- GAAGACAGGTTTATTGGGATGC- 3' (Antisense)	144 bp	-3.2 kb
HNF4 α Site 3	5'- TTTGAGAGTTGCCCCATAGTG-3' (Sense) 5'-GAATCTCCTAGCCCTTGTGG- 3' (Antisense)	120 bp	-5.2 kb

Primer sets used for detecting HNF4 α and SMRT binding at the Ect2 promoter region. Primer sequences used for chromatin immunoprecipitation of the HNF4 α /SMRT binding sites at the Ect2 promoter region.

recruiter, SMRT. Nuclear protein was isolated from fresh liver tissue using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific; Rockford, IL; Cat. No. 78835) following the manufacturer's protocol. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay reagents (Pierce Chemical; Rockford, IL; Cat. No. 23225). Equivalent amounts of nuclear protein (100 ug) from three BL/6 mice were used for the IP. An IP was performed using antibodies for SMRT/NCOR2 (10 ug; Abcam; Cambridge, MA; Cat. No. ab2781) and HNF4 α (10 ug; R&D Systems, Minneapolis, MN; Cat. No. PP-H1415-00).

4.3.5

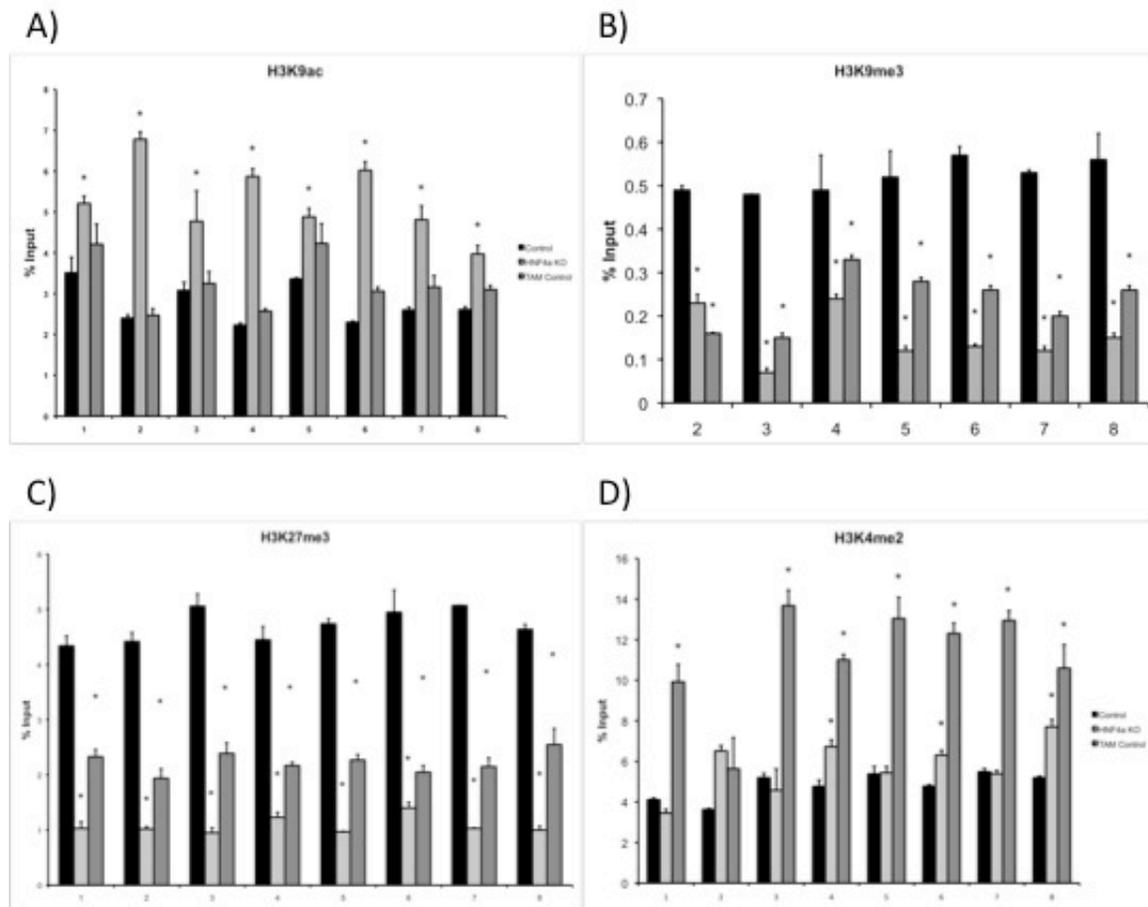
Statistical Analysis. For all experiments the results are expressed as mean \pm standard deviation. Student's t test was applied to all analyses with $P < 0.05$ being considered significant.

4.4 Results

4.4.1 Loss of HNF4 α results in a switch in the epigenetic signature at the Ect2 oncogene

We have utilized our previously published model of acute HNF4 α deletion in order to determine if a loss of HNF4 α affects the state of epigenetic marks throughout the Ect2 promoter region. Treatment of HNF4 α ^{F1/F1}, AlbERT2-Cre⁺ mice with TAM resulted in a loss of HNF4 α as demonstrated by Western blot analysis (Fig. 2.4.1.1B). The data show an ~80-90% decrease in HNF4 α protein level as compared to controls. Interestingly, deletion of HNF4 α resulted in a significant increase in the epigenetic modification H3K9ac (Fig. 4.4.1.1A), a known marker for transcriptional activation. The increase in H3K9ac can be seen throughout the entire 9 kb region we investigated. We also investigated the effect of an HNF4 α deletion on the epigenetic modifications H3K27me3, H3K4me2, and H3K9me3. These marks are typically associated with transcriptional repression. The marks H3K9me3 and H3K27me3 both followed a similar trend. The TAM control group showed a significant decrease in each mark, but a combination of TAM with a loss in HNF4 α resulted in further decrease as compared to the TAM control group (Fig. 4.4.1.1B and 4.4.1.1C). The only site that did not follow this trend was site 2 for H3K9me3. The histone mark H3K4me2 followed a different trend. The TAM control group showed a significant increase in this mark, but a combination with a loss in HNF4 α brought it back down to levels near the control (Fig. 4.4.1.1D). This was true for all sites except site 2.

Figure 4.4.1.1



Histone Modifications at the Ect2 Promoter following Deletion of HNF4α.

Changes in the histone modifications (A) H3K9ac, (B) H3K9me3, (C) H3K27me3, and H3K4me2 at the *Ect2* promoter following deletion of HNF4α. We observed an increase in the H3K9ac (A), a marker for increased transcriptional activation. We observed a decrease in the repressive marks H3K9me3 (B) and H3K27me3 (C). There was an increase in the TAM-treated sample for the mark H3K4me2 (D), which was partially blocked by a deletion of HNF4α.

4.4.2 HNF4 α interacts directly with the histone deacetylase recruiter, SMRT

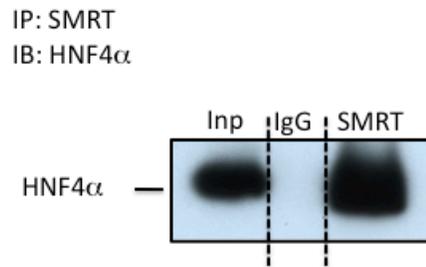
Because HNF4 α had an effect on the acetylation state of the Ect2 promoter region, we investigated whether or not HNF4 α can interact with the known histone deacetylase recruiter, SMRT. We chose to investigate SMRT because of its known function to recruit histone deacetylases and because data support the ability of SMRT to interact with HNF4 α (Ruse, Privalsky et al. 2002).

In order to show a direct interaction between HNF4 α and SMRT, we performed a protein immunoprecipitation experiment. We show that upon immunoprecipitation, SMRT is detected bound to HNF4 α via immunoblot (Fig. 4.4.2.1) suggesting a direct interaction between the two proteins in our model.

4.4.3 HNF4 α and SMRT immunoprecipitate the same region of the Ect2 oncogene

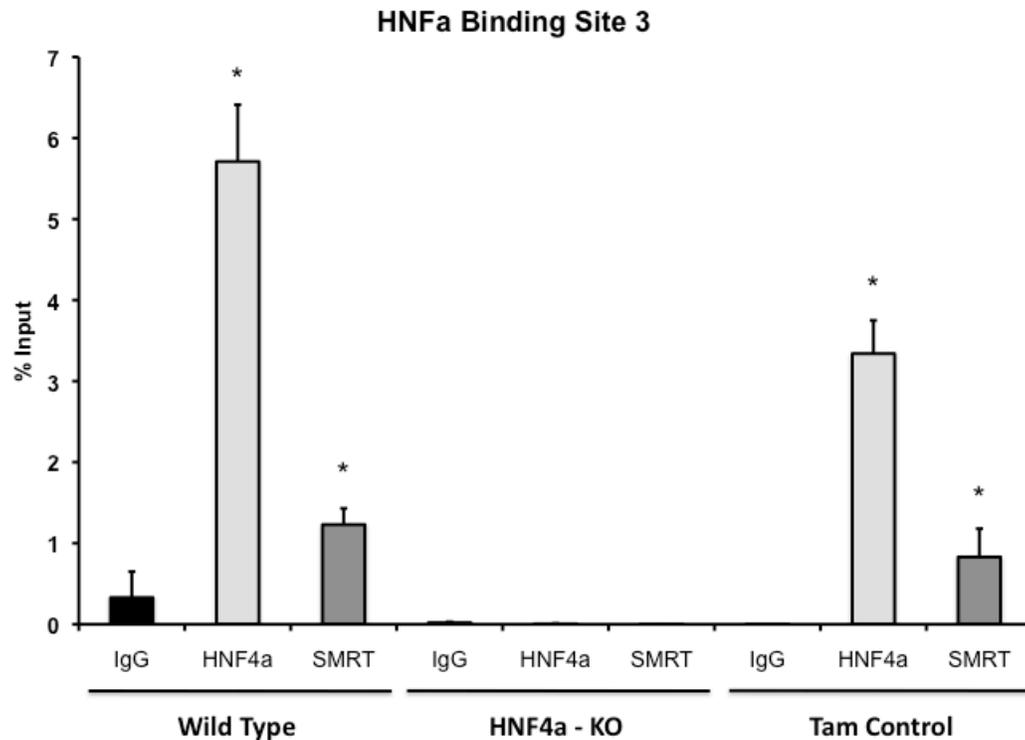
We performed ChIP in order to determine if HNF4 α and SMRT interact at the promoter region of the Ect2 oncogene. Primers were designed for three putative HNF4 α binding sites found within 16 kb surrounding the Ect2 transcriptional start site using the HNF4 α motif finder. HNF4 α and SMRT did not show significant binding at sites 1 and 2. Site 3, which is found 5.2 kb upstream of the TSS for Ect2, showed a significant amount of binding of both HNF4 α and SMRT (Fig. 4.4.3.1) in the control and TAM-control group. HNF4 α and SMRT were no longer bound to site 3 following deletion of

Figure 4.4.2.1



Direct interaction between HNF4 α and SMRT. Detection of HNF4 α by immunoblot following an immunoprecipitation of SMRT. The immunoblot suggests a direct interaction between the two proteins.

Figure 4.4.3.1



Binding of HNF4 α and SMRT to the Ect2 promoter region. Chromatin immunoprecipitation of HNF4 α and SMRT at the Ect2 promoter. HNF4 α and SMRT each show a statistically significant pull down of the HNF4 α binding site 3 region in the Wild Type and Tam Control groups. There was almost no detectable signal in the HNF4 α – KO group suggesting that HNF4 α is essential for SMRT binding to the region.

HNF4 α . Our results suggest an interaction between HNF4 α and SMRT at site 3 within normal, non-proliferating hepatocytes.

4.5 Discussion

Recent studies highlighted an important role of HNF4 α in the inhibition of hepatocyte proliferation; little is known about the mechanism by which HNF4 α accomplishes this. Loss of HNF4 α results in increased hepatocyte proliferation with an up-regulation of pro-mitogenic gene expression (Bonzo, Ferry et al. 2012; Walesky, Edwards et al. 2013; Walesky, Gunewardena et al. 2013). In this study we investigated a potential mechanism by which HNF4 α inhibits pro-mitogenic genes. We hypothesized that HNF4 α inhibits pro-mitogenic genes through the regulation of epigenetic modifications.

Acetylation neutralizes the positive charge associated with lysine residues, weakening the interaction between histones and the nucleosomal DNA. This increases the accessibility of DNA allowing for protein complexes to bind, such as the transcriptional machinery, initiating transcription (Zentner and Henikoff 2013). In our model, we believe that HNF4 α limits histone acetylation at select pro-mitogenic genes. In order to investigate this possibility, we selected a model pro-mitogenic gene that is up-regulated following deletion of HNF4 α and that exhibits a role in hepatocyte proliferation, the oncogene *Ect2*.

Ect2 is up-regulated in regenerating liver, HCC, and in actively proliferating hepatocytes within our HNF4 α -deletion model (Tatsumoto, Xie et al. 1999; Sakata, Rubin et al. 2000; Tatsumoto, Sakata et al. 2003; Liu, Ishida et al. 2004; Kim, Billadeau et al. 2005; Ocegüera-Yanez, Kimura et al. 2005; Fields and Justilien 2010; Wang, Ooi et al. 2011; Walesky, Edwards et al. 2013; Walesky, Gunewardena et al. 2013). We have previously shown, using ChIP,

that HNF4 α can bind directly to the Ect2 promoter region (Walesky, Gunewardena et al. 2013). Because Ect2 is up-regulated in our HNF4 α -deletion model and it is a direct target of HNF4 α , we thought it to be a great model gene to use to test our hypothesis.

We investigated four histone modifications in total; 3 modifications typically associated with transcriptional repression: histone H3 lysine 4 tri-methylation (H3K4me3), histone H3 lysine 27 tri-methylation (H3K27me3), and histone H3 lysine 9 di-methylation (H3K9me2); as well as one modification typically associated with transcriptional activation: histone H3 lysine 9 acetylation (H3K9ac). Interestingly, a loss of HNF4 α caused a significant increase in histone H3K9 acetylation.

The results we obtained for the histone methylation marks we investigated were not as easy to interpret. The histone marks H3K9me3 and H3K27me3 both followed a similar trend. There was a large TAM-dependent effect. TAM alone was enough reduce these marks ~50%. It is interesting, though, that TAM in combination with a loss in HNF4 α caused a further significant decrease in each of these marks. These data suggest that HNF4 α is increasing methylation on these residues promoting transcriptional repression. The histone mark H3K4me2 also showed a significant TAM-dependent effect in that the TAM-control group showed a large increase in this mark. In combination with a loss of HNF4 α , this modification dropped to levels near the control. It is possible that HNF4 α has a role in influencing each of these marks in hepatocytes, but a different model system would need to be used to better interpret its effect. At this point we are

confident in our conclusion regarding histone acetylation, but more work needs to be done on the influences of HNF4 α on histone methylation in order to reach a more solid conclusion.

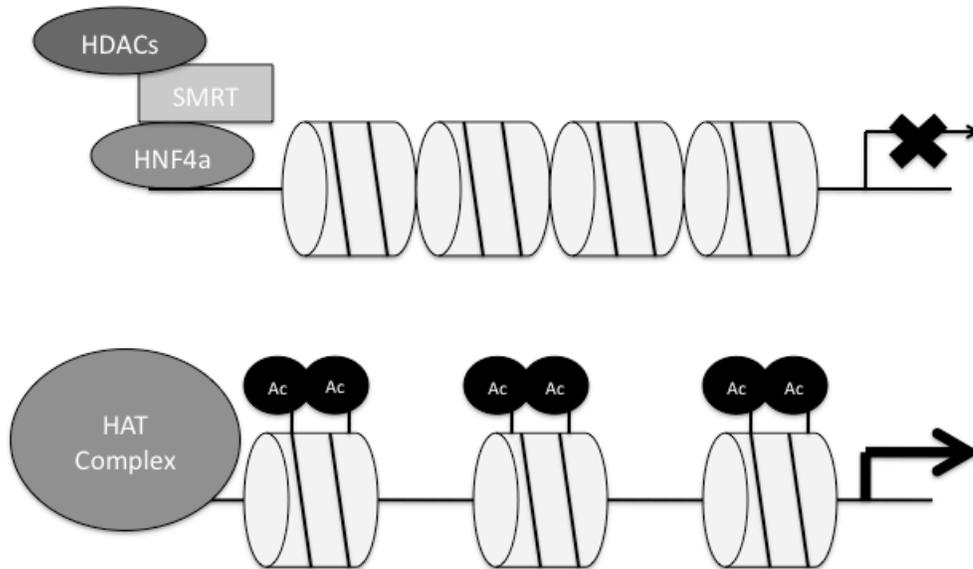
Because of our findings, we wanted to investigate if HNF4 α can bind to the Ect2 promoter in normal, non-proliferating hepatocytes and potentially recruit histone deacetylases in order to limit histone acetylation as a means of transcriptional repression. We decided to investigate the histone deacetylase recruiter, SMRT, because of past evidence that it can directly interact with HNF4 α (Ruse, Privalsky et al. 2002; Ungaro, Teperino et al. 2010). We performed protein immunoprecipitation and found that SMRT and HNF4 α the two proteins potentially interact directly in vivo.

We next wanted to determine if HNF4 α and SMRT interact at the Ect2 promoter region. In order to achieve this, we performed ChIP for HNF4 α and SMRT at putative HNF4 α binding sites at the Ect2 promoter. We utilized the HNF4 α motif finder, developed and maintained by Dr. Frances Sladek's laboratory (<http://nrmotif.ucr.edu/fuzzhtmlform.html>), in order to identify putative HNF4 α binding sites within the Ect2 promoter. The HNF4 α motif finder identified three putative HNF4 α binding sites. After performing ChIP for HNF4 α and SMRT, we found that HNF4 α binding site 3 (8 kb upstream of the TSS for Ect2) has a significant amount of HNF4 α and SMRT bound at the same time.

We conclude that HNF4 α and SMRT are forming a complex to potentially recruit HDACs in order to keep the Ect2 gene hypoacetylated and in a repressed state. Following deletion of HNF4 α , HNF4 α is no longer bound to the Ect2 promoter

disrupting the formation of the HNF4 α :SMRT:HDAC complex allowing for hyperacetylation of Ect2, chromatin remodeling, and transcriptional activation (Fig. 4.5.1). These data provide a potential mechanism by which HNF4 α may be inhibiting pro-mitogenic gene expression, keeping hepatocytes in a quiescent state. Elucidating the mechanism by which HNF4 α controls pro-mitogenic genes is important because it provides an avenue by which this mechanism may be targeted in influencing hepatocyte proliferation in specific liver pathologies such as hepatocellular carcinoma.

Figure 4.5.1



Model for HNF4 α -dependent transcriptional inhibition of Ect2. This study suggests a potential model for the inhibition of select pro-mitogenic genes dependent on the presence of HNF4 α . HNF4 α can bind to the promoter region of select genes resulting in the recruitment of the histone deacetylase recruiter, SMRT. The presence of SMRT results in gene silencing by promoting a closed conformation of chromatin through the inhibition of histone acetylation (top panel). When HNF4 α is not present at the gene, SMRT is no longer recruited which allows for the acetylation of histones and chromatin remodeling. This results in an open, more accessible state of the chromatin, allowing for transcription to take place (lower panel).

Chapter 5

Conclusions and Future Directions

HNF4 α is the master regulator of hepatic differentiation, but recent evidence suggests a novel role for HNF4 α in the inhibition of hepatocyte proliferation. Our studies outlined in this dissertation were designed to determine the role of HNF4 α on proliferation of mature hepatocytes, the role of HNF4 α in regulation of pathogenesis of liver cancers, and to characterize the potential mechanisms involved.

Generation of Novel Models of HNF4 α Deletion: Because of the importance of HNF4 α in liver development, a true HNF4 α knockout results in embryonic lethality. Therefore, the Cre-Lox system under the albumin promoter was previously utilized to create a liver-specific KO of HNF4 α (Hayhurst, Lee et al. 2001). This model provided some early evidence that lack of HNF4 α results in increased hepatocyte proliferation supported by an increase in liver-to-body weight ratio present in HNF4 α mice, which is observed in many models where hepatocyte proliferation is increased.

One problem with approaching the question of increased hepatocyte proliferation using this model is that the mice display severe hepatic metabolic disruption and die by 6-8 weeks of age. Another problem with this model is that the deletion of HNF4 α happens early in life, when the liver is still growing and differentiating. Many gene changes are occurring throughout this time period, which would make the elucidation of the mechanism extremely difficult. One approach for the question of whether HNF4 α has a direct effect on hepatocyte

proliferation is to use an inducible KO model. Therefore, we developed two inducible HNF4 α KO models. We utilized AAV8 to deliver the Cre recombinase or tamoxifen to induce Cre expression.

In each of these models we found that deletion of HNF4 α after the liver has fully matured resulted in hepatomegaly and steatosis. We further showed that hepatomegaly is due to hyperplasia as seen with an increase in PCNA staining, Ki-67 staining, and an observed increase in liver-to-body weight ratio. Taken together, these studies provide evidence that the presence of HNF4 α inhibits hepatocyte proliferation.

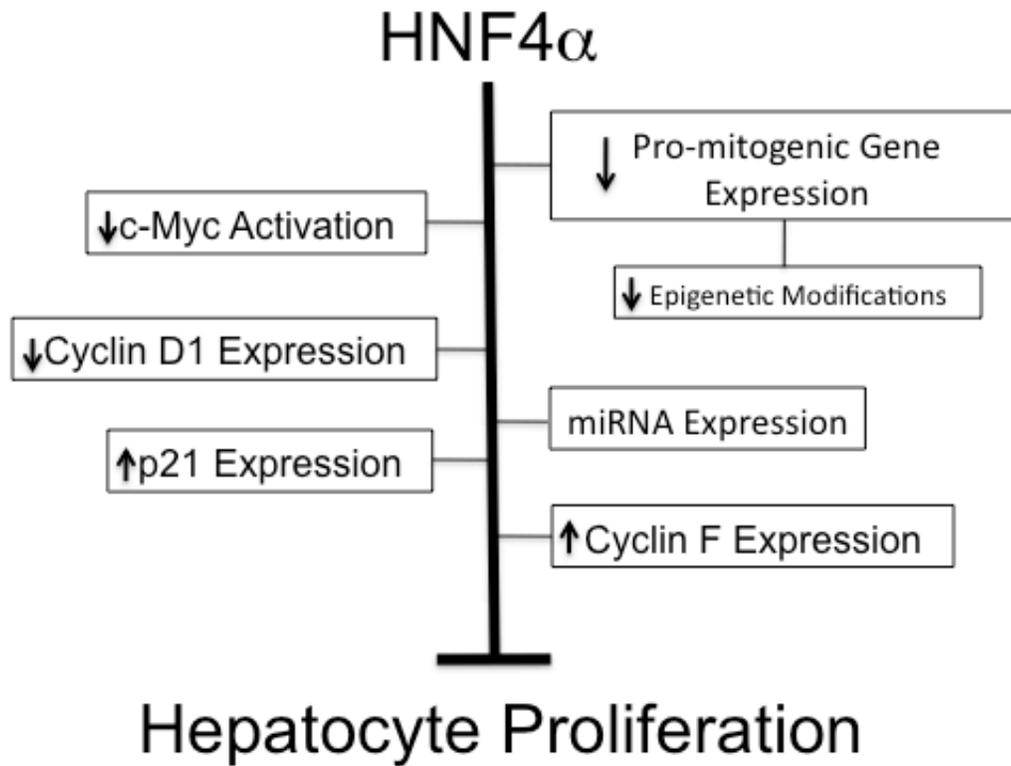
Role of HNF4 α in Liver Cancer Pathogenesis: We also looked at the effect of deletion of HNF4 α in a chemical-induced model of HCC (Walesky, Edwards et al. 2013). In this study, we treated mice with the known hepatic carcinogen diethylnitrosamine, or DEN, at post-natal day 15 in order to transform hepatocytes. At 8 months of age, we treated the mice with tamoxifen to delete HNF4 α and waited two more months to see how a loss of HNF4 α would effect the progression of the already initiated HCC. We found that a loss of HNF4 α in combination with DEN caused a large expansion in tumor number and tumor size with an almost 2-fold increase in liver-to-body weight ratio. We also highlighted the up-regulation of Cyclin D1 and the known oncogene, c-Myc, within the HNF4 α -depleted tumors.

Mechanisms of Increased Cell Proliferation after HNF4 α Deletion: Recent data suggest the involvement of multiple mechanisms by which HNF4 α may be inhibiting hepatocyte proliferation (Fig. 5.1). Because HNF4 α functions as a transcription factor, we looked at global gene expression changes by microarray analysis and RNA-sequencing (Chapter 2). We made the observation that many of the genes which are down-regulated are genes involved with differentiation, many of which are known HNF4 α target genes. Interestingly, many of the genes which are up-regulated are genes involved with cell proliferation, cell cycle progression, and cancer.

It is probable that HNF4 α 's influence on hepatocyte proliferation is due to multiple signaling pathways being affected because of the large number of targets HNF4 α has within the liver. It is not clear whether this effect is due to a direct or indirect mechanism. We first approached this question by comparing global gene expression changes to HNF4 α binding data (RNA-Seq vs. ChIP-seq). We found that nearly half of the genes that changed following a deletion of HNF4 α have a putative HNF4 α binding site. Further, we confirmed three of these binding sites by ChIP: *Ect2*, *Osgin1*, and *Hjurp*.

Mechanisms of HNF4 α -mediated Gene Repression: HNF4 α is generally thought to be a positive transcriptional activator (Sladek, Zhong et al. 1990; Gonzalez 2008). Our data suggest that HNF4 α may be functioning to repress genes involved in hepatocyte proliferation. A possible mechanism for the repression of these genes is through decrease in histone acetylation mediated

Figure 5.1



Schematic of possible mechanisms by which HNF4α may be inhibiting hepatocyte proliferation.

via recruitment of histone deacetylases. Acetylation of histones is thought to be a signal for the activation of transcription by influencing the conformation of chromatin promoting an open state that allows for the binding of transcription factors and the polymerase machinery. HNF4 α may be silencing these genes by recruiting histone deacetylases and keeping the chromatin in a closed state. HNF4 α has been shown in the past to interact with the known histone deacetylase recruiter, SMRT (Ruse, Privalsky et al. 2002; Ungaro, Teperino et al. 2010). Ungaro, et al. were the first to show that HNF4 α may be using this mechanism to inhibit the transcription of the gene *PED*. They showed that HNF4 α recruits SMRT to the *PED* promoter region leading to histone deacetylation and remodeling of chromatin to a heterochromatic state (Ungaro, Teperino et al. 2010).

In our studies, we confirmed a switch in the acetylation state of the promoter region of *Ect2* (Chapter 4), a novel target gene of HNF4 α , which we identified in our initial studies. We found that a deletion of HNF4 α results in an increase in acetylation throughout the *Ect2* promoter. We also found that HNF4 α can bind to the HDAC recruiter, SMRT, and that HNF4 α and SMRT are both found at the *Ect2* promoter when the acetylation state of the promoter is low. Therefore, we developed an HNF4 α -dependent model for the recruitment of HDACs to pro-mitogenic genes.

Other potential mechanisms involve activation of c-Myc, change in miRNAs, and post-translational modifications of HNF4 α , all of which need further investigation. c-Myc is an oncogene primarily associated with cell proliferation. It

is up-regulated during development and in proliferating cells, and is commonly up-regulated in human tumors giving it the designation as an oncoprotein. Elevated levels of c-Myc are known to cause cell cycle progression (Eilers, Schirm et al. 1991; Amati, Alevizopoulos et al. 1998) and cellular immortalization (Penn, Laufer et al. 1990). Hwang-Versleus and Sladek first showed an interaction between c-Myc and HNF4 α for control of the p21 promoter (Hwang-Versleus and Sladek 2008). In these studies they showed that HNF4 α can up-regulate p21, a known inhibitor of cell proliferation, by directly binding to specific regions at the p21 promoter. Further, they provide evidence that HNF4 α and c-Myc can interact and compete for binding at the p21 promoter.

We found a significant increase in c-Myc in the liver specific HNF4 α KO mice. RNA-Seq studies revealed that c-Myc-regulated gene network, which involves many pro-mitogenic genes such as *Fus*, *Set*, *Ccnb1*, *Ccnb2*, *Rrm2*, and *Myc*, was highly activated upon deletion of HNF4 α . Further studies in the DEN-induced HCC model revealed a correlation between a depletion of HNF4 α and an increase in c-Myc along with an increase in Cyclin D1 in the tumors. These data help to support the c-Myc/Cyclin D1/HNF4 α loop proposed by Sladek et al. as a possible mechanism controlling proliferation and differentiation within hepatocytes (Sladek 2012). Recent work by Hanse, et al. (Hanse, Mashek et al. 2012) provided evidence on the effect Cyclin D1 has on HNF4 α and its transcriptional activity at select target genes. They found that Cyclin D1 can inhibit HNF4 α 's binding at select genes causing a down-regulation in gene expression. They conclude that this is due to a direct interaction between Cyclin

D1 and HNF4 α . Dr. Sladek argues that their data supports the hypothesis of a Myc/Cyclin D1/HNF4 α loop.

Hatziapostolou, et al. demonstrated that an HNF4 α -driven miRNA feedback loop could be the potential mechanism for inhibiting proliferation within the liver. They show a loop consisting of miR-124, IL6R, STAT3, miR-24, and miR-629 contribute to maintain a transformed phenotype in the liver (Hatziapostolou, Polytarchou et al. 2011). In their studies, they use non-transformed, immortalized human hepatocytes and provide evidence for a role of HNF4 α in inhibiting proliferation of the cells. Following disruption of HNF4 α , they found that the hepatocytes were transformed, had increased invasiveness, and could promote tumor formation in immunodeficient mice. The tumors showed continued suppression of HNF4 α at day 55 suggesting to them that the inhibition of HNF4 α may initiate a feedback loop that continuously suppresses the gene. They identified two miRNAs, miR-24 and miR-629, which are direct regulators of HNF4 α expression. They found that miR-24 and miR-629 can be activated by STAT3, which is in turn activated by IL6R. They further characterize miR-124 as an HNF4 α target that inhibits IL6R.

We investigated this mechanism in our model. We could not corroborate these results at the level of IL6R and STAT3 up-regulation of activation. This could however be a limitation of the models used in either of the experiments, or a species dependent mechanism.

Recently, Yin, et al. (Yin, Wang et al. 2013) identified that HNF4 α can induce the expression of the miR-379-656 cluster in the DLK-DIO3 region and

regulate the transcription of this region. Further, they found that miR-134, a key member of this cluster, suppresses HCC cell proliferation through a KRAS-dependent mechanism. They also showed that low miR-134 levels correlate with low HNF4 α levels in highly aggressive human HCCs. We have not investigated this using our HNF4 α KO model but plan to do this in future studies.

Post-translational modification of proteins is an efficient biological signal for alteration of proteins playing a key role in functions such as protein activation/deactivation, protein localization, protein transportation, and transcriptional activity (Witze, Old et al. 2007; Sims and Reinberg 2008). Little is known about post-translational modification of HNF4 α and the involved functions. A recent study by Yokoyama, et al. (Yokoyama, Katsura et al. 2011) has identified 8 modification sites and created point mutations of the sites to investigate any role in transcriptional activation. In their study they identified phosphorylation (S142, T166, S167, T432, and S436), ubiquitination (K234 and K307), and acetylation (K458) sites. Following the introduction of point mutations at those sites, they found that a point mutation of S142D caused a down-regulation of HNF4 α transcriptional activity through a reduction in DNA-binding, which was consistent with previous studies (Viollet, Kahn et al. 1997). They also found that the point mutation K458R, which leads to an acetylation negative HNF4 α , caused a significantly higher transcriptional activity. Conversely, the K458Q acetylation-mimicking mutant showed weak transcriptional activity.

HNF4 α is a homodimeric transcription factor which binds to a response element with the half-site of AGGTCA with a one base pair spacer, known as a

DR1 binding site (Wallerman, Motallebipour et al. 2009; Fang, Mane-Padros et al. 2012). It has been shown that post-translational modifications of HNF4 α can cause a reduction in DNA-binding capacity (Viollet, Kahn et al. 1997). Another study, by Ktistaki, et al. (Ktistaki, Ktistakis et al. 1995) showed that tyrosine phosphorylation can affect the subnuclear localization, DNA-binding, and transactivation of HNF4 α . Also Li, et al (Li, Salisbury-Rowswell et al. 2002) showed that phosphorylation of HNF4 α can reduce transcriptional activity by reducing DNA-binding through a JAK2-dependent mechanism.

It is possible that post-translational modifications can also influence the recruitment of co-activators and co-repressors in order to modify the transcriptional influence HNF4 α has on genes in which it is bound. One possibility is that a given post-translational modification may recruit co-activators that further recruit the transcription machinery; whereas, another post-translational modification may inhibit this recruitment. As mentioned previously, a new hypothesis is that HNF4 α may be functioning to recruit co-factors that influence the conformation of chromatin. It is possible that post-translational modifications are influencing which co-factors are being recruited, either co-activators or co-repressors, by altering protein-protein interactions between its binding partners.

Significance of findings: The recent evidence that HNF4 α inhibits proliferation of hepatocytes, as well as other cell types throughout the body, has highlighted it as a potential target for drug development/target and/or its use as a prognostic

marker. HNF4 α is a member of the nuclear receptor superfamily of ligand-dependent transcription factors. For a long time, HNF4 α was classified as an orphan nuclear receptor, but recent evidence has shown that fatty acids, such as linoleic acid, can bind in the ligand-binding domain (Yuan, Ta et al. 2009). However, the researchers saw a moderate influence on HNF4 α transcriptional activity when linoleic acid was not present. Still, this is some of the first evidence suggesting that a molecule can bind reversibly in the ligand-binding pocket of HNF4 α giving confidence that it may be able to be targeted therapeutically.

Further work on how HNF4 α is targeting genes for promotion or inhibition will be needed in order to selectively target genes in a given disease state. It would be beneficial to inhibit pro-mitogenic genes by activating or up-regulating HNF4 α in a disease state such as HCC in order to inhibit proliferation and promote differentiation. This could be beneficial by slowing tumor growth and making tumors less invasive. In contrast, promoting pro-mitogenic gene expression by inhibiting HNF4 α in patients with acute liver failure may aid in the regenerative response. Since HNF4 α functions to influence many genes throughout the genome, adverse effects are likely. This is why understanding the mechanisms by which HNF4 α is controlling transcription is of utmost importance prior to development of HNF4 α -targeted therapeutic strategies.

Further, it is highly likely that HNF4 α could be used as a prognostic marker for diseases such as HCC. We are currently developing an “HNF4 α gene signature” that can be utilized to characterize gene expression in patients that may have a deregulation in HNF4 α . It is probable that there is a population of

patients that have near normal levels of HNF4 α protein that may not be functioning in a proper manner to inhibit pro-mitogenic gene expression. We believe that looking at an end-point, such as the expression of a particular set of HNF4 α -dependent genes, would allow clinicians to identify these patients and better diagnose their disease. This could allow for the identification of patients that may require a more aggressive treatment strategy.

In closing, we have established a novel function for HNF4 α in the inhibition of hepatocyte proliferation. We hope that our work has provided a stepping-stone for future understanding of these mechanisms.

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