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Increased Expression of Hepatocyte Nuclear Factor 6 Stimulates Hepatocyte Proliferation during Mouse Liver Regeneration

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Abstract

Background & Aims—The Hepatocyte Nuclear Factor 6 (HNF6 or ONECUT-1) protein is a cell-type specific transcription factor that regulates expression of hepatocyte-specific genes. Using hepatocytes for Chromatin Immunoprecipitation (ChIP) assays, the HNF6 protein was shown to associate with cell cycle regulatory promoters. Here, we examined whether increased levels of HNF6 stimulate hepatocyte proliferation during mouse liver regeneration.

Methods—Tail vein injection of adenovirus expressing the HNF6 cDNA (AdHNF6) was used to increase hepatic HNF6 levels during mouse liver regeneration induced by partial hepatectomy, and DNA replication was determined by Bromodeoxyuridine incorporation. Cotransfection and ChIP assays were used to determine transcriptional target promoters.

Results—Elevated expression of HNF6 during mouse liver regeneration causes a significant increase in the number of hepatocytes entering DNA replication (S-phase) and mouse hepatoma Hepa1-6 cells diminished for HNF6 levels by siRNA transfection exhibit a 50% reduction in S-phase following serum stimulation. This stimulation in hepatocyte S-phase progression was associated with increased expression of the hepatocyte mitogen Tumor Growth Factor α (TGF α) and the cell cycle regulators Cyclin D1 and Forkhead Box m1 (Foxm1) transcription factor. Cotransfection and ChIP assays show that TGF α , Cyclin D1, and HNF6 promoter regions are direct transcriptional targets of the HNF6 protein. Co-immunoprecipitation assays with regenerating mouse liver extracts reveal association between HNF6 and Foxm1 proteins and cotransfection assays show that HNF6 stimulates Foxm1 transcriptional activity.

Conclusion—These mouse liver regeneration studies show that increased HNF6 levels stimulate hepatocyte proliferation through transcriptional induction of cell cycle regulatory genes.

Abbreviations

HNF6, Hepatocyte Nuclear Factor 6; OC-1; ONECUT 1; Foxm1, Forkhead Box m1; TGF α , Tumor Growth Factor α ; AdHNF6, adenovirus expressing HNF6; AdLacZ, adenovirus expressing β -Galactosidase; ChIP, Chromatin Immunoprecipitation; BrdU, Bromodeoxyuridine; S-phase, DNA replication; PHx, partial hepatectomy; Co-IP, co-immunoprecipitation; siRNA, small interfering RNA; GH, growth hormone; CREB, cAMP Responsive Element Binding protein; CBP, CREB Binding Protein; CDKs, Cyclin-dependent kinases; CDKI proteins, CDK inhibitor proteins; RPA, RNase protection assays

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Introduction

Liver regeneration induced by two thirds partial hepatectomy (PHx) results in synchronous induction of hepatocyte proliferation through collaborative stimulation between the hepatocyte mitogens Hepatocyte Growth Factor (HGF) and Tumor Growth Factor α (TGF α) and the cytokine Interleukin 6¹⁻³. Activation of the Ras-Mitogen Activated Protein Kinase (MAPK) signaling pathway drives cell cycle progression by temporal expression of Cyclin regulatory subunits, which activate their corresponding Cyclin-dependent kinases (CDKs) through complex formation⁴. Progression into DNA replication (S-phase) requires phosphorylation of the Retinoblastoma (RB) protein by either the Cdk4/Cdk6 proteins in complex with Cyclin D or Cdk2 in complex with Cyclin E⁴. Hyper-phosphorylated RB dissociates from the E2F transcription factor and alleviates inhibition of E2F to allow transcriptional stimulation of Sphase promoting genes. Earlier expression of Cyclin D1 is associated with stimulating hepatocyte DNA replication in both rodent liver regeneration models and primary hepatocyte cultures⁵⁻⁷. Stimulation of the Cdk1-Cyclin B complex and expression of Polo-like Kinase I and Aurora kinases are required for mitotic progression by phosphorylating protein substrates essential for orchestrating mitosis^{4, 8, 9}. In addition to assembly with a cyclin regulatory subunit, activation of CDK kinase activity requires CDK dephosphorylation by the Cdc25 protein phosphatase family in which Cdc25A activates Cdk2 during G1/S transition and both Cdc25B and Cdc25C activate Cdk1 for progression into mitosis¹⁰. The CIP/KIP family of CDK inhibitor (CDKI) proteins p21^{Cip1} and p27^{Kip1} also negatively regulates CDK activity through protein complex formation^{4, 11}. To facilitate S-phase progression, the CDKI proteins are phosphorylated by the Cdk2-Cyclin E complex and are subsequently targeted for degradation by the ubiquitin mediated proteasome pathway^{12, 13}.

The mammalian Forkhead Box (Fox) family of transcription factors consists of more than 50 proteins¹⁴ that share homology in the winged helix DNA binding domain^{15, 16}. Expression of Foxm1 is found in all proliferating mammalian cells and tumor derived cell lines and its expression is extinguished in terminally differentiated cells that exit the cell cycle¹⁷⁻²⁰. Transcription of the mouse *Foxm1* locus results in three differentially spliced mRNAs that are almost identical in sequence, but differ by the addition of two small exons: the Foxm1b isoform (HFH-11B or human FoxM1b) contains no additional exons, while the Foxm1c (Trident, WIN, or MPP2) and Foxm1a (HFH-11A) isoforms contain one or two additional exons, respectively¹⁸⁻²¹. During mouse liver regeneration, expression of the Foxm1 transcription factor is induced in mid-G1 phase of the cell cycle and its expression continues during S-phase and mitosis^{20, 22}. Liver regeneration studies with mice in which the Albumin promoter-enhancer Cre Recombinase (Alb-Cre) mediated conditional deletion of the *Foxm1* LoxP/LoxP (fl/fl) targeted allele in adult hepatocytes demonstrated that *Foxm1* is required for high levels of regenerating hepatocyte DNA replication and is essential for mitosis²³. The Foxm1 protein was shown to be essential for diminishing nuclear accumulation of CDK inhibitor (CDKI) proteins p21^{Cip1} and p27^{Kip1} and for transcription of Cdc25B phosphatase required for activating Cdk1^{23, 24}. Moreover, Alb-Cre *Foxm1* $-/-$ hepatocytes fail to proliferate and are highly resistant to formation of hepatocellular carcinoma in response to a Diethylnitrosamine/Phenobarbital liver tumor induction protocol²⁴. *Foxm1* $-/-$ mouse embryos die *in utero* between 13.5 and 17.5 days of gestation due to severe defects in liver development and a failure to form intra-hepatic bile ducts²⁵. These phenotypes were associated with a 75% reduction in the number of hepatoblasts due to defective mitotic progression.

The Hepatocyte Nuclear Factor 6 (HNF6) or ONECUT 1 (OC-1) transcription factor binds to DNA as a monomer utilizing a C-terminal DNA binding domain consisting of a single Cut and Homeodomain, which is also known as the "ONECUT" DNA binding domain²⁶⁻²⁹. Recent NMR studies of the HNF6 DNA binding domain demonstrated that the HNF6 Cut domain folds into a topology homologous to the Oct-1 Pou DNA binding domain, even though there

is no sequence homology between the Cut and Pou domain sequences³⁰. Interestingly, the Pou-Homeodomain GHF1/Pit1, Brn1, Oct-2 and Oct-3 transcription factors play essential roles in stimulating cellular proliferation and regulating expression of cell-type specific genes^{31–34}. Published hepatocyte Chromatin Immunoprecipitation (ChIP) assays demonstrate that the HNF6 transcription factor occupied endogenous promoters of the cell cycle regulatory genes Cdc25A, Cdk2 and E2F1³⁵, suggesting the hypothesis that HNF6 regulates hepatocyte proliferation during liver regeneration.

Mouse genetic studies demonstrated that *Hnf6* ^{−/−} embryos fail to develop a functional endocrine pancreas, gall bladder and extra-hepatic bile ducts^{36–38}. Interestingly, similar to the Foxm1 transcription factor, HNF6 is also required for development of intra-hepatic bile ducts in the developing liver³⁶. In the adult mouse liver, HNF6 protein continues to be expressed in hepatocytes with increased levels of the HNF6 protein in the biliary epithelial cells^{36, 39}. The HNF6 transcription factor regulates the *in vivo* hepatic expression of the Glucokinase⁴⁰, Glucose Transporter 2⁴¹, Protein C⁴², and Cholesterol 7 α hydroxylase genes⁴³. HNF6 transcriptional activity requires an N-terminal STP Box that is rich in Serine, Threonine and Proline residues and the Cut-Homeodomain DNA binding domain sequences mediate recruitment of the CREB Binding Protein (CBP) histone acetyltransferase⁴⁴. Published cotransfection studies demonstrated that formation of complexes between the DNA binding domains of the HNF6 and Foxa2 transcription factors resulted in Foxa2 transcriptional activity through recruitment of the CBP coactivator protein by the HNF6 Cut-Homeodomain sequences⁴⁵.

In this current study, mice were infected with adenovirus expressing HNF6 (AdHNF6) to increase hepatic expression of HNF6 during liver regeneration. Increased hepatic levels of HNF6 significantly stimulated the number of regenerating mouse hepatocytes entering S-phase following partial hepatectomy (PHx). HNF6 mediated stimulation in hepatocyte S-phase progression was associated with increased expression of the hepatocyte mitogen TGF α and cell cycle regulators Cyclin D1, Cdk2 and the Foxm1 transcription factor. Cotransfection and ChIP assays demonstrated that TGF α , Cyclin D1 and HNF6 promoters are direct transcriptional targets of HNF6 and that combining HNF6 and FoxM1b further stimulated transcription of the TGF α promoter. We also show that HNF6 associates with Foxm1 protein to stimulate Foxm1 transcriptional activity in cotransfection assays. Furthermore, cotransfection and ChIP assays demonstrate that both Foxm1 and HNF6 proteins activate transcription of the endogenous HNF6 promoter region. These liver regeneration studies demonstrate for the first time that the liver-enriched HNF6 transcription factor is capable of stimulating proliferation of regenerating hepatocytes through transcriptional activation of cell cycle regulatory genes.

Materials and Methods

Generation, purification, and in vivo administration of recombinant adenoviruses

Generation of the adenovirus containing the CMV promoter driving expression of the mouse HNF6 cDNA (AdHNF6) and adenovirus expressing the bacterial LacZ or β -Galactosidase gene (AdLacZ) was described previously⁴¹. Recombinant adenoviruses were used to infect QBO-293 cells (Quantum Biotechnologies, Montreal, Canada) and cell lysates were harvested at 72 hours post infection (PI). Adenovirus particles were purified from this cell lysate by CsCl centrifugation and dialyzed to remove the CsCl as described previously^{46, 47}. Two days prior to the partial hepatectomy (PHx) operation, 2-month old CD-1 mice were subjected to tail vein injection of 200 μ l of PBS containing 1×10^{11} purified adenovirus particles (AdHNF6 or AdLacZ).

Partial hepatectomy surgery, immunohistochemical staining, Western blot and coimmunoprecipitation assays

Two days after infection, all the CD-1 mice were subjected to partial hepatectomy (PHx) to induce liver regeneration as described previously ^{22, 23, 48}. Three mice at each time point were sacrificed using CO₂ asphyxiation at 24, 32, 36, 40, 44 or 48 hours following PHx. An intraperitoneal (IP) injection of a PBS solution containing 10 mg/ml of 5- bromo-2'-deoxyuridine (BrdU, Sigma; 50mg/g body weight) was administered two hours prior to harvesting the remnant regenerating liver. The regenerating livers were harvested and divided into three portions: One to isolate total RNA ²², one to isolate total protein extract ⁴⁹, and one utilized for paraffin embedding ^{23, 48}. Determination of the number of hepatocytes undergoing DNA synthesis was performed by monoclonal antibody detection of BrdU incorporation (Roche) of regenerating liver (5 μ m paraffin sections) using the microwave antigen retrieval method described previously ²². Using three regenerating livers per time point, we counted the number of BrdU positive nuclei per 1000 hepatocytes to calculate the mean number of BrdU positive cells ($\pm$ SD) as described previously ²². Three regenerating liver sections per time point (36, 40, 44 and 48 hours post PHx) were stained with Hematoxylin and Eosin and examined for mitotic figures (mitosis). Hepatocyte mitosis is expressed as the mean of the number of mitotic figures found per 1000 hepatocytes $\pm$ SD as described previously ²². Adenovirus infected mice undergoing liver regeneration were given free access to food and water.

The rabbit antibody specific to the HNF6 amino terminus ⁴⁹ was used for immunohistochemical detection of paraffin embedded 5 μ m sections of regenerating liver using methods described previously ⁴¹. For Western blot analysis, 100 μ g of total liver protein ⁴⁹ were separated on SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Protran membrane (Schleicher & Schuell, Keene, NH) and then incubated with either mouse monoclonal Cyclin D1 antibody or rabbit antibodies specific to either Cdk2 (Santa Cruz), HNF6 ⁴⁹ or Foxm1 ⁵⁰. Liver β -Actin levels were used for normalization control for Western blots. For coimmunoprecipitation assays, 750 μ g of total protein extract from quiescent mouse liver (0h) or regenerating mouse liver was immunoprecipitated with HNF6 antibody and then Western blotted for FoxM1 protein using FoxM1 antibody detected by Peroxidase conjugated ImmunoPure Recombinant Protein A/G (Pierce, Rockford, IL, product # 32490). The signal from the antigen-antibody reaction was amplified by horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG (Bio-Rad, Hercules, CA), and signals were detected with Enhanced Chemiluminescence Plus (ECL-plus, Amersham Pharmacia Biotech, Piscataway, NJ). The antigen-antibody signals from Chemiluminescence were detected by either autoradiography or scanning with the Storm Phosphorimager (Amersham Pharmacia Biotech, Piscataway, NJ). Quantitation of expression levels was determined with Tiff files from scanned films by using the BioMax 1D program (Kodak).

The Glutathione-S-Transferase (GST)-FoxM1b N-terminal (N) fusion protein containing FoxM1b amino acids 1 to 138 was described previously ²⁰. The GST-FoxM1b DNA binding domain (DBD) fusion protein containing amino acids 221 to 355 was amplified from the human FoxM1b cDNA by PCR using the following primers: GST-(EcoR1) Foxm1b 221 Forward: 5'-TCTGAATTCGAGCCTTCGAGACCATCA-3' and GST (Xho1) Foxm1b 355 Reverse: 5'-TCTCTCGAGCAGTGGCTTCATCTCCG-3'. Production and isolation of GST fusion proteins from BL21 E. coli and GST fusion proteins were separated on SDS-PAGE followed by staining with Commassie Blue was described previously ⁵¹. We used *in vitro* transcription and translation (IVT) of the HNF6 Cut-Homeodomain expression plasmid to synthesize ³⁵S Methionine labeled HNF6 Cut-Homeodomain protein as described previously ⁴⁵. The binding buffer and wash buffer for the *in vitro* GST pull-down assay was performed as described

previously⁴⁵. The bound labeled HNF6 Cut-Homeodomain protein was eluted with SDS loading buffer and eluted proteins was separated on SDS-PAGE followed by autoradiography.

Isolation of total liver RNA and RNase protection assay

Total RNA was prepared from mouse liver at indicated hours post PHx using RNA-STAT-60 (Tel-Test "B" Inc. Friendswood, TX) and used for RNase protection assays with antisense { α -32P} UTP labeled probes specific to cell cycle regulatory genes as described previously²³. Twenty μ g of total liver RNA was used for RNase protection assays (RPA) with antisense { α -32P} UTP labeled RNA probes made from cyclin mCyc-1 template (BD Pharmingen; catalog # 45620P) to examine Cyclin D1 as described previously²³. RNase protection assays to measure Foxm1b mRNA levels were performed with antisense labeled RNA synthesized from the mouse Foxm1b genomic probe or with the mouse HNF6 cDNA probe as described previously^{23, 41}. The mouse Tumor Growth Factor α (TGF α) RNase protection probe (TGF α cDNA 600 to 820 nucleotides) was obtained by PCR amplification of mouse genomic DNA with the following primers: sense 5'-gcctatgacagccaacc-3' and antisense 5'-aatgtgtccactggcct-3'. The resulting TGF α PCR product was cloned into the HindIII and XbaI sites of pBlueScript plasmid (pBS, Stratagene). Radioactively labeled antisense RNA probe was synthesized with T3 RNA polymerase using a XhoI digested TGF α cDNA pBS DNA template. Quantitation of expression levels was determined with Tiff files from scanned films by using the BioMax 1D program (Kodak). Ribosomal large subunit 32 (L32) mRNA or Cyclophilin expression signals were used for normalization control between different liver samples.

Promoter Luciferase plasmids and U2OS cell cotransfection assays

The mouse -1647 to +79 Tumor Growth Factor α (TGF α) promoter region was PCR amplified from mouse DNA with the following primers: mTGF α -1647XhoI: 5'-TCTCTCGAGGCCAGATGAGACTACATA and mTGF α +79HindIII: 5'-TCTAAGCTTAGGGTGCTGCAGCCACCG and cloned into the corresponding XhoI and HindIII sites of the pGL3 basic Luciferase vector (Promega). The human -1.4 KB or -1.3 KB HNF6 promoter region was PCR amplified from human U2OS DNA with the following primers: hHNF6 -1.4kb XhoI: 5'-TCTCTCGAGAGGCCCAAAGACCAGGTT or hHNF6 -1.3kbXhoI: 5'-TCTCTCGAGTCGGGGACGCCGGGAAAA and hHNF6 +2XhoI: 5'-TCTCTCGAGACTCTGTGGCCGCGGTTC and cloned into the XhoI site of the pGL3 basic Luciferase vector (Promega). We thank Dr. Richard Pestell for providing us with the -1745 Cyclin D1 human promoter Luciferase construct⁵². Human HepG2 cells were transfected with 200 ng of either CMV-FoxM1b cDNA^{20, 53}, CMV HNF-6 cDNA^{41, 45} or CMV empty expression vectors and 1500 ng of either the Luciferase reporter constructs containing either the -1.7 Kilobase pairs (KB) mouse TGF α promoter, the -1.7 KB human Cyclin D1 promoter, -1.4 KB human HNF6 or -1.3 KB human HNF6 promoter using Fugene6 (Roche). We also performed cotransfection studies with CMV-FoxM1b and 6X Foxm1 TATA Luciferase⁵³ or -2.7 KB mouse Cdc25B promoter Luciferase²³ with increasing amounts of CMV-HNF6 expression vector. Protein extracts were prepared from transfected HepG2 cells at 24 hours following DNA transfection and the Dual-Luciferase Assay System (Promega) was used to measure Luciferase enzyme activity as described previously²³. Results are presented as mean fold induction of promoter activity $\pm$ SD from two separate experiments in triplicate, with the CMV empty value set at 1.0. Dharmacon Research designed and synthesized a 21-nucleotide siRNA duplex specific to either human or mouse HNF6 RNA containing symmetric 2-uracil (U) 3' overhangs. Sequence for siRNA specific to human/mouse HNF6 cDNA (1464 to 1482): 5'-GACGAGGGCAGCTCCAATT-3', which efficiently decreased HNF6 expression. The control siRNA was made against human HNF6 cDNA (597 to 615): 5'-GGGCTGGCCTCCATGAATA, which was unable to suppress HNF6 expression. The HNF6 siRNA duplex (100 nM) was transfected into mouse hepatoma Hepa1-6 cells using

Lipofectamine™ 2000 reagent (Invitrogen) in serum free tissue culture media following the manufacturer's protocol. Four hours after transfection, the cells were serum starved for 48 hours and then serum stimulated with DMEM media containing 10% FCS and then BrdU was added for one hour at 16 hours following serum addition and then cells were harvested to determine BrdU incorporation (DNA replication) by immunostaining.

Infection of hepatoma Hepa1-6 or HepG2 cells with recombinant adenoviruses and Chromatin Immunoprecipitation (ChIP) Assays

Mouse hepatoma Hepa1-6 cells or human hepatoma HepG2 cells (5×10^6 cells per 100-mm dish) were infected at a multiplicity of infection (MOI) of 10 infectious units per cell with either AdHNF6, AdFoxM1b or AdGFP control virus or mock infected. To infect the Hepa1-6 or HepG2 cells, Adenovirus was applied in 1 ml of complete media per 100-mm plate of sub-confluent cells and incubated for 60 minutes in a 37° C humidified incubator containing a 5% CO₂ atmosphere. Complete media was then added to the plates to a total volume of 10 ml and then 24 hours after infection the cells were processed for Chromatin Immunoprecipitation (ChIP) assays as described previously^{50, 54}. The AdHNF6 infected Hepa1-6 or HepG2 cells were also used to harvest total RNA for RNase protection (RPA) assays with HNF6 or TGF α RPA probes as described above.

For ChIP assays, infected Hepa1-6 or HepG2 cells were cross-linked in situ by addition of 37% formaldehyde (Fisher Scientific) to a final concentration of 1% (w/v) and incubated at 25° C for 10 minutes with gentle swirling. The cross linking reaction was stopped by the addition of 2.5 M glycine to a final concentration of 0.125 M followed by an additional 5 minutes of gentle swirling. Cells were washed once in a 1 X solution of 4° C sterile phosphate-buffered saline (PBS). Cells were then collected by adding 1 ml of 4° C sterile PBS containing protease inhibitors (Roche, Mannheim, Germany), scraping the cells from the dish with a razor blade, and transferring the suspended cells by pipetting them into a 1.5 ml Eppendorf tube, which was centrifuged at 2000 g for 10 minutes. The cell pellet was then resuspended in a 2 X pellet volume of SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris, pH 8.1) and placed on ice for 10 minutes.

The resulting extract was subsequently sonicated using a Misonix 600W Sonicator (Misonix Inc., Farmingdale, NY) fitted with a 3 mm stepped micro-tip for 10 pulses of 15 seconds at a power setting of 30%. Between each pulse, the extract was incubated on ice for 1 minute. At this stage, the processing of all experimental samples and total input (TI) was carried out according to the Upstate Cell ChIP assay protocol (Lake Placid, NY catalog # 17-295). For the immunoprecipitation, antibody (specific amounts as indicated) was added to the precleared and clarified sample, which was incubated at 4°C with rotation for 12–16 hours and washed according to Upstate ChIP assay protocol. The following antibodies were used in the indicated amounts: 10 μ l of rabbit antiserum specific for FoxM1 protein (amino acids 365–748), 10 μ l of rabbit antisera (Vector Laboratories) or 10 μ l of rabbit antiserum specific for HNF6 protein⁴⁹. Crosslinks were reversed on all samples, including 20% input, by addition of 100 μ l TE (1 mM EDTA, 10mM Tris-HCl, pH 7.4) containing 10 μ g of RNase A and then incubated for 15 min at 25°C. Proteinase K (10 μ g) and NaCl (4 μ l of 5M solution) was then added and samples were digested for 16 hours at 65°C. DNA was extracted from the digested samples using PCR purification columns following manufacturer's instructions (Qiagen, Maryland). We then used 2 μ l of a total of 50 μ l ChIP DNA sample in the subsequent 25 μ l Real-Time PCR reaction. The total input sample was diluted 1:10 and 2 μ l was used for Real-Time PCR (10% total input).

PCR primers and reaction conditions for ChIP assay

The primers used to amplify the following human or mouse gene promoter fragments are annotated with the binding position upstream of the transcription start site, annealing

temperature (Ta) and whether they are in the sense (S) or antisense (AS) orientation: mouse TGF α promoter: -1647S 5'-GCCAGATGAGACTACATA and -1495AS 5'-GCCCAACCAAGCAGCAAA, (Ta: 54°C); human HNF6 promoter: -1499S 5'-AAAGAAAGCCGCGGCATG and -1337AS 5'-GCCTCCAGATTTGAAAAT, (Ta: 58°C); mouse Cyclin D1 promoter: -890S 5'-CGTGTCTCACCTTTTCTTCAACG and -733AS 5'-TTTCATCTATTCCTCCTCGCTGG (Ta: 58°C). The following reaction mixture was used for all PCR samples: 1X of IQ SybrGreen Supermix (Biorad, Carlsbad, CA), 100 nM of each primer, and 2 μ l of each purified ChIP extract in a 25 μ l total volume. Reactions were amplified and analyzed in triplicate using a MyiQ Single Color Real-Time PCR Detection System (Biorad, Carlsbad, CA). Normalization was carried out using the $\Delta\Delta C_T$ method. Briefly, IP samples and total input threshold cycles (CT) for each treatment were subtracted from the CT of the corresponding serum control IP (Rabbit antisera). The resulting corrected experimental IP value was then subtracted from the corrected value for the total input ($\Delta\Delta CT$), and these values were raised to the power of two ($2^{\Delta\Delta CT}$). These values were then expressed as a relative promoter binding $\pm$ SD.

Statistical analysis

We used Microsoft Excel Program to calculate SD and statistically significant differences between samples using the Student T Test. The asterisks in each graph indicates statistically significant changes with P values calculated by Student T Test: *P < 0.05, **P $\leq$ 0.01 and P $\leq$ 0.001. P values < 0.05 were considered statistically significant.

Results

Adenovirus mediated increase in HNF6 levels stimulates hepatocyte proliferation during mouse liver regeneration

Published hepatocyte Chromatin Immunoprecipitation (ChIP) assays demonstrated that the HNF6 transcription factor associated with the endogenous promoter regions of the cell cycle regulatory genes Cdk2, E2F1, and Cdc25A³⁵. These studies suggest the hypothesis that HNF6 stimulates hepatocyte proliferation during liver regeneration. In order to test this hypothesis, we used mouse tail vein injection to infect CD-1 mouse liver with replication defective adenovirus expressing either the mouse HNF6 cDNA (AdHNF6) or the control bacterial LacZ (β -Galactosidase; AdLacZ) gene and two days later these infected mice were subjected to partial hepatectomy (PHx) as described previously⁴⁸. The remnant regenerating livers were harvested at four-hour intervals between 32 and 48 hours (h) following surgery and hepatocyte DNA synthesis was monitored by immunohistochemical staining of 5-bromo-2'-deoxyuridine (BrdU) incorporation into DNA as described previously⁴⁸. To examine expression of cell cycle genes, we also use the regenerating liver tissue to isolate total RNA for RNase protection assays (RPA) and total protein extracts for Western blot analysis. RPA with regenerating liver RNA demonstrated that AdHNF6 infected mice exhibited increased hepatic levels of HNF6 mRNA compared to AdLacZ infected control liver (Fig. 1A). Consistent with our previous studies^{39, 41}, AdHNF6 infected mice displayed increased hepatocyte nuclear levels of HNF6 protein compared to AdLacZ infected control liver as determined by HNF6 immunostaining of regenerating liver sections (Fig. 1B-I). This analysis also showed that expression of HNF6 mRNA and protein is maintained during liver regeneration in AdLacZ infected mice.

Compared to regenerating liver of AdLacZ infected mice, AdHNF6 infected mice displayed a 1.8-fold increase in the number of hepatocytes entering DNA replication (S-phase) at 40 hours following PHx as evidenced by elevated hepatocyte immunostaining of BrdU incorporation in regenerating liver sections (Fig. 2A-C). Regenerating livers of AdHNF6 infected mice also displayed a statistically significant increase in the number of cells that have incorporated BrdU at both 32 and 36 hours following PHx compared to AdLacZ infected control mice at similar

time points following PHx (Fig. 2A). In addition, AdHNF6 infected mice exhibited a significant increase in the number of regenerating hepatocytes undergoing mitosis compared to AdLacZ infected regenerating liver controls (Fig. 2D). These results suggest that increased hepatic levels of the HNF6 transcription factor stimulated the number of hepatocytes undergoing DNA replication and mitosis during mouse liver regeneration.

Regenerating livers of AdHNF6 infected mice exhibit increased expression of Cyclin D1, Cdk2, Foxm1 and Tumor Growth Factor α

RPA were performed with regenerating liver RNA from AdHNF6 or AdLacZ infected mice and antisense RNA probes specific to the proliferation-specific Foxm1 transcription factor, Cyclin D1 or the hepatocyte mitogen Tumor Growth Factor α (TGF α) gene (Fig. 3). These RPA revealed increased levels of the Foxm1b transcription factor, Cyclin D1 and TGF α mRNAs in regenerating mouse livers of AdHNF6 infected mice compared to that of AdLacZ infected mice (Fig. 3A-B). Western blot analysis with AdHNF6 infected liver extracts at 32 hours after PHx showed an increase in expression of the S-phase promoting Cyclin D1 and Cdk2 proteins compared to that found in AdLacZ infected regenerating liver controls (Fig. 3D). These results demonstrated that increased HNF6 levels in regenerating liver stimulated hepatic expression of Foxm1, Cdk2, Cyclin D1 and TGF α genes, all of which are critical for regenerating hepatocyte proliferation^{1, 6, 7, 23, 55–57}.

TGF α and Cyclin D1 promoters are direct transcriptional targets of the HNF6 and Foxm1 proteins as determined by cotransfection and ChIP assays

AdHNF6 infection of human hepatoma HepG2 cells also caused increased expression of human TGF α mRNA (Fig. 4A). We therefore searched the mouse TGF α promoter region for DNA binding consensus sequences of either the HNF6²⁹ or Foxm1²⁰ proteins and found five overlapping Foxm1 binding sites between –1561 to –1531 base pairs (bp) and two HNF6 binding sites between –1608 to –1591 bp, which were conserved with the human TGF α promoter. Published cotransfection assays demonstrated that the CMV FoxM1b expression vector could activate transcription of the –1.7 KB human Cyclin D1 promoter region⁵⁸. A potential HNF6 binding site was between –1095 to –1083 bp of the human Cyclin D1 promoter region that was conserved in the mouse Cyclin D1 promoter (–1064 to –1049 bp), yet no potential Foxm1 binding sites were found in these promoter sequences. However, published studies demonstrate that the Foxm1 (WIN) protein is also capable of recognizing HNF6 binding sequences¹⁹ and Foxm1 is therefore predicted to recognize the HNF6 binding site of the Cyclin D1 promoter region.

Based on these potential Foxm1 and HNF6 binding sites, we performed cotransfection assays in HepG2 cells with the CMV HNF6 expression vector and Luciferase reporter constructs containing either the –1.7 KB Kilobase pairs (KB) mouse TGF α promoter region or the –1.7 KB human Cyclin D1 promoter region. We also examined whether these promoter regions could be stimulated by cotransfection with the CMV FoxM1b expression vector and whether FoxM1b could provide transcriptional synergy with HNF6. Protein extracts were prepared from HepG2 cells at 24 hours following DNA transfection and promoter expression was determined in these extracts by measuring Dual-Luciferase enzyme levels as described previously⁴¹. Cotransfection of either HNF-6 or FoxM1b expression vector elicited a 4-fold increase in the TGF α promoter expression, while combining both Foxm1b and HNF6 expression vectors provided only additive transcriptional activation of the TGF α promoter region (Fig. 4B). These results suggest that HNF6 and FoxM1b provided independent transcriptional activation of the TGF α promoter region. Cotransfection of either HNF-6 or FoxM1b expression vector separately provided a 3- fold increase in the Cyclin D1 promoter expression, yet no further activation of the Cyclin D1 promoter was observed when Foxm1b and HNF6 expression vectors were combined (Fig. 4C).

The mouse TGF α promoter region contains distinct DNA binding sequences for the HNF6 protein (–1608 to –1591 bp) and the FoxM1 protein (–1561 to –1531 bp), whereas these transcription factors bind to the same DNA sequence in the mouse Cyclin D1 promoter region (–1064 to –1049 bp). We therefore used Chromatin Immunoprecipitation (ChIP) assays to examine whether HNF6 and Foxm1 proteins bound to these endogenous mouse promoter regions. For these ChIP assays, mouse hepatoma Hepa1-6 cells were mock infected or infected with AdHNF6 or adenovirus expressing Green Fluorescent Protein (AdGFP). To examine FoxM1 binding to the Cyclin D1 promoter by ChIP assays (Fig. 5D), we also infected Hepa1-6 cells with AdHNF6 or AdFoxM1b (AdM1b; adenovirus expressing human FoxM1b) separately or together. At 24 hours after infection the chromatin was cross-linked, sonicated to DNA fragments of 500 to 1000 nucleotides in length, and then processed for quantitative ChIP assays^{50, 54}. The cross-linked and sonicated mouse chromatin was immunoprecipitated (IP) with antibodies specific to either HNF6 protein or FoxM1 protein or with rabbit antisera (control) and the amount of promoter DNA associated with the IP chromatin was quantitated by Real-Time PCR with primers specific to either the –1647 to –1495 bp mouse TGF α promoter region or the –890 to –733 bp mouse Cyclin D1 promoter region. This quantitative ChIP assay revealed that the HNF6 protein bound to the Hepa1-6 mouse TGF α and Cyclin D1 promoter regions and that AdHNF6 infection significantly increased HNF6 association with these endogenous promoter regions (Fig. 5A–B). This result is consistent with increased expression of the TGF α and Cyclin D1 genes in regenerating livers of AdHNF6 infected mice and hepatoma cell lines (Fig. 3 and 4A). These quantitative ChIP assays also revealed that Foxm1 protein bound to the endogenous TGF α promoter region, but increasing HNF6 levels did not stimulate FoxM1 binding to the endogenous TGF α promoter region (Fig. 5C). These results suggest that binding of FoxM1 and HNF6 are independent on the endogenous TGF α promoter region, a finding consistent with additive transcriptional activation of the TGF α promoter region by HNF6 and FoxM1b in cotransfection assays. In contrast, quantitative ChIP assays with Hepa1-6 cells infected with AdHNF6 stimulated FoxM1 binding to the endogenous mouse Cyclin D1 promoter region and increasing both FoxM1b and HNF6 levels further increased recruitment of FoxM1 to the endogenous Cyclin D1 promoter region (Fig. 5D). These results suggest that the FoxM1 and HNF6 transcription factors may interact and stimulate their recruitment to the same binding site in the endogenous Cyclin D1 promoter region.

Association of the HNF6 and Foxm1 protein results in stimulation of Foxm1 transcriptional activity

The previous cotransfection and ChIP assays suggested the hypothesis that the HNF6 and Foxm1 transcription factors may functionally interact during liver regeneration. In order to test this hypothesis, we performed co-immunoprecipitation (Co-IP) assays with protein extracts from quiescent mouse liver (0h) that lack detectable expression of Foxm1 protein or regenerating mouse liver isolated at 40 hours following PHx (40h), which expresses abundant levels of Foxm1 protein (Fig. 6A; Foxm1 Input compare 0h and 40h lanes). Quiescent or regenerating mouse liver extracts were Co-IP with the HNF6 antibody and then the IP proteins were subjected to Western blot analysis with the Foxm1 C-terminal Antibody. This Co-IP experiment demonstrated that HNF6 and Foxm1 proteins formed a stable complex in liver extracts from regenerating liver but not from quiescent liver, which does not express the Foxm1 protein (Fig. 6A; IP:HNF6 Ab). We previously showed that the FoxA2 and HNF6 proteins associated with each other through their DNA binding domain (DBD) sequences⁴⁵. To determine whether the HNF6 and FoxM1b proteins associated through their DBD sequences *in vitro*, we performed pull-down experiments with Glutathione-S-Transferase (GST) FoxM1b fusion proteins and ³⁵S Methionine labeled HNF6 Cut-Homeodomain DBD protein synthesized by *in vitro* transcription and translation (IVT). The radioactively labeled HNF6 DBD protein was utilized for binding reactions with GST alone, GST-FoxM1b N-terminal (N; amino acids 1-138) or GST-FoxM1b DBD (amino acids 221 to 355) fusion proteins (Fig. 6B;

right panel) immobilized on Glutathione-Sepharose beads as described previously⁴⁵. These GST-FoxM1b pull-down assays demonstrated that labeled HNF6 Cut-Homeodomain DBD protein bound efficiently to the GST-FoxM1b DBD fusion protein but not to the GST alone or to the GST-FoxM1b N-terminal fusion protein (Fig. 6B, left panel). Taken together, these studies demonstrate that the proliferation-specific Foxm1 transcription factor associates with the HNF6 protein in regenerating mouse liver and *in vitro* through their DNA binding domain sequences.

In order to examine the functional significance of the HNF6 and Foxm1 protein interaction we performed cotransfection studies in HepG2 cells or osteosarcoma U2OS cells with CMV HNF6 and CMV FoxM1b expression constructs and the Foxm1 reporter gene 6X Foxm1 TATA Luciferase plasmid⁵³. Cotransfection of CMV-HNF6 expression vector could not stimulate expression of the 6X Foxm1 TATA Luciferase reporter gene in either HepG2 or U2OS cells (Fig. 6C–D). Cotransfection of CMV FoxM1b expression vector showed only slight stimulation of the 6X Foxm1 TATA Luciferase reporter gene in HepG2 cells, whereas FoxM1b provided a 25-fold stimulation of this Foxm1 reporter gene in U2OS cells (Fig. 6C–D). However, increasing concentration of the HNF6 expression vector in HepG2 or U2OS cell cotransfection assays stimulated FoxM1b's ability to activate expression of its reporter gene (Fig. 6C–D). Likewise, cotransfection of both HNF6 and FoxM1b expression vectors in U2OS cells stimulated FoxM1b's ability to activate transcription of the –2.7 KB Cdc25B promoter Luciferase gene (Fig. 6E), which is a known transcriptional target gene of Foxm1²³. Taken together, these studies suggest that formation of the HNF6-FoxM1b protein complex stimulated FoxM1b transcriptional activity in cotransfection assays.

HNF6 and Foxm1 proteins collaborate to activate transcription of the HNF6 promoter region

RPA with the HNF6 probe and RNA isolated from AdHNF6 infected HepG2 cells revealed that increased levels of adenovirus-generated mouse HNF6 (Adeno mHNF6) stimulated expression of the endogenous human HNF6 gene (Fig. 7A, hHNF6). An HNF6 binding site was found in the –1361 to –1348 bp region of the human HNF6 promoter (TgaATTGATTTC) and this HNF6 recognition sequence was completely conserved in the mouse HNF6 promoter sequence at –1030 to –1017 bp (TgaATTGATTTC). We performed electrophoretic mobility shift assays (EMSA) with AdHNF6 infected HepG2 cell extracts and radioactively labeled double stranded oligonucleotide synthesized to the –1368 to –1329 bp region of the human HNF6 promoter. This EMSA showed that the –1368 to –1329 bp HNF6 promoter region formed a specific HNF6 protein-DNA complex, which was inhibited by including either a 100-fold excess of cold homologous oligonucleotide or HNF6 antisera in the binding reaction (Fig. 7B). Cotransfection assays with –1.4 KB human HNF6 promoter Luciferase plasmid demonstrated that both the CMV-HNF6 and CMV-FoxM1b expression vectors stimulated HNF6 promoter expression and that combining these expression vectors provided additive transcriptional increase of the HNF6 promoter region (Fig. 7C). The –1.3 KB HNF6 promoter region, which deleted this HNF6 binding site, showed significant reduction in transcriptional activation by both HNF6 and FoxM1b expression vectors in cotransfection assays (Fig. 7C). These results suggest that this HNF6 binding site was mediating transcriptional activation of the –1.4 KB HNF6 promoter region. Quantitative ChIP assays revealed that FoxM1 and HNF6 proteins bind to the endogenous human HNF6 promoter region (Fig. 7D–E) and that AdHNF6 infection stimulated HNF6 binding to the its own endogenous promoter region, but did not stimulate FoxM1 binding to the endogenous HNF6 promoter region (Fig. 7D). Taken together, these studies demonstrate for the first time that HNF6 auto-activates its own promoter region and that HNF6 and Foxm1 proteins independently stimulate transcription of the HNF6 promoter region.

HNF6 is required for normal levels of DNA replication in Hepa1-6 cells

In order to determine whether reduced expression of HNF6 would diminish DNA replication, mouse hepatoma Hepa1-6 cells were transfected with HNF6 siRNA, control siRNA or left untransfected, serum starved for 48 hours and then stimulated to reenter the cell cycle with the addition of 10% fetal calf serum (FCS). RPA assays and Western Blot analysis demonstrated that transfection of Hepa1-6 cells with HNF6 siRNA caused a 50% reduction in levels of HNF6 mRNA and protein, but HNF6 expression was not influenced by transfection with a control siRNA (Fig. 8A–B). These siRNA transfected or untreated Hepa1-6 cells were subjected to a one-hour pulse label with BrdU prior to harvesting them at 16 hours after serum stimulation and then DNA replication was determined by immunostaining of cells with BrdU antibody. Quantitation of the BrdU staining demonstrated that diminished HNF6 expression in siRNA transfected Hepa1-6 cells caused a 50% decrease in the number of cells that have incorporated BrdU compared to cells transfected with control siRNA or left untransfected (Fig. 8C; $P < 0.001$). Taken together, these studies demonstrate that diminished expression of HNF6 reduces DNA replication in hepatoma cells following serum stimulation suggesting that HNF6 contributes significantly to S-phase progression.

Discussion

The Hepatocyte Nuclear Factor 6 (HNF6) protein is a cell-type specific transcription factor that is known to regulate *in vivo* expression of hepatocyte-specific genes such as Glucokinase⁴⁰, Glucose Transporter 2⁴¹, Protein C⁴², and Cholesterol 7 α hydroxylase⁴³. Published hepatocyte Chromatin Immunoprecipitation (ChIP) assays demonstrated that the HNF6 transcription factor associated with the endogenous promoter regions of the cell cycle regulatory genes Cdk2, E2F1, and Cdc25A³⁵, suggesting the hypothesis that HNF6 may stimulate hepatocyte proliferation during liver regeneration. In this current study, we demonstrated that adenovirus mediated increase in hepatic expression of the HNF6 transcription factor (AdHNF6) stimulated the number of hepatocytes entering S-phase following partial hepatectomy (PHx). Our current liver regeneration studies are the first to demonstrate that the liver-enriched HNF6 transcription factor activates expression of the TGF α , Cdk2, and Cyclin D1 genes and the Foxm1 transcription factor, each of which is necessary to stimulate hepatocyte proliferation during liver regeneration^{1, 6, 7, 22, 55–57}. Cotransfection and ChIP assays demonstrated that HNF6 stimulates transcription of the TGF α , Cyclin D1 and HNF6 promoters. ChIP assays demonstrated that increased HNF6 levels stimulated FoxM1 binding to the endogenous mouse Cyclin D1 promoter region and increasing both FoxM1b and HNF6 levels further increased recruitment of FoxM1 to the endogenous Cyclin D1 promoter region. These results suggest that the FoxM1 and HNF6 transcription factors may interact and stimulate their recruitment to the same binding site in the endogenous Cyclin D1 promoter region. Consistent with these findings, co-immunoprecipitation assays with regenerating mouse liver extracts show a functional association between HNF6 and Foxm1 transcription factors. We have also used *in vitro* GST pulldown assays to demonstrate that HNF6 and FoxM1b proteins directly interact through their DNA binding domains. Cotransfection assays also demonstrated that formation of the HNF6-FoxM1 protein complex stimulated expression of FoxM1-dependent target genes, which are known to be critical for cell cycle progression during liver regeneration^{23, 56}.

Earlier expression of Cyclin D1 is associated with acceleration in the onset of hepatocyte DNA replication in both liver regeneration rodent models and primary hepatocyte cultures^{5–7}. In the absence of growth factor stimulation, ectopic expression of Cyclin D1 is sufficient to drive primary hepatocytes into DNA replication at levels similar to mitogen-stimulated hepatocytes⁵. Administration of the hepatocyte mitogen 1,4-Bis-2-(3,5-Dichloropyridyloxy)-benzene (TCPOBOP) to mice caused earlier expression of Cyclin D1, which was associated with

accelerated onset of hepatocyte DNA replication compared to PHx induced liver regeneration⁶. In a manner similar to these other liver regeneration studies, increased expression of HNF6 was sufficient to stimulate transcription of Cyclin D1, which is consistent with stimulation of S-phase progression of regenerating hepatocytes.

In addition to the direct transcriptional activation of the Cyclin D1 and TGF α by HNF6, increased levels of HNF6 in regenerating mouse liver also stimulated expression of Foxm1, a transcription factor known to regulate expression of genes essential for hepatocyte proliferation during liver regeneration^{23, 56}. This coincided with increased expression of epidermal growth factor receptor ligand, TGF α , which activates the Ras-MAPK pathway to stimulate expression of the Foxm1 gene^{17, 20, 21}. In addition, Foxm1 transcriptional activity requires binding of activated Cdk-Cyclin complexes to mediate phosphorylation-dependent recruitment of the CREB binding protein (CBP) transcriptional coactivator⁵³. HNF6 mediated increase in TGF α levels will also stimulate FoxM1 transcriptional activity through activation of the Cdk-Cyclin complexes¹. Co-immunoprecipitation experiments with regenerating liver extracts and cotransfection assays in both human hepatoma HepG2 and osteosarcoma U2OS cells demonstrated that formation of the HNF6-Foxm1 protein complex stimulated transcriptional activity of the Foxm1 protein. Published studies demonstrate that formation of the HNF6-Foxa2 complex stimulates transcriptional activity of Foxa2 by recruiting the CBP transcriptional coactivator protein⁴⁵. One potential mechanism by which HNF6 stimulates Foxm1 transcriptional activity is through HNF6 mediated recruitment of CBP coactivator to the Foxm1 protein, which is required to stimulate its transcriptional activity⁵³. Taken together, these studies suggest the hypothesis that HNF6 stimulates transcriptional activity of the Foxm1 protein and may therefore promote cell cycle progression through increased transcription of Foxm1 cell cycle target genes.

Functional analysis of the *Hnf6* promoter region demonstrates that HNF6 promoter activity requires binding of the HNF4 α transcription factor⁵⁹. It is interesting to note that proliferating adult hepatocytes exhibit a significant decrease in HNF4 α expression⁶⁰ and this decrease in HNF4 α transcription factor may lead to reduced levels of HNF6 during liver regeneration. Our results suggest that induction of FoxM1 levels during liver regeneration and its activation of the HNF6 promoter may prevent decreased expression of HNF6 resulting from diminished levels of HNF4 α in regenerating liver. In addition, HNF6 will reinforce transcription of its own promoter region through auto-activation. Our data also suggest the model that maintaining HNF6 expression during liver regeneration will allow HNF6 to stimulate transcription of cell cycle regulators Cyclin D1 and TGF α as well as potentiate Foxm1-mediated transcription of cell cycle genes required for hepatocyte proliferation.

A number of Pou-Homeodomain transcription factors play an essential role in stimulating both cellular proliferation and cell-type specific differentiation³¹⁻³⁴. Interestingly, recent NMR studies of the HNF6 DNA binding domain demonstrated that the Cut domain folds into a topology homologous to the Pou DNA binding domain, even though there is no sequence homology between the Cut and Pou domain sequences³⁰. In a manner similar to the Pou-Homeodomain family, HNF6 is involved in stimulating transcription of both hepatocyte-specific genes and cell cycle regulatory genes. Mouse genetic studies demonstrate that the *Hnf6* gene is essential for development of the pancreas, gall bladder and both intrahepatic and extra hepatic bile ducts³⁶⁻³⁸. Similar defects in the hepatic biliary tree were also observed in embryos in which the Pou-Homeodomain HNF1 β transcription factor was conditionally deleted in hepatoblasts at early stages of liver development⁶¹. At 15 days of gestation, bipotential hepatoblasts that coalesce around the portal mesenchyme begin to express biliary cytochrome proteins and then undergo a complex morphogenesis process culminating in the formation of intra-hepatic bile ducts by 17.5 days of mouse embryonic development⁶². It is therefore tempting to speculate that HNF6 and HNF1 β may play a proliferative role for biliary

hepatoblasts during the development of intrahepatic bile ducts. Consistent with this speculation, embryonic *Foxm1* $-/-$ livers display a significant decrease in hepatoblast proliferation causing a failure in development of the intrahepatic bile ducts from biliary hepatoblast precursor cells ²⁵. Our current studies lead us to propose the hypothesis that HNF6 transcription factor may regulate proliferation-specific genes in hepatoblasts during liver morphogenesis, which may in turn be critical for proper development of the intrahepatic bile ducts from biliary hepatoblasts coalescing around the portal mesenchyme.

In summary, these liver regeneration studies demonstrate for the first time that the liver-enriched HNF6 transcription factor is capable of stimulating proliferation of regenerating hepatocytes through transcriptional activation of cell cycle regulatory genes Cyclin D1, Cdk2, and the Foxm1 transcription factor, and the hepatocyte mitogen TGF α . Cotransfection and ChIP assays demonstrated that TGF α , Cyclin D1 and HNF6 promoters are direct transcriptional targets of HNF6 and Foxm1. Consistent with these findings, co-immunoprecipitation with regenerating mouse liver extracts and cotransfection assays show a functional association between HNF6 and Foxm1 transcription factors and that HNF6 potentiated Foxm1 transcriptional activity.

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Figure 1; Tan et al.

Fig. 1. Adenovirus mediated increase in hepatic HNF6 expression following partial hepatectomy
Two-month-old CD-1 mice were subjected to tail vein injections of either adenovirus CMV-LacZ (AdLacZ) or adenovirus CMV-HNF6 (AdHNF6). Two days after injection, mice were subjected to partial hepatectomy (PHx) operation and remnant regenerating livers were harvested at indicated time points following PHx. At least three regenerating mouse livers were used for each time point. (A) AdHNF6 infected regenerating liver displayed increased levels of HNF6 mRNA. RNase protection assays were performed to detect mouse HNF6 expression in either AdLacZ infected or AdHNF6 infected livers at the indicated time points following PHx and Cyclophilin levels were used as a loading. (B-I) AdHNF6 infection increases nuclear levels of HNF6 protein in regenerating hepatocytes. We used affinity purified HNF6 antibody

for immunohistochemical staining of liver sections at the indicated hours following PHx from mice infected with either AdLacZ (B, D, F and H) or AdHNF6 (C, E, G and I). Micrographs are at 200X magnification.

Figure 2; Tan et al.

Fig. 2. Adenovirus mediated increase in hepatic HNF6 expression stimulates levels of hepatocyte DNA replication and mitosis following partial hepatectomy
 Adenovirus infections and partial hepatectomy (PHx) were performed as described in Fig. 1 legend and in Materials and Methods. (A) Graph depicting increased hepatocyte DNA replication in AdHNF6 infected mice following PHx. Mice undergoing liver regeneration were injected with 5-bromo- 2'-deoxyuridine (BrdU) 2 hours prior to sacrifice. DNA replication was determined from the number of hepatocytes that incorporated BrdU measured by immunohistochemical staining of regenerating liver sections with BrdU antibody. DNA replication was determined by counting the number of BrdU stained hepatocytes from 1000 cells using three regenerating mouse livers per time point after PHx. Graphic representation

of the mean number of the BrdU positive hepatocytes per 1000 cells ($\pm$ SD) from mice undergoing liver regeneration infected with either AdLacZ (circles) or AdHNF6 (Squares, thick line) was plotted against the hours following PHx. Also shown immunohistochemical staining of liver sections at 40 hours following PHx with BrdU antibody from mice infected with either AdLacZ (B) or AdHNF6 (C). (D) Increased hepatocyte mitosis in AdHNF6 infected mice following PHx. Regenerating liver sections were stained with H&E and used to detect mitotic figures (mitosis) between 36 and 48 hours following PHx from three regenerating mouse livers per time point was determined as described above for BrdU incorporation rate. Graphic representation of the mean number of mitotic figures per 1000 hepatocytes ($\pm$ SD) from mice undergoing liver regeneration infected with either AdLacZ (open bar) or AdHNF6 (solid bar) was plotted against the hours following PHx. The asterisks indicate statistically significant changes: * $P \leq 0.05$ and ** $P \leq 0.01$.

Figure 3; Tan et al.

Fig. 3. Increased expression of Cyclin D1, Forkhead Box m1, Tumor Growth Factor α and Cdk2 during liver regeneration in AdHNF6 infected mice

(A–B) Increased hepatic mRNA levels of Forkhead Box m1 (Foxm1) transcription factor, Cyclin D1 and Tumor Growth Factor α (TGF α) in regenerating livers from AdHNF6 infected mice. Total RNA was isolated from regenerating liver of mice that were infected with either AdLacZ or AdHNF6 and analyzed for mRNA expression by RNase protection assays (in triplicate) with probes specific to either HNF6 (B), TGF α (B), Foxm1 (A) or Cyclin D1 (A). Note that representative duplicate samples are shown and that Cyclophilin or L32 levels were used to normalize expression levels. Mean mRNA expression levels of 2-month old CD-1 mouse liver at either 0 hours (A) or 32 hours post PHx (B) were set at 1.0. (C) Increased protein

levels of Cyclin D1 and Cdk2 during regenerating livers in AdHNF6 infected mice. Total protein extracts were isolated from regenerating liver and analyzed for Cdk2 and Cyclin D1 expression by Western blot analysis. Note that representative duplicate samples from two distinct regenerating mouse livers are shown and that β -Actin protein band was used to normalize expression levels. Quiescent mock-infected liver (MI) was set at 1.0. The asterisk indicates statistically significant changes: $*P \leq 0.05$.

Figure 4; Tan et al.

Fig. 4. HNF6 and Foxm1 proteins activate transcription of the Cyclin D1 and Tumor Growth Factor α promoter regions in cotransfection assays

(A) AdHNF6 infection of HepG2 cells increases mRNA expression of endogenous human Tumor Growth Factor α (TGF α) gene. HepG2 cells were either mock infected (MI) or infected with AdLacZ (LacZ) or AdHNF6 (H6) and 24 hours after infection, RNA was isolated and analyzed for mRNA expression by RNase protection assays (RPA) with probes specific to either HNF6 or TGF α . (B–C) CMV-HNF6 and CMV-FoxM1b expression vectors activate transcription of the Cyclin D1 and Tumor Growth Factor α promoter regions in cotransfection assays. HepG2 cells were transfected with the –1.7 Kilobase pair (KB) mouse TGF α promoter (B) or the –1.7 KB human Cyclin D1 promoter (C) Luciferase plasmid and either CMV HNF-6

cDNA (HNF6) or CMV empty expression vectors. At 24 hours after transfection, the cells were used to prepare cell extracts, which were then analyzed for dual Luciferase enzyme activity as described in Materials and Methods. We also examined whether cotransfection of these promoters with the CMV human FoxM1b expression vector (FoxM1b) could stimulate expression of these promoter regions and synergize with the HNF6 transcription factor (HNF6 + FoxM1b). Results are presented as mean fold induction of promoter activity $\pm$ SD from two separate experiments in triplicate, with the CMV empty value set at 1.0. The asterisks indicate statistically significant changes: * $P \leq 0.05$ and ** $P \leq 0.01$.

Figure 5; Tan et al.

Fig. 5. Chromatin Immunoprecipitation assay demonstrates that HNF6 and Foxm1 proteins bind to the endogenous TGF α and Cyclin D1 promoter regions

The mouse TGF α promoter region contains distinct DNA binding sequences for the HNF6 protein (-1608 to -1591 bp) and the FoxM1 protein (-1561 to -1531 bp), whereas these transcription factors bind to the same DNA sequence in the mouse Cyclin D1 promoter region (-1064 to -1049 bp). Mouse hepatoma Hepa1-6 cells were mock infected or infected with AdHNF6 or adenovirus expressing Green Fluorescent Protein (AdGFP). For the Cyclin D1 promoter ChIP assays (Panel D), we also infected Hepa1-6 cells with AdHNF6 or AdM1b (adenovirus expressing human FoxM1b) separately or together. At 24 hours after infection the chromatin was cross-linked, sonicated to DNA fragments of 500 to 1000 nucleotides in length, and then processed for ChIP assays as described in the Materials and Methods. The cross-

linked and sonicated mouse chromatin was immunoprecipitated (IP) with antibodies specific to either HNF6 (+, A and B) or FoxM1 (+, C and D) or with rabbit antisera (–) and the amount of promoter DNA associated with the IP chromatin was quantitated by Real-Time PCR with primers specific to either the –1647 to –1495 bp mouse TGF α promoter region (A and C) or the –890 to –733 bp mouse Cyclin D1 promoter region (B and D). The asterisks indicate statistically significant changes: *P $\leq$ 0.05, **P $\leq$ 0.01 and P $\leq$ 0.001.

Figure 6; Tan et al.

Fig. 6. Association of the HNF6 and Foxm1 proteins results in stimulation of Foxm1 transcriptional activity

(A) Co-immunoprecipitation assays with regenerating liver extracts demonstrate functional association between the HNF6 and Foxm1 transcription factors. We performed co-immunoprecipitation (Co-IP) assays with protein extracts from quiescent mouse liver (0h) that lack detectable expression of Foxm1 protein or regenerating mouse liver isolated at 40 hours following PHx (40h), which express abundant levels of Foxm1 protein (Foxm1 Input). Quiescent or regenerating mouse liver extracts were Co-IP with the HNF6 antibody and then IP proteins were subjected to Western blot analysis with the FoxM1 C-terminal Antibody. (B) In vitro GST pull-down assays demonstrated that HNF6 Cut-Homeodomain protein interacts

with the FoxM1b winged helix domain. Right panel shows the expression levels of the Glutathione-S-Transferase (GST), GST-FoxM1b N-terminal (N; amino acids 1-138) or GST-FoxM1b binding domain (DBD; amino acids 221 to 355) fusion proteins separated by SDS-PAGE and stained with Commassie Blue. The HNF6 Cut-Homeodomain DBD plasmid was used to synthesize radioactively labeled HNF6 DBD protein using in vitro transcription and translation (IVT) with ³⁵S Methionine and used for the in vitro GST-FoxM1b pull-down assays and bound labeled HNF6 protein was separated by SDS-PAGE and visualized by autoradiography (left panel). (C–D) Cotransfection studies demonstrate that HNF6 stimulates FoxM1b's ability to activate Foxm1-dependent target promoters. Human hepatoma HepG2 (C) or osteosarcoma U2OS (D) cells were cotransfected with the Foxm1 reporter gene 6X Foxm1 TATA Luciferase reporter gene⁵³ and increasing amounts of CMV HNF6 expression vector and constant amount of CMV FoxM1b expression vectors (numbers represent ng transfected). Cotransfection of CMV-HNF6 expression vector could not stimulate expression of the FoxM1 reporter gene in either HepG2 or U2OS cells. (E) Cotransfection assays demonstrated that HNF6 also stimulated FoxM1b ability to stimulate transcription of the -2.7 KB Cdc25B promoter region, which is a known transcriptional target of Foxm1²³. The asterisks indicate statistically significant changes: *P ≤ 0.05 and **P ≤ 0.01.

Figure 7; Tan et al.

Fig. 7. HNF6 and Foxm1 regulate transcription of the HNF6 promoter as determined by cotransfection and ChIP assays

(A) Infection of HepG2 cells with AdHNF6 stimulated expression of the endogenous human HNF6 gene. HepG2 cells were infected with AdHNF6 and RNA was isolated at different times after AdHNF6 infection and analyzed for HNF6 expression by RPA. (B) HNF6 protein binds to its own promoter region. Electrophoretic mobility shift assays with AdHNF6 infected HepG2 cell extracts and radioactively labeled double stranded oligonucleotide synthesized to the -1368 to -1329 bp human HNF6 promoter region forms a specific HNF6 protein-DNA complex that was inhibited by including in the binding reaction either a 100-fold excess of cold homologous oligonucleotide or HNF6 antisera. (C) HepG2 cell cotransfection assays shows

that HNF6 and FoxM1b stimulates transcription of the -1.4 KB human HNF6 promoter region. HepG2 cells were cotransfected with either -1.4 KB or -1.3 KB human HNF6 promoter Luciferase plasmid and CMV-HNF6 and CMV-FoxM1b expression vectors either alone or together. At 24 hours after transfection, the cells were used to prepare cell extracts, which were then analyzed for dual Luciferase enzyme activity as described in Materials and Methods. (D-E) Quantitative ChIP assays revealed that Foxm1 and HNF6 proteins bind to the endogenous human HNF6 promoter region. HepG2 cells were mock infected or infected with either AdHNF6 or AdGFP and 24 hours after infection the chromatin was cross-linked and processed for ChIP assays as described in the Materials and Methods. The cross-linked and sonicated mouse chromatin was immunoprecipitated (IP) with antibodies specific to either HNF6 (+, D) or FoxM1 (+, E) or with rabbit antisera (-) and the amount of promoter DNA associated with the IP chromatin was quantitated by Real-Time PCR with primers specific to -1499 to -1337 bp of the human HNF6 promoter region. The asterisks indicate statistically significant changes: * $P \leq 0.05$, ** $P \leq 0.01$ and $P \leq 0.001$.

Figure 8; Tan et al.

Fig. 8. Diminished HNF6 levels in Hepa1-6 cells by siRNA transfection causes a significant decrease in DNA replication following serum stimulation

Mouse hepatoma Hepa1-6 cells were transfected with HNF6 siRNA, control siRNA or left untransfected, serum starved for 48 hours and then stimulated to reenter the cell cycle with the addition of 10% fetal calf serum (FCS). These siRNA transfected or untreated Hepa1-6 cells were subjected to a one-hour pulse label with BrdU prior to harvesting them at 16 hours after serum stimulation and then DNA replication was determined by immunostaining of cells with BrdU antibody. (A–B) RNase protection assays and Western blot analysis demonstrated that transfection of Hepa1-6 cells with HNF6 siRNA caused a 50% reduction in levels of HNF6 mRNA and protein, but HNF6 expression was not influenced by transfection with a control siRNA. (C) Graph depicting reduced number of BrdU positive Hepa1-6 cells depleted in HNF6 levels following serum stimulation. Diminished number of BrdU positive Hepa1-6 cells after transfection of HNF6 siRNA to diminish HNF6 expression compared to Hepa1-6 cells transfected with control siRNA or left untransfected. Quantitation of the BrdU incorporation rates by counting the number of BrdU positive Hepa1-6 cells in 5 fields from three different HNF6 siRNA transfected cell plates. Student T-test determines statistically significant decrease

in BrdU incorporation. The asterisks indicate statistically significant changes: ** $P \leq 0.01$ and $P \leq 0.001$.