

Basic Study

## Regulation of hepatic microRNA expression by hepatocyte nuclear factor 4 alpha

Hong Lu, Xiaohong Lei, Jerry Liu, Curtis Klaassen

Hong Lu, Xiaohong Lei, Department of Pharmacology, SUNY Upstate Medical University, Syracuse, NY 13210, United States

Jerry Liu, Curtis Klaassen, Department of Medicine, University of Kansas Medical Center, Kansas City, KS 66160, United States

**Author contributions:** Lu H wrote the paper; Lu H, Lei X and Liu J performed the experiments and analyzed the data; Lu H and Klaassen C conceived and designed the experiments.

Supported by NIH grant ES019487 in part.

**Institutional animal care and use committee statement:** All animal procedures in the study were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. The animal protocol was designed to minimize the pain or distress to the mice. Age-matched young-adult HNF4 $\alpha$  Liv-KO mice and their wild-type control littermates were fed rodent chow (#8064, Teklad; Harlan, Indianapolis, IN). Mice were housed at an ambient temperature of 22 °C with alternating 12-h light/dark cycles and allowed water and feed ad libitum.

**Conflict-of-interest statement:** The authors have no conflict of interest to declare.

**Data sharing statement:** No additional data are available.

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**Manuscript source:** Invited manuscript

**Correspondence to:** Hong Lu, PhD, Assistant Professor, Department of Pharmacology, SUNY Upstate Medical University, 750 E Adams ST, Syracuse, NY 13210, United States. [luh@upstate.edu](mailto:luh@upstate.edu)  
Telephone: +1-315-4647978

Fax: +1-315-4648008

Received: July 30, 2016

Peer-review started: August 2, 2016

First decision: September 8, 2016

Revised: October 2, 2016

Accepted: December 1, 2016

Article in press: December 2, 2016

Published online: February 8, 2017

### Abstract

#### AIM

To uncover the role of hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ) in regulating hepatic expression of microRNAs.

#### METHODS

Microarray and real-time PCR were used to determine hepatic expression of microRNAs in young-adult mice lacking Hnf4 $\alpha$  expression in liver (Hnf4 $\alpha$ -LivKO). Integrative genomics viewer software was used to analyze the public chromatin immunoprecipitation-sequencing datasets for DNA-binding of HNF4 $\alpha$ , RNA polymerase- II, and histone modifications to loci of microRNAs in mouse liver and human hepatoma cells. Dual-luciferase reporter assay was conducted to determine effects of HNF4 $\alpha$  on the promoters of mouse and human microRNAs as well as effects of microRNAs on the untranslated regions (3' UTR) of two genes in human hepatoma cells.

#### RESULTS

Microarray data indicated that most microRNAs remained unaltered by Hnf4 $\alpha$  deficiency in Hnf4 $\alpha$ -LivKO mice. However, certain liver-predominant microRNAs were down-regulated similarly in young-adult male and female Hnf4 $\alpha$ -LivKO mice. The down-regulation of miR-101, miR-192, miR-193a, miR-194, miR-215, miR-802, and miR-122 as well as induction of miR-34 and miR-29 in male Hnf4 $\alpha$ -LivKO mice were confirmed by real-time

PCR. Analysis of public chromatin immunoprecipitation-sequencing data indicates that HNF4 $\alpha$  directly binds to the promoters of miR-101, miR-122, miR-194-2/miR-192 and miR-193, which is associated with histone marks of active transcription. Luciferase reporter assay showed that HNF4 $\alpha$  markedly activated the promoters of mouse and human miR-101b/miR-101-2 and the miR-194/miR-192 cluster. Additionally, miR-192 and miR-194 significantly decreased activities of luciferase reporters for the 3'UTR of histone H3F3 and chromodomain helicase DNA binding protein 1 (CHD1), respectively, suggesting that miR-192 and miR-194 might be important in chromosome remodeling through directly targeting H3F3 and CHD1.

## CONCLUSION

HNF4 $\alpha$  is essential for hepatic basal expression of a group of liver-enriched microRNAs, including miR-101, miR-192, miR-193a, miR-194 and miR-802, through which HNF4 $\alpha$  may play a major role in the post-transcriptional regulation of gene expression and maintenance of the epigenome in liver.

**Key words:** Liver; Hepatocyte nuclear factor 4 alpha; Knockout; Mice; Human; miR-122; miR-192; miR-194; miR-101; miR-802

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**Core tip:** Hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ) is a liver-enriched master regulator of liver development and function. HNF4 $\alpha$  plays a key role in regulating hepatic transcriptome and epigenome. However, little was known about the role of HNF4 $\alpha$  in regulating hepatic expression of microRNAs, essential modulators of the transcriptome and epigenome. Results from this study uncover species differences and similarities between humans and mice in the role of HNF4 $\alpha$  in regulating hepatic expression of certain important microRNAs. Such novel knowledge will help understand the role of HNF4 $\alpha$  in post-transcriptional regulation of gene expression and maintenance of the normal epigenome and physiology in mouse and human liver.

Lu H, Lei X, Liu J, Klaassen C. Regulation of hepatic microRNA expression by hepatocyte nuclear factor 4 alpha. *World J Hepatol* 2017; 9(4): 191-208 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i4/191.htm> DOI: <http://dx.doi.org/10.4254/wjgh.v9.i4.191>

## INTRODUCTION

Hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ) is a master regulator of liver development and function<sup>[1]</sup>. HNF4 $\alpha$  is essential for hepatocyte differentiation in fetal liver<sup>[2-4]</sup>, maintenance of liver function in adult<sup>[5,6]</sup>, and protection against liver cirrhosis and liver cancer<sup>[7,8]</sup>. HNF4 $\alpha$  is

critical in regulating hepatic metabolism of fatty acids, bile acids, and ureagenesis<sup>[5,9-11]</sup>. Moreover, HNF4 $\alpha$  is essential in regulating hepatic expression of drug processing genes, namely cytochrome P450s, phase-II conjugation enzymes, and transporters<sup>[1,12,13]</sup>.

There are very large individual variations in hepatic basal expression of HNF4 $\alpha$  in humans<sup>[14]</sup>, and mutation of HNF4 $\alpha$  causes maturity onset diabetes of young humans<sup>[15]</sup>. The expression and/or transcriptional activity of HNF4 $\alpha$  is decreased markedly in severe cirrhotic livers, alcoholic liver disease, tumor necrosis factor- $\alpha$ -induced hepatotoxicity, and hepatoma progression<sup>[16-19]</sup>. Thus, it is important to understand how HNF4 $\alpha$  deficiency affects hepatic gene expression and its underlying mechanism.

Interestingly, overexpression of HNF4 $\alpha$  in hepatocellular carcinoma (HCC) markedly decreases the stemness of gene expression and the percentage of cancer stem cells in HCC<sup>[7]</sup>; however, the underlying mechanism is unknown. Epigenetic modifications play key roles in regulating gene expression and stem cell differentiation. Our recent study demonstrates that *Hnf4 $\alpha$*  deficiency in young-adult mouse livers causes marked alteration in histone methylation and acetylation, which is associated with induction of certain key epigenetic enzymes, including enhancer of zeste homolog 2 (EZH2), G9a and DNA methyltransferase (cytosine-5) 1 (Dnmt1)<sup>[20]</sup>. EZH2 plays a key role in maintaining the stemness of stem cells<sup>[21]</sup>. Therefore, establishment and maintenance of the epigenome of differentiated hepatocytes may be a key mechanism in the regulation of gene expression and cell differentiation by HNF4 $\alpha$ .

The importance of HNF4 $\alpha$  in regulating hepatic expression of mRNAs has been well established, however, the underlying mechanism remains less clear. HNF4 $\alpha$  directly binds to a large number of gene promoters in human and mouse liver<sup>[22-24]</sup>. *Hnf4 $\alpha$*  deficiency in young-adult mouse liver caused induction of certain key epigenetic modifiers<sup>[20]</sup>. However, our analysis of published data of chromatin immunoprecipitation-sequencing (ChIP-seq) of *Hnf4 $\alpha$*  in adult mouse liver<sup>[25]</sup> revealed no binding of *Hnf4 $\alpha$*  to these epigenetic modifiers, suggesting indirect regulation of these epigenetic modifiers by *Hnf4 $\alpha$*  in liver. microRNAs are important post-transcriptional regulators of gene expression, and deregulation of microRNAs is common in human hepatocarcinogenesis<sup>[26]</sup>. Through binding to the untranslated regions (UTRs, usually the 3'UTR) of mRNAs, microRNAs affect the stability/translation of mRNAs and thus the mRNA and/or protein levels of their target genes. We hypothesized that HNF4 $\alpha$  can indirectly regulate hepatic gene expression through directly regulating hepatic expression of certain microRNAs. Thus, the purpose of this study was to uncover the role of HNF4 $\alpha$  in regulating hepatic expression of microRNAs. We used microarray and real-time PCR to determine hepatic expression of microRNAs in young-adult mice lacking *Hnf4 $\alpha$*  expression in liver (*Hnf4 $\alpha$* -LivKO). We used integrative genomics viewer (IGV) software to analyze the public ChIP-seq datasets

for DNA-binding of HNF4 $\alpha$ , RNA polymerase- II, and histone modifications to loci of microRNAs in mouse liver and human hepatoma cells. Additionally, we conducted dual-luciferase reporter assay to determine effects of HNF4 $\alpha$  on the promoters of mouse and human microRNAs as well as effects of microRNAs on the 3'UTR of two putative target genes in human hepatoma cells.

## MATERIALS AND METHODS

### Preparation of liver samples

The livers of male and female young-adult mice with liver-specific knockout of *Hnf4 $\alpha$*  (*Hnf4 $\alpha$ -LivKO*) (*Hnf4 $\alpha$*  flox/flox, Alb-cre/+) and age-matched wild-type (*Hnf4 $\alpha$*  flox/flox, Alb-cre/-) littermates at the age of 45 d were collected in the previous study<sup>[27]</sup> and stored at -80 °C until use. All animal procedures in the study were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center<sup>[27]</sup>.

### Microarray profiling of microRNA expression in *Hnf4 $\alpha$ -LivKO* mice

Pooled total RNAs from livers of young-adult (42-45 d old) male and female *Hnf4 $\alpha$ -LivKO* and their age-matched wild-type littermates ( $n = 5-6$ ) were used for microarray analysis of microRNAs, utilizing miRCURY™ LNA array version 11.0 (Exiqon, Denmark), which contains probes targeting all mouse microRNAs registered in the miRBASE version 13.0. Background correction was conducted utilizing normexp plus offset method with offset value 10<sup>[28]</sup>. The non-linear regression method was used for data normalization to remove certain systematic biases from microarray data, such as dye effects or intensity dependence.

### Heat map and unsupervised hierarchical clustering of microRNAs

The heat map diagram shows the result of the 2-way hierarchical clustering of microRNAs and samples<sup>[29]</sup>. Each row represents a microRNA and each column represents a pooled liver sample. The microRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. The color scale shown at the bottom illustrates the relative expression level of a microRNA across all samples: Red color represents an expression level above mean, blue color represents expression lower than the mean. The clustering is performed on log<sub>2</sub>(Hy3/Hy5) ratios which passed the filtering criteria on variation across samples; LogMedianDRatios differences > 0.58, corresponding to 50% differential expression.

### Quantification of microRNAs using real-time PCR

miRCURY LNA™ Universal RT microRNA PCR (Exiqon) was used to quantify microRNAs in individual RNA samples from livers of male *Hnf4 $\alpha$ -LivKO* mice. All PCR reagents and specific LNA-modified PCR primer sets were purchased from Exiqon. The PCR primer sets for mmu-miR-19b, 26a, 29b, 34a, 122, 192, 193a-3p, 194 and

195 target both human and mouse microRNA homologs, whereas PCR primer sets for mmu-miR-101b, 215, and 802 were specific for mouse microRNAs. The relative expression of each microRNA was normalized by 5s rRNA and U6 rRNA with values of wild-type mice set at 100.

### Use of public database to analyze DNA-binding of HNF4 $\alpha$ and the chromatin status of microRNAs in mouse liver, intestine, and human hepatoma HepG2 cells

Actively transcribed genes typically remain in loosely-packed euchromatin, where DNA is more accessible to the transcriptional machinery. DNase- I hypersensitive sites (DHSs), determined by DNase-sequencing (DNase-seq), is a key determining factor of the chromatin accessibility of transcription factors. DNA-binding of RNA polymerase 2 (Pol2) is widely used as a marker of active transcription. Histone H3 trimethylation at lysine-4 (H3K4me3) is enriched around the transcription start sites (TSS) and correlates tightly with active gene transcription<sup>[30,31]</sup>, whereas H3 trimethylation at lysine-36 (H3K36me3) along the gene coding regions after TSSs correlated highly with transcription elongation<sup>[32]</sup>. Our previous study shows that alterations of H3K4me3 correlate bi-directionally with mRNA expression in HNF4 $\alpha$ -null livers<sup>[20]</sup>. Conversely, Histone H3 trimethylation at lysine-27 (H3K27me3) and at lysine-9 (H3K9me3) are well-established epigenetic signatures of gene silencing<sup>[31,33]</sup>. The public genome-wide datasets of DNase-seq (GSM1003818) as well as ChIP-seq of H3K4me3 (GSM769014), H3K36me3 (GSM1000151), H3K9me3 (GSM1087075), H3K27me3 (GSM1087069), Pol2 (GSM722763) and HNF4 $\alpha$  (GSM1390711) in wild-type mouse liver were retrieved from GEO DataSets and uploaded into the IGV software<sup>[34]</sup> to visualize the DNA-binding of HNF4 $\alpha$ , Pol2 and these epigenetic signatures in each microRNA locus in mouse liver. Similarly, the public genome-wide datasets of DNase-seq (GSM816662) as well as ChIP-seq of H3K4me3 (GSM945182), H3K36me3 (GSM945211), H3K9me3 (GSM1003519), H3K27me3 (GSM945231), Pol2 (GSM935543), and HNF4 $\alpha$  (GSM935619) in HepG2 cells were retrieved from GEO DataSets for their visualization in the IGV software. Additionally, to determine the role of tissue-specific binding of HNF4 $\alpha$  in the tissue-specific regulation of miRs, ChIP-seq data for DNA-binding of HNF4 $\alpha$  in the mouse liver (GSM1390711) and small intestinal villus cells (GSM851120) were compared using the IGV software.

### Generation of expression vectors for wildtype and mutant mouse *Hnf4 $\alpha$* 1

The mouse *Hnf4 $\alpha$* 1 cDNA was synthesized by Integrated DNA Technologies, Inc (IDT, Coralville, IA) and cloned into the pcDNA3 backbone to generate the expression vector for wildtype *Hnf4 $\alpha$* 1, which was named as pcDNA3-*Hnf4 $\alpha$* 1. The expression vector for the 304 serine to aspartic acid (S304D) mutant of *Hnf4 $\alpha$* 1 was generated using pcDNA3-*Hnf4 $\alpha$* 1 and the Q5® Site-Directed Muta-

genesis Kit (New England Biolabs), and verified by sequencing.

#### **Generation of reporter constructs for the promoters of human miR-101-2 and mouse miR-101b**

miR-101 is mainly transcribed from the human miR-101-2 and mouse miR-101b loci<sup>[35]</sup> which are located in the intron8-9 of RNA terminal phosphate cyclase-like 1 (*RCL1*) gene. The first base of the pre-miR-101-2 was assigned as chr9:4840297<sup>[35]</sup>, located within intron5-6 of *RCL1*, around where prominent peaks of H3K4me3 and DNA-binding of Pol2 and HNF4 $\alpha$  were identified. Thus, we PCR cloned a 739-bp fragment of miR-101-2 proximal promoter (-926 to -190 bp), located within the intron5-6 of *RCL1*, into the KpnI/MluI sites of pGL3-Basic reporter vector, which was named as pGL3-miR-101-2. In mice, miR-101b is predominantly expressed in the liver<sup>[35]</sup>. Similar to its human ortholog miR-101-2, we found prominent peaks of HNF4 $\alpha$ , H3K4me3 and Pol2 that start at the intron5-6 of *Rcl1* and extend to intron7-8 and intron8-9 of *Rcl1*. Thus, we PCR cloned a 933-bp fragment of the miR-101b promoter, located within intron5-6 of *Rcl1* that contains the peaks of HNF4 $\alpha$  and Pol2, into the KpnI/MluI sites of pGL3-Basic reporter vector, which was named as pGL3-miR-101b.

#### **Generation of reporter constructs for the proximal and/or distal promoters of human and mouse miR-194-2/miR-192 cluster**

Mouse miR-194-1/miR-215 and miR-194-2/miR-192 forms gene clusters in chromosome 1 and 19, respectively. The miR-194-1/miR-215 loci is expressed lowly in mouse liver<sup>[36]</sup>. In mouse liver, we found prominent peaks of DHSs, HNF4 $\alpha$ , Pol2 and H3K4me3 located approximately 1.6 kb upstream of the miR-194-2. Thus, we PCR cloned a 1973 bp fragment (-1694 to + 279 bp) of the promoter of the mouse miR-194-2/miR-192 cluster into the MluI/XhoI site of pGL3-Basic reporter vector, which was named as pGL3-mmIR-194-2. The sequences of all the primers used for PCR cloning of miR promoters are listed in Supplemental Materials.

A previous study indicates that a single approximately 2.4 kb transcript contains the human pri-miR-194-2 transcript and a 5' AK092802 cDNA. In the human colon cancer Caco-2 cells, HNF1 $\alpha$  binds to a HNF1 site located between -70 and -52 bp upstream of the transcription start site (TSS) of AK092802 to activate the promoter of pri-miR-194-2<sup>[37]</sup>. The upstream genomic region close to the TSS of pri-miR-194-2 contains some highly conserved regions between humans and mice<sup>[37]</sup>. We found prominent peaks of DHSs, HNF4 $\alpha$ , Pol2 and H3K4me3 within a 350 bp fragment from -329 to +21 bp upstream of the TSS of AK092802, which was PCR cloned into the KpnI/MluI sites of pGL3-Basic reporter vector and named as pGL3-hmiR-194-2-Dist. Genomic DNA prepared from C57BL/6 mouse liver and human embryonic kidney 293 cells were used as the PCR templates. In addition to the prominent peaks of HNF4 $\alpha$  and Pol2 identified in

approximately 2 kb upstream of the human miR-194-2 loci, smaller peaks of HNF4 $\alpha$  and Pol2 were also found in the proximal promoter of human miR-194-2. A DNA fragment of 417 bp that contains 5' KpnI and 3' Hind III restriction sites as well as a wild-type and mutant 405 bp human miR-194-2 promoter (from -405 to +1) were synthesized and verified by sequencing (GenScript United States Inc., Piscataway, NJ), and ligated into the KpnI/HindIII site in the pGL3-basic vector, which was named as pGL3-hmiR-194-2-Pro and pGL3-hmiR194-2 TriM. The mutant 405-bp human miR-194-2 promoter had mutations of 3 putative HNF4-binding sites predicted by software of NHR-scan<sup>[38]</sup> and HNF4 Binding Site Scanner<sup>[39]</sup> (for DNA sequences see Supplemental Materials).

#### **Generation of reporter construct for the mouse miR-802 promoter**

We PCR cloned a 2 kb fragment of the mouse miR-802 promoter (-2004 to -1 bp) into the MluI/XhoI sites of pGL3-Basic to generate the reporter vector for mouse miR-802 promoter, which was named as pGL3-mmIR-802 Pro.

#### **Determination of effect of HNF4 $\alpha$ on the promoter activities of human and mouse miRs**

Human hepatocellular adenoma HepG2 cells were maintained in D-MEM with 5% FBS. Cells were added to 96-well plates and grown to approximately 80% confluence. Plasmid DNA including pGL3 reporter vectors, the pRL-CMV luciferase (as control for transfection efficiency), pCDNA3-HNF4 $\alpha$ 2 (Addgene), pCMV-CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ) (gift from Dr. Magnus Nord, Karolinska Institute), or pCDNA3 were complexed with Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) and applied to individual wells, according to the manufacturer's protocol. Transfected cells were lysed with passive lysis buffer (Promega) 24 h after transfection. Promoter activities of cell lysates were quantified by Dual-Glo<sup>TM</sup> luciferase assay (Promega) with the control values of pGL3-Basic vs pRL-CMV set at 1.0. To study the role of SP1 in mediating the transactivation of human miR-194-2 proximal promoter by HNF4 $\alpha$ , the SP1 inhibitor mithramycin was added 1 h after transfection and cells were lysed 24 h after transfection for dual-luciferase assay.

#### **Generation of reporter construct for the 3'UTR of mouse chromodomain helicase DNA binding protein 1 (*Chd1*) and *H3f3* mRNAs**

The chromatin remodeling factor Chd1 is required to maintain the open chromatin and pluripotency of mouse embryonic stem cells<sup>[40]</sup>. DNA sequence containing 48 bp of the 3'UTR of mouse Chd1 mRNA (NM\_007690.3, 6708-6756, in bold), namely CTAGTGATTGGCTTT AATATAAAACTGTTACAGTACACACTGATTGTATATA CGCGTA, and its antisense sequence AGCTTACGCG TATATACAATCAGTGTGTACTGTAACAGTTTTTATATTTAA

GCCAATCA were synthesized by IDT. DNA sequence containing 48 bp of the 3'UTR of mouse H3f3b mRNA (NM\_008211.3, 1593-1639, in bold), namely CTAGTAA GTATCCTATTGAAGTTTTAGGTCAATTATGTATGTTGA CTAATACGCGTA, and its antisense sequence AGCTT ACGCGTATTTAGTCAACATACATAATTGACCTAAAAA CTTCAATAGGATACTTA were synthesized by IDT. The two sense and antisense oligos were annealed and ligated into the Spe I /HindIII site between the luciferase cDNA and SV40 polyA in pMIR-REPORT™ microRNA Expression Reporter Vector (Applied Biosystems/Ambion, Austin, TX), which was named pMIR-Chd1 and pMIR-H3f3, respectively. The correctness of pMIR-Chd1 and pMIR-H3f3 was verified by the unique restriction site (ACGCGT) for MluI that was introduced into the synthetic oligo.

#### **Determination of effect of miR-194 and miR-192 on the stability of mouse Chd1 and H3f3 3'-UTR using dual-luciferase assay**

HepG2 human hepatocellular adenoma cells were maintained in D-MEM with 5% FBS. Cells were added to 96-well plates and grown to approximately 80% confluence. Plasmid DNA including pmiR-Chd1 (or pmiR-H3f3), the pRL-CMV luciferase, and a synthetic mimic of miR-194/miR-192 (miScript miR-194/miR-192, QIAGEN Inc, Valencia, CA), or AllStars Negative Control siRNA (QIAGEN, as negative control for microRNAs) were co-transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol of DNA-RNAi co-transfection. Transfected cells were lysed with passive lysis buffer (Promega) 24 h after transfection. Promoter activities of cell lysates were quantified by Dual-Glo™ luciferase assay (Promega) with the control values of pmiR-Chd1/pmiR-H3f3 vs pRL-CMV set at 1.0.

**Animal care and use statement:** The animal protocol was designed to minimize the pain or distress to the mice. Age-matched young-adult HNF4 $\alpha$  Liv-KO mice and their wild-type control littermates were fed rodent chow (#8064, Teklad; Harlan, Indianapolis, IN). Mice were housed at an ambient temperature of 22 °C with alternating 12-h light/dark cycles and allowed water and feed *ad libitum*.

#### **Statistical analysis**

Data are presented as mean  $\pm$  SE. Differences between two groups were determined using Student's *t*-test. For multiple comparisons, analysis of variance was performed, followed by the Student-Newman-Keuls Method in SigmaPlot 12.5, with significance set at  $P < 0.05$ .

## **RESULTS**

#### **Results of microarray analysis of microRNAs in pooled young-adult male and female Hnf4 $\alpha$ -LivKO mouse livers**

Generally, there were few gender differences in hepatic expression of microRNAs in mice (Figure 1), which is similar to that in rats<sup>[41]</sup>. Hepatic expression of most microRNAs

remained unchanged ( $< 50\%$  differential expression among the 4 pooled samples) in *Hnf4 $\alpha$ -LivKO* mice (data not shown). However, *Hnf4 $\alpha$ -LivKO* mouse livers had up- or down-regulation of a small portion of microRNAs that are important in regulating cell proliferation, differentiation, and apoptosis (Figure 1). Thirty microRNAs were found to have  $\geq 50\%$  differential expression among the 4 pooled samples, namely male WT and *Hnf4 $\alpha$ -LivKO* as well as female WT and *Hnf4 $\alpha$ -LivKO* mice. Fourteen microRNAs had  $> 50\%$  lower expression in *Hnf4 $\alpha$ -LivKO* mice than in WT mice (Figure 1A). Among them, the 4 liver-predominant microRNAs miR-194, miR-192, miR-215 and miR-193 were 71%, 72%, 70% and 70% lower, respectively, in *Hnf4 $\alpha$ -LivKO* male mouse livers than WT males (WTM). miR-101a and 101b, which are expressed moderately in liver, also decreased  $> 50\%$  in male *Hnf4 $\alpha$ -LivKO* mice. Female *Hnf4 $\alpha$ -LivKO* mouse livers had very similar lower expression of these microRNAs than WT females (Figure 1A). In contrast, two microRNAs that are expressed highly in liver, namely miR-122 and miR-26a<sup>[42,43]</sup>, had less than 50% differential expression in all the groups (Supplemental Table 1).

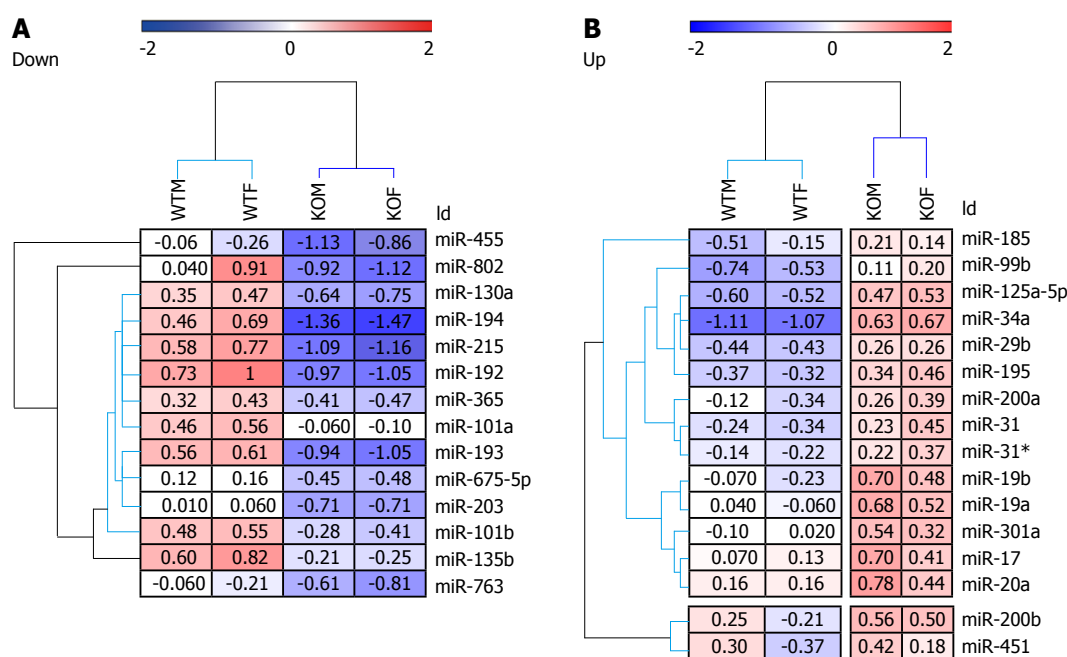
In contrast to the down-regulation of certain liver-predominant microRNAs, hepatic expression of 16 microRNAs were  $> 50\%$  higher in *Hnf4 $\alpha$ -LivKO* mice than in WT mice (Figure 1B). The tumor-suppressor miR-34a<sup>[44]</sup> was expressed at relatively low levels in wild-type mouse liver, but was induced 2.6 fold in male *Hnf4 $\alpha$ -LivKO* mouse livers. Tumor-suppressor miR-29b and miR-195<sup>[45]</sup> were highly and modestly expressed in WT mouse livers, respectively, and were 90% and 70% higher, respectively, in male *Hnf4 $\alpha$ -LivKO* mouse livers than WTM (Figure 1B).

The oncogenic miR-17-92 locus encodes a cluster of 7 microRNAs transcribed as a single primary transcript<sup>[46]</sup>. Four miR-17-92 members, namely miR-17, 19a, 19b and 20 tended to be higher in *Hnf4 $\alpha$ -LivKO* mouse liver (Figure 1B).

#### **Verification of changes in hepatic microRNAs in male Hnf4 $\alpha$ -LivKO mice by real-time PCR**

To verify the changes in microRNAs detected by microarray in the pooled liver samples, real-time PCR was used to quantify 12 microRNAs in individual samples from *Hnf4 $\alpha$ -LivKO* mice (Figure 2). Because similar alterations of these microRNAs were found in male and female *Hnf4 $\alpha$ -LivKO* mice (Figure 1), only individual male *Hnf4 $\alpha$ -LivKO* liver samples were used in this study. The selection of these 12 microRNAs for verification was based on their relative expression levels (Supplemental Table 1) and their reported importance in cellular pathophysiology.

Compared to male WT mice, male *Hnf4 $\alpha$ -LivKO* mice had markedly lower levels of miR-101b (7% of WT values), miR-192 (24%), miR-193a (24%), miR-194 (16%), miR-215 (59%) and miR-802 (33%) (Figure 2A-B), but higher levels of miR-29b (190%) and miR-34a (244%) (Figure 2C). In contrast, hepatic levels of miR-26a and miR-195 were similar between male WT and *Hnf4 $\alpha$ -LivKO* mice (Figure 2C-D). Hepatic miR-122 was



**Figure 1** Heat map and unsupervised hierarchical clustering of hepatic microRNAs in male and female *Hnf4 $\alpha$* -LivKO mice. The heat map diagram shows the results of the 2-way hierarchical clustering of microRNAs and samples. Each row represents a microRNA and each column represents a pooled liver sample. The microRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. The color scale shown at the top illustrates the relative expression level of a microRNA across all samples: Red color represents an expression level above mean, blue color represents expression lower than the mean. The clustering is performed on log<sub>2</sub>(Hy3/Hy5) ratios which passed the filtering criteria on variation across samples; LogMedianDRatios differences > 0.58, corresponding to 50% differential expression. WTM: Wild-type male; WTF: Wild-type female; KOM: Knockout male; KOF: Knockout female.

modestly (30%) lower in male *Hnf4 $\alpha$* -LivKO mice than male WT mice (Figure 2D).

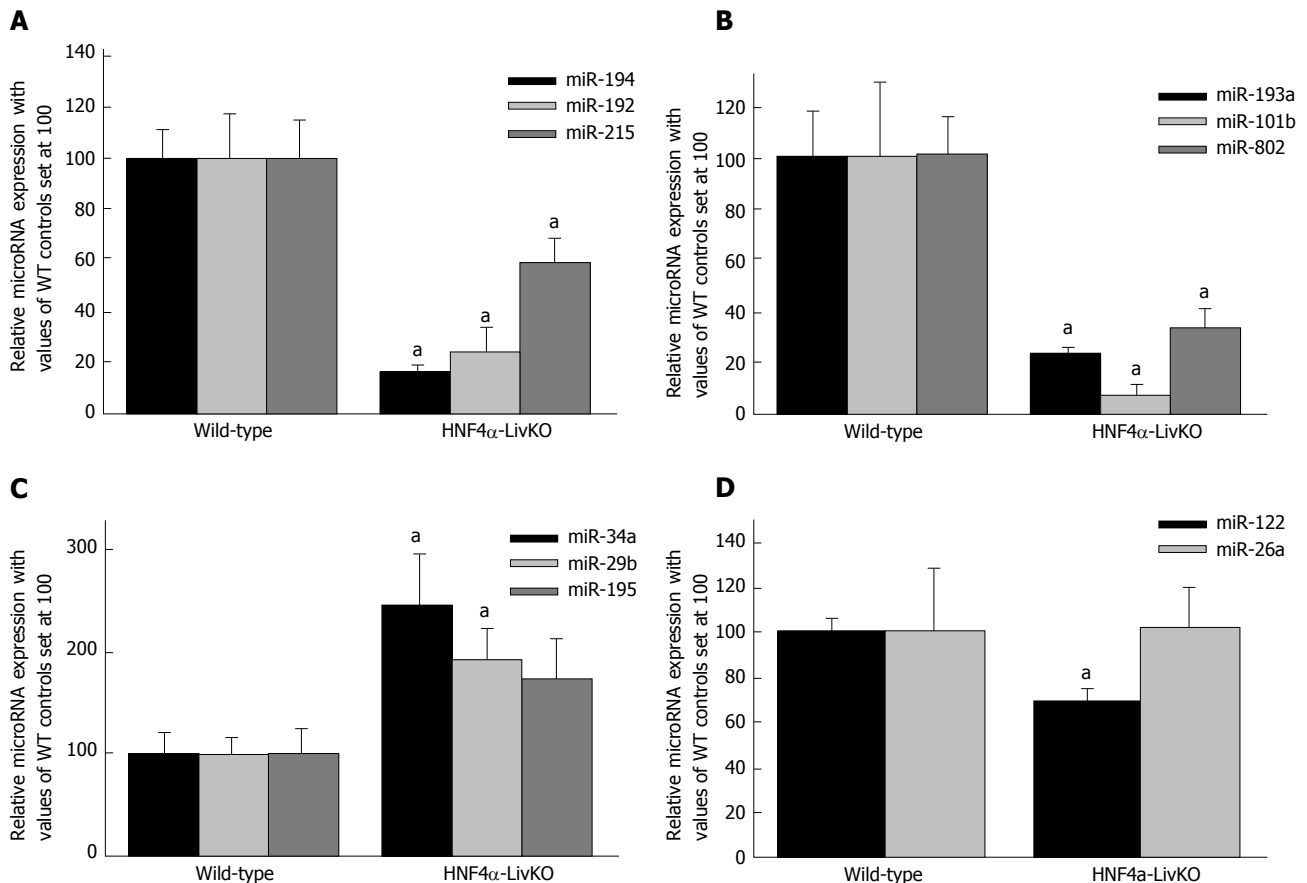
#### DNA-binding of HNF4 $\alpha$ in mouse liver and small intestine as well as the chromatin status of microRNAs in mouse liver

To understand the mechanism of regulation of microRNA expression by HNF4 $\alpha$  in mouse liver, we used IGV software to analyze the published genome-wide DNase-seq and ChIP-seq data on DNA-binding of HNF4 $\alpha$  as well as the presence of DHSs, Pol2 and active (H3K4me3 and H3K36me3) and suppressing (H3K9me3 and H3K27me3) epigenetic signatures, in the loci of several microRNAs in mouse liver and/or small intestine. Consistent with their high expression in mouse liver, miR-122a, miR-194-2/miR-192 and miR-101b had large peaks of DHSs in their gene loci, which were associated with sequential prominent peaks of HNF4 $\alpha$ , Pol2, H3K4me3 and H3K36me3 downstream (Figure 3A-3C). This strongly suggests that the binding of HNF4 $\alpha$  to the promoter of these miR genes causes the recruitment of Pol2 and the introduction of H3K4me3 and H3K36me3, the active marks of transcription initiation and elongation. Consistent with the liver-specific and liver-predominant expression of miR-122a and miR-101b, respectively, no binding of HNF4 $\alpha$  to the promoters of miR-122a and miR-101b was found in mouse small intestine (Figure 3A and 3C). In contrast, large peaks of HNF4 $\alpha$  were identified in the distal and proximal promoters of the miR-194-2/miR-192 cluster, consistent with their high expression in the mouse intestine<sup>[37]</sup>.

Similarly, peaks of DHSs, HNF4 $\alpha$ , Pol2 and H3K4me3 were also found in the gene loci of miR-193 and miR-802 (Figure 3D and E); however, the peaks were smaller and less sequential compared to those in the gene loci of miR-122, miR-194-2/miR-192 and miR-101b. In contrast, no clear peaks of H3K36me3 were found in regions that encode the mature transcripts of miR-193 and miR-802 (Figure 3D and E). Interestingly, the silencing mark H3K27me3 was found to span the whole locus of miR-802, whereas a peak of H3K9me3 was found 3' downstream of the miR-802 (Figure 3E). In summary, the data suggest that these five microRNAs might be directly regulated by HNF4 $\alpha$  in mouse liver.

Much smaller peaks of HNF4 $\alpha$  were found in the gene loci of miR-194-1/miR-215, miR26a-1 and miR26a-2, and DNA-binding of HNF4 $\alpha$  was not associated with prominent peaks of Pol2 or H3K4me3 in mouse liver (Figure 4A-C). Conversely, although prominent peaks of DHSs, HNF4 $\alpha$ , Pol2 and H3K4me3 were found in the miR-26b locus, the direction of HNF4 $\alpha$ , Pol2 and H3K4me3 peaks was toward the upstream of miR-26b, rather than the transcription initiation of miR-26b (Figure 4D). These data suggest that HNF4 $\alpha$  may not have a direct and/or important role in regulating hepatic expression of miR-194-1/miR-215, miR-26a and miR-26b. In contrast, large peaks of HNF4 $\alpha$  were found in the distal and proximal promoter of the miR-194-1/miR-215 cluster in mouse small intestine (Figure 4A), suggesting that HNF4 $\alpha$  may be important in regulating the high expression of the miR-194-1/miR-215 cluster in mouse small intestine<sup>[36]</sup>.

It was reported that HNF4 $\alpha$  binds to the proximal



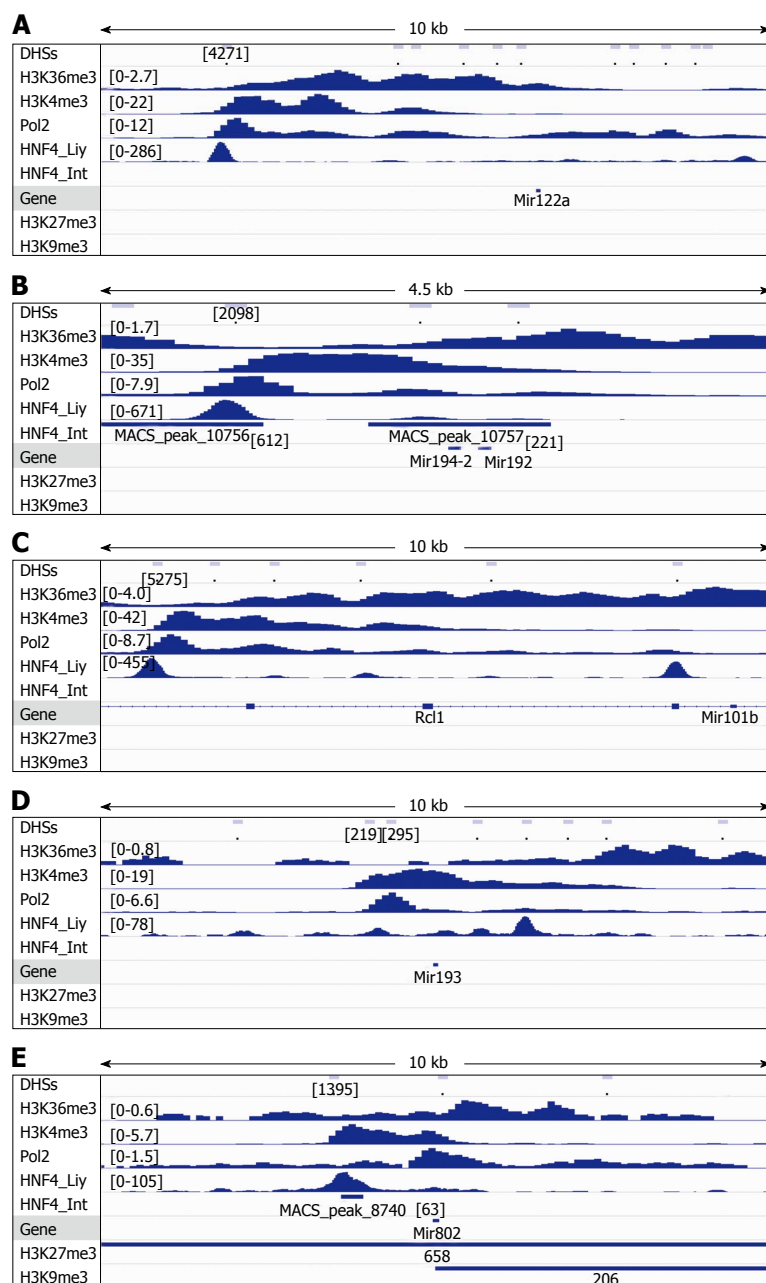
**Figure 2** Hepatic microRNA expression in young-adult male mice with liver-specific deletion of *Hnf4a* (*Hnf4a*-LivKO) (A-D). microRNAs in total RNA from livers of *Hnf4a*-LivKO and wild-type (WT) control mice ( $n = 5-6$ ) were determined by miRCURY LNA™ Universal RT microRNA PCR (Exiqon). Mean  $\pm$  SE.  $^*P < 0.05$  compared to WT control. HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha.

promoter of miR-29 a-b cluster in cultured mouse hepatocytes, and acute loss of HNF4 $\alpha$  decreased the levels of miR-29a and miR-29b in isolated hepatocytes and livers from mice on a mixed background of SvJ129/FVB<sup>[47]</sup>. However, only a small peak of HNF4 $\alpha$  was found within 10 kb of the mouse miR-29 a-b loci in adult liver from C57BL/6 mice, and the small HNF4 $\alpha$  peak was not associated with peaks of Pol2 or H3K4me3 (Figure 5A). In contrast, a larger peak of HNF4 $\alpha$  was found in the promoter of the miR-29 a-b loci in the small intestine (Figure 5A). Thus, the role of HNF4 $\alpha$  in regulating hepatic expression of miR-29 a-b cluster in mice may be strain and/or cell-context dependent.

Recent studies indicate that HNF4 $\alpha$  directly regulates miR-124 and miR-134 in human liver, and down-regulation of HNF4 $\alpha$  is associated with reduction of miR-124 and miR-134 in human HCC<sup>[48,49]</sup>. However, our microarray data showed that miR-124 and miR-134 were expressed very lowly in mouse liver, and *Hnf4a* deficiency had no effect on hepatic expression of miR-124 and miR-134 in mice (Supplemental Table 1). Consistently, there were no clear peaks of HNF4 $\alpha$ , Pol2, or the activating signatures H3K4me3, H3K36me3 in the loci of the 3 mouse miR-124 genes, namely miR-124a-1, 124a-2 and 124a-3 in livers of C57BL/6 mice (Figure 5B-D). In contrast, large peaks of the silencing mark H3K27me3 were found

in the whole loci of miR-124a-1, 124a-2 and 124a-3, and a large peak of H3K9me3 was found in the miR-124a-1 locus (Figure 5B-D). Similarly, there were no prominent peaks of DHSs, HNF4 $\alpha$ , Pol2, H3K4me3, or H3K36me3 detected in the locus of mouse *miR-134* gene, where the silencing mark H3K9me3 was found (Figure 5E). Taken together, the very low signal of miR-124s and miR-134 in the microarray data (Supplemental Table 1) and the lack of activating epigenetic signatures but enrichment of silencing epigenetic signatures in the loci of miR-124s and miR-134 strongly indicate that miR-124 and miR-134 are expressed very lowly in adult mouse liver, and they are not HNF4 $\alpha$ -target genes in mouse liver. Thus, there appear to be species differences between humans and mice in hepatic basal expression and regulation of miR-124 and miR-134 by HNF4 $\alpha$ .

Because our data of microRNA expression and analysis of public database for ChIP-seq strongly suggest that HNF4 $\alpha$  has a critical direct role in maintaining hepatic expression of miR-194/miR-192 and miR-101b in mice, we further examined DNA-binding of HNF4 $\alpha$  and chromatin status in the gene loci of miR-194-2/miR-192 and miR-101-2 in the human hepatoma HepG2 cells using the data from public database (Figure 6). Very similar to the mouse miR-194-2/miR-192 cluster (Figure 3B), starting from approximately 2 kb upstream of the



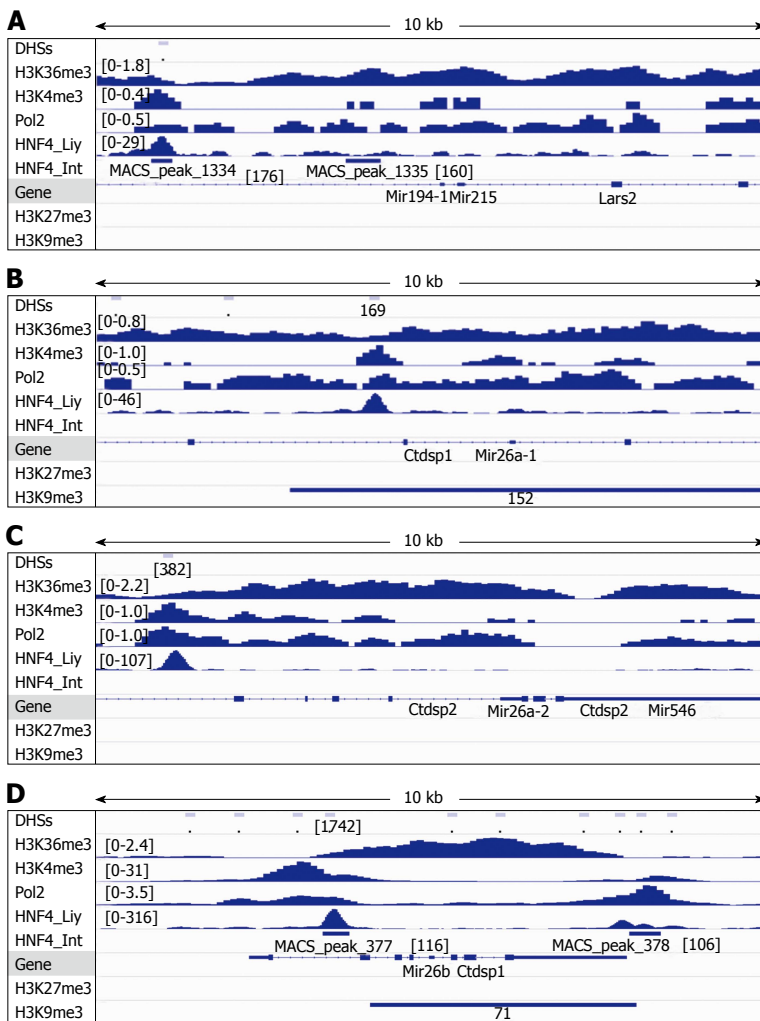
**Figure 3** Analysis of DNase-I hypersensitive sites as well as DNA-binding of HNF4 $\alpha$ , RNA polymerase II (Pol2), and methylated histones to loci of miR-122a (A), miR-194-2/miR-192 (B), miR-101b (C), miR-193 (D) and miR-802 (E) in wildtype mouse liver. DNA-binding of HNF4 $\alpha$  to these microRNA loci in the mouse small intestine (HNF4 $\alpha$ \_Int) was compared to those in the mouse liver (HNF4 $\alpha$ \_Liy). Data of DHSs (determined by DNase-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNase-I hypersensitive sites; HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.

human pri-miR-194-2, prominent sequential peaks of DHSs, HNF4 $\alpha$ , Pol2, H3K4me3 and H3K36me3 were identified in the human *miR-194-2/miR-192* gene cluster in HepG2 cells (Figure 6A). Very similar to the mouse miR-101b, the human *miR-101-2* gene body is located in the intron8-9 of the *RCL1* gene, and clear (but weaker than miR-194-2) sequential peaks of HNF4 $\alpha$ , Pol2, H3K4me3 and H3K36me3 were identified in the intron5-6 of *RCL1* (Figure 6B). These data strongly suggest that HNF4 $\alpha$  may also have a direct critical role in

regulating hepatic expression of miR-194-2/miR-192 and miR-101-2 in humans. In contrast, there were no clear peaks of HNF4 $\alpha$ , Pol2, or H3K4me3 (Figure 6C) in the miR-122 locus which is known to be silenced in HepG2 cells<sup>[42]</sup>.

#### Regulation of the mouse and human miR-194-2/miR-192 gene cluster by HNF4 $\alpha$

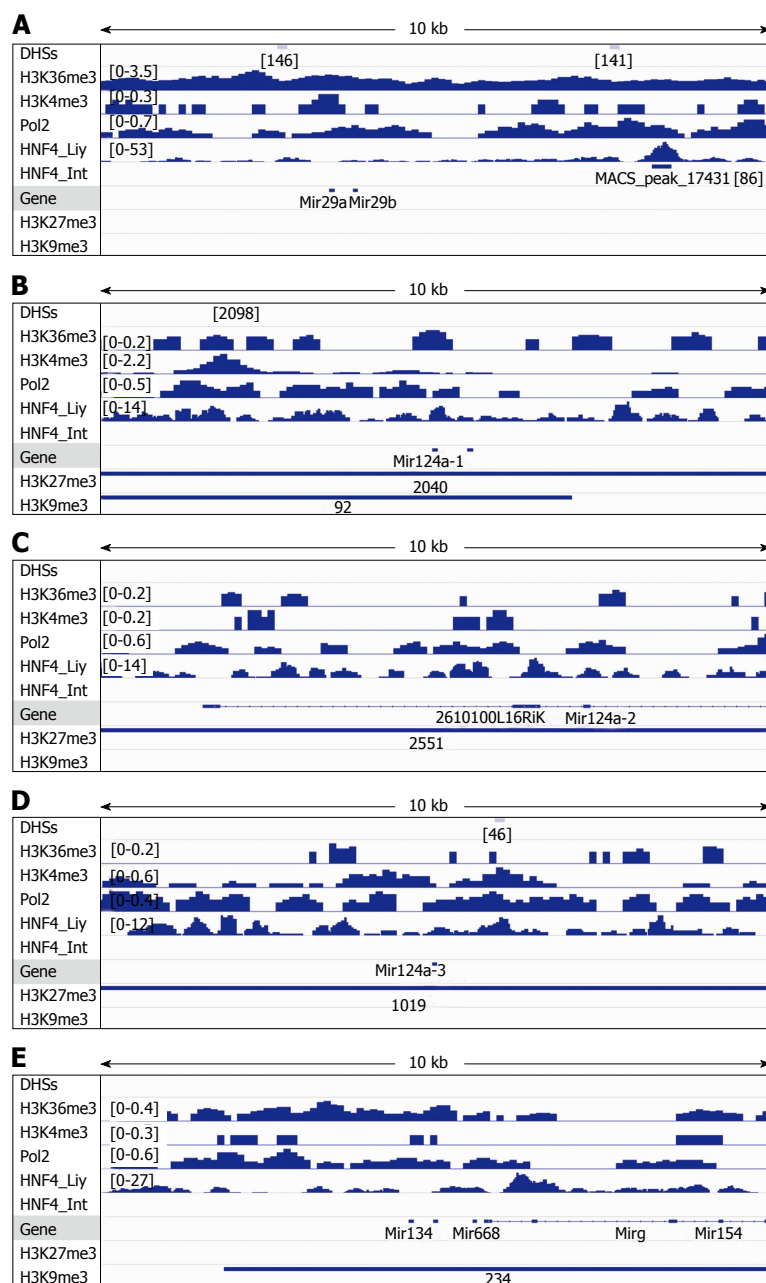
Hepatic expression of miR-194 is markedly down-regulated in mice null for *Hnf1 $\alpha$* <sup>[36]</sup>, a down-stream target



**Figure 4** Analysis of DNase-I hypersensitive sites as well as DNA-binding of HNF4 $\alpha$ , RNA polymerase II (Pol2), and methylated histones to loci of miR-194-1/miR-215 (A), miR-26a-1 (B), miR-26a-2 (C) and miR-26b (D) in wildtype mouse liver. DNA-binding of HNF4 $\alpha$  to these microRNA loci in the mouse small intestine (HNF4 $\alpha$ \_Int) was compared to those in the mouse liver (HNF4 $\alpha$ \_Liv). Data of DHSs (determined by DNase-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNase-I hypersensitive sites; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.

of HNF4 $\alpha$ . In small intestine, miR-194 is transcriptionally up-regulated by Hnf1 $\alpha$ <sup>[37]</sup>. Hepatic mRNA expression of Hnf1 $\alpha$  decreased modestly in *Hnf4 $\alpha$ -LivKO* mice<sup>[1]</sup>. We found that HNF1 $\alpha$  and HNF4 $\alpha$  modestly activated the reporter for the mouse *miR-194-2/miR-192* gene cluster 1.5 and 2.8 fold, respectively, and they synergistically activated mouse miR-194-2/miR-192 promoter 7.5 fold (Figure 7A). ChIP-seq results showed that HNF4 $\alpha$  bound strongly to the distal promoter but weakly to the proximal promoter of human miR-194-2/miR-192 cluster (Figure 6A). To determine the role of HNF4 $\alpha$  in regulating the *miR-194-2/miR-192* gene cluster in humans, we generated reporter vectors for the distal and proximal promoters of human miR-194-2/miR-192 cluster. Surprisingly, HNF4 $\alpha$  only modestly activated the distal promoter 3 fold, but very strongly activated the proximal promoter of human miR-194-2/miR-192 cluster by 200 fold (Figure 7B). To identify the critical cis-elements responsible for the very strong transactivation

of this proximal promoter by HNF4 $\alpha$ , we engineered luciferase reporter constructs for the mutated 400-bp proximal promoter of human *miR-194-2* gene cluster. Surprisingly, mutations of the 3 putative HNF4-binding sites (HNF4-RE) within the 400-bp miR-194-2 promoter had little effects on the transactivation of this promoter by HNF4 $\alpha$  (Figure 7C). HNF4 $\alpha$  can transactivate the human p21 promoter *via* physically interacting with the general transcription factor SP1, independent of DNA-binding of HNF4 $\alpha$ , because the S304D mutant of HNF4 $\alpha$  which has markedly decreased DNA-binding activity<sup>[50]</sup>, is equally active as the WT HNF4 $\alpha$  in transactivating p21<sup>[51]</sup>. Thus, we tested the hypothesis that HNF4 $\alpha$  can DNA-binding-independently transactivate the proximal human miR-194-2 promoter *via* interacting with SP1. We found that mithramycin, a widely used SP1 inhibitor<sup>[52]</sup>, dramatically suppressed the HNF4 $\alpha$ -transactivation of both the WT and HNF4RE-mutant miR-194-2 promoter by 94% and 95%, respectively (Figure 7C). Moreover,



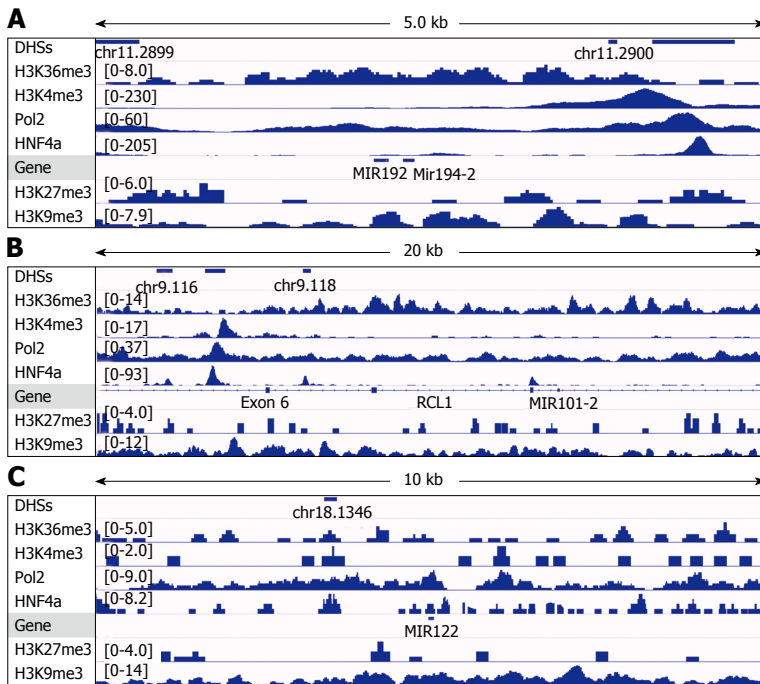
**Figure 5** Analysis of DNase-I hypersensitive sites as well as DNA-binding of HNF4 $\alpha$ , RNA polymerase II (Pol2), and methylated histones to loci of miR-29a/miR-29b (A), miR-124a-1 (B), miR-124a-2 (C), miR-124a-3 (D) and miR-134 (E) in wildtype mouse liver. DNA-binding of HNF4 $\alpha$  to these microRNA loci in the mouse small intestine (HNF4 $\alpha$ \_Int) was compared to those in the mouse liver (HNF4 $\alpha$ \_Liv). Data of DHSs (determined by DNase-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNase-I hypersensitive sites; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.

the S304D-mutant of HNF4 $\alpha$  was equally active as the WT HNF4 $\alpha$  in transactivating the proximal human miR-194-2 promoter (Figure 7D). Taken together, these data strongly indicate that HNF4 $\alpha$  can DNA-binding-independently transactivate the proximal human miR-194-2 promoter *via* interacting with SP1.

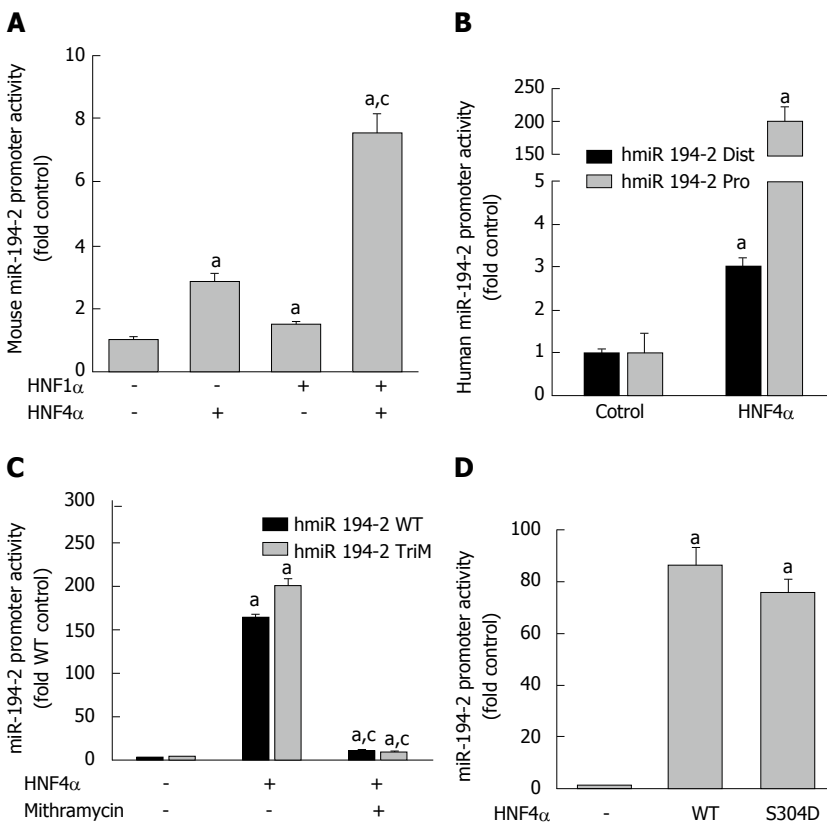
#### Regulation of mouse miR-101b and human miR-101-2 promoters by HNF4 $\alpha$

The mouse miR-101b promoter was moderately active in

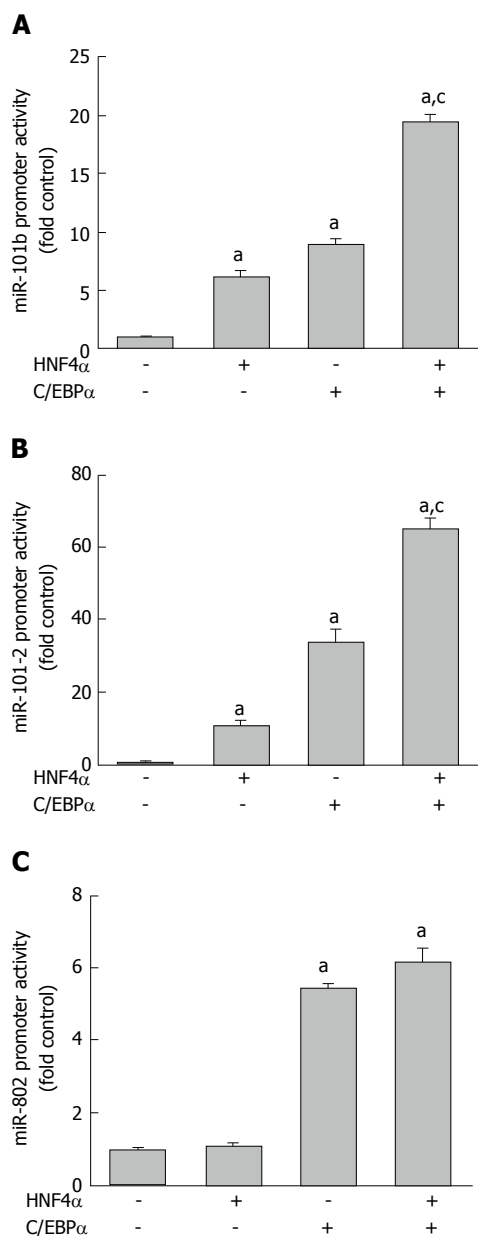
HepG2 cells (Figure 8A), whereas the human miR-101-2 promoter was largely inactive in HepG2 cells (Figure 8B). C/EBP $\alpha$ , a liver-enriched transcription factor, plays a key role in regulating liver-specific gene expression. The expression of C/EBP $\alpha$  is low in HepG2 cells, and re-expression of C/EBP $\alpha$  in HepG2 cells can reactivate certain liver-specific genes<sup>[53]</sup>. Our analysis of published ChIP-seq data for C/EBP $\alpha$  in mouse liver (GSM1037657) showed that C/EBP $\alpha$  bound to the miR-101b promoter, located in the Intron5-6 of Rcl1, in close proximity to



**Figure 6** Analysis of DNase-I hypersensitive sites as well as DNA-binding of HNF4 $\alpha$ , RNA polymerase II (Pol2), and methylated histones to loci of miR-194-2/miR-192 (A), miR-101-2 (B) and miR-122 (C) in human hepatoma HepG2 cells. Data of DHSs (determined by DNase-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNase-I hypersensitive sites; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.



**Figure 7** Activation of mouse (A) and human (B-D) miR-194-2/miR-192 promoter by HNF4 $\alpha$ . Human hepatoma HepG2 cells were transfected with firefly luciferase vectors containing wild-type and mutant miR-194-2 promoter, pRL-CMV, and an expression vector for HNF4 $\alpha$ /HNF1 $\alpha$ . Dual-luciferase reporter assay was conducted 24 h after transfection. The y-axis represents relative luciferase activity for microRNA promoter normalized by the renilla luciferase.  $n = 4$ , Mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  compared to vector control; <sup>b</sup> $P < 0.05$  compared to HNF4 $\alpha$  alone group. HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha.



**Figure 8** Activation of (A) mouse miR-101b, (B) human miR-101-2, and (C) mouse miR-802 promoter by HNF4 $\alpha$ . Human hepatoma HepG2 cells were transfected with firefly luciferase vectors containing microRNA promoter, pRL-CMV, and an expression vector for HNF4 $\alpha$  and/or C/EBP $\alpha$ . Dual-luciferase reporter assay was conducted 24 h after transfection. The Y-axis represents relative luciferase activity for microRNA promoter normalized by the renilla luciferase.  $n = 4$ , Mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  compared to vector control; <sup>c</sup> $P < 0.05$  compared to HNF4 $\alpha$  alone group. HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; C/EBP $\alpha$ : CCAAT/enhancer-binding protein  $\alpha$ .

HNF4 $\alpha$ . Moreover, putative C/EBP binding sites are highly enriched in the human miR-101-2 promoter, predicted by the Alibaba2 software. We found that HNF4 $\alpha$  and C/EBP $\alpha$  activated the mouse miR-101b promoter 6.2 and 8.9 fold, respectively, and they synergistically activated the miR-101b promoter 19 fold in HepG2 cells (Figure 8A). Similarly, HNF4 $\alpha$  and C/EBP $\alpha$  activated the human miR-101-2 promoter 11 and 33 fold, respectively, and they synergistically activated the miR-101-2 promoter 65 fold in HepG2 cells (Figure 8B).

### Regulation of mouse miR-802 promoter by HNF4 $\alpha$

Different from miR-101, HNF4 $\alpha$  had no effect on the 2 kb mouse miR-802 promoter, and HNF4 $\alpha$  did not enhance the transactivation of the miR-802 promoter by C/EBP $\alpha$  in HepG2 cells (Figure 8C).

### Regulation of mouse Chd1 and H3f3 by miR-194 and miR-192

TargetScan was used to identify potential targets of liver-predominant microRNAs down-regulated in *Hnf4 $\alpha$* -LivKO livers. miR-192/215 and miR-194 have a perfect match (8 mer) and very high context score percentile of 96%-99% with human and mouse histone H3f3b (H3.3b) and Chd1, respectively, indicating a very high likelihood of inhibition (Table 1). Therefore, we generated luciferase reporters for the 3'UTR of H3.3 and Chd1. Results of dual luciferase assay showed that miR-194 and miR-192 significantly decreased the luciferase activity for the 3' UTR of Chd1 (Figure 9A) and H3.3 (Figure 9B) by 37% and 36%, respectively, in HepG2 cells.

## DISCUSSION

The present study demonstrates that *Hnf4 $\alpha$*  is essential for hepatic expression of certain liver-predominant microRNAs, namely miR-101, miR-192, miR-193 and miR-194. HNF4 $\alpha$  transactivates these miRs *via* direct DNA-binding to the promoters and/or interacting with the general transcription factor SP1. These miRs target essential epigenetic modifiers, such as EZH2 (by miR-101), histone H3.3 (by miR-192) and Chd1 (by miR-194) (Figure 10).

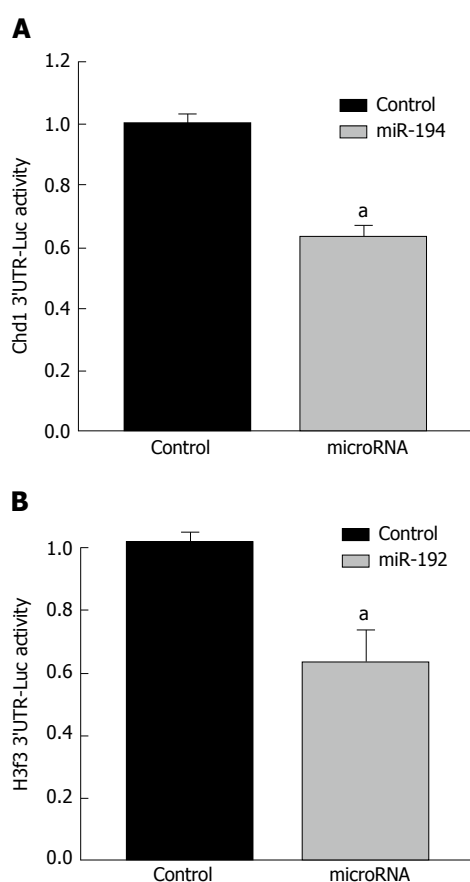
The present data provide the first evidence that HNF4 $\alpha$  is essential for hepatic expression of miR-194 in mice, and likely in humans. In both mice and humans, miR-194 is expressed highly in kidney and GI tract including liver and small intestine<sup>[37]</sup>. The tissue distribution of miR-194 parallels that of HNF4 $\alpha$ . In liver, miR-194 signals are detected in hepatocytes but not in non-parenchymal cells, and miR-194 is down-regulated during dedifferentiation of hepatocytes<sup>[54]</sup>. miR-194 inhibits the metastasis of mesenchymal-like liver cancer cells. Moreover, ChIP-seq results demonstrate direct binding of HNF4 $\alpha$  to the distal and proximal promoters of mouse and human miR-194-2 (Figure 3B and 6A). Furthermore, results of reporter assays indicate that HNF4 $\alpha$  potentially activates the promoter of mouse and human *miR-194-2/miR-192* gene cluster (Figure 7). Taken together, these data strongly indicate that HNF4 $\alpha$  plays a key role in maintaining hepatic expression of miR-194 in mice and humans.

Two recent studies of mice with inducible knockout of *Hnf4 $\alpha$*  demonstrate that acute loss of *Hnf4 $\alpha$*  in adult mouse liver triggers extensive hepatocyte proliferation, hepatomegaly, and increased HCC<sup>[55-57]</sup>. The increased intestinal cell proliferation in mice with specific loss of *Hnf4 $\alpha$*  in the adult intestinal epithelium is ascribed to the activation of the Wnt/beta-catenin system<sup>[58]</sup>. miR-194 negatively control expression of frizzled-6, which activates the beta-catenin pathway<sup>[36]</sup>. Therefore, *Hnf4 $\alpha$*  may

Table 1 Targeting of human and mouse genes by liver-predominant microRNAs predicted by TargetScan

	Predicted pairing of target region (top) and microRNA (bottom)	Seed match	Context score percentile
Position 1085-1091 of human H3F3B 3'UTR miR-192/215	5' ...AUUUACUGAAGUUUUUAGGUCAA...           3' CCGACAGUUAAGUAUCCAGUC	8 mer	96
Position 1064-1070 of mouse H3f3b 3'UTR miR-192/215	5' ...UCCUAUUGAAGUUUUUAGGUCAA...           3' CCGACAGUUAAGUAUCCAGUC	8 mer	99
Position 1109-1115 of human CHD1 3'UTR miR-194	5' ...GACUUUUAAUAUAAACUGUUACA...           3' AGGUGUACCUCAACGACAAUGU	8 mer	99
Position 1100-1106 of mouse Chd1 3'UTR miR-194	5' ...GCUUUAAUAUAAAAACUGUUACA...           3' AGGUGUACCUCAACGACAAUGU	8 mer	99

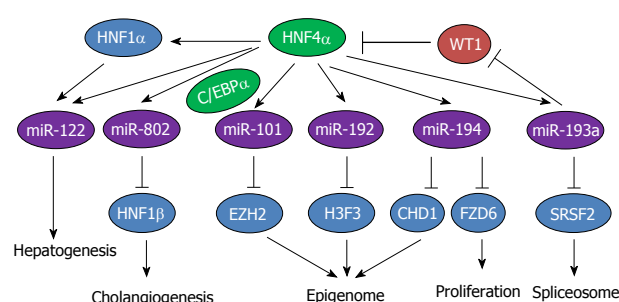
3'UTR: Untranslated regions.



**Figure 9 Effects of miR-194 and miR-192 on the activities of luciferase reporter vectors for the 3'UTR of mouse Chd1 and H3f3.** Human hepatoma HepG2 cells were transfected with plasmid DNA including pmir-Chd1 (or pmir-H3f3), the pRL-CMV luciferase, and a synthetic mimic of miR-194/miR-192, or AllStars Negative Control siRNA (as negative control for microRNAs) using Lipofectamine 2000. Dual-luciferase reporter assay was conducted 24 h after transfection. The Y-axis represents relative luciferase activity for the 3'UTR of Chd1 or H3f3 normalized by the renilla luciferase.  $n = 4$ , Mean  $\pm$  SE.  $^aP < 0.05$  compared to control (AllStars Negative Control siRNA). 3'UTR: Untranslated regions.

inhibit cell proliferation through the miR-194→frizzled-6→beta-catenin signaling pathway.

The chromatin remodeling factor CHD1 is required to maintain the open chromatin and pluripotency of mouse



**Figure 10** Diagram that illustrates the regulation of hepatic microRNA expression by Hnf4α in mouse liver. HNF4α: Hepatocyte nuclear factor 4 alpha; C/EBPα: CCAAT/enhancer-binding protein α.

embryonic stem cells<sup>[40]</sup>. CHD1 is required for chromatin incorporation of the histone variant H3.3, which is generally associated with active genes<sup>[59]</sup>. However, CHD1 may also repress gene expression *via* association with HDACs<sup>[60]</sup>. Overexpression of HNF4 $\alpha$  in hepatoma cells dramatically decreased the “stemness” gene expression and the percentage of cancer stem cells in HCC<sup>[8]</sup>; however, the underlying mechanism is unknown. HNF4 $\alpha$ , *via* regulating miR-194, might inhibit stemness gene expression by targeting the chromatin remodeling factor CHD1, which deposits the unmodified or altered histone H3.3 into chromatin and increases the stemness of *Hnf4 $\alpha$* -LivKO hepatocytes.

The present data indicate that Hnf4 $\alpha$  is essential for hepatic expression of miR-192, and the histone variant H3.3 is a direct target of miR-192. Thus, down-regulation of miR-192 may be the underlying mechanism of hepatic induction of H3.3 in young-adult Hnf4 $\alpha$ -LivKO mice<sup>[21]</sup>. The replacement H3 variant H3.3 is encoded by two genes termed H3.3A and H3.3B, both code for the same amino acid sequence, but differ in nucleotide sequences and gene organization<sup>[61]</sup>. H3.3 is the exclusive substrate for replication-independent deposition, which provides a mechanism for the immediate activation of genes that are silenced by histone modification<sup>[62,63]</sup>, and H3.3 is important in epigenetic memory<sup>[64]</sup>. H3.3/H2A.Z double variant-containing nucleosomes mark “nucleosome-free regions” of active promoters and other regulatory

regions<sup>[65]</sup>. Deposition of H3.3 can rapidly derepress gene silencing<sup>[66]</sup>. Taken together, Hnf4 $\alpha$  directly regulates miR-192, and the down-regulation of miR-192 in *Hnf4 $\alpha$ -LivKO* livers may be the underlying mechanism of hepatic induction of H3.3, which contributes to the marked alteration of epigenome and transcriptome in *Hnf4 $\alpha$ -LivKO* livers<sup>[21]</sup>.

The present study indicates that Hnf4 $\alpha$  is required for hepatic expression of the tumor-suppressor miR-101. miR-101 is predominantly expressed in the liver<sup>[35]</sup>. miR-101 is down-regulated in HCC<sup>[67]</sup> and miR-101 directly represses EZH2<sup>[68,69]</sup>, a protooncogene that silences the expression of tumor-suppressors *via* H3K27me3. Down-regulation of miR-101 in *Hnf4 $\alpha$ -LivKO* mouse livers might be the underlying mechanism of induction of EZH2 and increased H3K27me3 observed previously<sup>[21]</sup>.

The present data indicate that Hnf4 $\alpha$  is important for hepatic basal expression of the tumor-suppressor miR-193a. miR-193a and miR-365 closely cluster in chromosome 11 in mice. The tumor-suppressor miR-193a is down-regulated in the majority of HCC in humans<sup>[70]</sup> and miR-193a prevents the resistance of HCC to 5-fluorouracil *via* repressing the expression of serine/arginine-rich splicing factor 2 (SRSF2)<sup>[71]</sup>. Through maintaining hepatic expression of miR-193a, HNF4 $\alpha$  might regulate expression of SRSF2 and the splicing of transcripts in liver. Interestingly, miR-193a also targets directly Wilms' tumor protein 1 (WT1)<sup>[72]</sup>. WT1 is overexpressed in cirrhotic liver and HCC<sup>[18,73]</sup>, and induction of WT1 down-regulates HNF4 $\alpha$  expression in liver<sup>[18]</sup>. The putative feedback regulatory loop of HNF4 $\alpha$ →miR-193a→WT1 and its significance in liver cirrhosis and carcinogenesis warrant further investigation.

The present data provide the first evidence that Hnf4 $\alpha$  is important for hepatic expression of miR-802 (Figure 2). Results of reporter assay (Figure 8C) suggest that HNF4 $\alpha$  may indirectly regulate hepatic miR-802 expression *via* C/EBP $\alpha$ , whose DNA-binding activity decreased in *Hnf4 $\alpha$ -LivKO* mice<sup>[27]</sup> and human hepatoma cells. Interestingly, the miR-802 locus is marked with both the activating signature H3K4me3 and the silencing signature H3K27me3, a feature of bivalent chromatin which allows a low basal expression but timely activation of developmentally-regulated genes<sup>[74]</sup>. Hnf1 $\beta$  is a direct target of miR-802<sup>[75]</sup>, and Hnf1 $\beta$  is overexpressed in adult *Hnf4 $\alpha$ -LivKO* mouse livers<sup>[4]</sup>. In mouse liver, miR-802 is expressed at 10-fold higher levels in hepatocytes than non-hepatocytes<sup>[75]</sup>. In contrast, Hnf1 $\beta$  is strongly expressed in cholangiocytes but weakly in hepatocytes, and Hnf1 $\beta$  plays a key role in bile-duct morphogenesis and glucose homeostasis<sup>[76]</sup>. Thus, the putative HNF4 $\alpha$ →C/EBP $\alpha$ →miR-802→HNF1 $\beta$  pathway might play a role in controlling cell-specific expression of HNF1 $\beta$  and liver morphogenesis during liver development.

The tumor-suppressor microRNAs miR-34a, miR-192, miR-215 and miR-194 are all p53-inducible microRNAs<sup>[77]</sup>. The induction of the p53-target gene p21 in *Hnf4 $\alpha$ -nul* mouse livers<sup>[13]</sup> suggests that p53 is activated by *Hnf4 $\alpha$*  deficiency, which may contribute to the induction of the p53-target miR-34a and miR-29b (Figure 2B). However,

hepatic expression of other p53-target microRNAs miR-192, miR-215 and miR-194 are markedly down-regulated in *Hnf4 $\alpha$ -LivKO* mice. It is interesting that HNF4 $\alpha$  can transactivate two p53-target genes, p21 and miR-194 (Figure 7), independent of DNA-binding of HNF4 $\alpha$  to the promoter. The AMP-activated protein kinase (AMPK) phosphorylates HNF4 $\alpha$  at S304, resulting in a marked decrease in the DNA-binding activity and decreased transactivation of apolipoprotein C3<sup>[50]</sup>. AMPK suppresses lipogenesis and carcinogenesis in liver<sup>[78,79]</sup>. The contribution of selective modulation of HNF4 $\alpha$ -target lipogenic genes and tumor-suppressors (p21 and miR-194) to the physiological and pharmacological roles of AMPK in liver diseases warrants further investigation.

miR-29 is broadly expressed at high levels in normal tissues. miR-29 sensitizes cholangiocarcinoma cells to TNF-induced cytotoxicity<sup>[80]</sup> and miR-29 activates p53<sup>[81]</sup>. miR-29 induces global DNA hypomethylation and tumor suppressor gene reexpression in lung cancer and acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1<sup>[82,83]</sup>. miR-29 also directly inhibits Dnmt3a and Dnmt3b in mice<sup>[84]</sup>. Thus, induction of miR-29b might contribute to the lack of global changes in hepatic DNA methylation, despite an induction of Dnmt1, in the young-adult *Hnf4 $\alpha$ -LivKO* mice<sup>[21]</sup>. Currently, the mechanism of induction of miR-29 in the young-adult *Hnf4 $\alpha$ -LivKO* mice remains unknown. miR-29 can be transactivated by p53<sup>[85]</sup>. Thus, activation of p53 might contribute to hepatic induction of miR-29b in *Hnf4 $\alpha$ -LivKO* mice.

The liver-specific miR-122 is important in regulating hepatic cholesterol and lipid metabolism<sup>[86,87]</sup>, and down-regulation of miR-122 contributes to HCC malignancy<sup>[88-90]</sup>. HNF4 $\alpha$  can directly activate the expression of miR-122 in mouse liver<sup>[91]</sup>. However, knockdown of HNF4 $\alpha$  does not affect the high expression of miR-122 in a HCC cell line, although miR-122 expression correlates strongly with HNF4 $\alpha$ <sup>[88]</sup>. In contrast, hepatic miR-122 expression is regulated by Hnf1 $\alpha$ <sup>[88]</sup>. The moderate down-regulation of miR-122 in *Hnf4 $\alpha$ -LivKO* mouse livers parallels the moderate decrease of Hnf1 $\alpha$  in these mice<sup>[27]</sup>. Taken together, these data suggest that HNF4 $\alpha$  has a positive but limited role in regulating hepatic expression of miR-122.

The present study demonstrates species differences between humans and mice in hepatic basal expression and regulation of miR-124 and miR-134 by HNF4 $\alpha$ . Interleukin-6 (IL6) plays a key role in inflammation and hepatocarcinogenesis<sup>[92]</sup>. Interestingly, HNF4 $\alpha$  exerts anti-inflammatory effects in human hepatocytes *via* the miR-124-IL6R-STAT3 pathway; knockdown of HNF4 $\alpha$  in human hepatocytes leads to down-regulation of miR-124, induction of IL6R and IL6, and activation of STAT3<sup>[49]</sup>. However, there is no induction of IL-6 or activation of STAT3 in adult mice with acute loss of HNF4 $\alpha$ <sup>[56]</sup>. Thus, there may be species difference between humans and mice regarding the interaction of HNF4 $\alpha$  with miR-regulated inflammatory and carcinogenic pathways in the liver.

Our previous study found that Hnf4 $\alpha$  deficiency in

young-adult mice causes marked alteration of histone modifications, which is associated with induction of epigenetic modifiers such as Ezh2 and histone H3.3<sup>[20]</sup>. However, ChIP-seq data reveal no direct binding of Hnf4 $\alpha$  to these epigenetic modifiers in adult mouse livers, suggesting that these epigenetic modifiers may not be directly regulated by Hnf4 $\alpha$ . The present study provides the first evidence of the essential role of Hnf4 $\alpha$  in maintaining hepatic expression of certain microRNAs, including miR-101, miR-192, miR-193a, miR-194 and miR-802. These microRNAs target certain key proteins in gene regulation and epigenetic modifications, such as WT1 (by miR-193a)<sup>[72]</sup>, HNF1 $\beta$  (by miR-802)<sup>[75]</sup>, CHD1 (by miR-194) (Figure 9), EZH2 (by miR-101)<sup>[69]</sup>, SRSF2 (by miR-193a)<sup>[71]</sup>, and histone H3.3 (by miR-192) (Figure 9). Establishment and maintenance of hepatic expression of these microRNAs by HNF4 $\alpha$  may play a key role in the indirect regulation of hepatic transcriptome and epigenome by HNF4 $\alpha$  (Figure 10).

## ACKNOWLEDGMENTS

The authors would like to thank the members of Sequencing Core at SUNY Upstate Medical University for their technical support.

## COMMENTS

### Background

Hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ) is a liver-enriched master regulator of liver development and function. HNF4 $\alpha$  plays a key role in regulating hepatic transcriptome and epigenome. However, little was known about the role of HNF4 $\alpha$  in regulating hepatic expression of microRNAs, essential modulators of the transcriptome and epigenome. Additionally, HNF4 $\alpha$  deficiency causes marked induction of a large number of genes in mouse liver; however, the mechanism of suppression of hepatic gene expression by HNF4 $\alpha$  remains poorly understood.

### Research frontiers

Previous studies demonstrate that HNF4 $\alpha$  regulates hepatic expression of miR-122, miR-124 and miR-29.

### Innovations and breakthroughs

This is the first study to use microarray and liver-specific knockout mice to determine the genome-wide role of HNF4 $\alpha$  in the regulation of hepatic expression of microRNAs in mice. The key changes in hepatic microRNA expression induced by HNF4 $\alpha$  deficiency were verified by real-time polymerase chain reaction. Moreover, hepatic microRNA expression were correlated with chromatin accessibility as well as DNA-binding of HNF4 $\alpha$ , RNA polymerase II, and activating/silencing epigenetic signatures to determine the role of HNF4 $\alpha$  in regulating hepatic expression of these microRNAs. The novel key role of HNF4 $\alpha$  in regulating liver-predominant expression of miR-101-2/miR-101b and the miR-194-2/miR-192 cluster was confirmed by luciferase reporter assay.

### Applications

Results from this study uncover species differences and similarities between humans and mice in the role of HNF4 $\alpha$  in regulating hepatic expression of certain important microRNAs. Such novel knowledge will help understand the role of HNF4 $\alpha$  in post-transcriptional regulation of gene expression and maintenance of the normal epigenome and physiology in mouse and human liver.

### Terminology

Epigenetic signatures/marks are modifications of the genome that do not change

the underlying DNA sequence but can switch genes on and off and thus affect how cells express genes. Typical epigenetic signatures/marks include DNA methylation and histone modifications.

## Peer-review

The study appears to be properly conducted and written. No major criticisms and/or weaknesses were noted.

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