



# An HNF4 $\alpha$ -microRNA-194/192 signaling axis maintains hepatic cell function

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Hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) controls the expression of liver-specific protein-coding genes. However, some microRNAs are also modulated by HNF4 $\alpha$ , and it is not known whether they are direct targets of HNF4 $\alpha$  and whether they influence hepatic function. In this study, we found that HNF4 $\alpha$  regulates microRNAs, indicated by marked down-regulation of miR-194 and miR-192 (miR-194/192) in liver-specific *Hnf4a*-null (*Hnf4a*<sup>ΔH</sup>) mice. Transactivation of the shared miR-194/192 promoter was dependent on HNF4 $\alpha$  expression, indicating that miR-194/192 is a target gene of HNF4 $\alpha$ . Screening of potential mRNAs targeted by miR-194/192 revealed that expression of genes involved in glucose metabolism (glycogenin 1 (*Gyg1*)), cell adhesion and migration (activated leukocyte cell adhesion molecule (*Alcam*)), tumorigenesis and tumor progression (*Rap2b* and epiregulin (*Ereg*)), protein SUMOylation (*Sumo2*), epigenetic regulation (*Setd5* and Cullin 4B (*Cln4b*)), and the epithelial-mesenchymal transition (moesin (*Msn*)) was up-regulated in *Hnf4a*<sup>ΔH</sup> mice. Moreover, we also found that miR-194/192 binds the 3'-UTR of these mRNAs. siRNA knockdown of HNF4 $\alpha$  suppressed miR-194/192 expression in human hepatocellular carcinoma (HCC) cells and resulted in up-regulation of their mRNA targets. Inhibition and overexpression experiments with miR-194/192 revealed that *Gyg1*, *Setd5*, *Sumo2*, *Cln4b*, and *Rap2b* are miR-194 targets, whereas *Ereg*, *Alcam*, and *Msn* are miR-192 targets. These findings reveal a novel HNF4 $\alpha$  network controlled by miR-194/192 that may play a critical role in maintaining the hepatocyte-differentiated state by inhibiting expression of genes involved in dedifferentiation and tumorigenesis. These insights may contribute to the development of diagnostic markers for early HCC detection, and targeting of the miR-194/192 pathway could be useful for managing HCC.

Liver has diverse functions such as metabolism of carbohydrates, lipids, and proteins; detoxification of ammonia and xenobiotics; and synthesis of bile acids and blood coagulation factors. Various liver-enriched transcription factors such as hepatocyte nuclear factor 1 (HNF1),<sup>2</sup> HNF3, HNF4, and HNF6 are needed to maintain differentiated liver-specific functions (1). Of these, HNF4 $\alpha$  is a member of the nuclear receptor superfamily and a key regulator involved in the transcription regulatory network in the liver (2, 3), and re-expression of HNF4 $\alpha$  in dedifferentiated hepatoma cells has the potential to revert cells back to the differentiated state (4). As *Hnf4a*-null mice are embryonic lethal during gastrulation (5), two types of liver-specific *Hnf4a*-null mice (*Hnf4a*<sup>ΔH</sup>) were established (6, 7). Disruption of HNF4 $\alpha$  in the fetal livers showed that hepatic HNF4 $\alpha$  is essential for formation of a hepatic epithelium, liver morphogenesis, and liver homeostasis by positive regulation of a large number of genes during development into the mature liver (6). Disruption of HNF4 $\alpha$  in the *Hnf4a*<sup>ΔH</sup> mice led to diverse phenotypes such as fatty livers and elevation of serum bile acids and ammonia (7–9). *Hnf4a*<sup>ΔH</sup> mice also exhibit increased spontaneous hepatocyte proliferation and increased susceptibility to diethylnitrosamine-induced liver cancer, indicating that HNF4 $\alpha$  has tumor suppressor activity, although the mechanism has not been established (10, 11). Because HNF4 $\alpha$  regulates many liver-enriched genes via HNF4 $\alpha$ -binding sites in the promoter regions of the target genes and HNF4 $\alpha$  is bound to about 12% of the gene promoters expressed in human hepatocytes (12), the HNF4 $\alpha$ -derived transcriptional network in the liver is expected to be considerably complicated.

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This article contains supplemental Figs. S1 and S2 and Tables S1–S5.

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<sup>2</sup> The abbreviations used are: HNF, hepatocyte nuclear factor; miRNA, microRNA; miR-194/192, miR-194 and miR-192; *Hnf4a*<sup>ΔH</sup> mice, liver-specific *Hnf4a*-null mice, *Hnf4a*<sup>fl/fl</sup> mice, *Hnf4a*-floxed mice; HCC, hepatocellular carcinoma; EMT, epithelial-mesenchymal transition; *Otc*, ornithine transcarbamylase; *Gyg1*, glycogenin 1; *Fzd6*, frizzled-6; *Alcam*, activated leukocyte cell adhesion molecule; *Ereg*, epiregulin; *Cln4b*, Cullin 4B; *Msn*, moesin; *Cdh2*, N-cadherin; *Hbegf*, heparin-binding epidermal growth factor; *Arfgap2*, tyrosine protein phosphatase non-receptor type 2; *Itga9*, integrin  $\alpha$ 9; *Socs2*, suppressor of cytokine signaling 2; *Dnmt3a*, DNA methyltransferase 3A; *Tln2*, talin 2; *Hook3*, hook microtubule-tethering protein 3; *Seps1*, selenophosphate synthetase 1; SUMO, small ubiquitin-like modifier; *Arfgap1*, ADP-ribosylation factor guanine nucleotide-exchange factor 1; *Rsad2*, radical S-adenosylmethionine domain-containing 2; qRT-PCR, quantitative RT-PCR; pri-miR, primary miRNA; C/EBP $\alpha$ , CCAAT-enhancer-binding protein  $\alpha$ ; RIPA, radioimmune precipitation assay; qPCR, quantitative PCR.

The gene expression pattern in the livers of *Hnf4a*<sup>ΔH</sup> mice is markedly changed largely due to loss of expression of direct HNF4 $\alpha$  target genes that are down-regulated, giving rise to the altered phenotypes of these mice (13). For instance, reduced expression of ornithine transcarbamylase (*Otc*), encoded by a HNF4 $\alpha$  target gene, caused elevated serum ammonia due to the inability to degrade ammonia (8). In contrast, many mRNAs are up-regulated in *Hnf4a*<sup>ΔH</sup> mice. Although direct transcriptional repression by HNF4 $\alpha$  itself could be considered as a mechanism by which these mRNAs are reduced in *Hnf4a*<sup>ΔH</sup> mice, another possibility is the involvement of microRNAs (miRNAs) that could be under direct transcriptional control of HNF4 $\alpha$ . miRNAs are small non-coding RNAs involved in various physiological functions such as development, differentiation, inflammation, and tumorigenesis through their ability to down-regulate protein-coding gene expression by binding to target mRNAs and either promoting mRNA degradation or inhibiting mRNA translation (14, 15). miRNAs have important roles in liver diseases such as viral hepatitis, steatohepatitis, liver fibrosis, polycystic liver disease, and hepatocellular carcinoma (HCC) (16, 17).

Among the miRNAs, miR-122 is the most abundant in adult liver (18). Expression of miR-122 is reduced in HCC, and over-expression of miR-122 suppresses cell proliferation (19, 20). Thus, miR-122 could function as a tumor suppressor gene against HCC. Expression of miR-122 positively correlates with HNF4 $\alpha$  expression in HCC cell lines, and miR-122 is directly regulated by HNF4 $\alpha$ , indicating that the HNF4 $\alpha$ -directed miR-122 pathway could be essential for inhibition of hepatocyte proliferation and HCC development (21, 22). Likewise, HNF4 $\alpha$  directly regulates the expression of miR-134 and miR-134-suppressed oncogenic KRAS expression, revealing that miR-134 has a suppressive effect on HCC progression (23). Moreover, the following HNF4 $\alpha$ -miRNA inflammatory feedback circuits in human HCC have been proposed. HNF4 $\alpha$  positively regulates miR-7 and miR-124 followed by suppression of IL6R, STAT3, and RelA-mediated NF- $\kappa$ B pathways, eventually resulting in the up-regulation of HNF4 $\alpha$  through down-regulation of miR-24, miR-629, or miR-21 (24, 25). These results also indicate that lack of HNF4 $\alpha$ -controlled miRNAs could result in HCC development by activation of inflammatory signaling. These miRNAs were identified from studies using immortalized hepatocytes or HCC cell lines and thus could be biomarkers for HCC diagnosis and candidates for new target genes for HCC therapy. However, miRNAs regulated by HNF4 $\alpha$  in normal hepatocytes but not in immortalized or transformed hepatocytes remain to be identified.

In this study, the miRNAs regulated by HNF4 $\alpha$  were examined using *Hnf4a*<sup>ΔH</sup> mice. miR-194 and miR-192, controlled by the same promoter, were down-regulated in *Hnf4a*<sup>ΔH</sup> mice, and HNF4 $\alpha$  directly bound to the promoter region of the miR-194-2 and miR-192 (miR-194/192) gene cluster. Furthermore, miR-194 and miR-192 directly targeted many mRNAs including frizzled-6 (*Fzd6*), *Gyg1*, *Setd5*, *Sumo2*, *Cln4b*, *Rap2b*, *Ereg*, *Alcam*, and *Msn*. The HNF4 $\alpha$ -miR-194/192 cascade was also conserved in the human HCC cell line. Consequently, the HNF4 $\alpha$ -miR-194/192 pathway could be a novel HNF4 $\alpha$  cas-

cade and a candidate for early detection of HCC and development of anti-HCC drugs.

## Results

### Down-regulation of hepatic miR-194 and miR-192 in *Hnf4a*<sup>ΔH</sup> mice

HNF4 $\alpha$  positively regulates the expression of many genes through HNF4 $\alpha$ -binding sites in promoter regions (1, 3). A marked change of the mRNA expression pattern was observed in the livers of *Hnf4a*<sup>ΔH</sup> mice (13), including many down-regulated mRNAs identified as encoded by direct HNF4 $\alpha$  target genes (8, 9, 26). In addition, several up-regulated genes were also detected in *Hnf4a*<sup>ΔH</sup> mice, but their mechanism of increase is not known. Although transcriptional repression of genes such as *Snail*, *Slug*, and *HMG2* by HNF4 $\alpha$  was demonstrated (27), the mechanism of negative regulation of other genes by HNF4 $\alpha$  has not been established. Another possibility for negatively regulated mRNAs is indirectly through miRNA. Indeed, HNF4 $\alpha$  is known to regulate the expression of miR-7, miR-122, miR-124, and miR-134 (22–25). Thus, expression profiling of hepatic miRNAs in *Hnf4a*-floxed (*Hnf4a*<sup>f/f</sup>) and *Hnf4a*<sup>ΔH</sup> mice was investigated by microarray analysis (supplemental Table S1). Of 610 miRNAs, expression of 24 and 19 miRNAs was down-regulated or up-regulated more than 2-fold in *Hnf4a*<sup>ΔH</sup> mice, respectively (Table 1). In general, because HNF4 $\alpha$  positively regulates the target genes, down-regulated miRNAs in *Hnf4a*<sup>ΔH</sup> mice could be direct target genes for HNF4 $\alpha$ . Although other miRNAs, including miR-34a and miR-301a, were increased in *Hnf4a*<sup>ΔH</sup> mice, they were not investigated in the current study. They could be either directly repressed by HNF4 $\alpha$  or the result of downstream events mediated by loss of expression of other direct HNF4 $\alpha$  target genes.

The expression of miR-194 was decreased by 90% in *Hnf4a*<sup>ΔH</sup> mice compared with *Hnf4a*<sup>f/f</sup> mice. miR-194 was also expressed at considerably higher levels in *Hnf4a*<sup>f/f</sup> mice when compared with other miRNAs. Similarly, the expression of miR-192 was also higher in *Hnf4a*<sup>f/f</sup> mice and markedly lower in *Hnf4a*<sup>ΔH</sup> mice. Indeed, qRT-PCR quantification showed that hepatic expression of miR-194 and miR-192 was suppressed by about one-tenth in *Hnf4a*<sup>ΔH</sup> mice (Fig. 1A). Expression of other liver-enriched miRNAs such as miR-21, -101b, -130a, -193, -193b, -455, and -805 was also decreased in *Hnf4a*<sup>ΔH</sup> mice (Fig. 1A). Others have reported that the expression of miR-194 and miR-192 is regulated under the same promoter, and tissue distribution of miR-194 and miR-192 is almost equivalent to that of HNF4 $\alpha$  (28, 29), suggesting that HNF4 $\alpha$  could positively regulate the expression of miR-194 and miR-192. Therefore, further studies were performed on miR-194 and miR-192. No significant difference in hepatic expression of miR-7, miR-122, miR-124, and miR-134, which were reported as the target miRNAs for HNF4 $\alpha$  (22–25), was observed in *Hnf4a*<sup>ΔH</sup> mice (supplemental Table S1).

In mice, mature miR-194 is located on two chromosomes with miR-194-1 and miR-194-2 clustered with miR-215 and miR-192, respectively. Expression of miR-215 was unchanged in *Hnf4a*<sup>ΔH</sup> mice, and the expression level was very low as compared with miR-194 and miR-192 (Table 1). Thus, miR-194-2,

## Regulation of miR-194/192 by hepatic HNF4 $\alpha$

**Table 1**

**Expression profiling of hepatic miRNAs in *Hnf4a* <sup>$\Delta$ H</sup> mice**

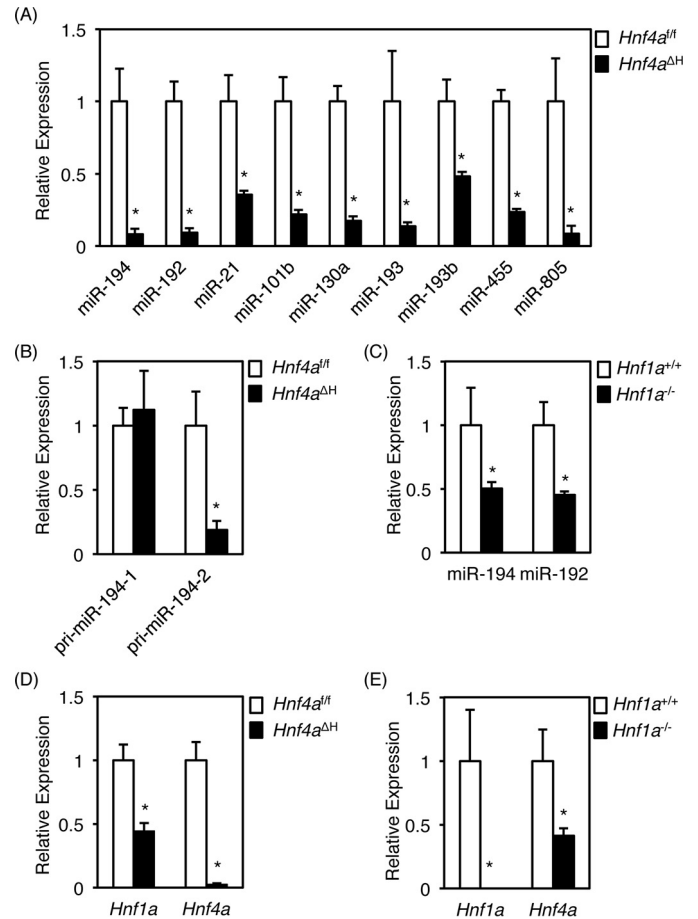
miRNAs down-regulated or up-regulated more than 2-fold in *Hnf4a* <sup>$\Delta$ H</sup> mice are listed. No significant change of miR-215 and miR-122 was detected. Raw data of each miRNA are given in parentheses.

miRNA	Fold change ( <i>Hnf4a</i> <sup><math>\Delta</math>H</sup> / <i>Hnf4a</i> <sup>+/+</sup> )
<b>Down-regulated</b>	
miR-194	0.11 (77.4/698.7)
miR-455	0.11 (1.4/12.5)
miR-805	0.16 (21.8/137.6)
miR-192	0.17 (96.5/559.5)
miR-193	0.25 (22.3/89.5)
miR-365	0.27 (4.4/16.1)
miR-193b	0.31 (19.8/64.1)
miR-203	0.31 (6.9/22.1)
miR-130a	0.32 (55.3/171.8)
miR-467a*, -d*	0.32 (1.3/4.1)
miR-377	0.33 (1.3/4.0)
miR-220	0.37 (1.1/3.0)
miR-466b-3-3p	0.38 (1.0/2.6)
miR-101b	0.39 (29.6/75.5)
miR-323-5p	0.43 (1.6/3.7)
miR-299*	0.43 (1.3/3.0)
miR-574-3p	0.44 (8.3/19.0)
miR-669e	0.46 (1.7/3.7)
miR-21	0.47 (127.8/270.0)
miR-680	0.47 (3.7/7.8)
miR-467f	0.47 (2.8/6.0)
miR-802	0.47 (2.1/4.5)
miR-425*	0.48 (1.2/2.5)
miR-290-3p	0.48 (1.0/2.1)
<b>Up-regulated</b>	
miR-34a	5.99 (53.9/9.0)
miR-301a	4.59 (7.8/1.7)
miR-28	3.34 (9.7/2.9)
miR-497	3.06 (19.9/6.5)
miR-484	2.97 (11.3/3.8)
miR-181a	2.92 (11.1/3.8)
miR-689	2.56 (4.6/1.8)
miR-350	2.41 (14.0/5.8)
miR-500	2.41 (7.0/2.9)
miR-152	2.32 (24.6/10.6)
miR-125a-5p	2.31 (65.1/28.2)
miR-31	2.29 (151.7/66.2)
miR-195	2.28 (50.9/22.3)
miR-335-5p	2.27 (11.8/5.2)
miR-31*	2.26 (4.3/1.9)
miR-142-5p	2.23 (13.6/6.1)
miR-140*	2.16 (14.9/6.9)
miR-151-5p	2.10 (26.1/12.4)
miR-200b	2.05 (19.5/9.5)
miR-215	1.38 (2.5/1.8)
miR-122	0.93 (7459/8046)

but not miR-194-1, could be highly expressed in normal liver and preferentially suppressed in *Hnf4a* <sup>$\Delta$ H</sup> mice. As expected, hepatic expression of pri-miR-194-1 was unchanged, whereas expression of pri-miR-194-2 was markedly suppressed in *Hnf4a* <sup>$\Delta$ H</sup> mice (Fig. 1B). These data indicate that HNF4 $\alpha$  could directly activate the miR-194/192 gene via a single promoter.

### Regulation of hepatic miR-194/192 by HNF4 $\alpha$ and HNF1 $\alpha$

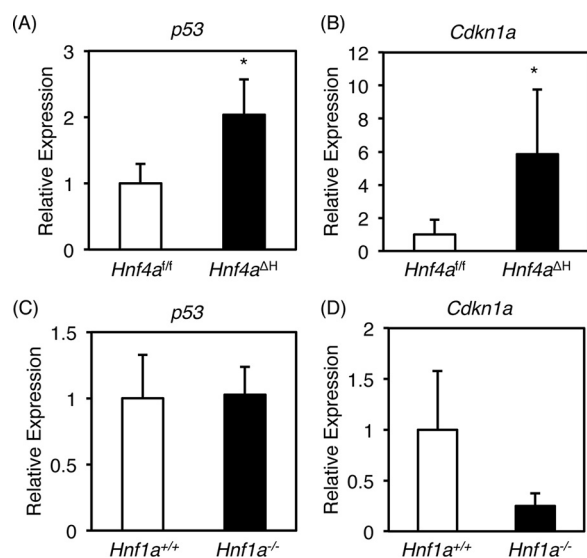
HNF1 $\alpha$ , another liver-enriched transcription factor, directly regulates the miR-194/192 gene through a binding site in the miR-194/192 promoter region (28). Accordingly, qRT-PCR analysis using the livers of *Hnf4a* <sup>$\Delta$ H</sup> mice and *Hnf1a*-null mice was performed to determine which factor, HNF1 $\alpha$  or HNF4 $\alpha$ , predominates in controlling expression of the miR-194/192 gene. Hepatic expression of both miRNAs in *Hnf1a*-null mice was reduced to 50%, and the remnant levels were much higher when compared with *Hnf4a* <sup>$\Delta$ H</sup> mice (Fig. 1C). Because HNF4 $\alpha$  positively regulates *Hnf1a* in the liver (2), decreased expression



**Figure 1. Down-regulation of hepatic miRNAs in *Hnf4a* <sup>$\Delta$ H</sup> mice.** Real-time PCR data for miR-194, miR-192, miR-21, miR-101b, miR-130a, miR-193, miR-193b, miR-455, and miR-805 (A), pri-miR-194-1 and pri-miR-194-2 (B), and *Hnf1a* and *Hnf4a* (D) from total liver RNA of *Hnf4a* <sup>$\Delta$ H</sup> and *Hnf4a*<sup>+/+</sup> mice and for miR-194 and miR-192 (C) and *Hnf1a* and *Hnf4a* (E) from total liver RNA of *Hnf1a*<sup>-/-</sup> and *Hnf1a*<sup>+/+</sup> mice are shown. Error bars represent S.D. Data are mean  $\pm$  S.D. of four independent mice. \*,  $p < 0.05$ .

of miR-194/192 in *Hnf4a* <sup>$\Delta$ H</sup> mice could be due to decreased expression of both *Hnf1a* and *Hnf4a*. Therefore, the expression of *Hnf4a* and *Hnf1a* mRNAs in *Hnf1a*-null mice was also investigated. The expression of *Hnf4a* was reduced to 3%, and the expression of *Hnf1a* mRNA was reduced to 45% in *Hnf4a* <sup>$\Delta$ H</sup> mice (Fig. 1D). Conversely, expression of *Hnf1a* mRNA was not detected, and expression of *Hnf4a* mRNA was reduced to 42% in *Hnf1a*-null mice (Fig. 1E), revealing that the remaining expression of *Hnf1a* in *Hnf4a* <sup>$\Delta$ H</sup> mice and *Hnf4a* in *Hnf1a*-null mice is similar. Hence, HNF4 $\alpha$  could be a more essential factor for controlling expression of miR-194/192 as compared with HNF1 $\alpha$  in the liver.

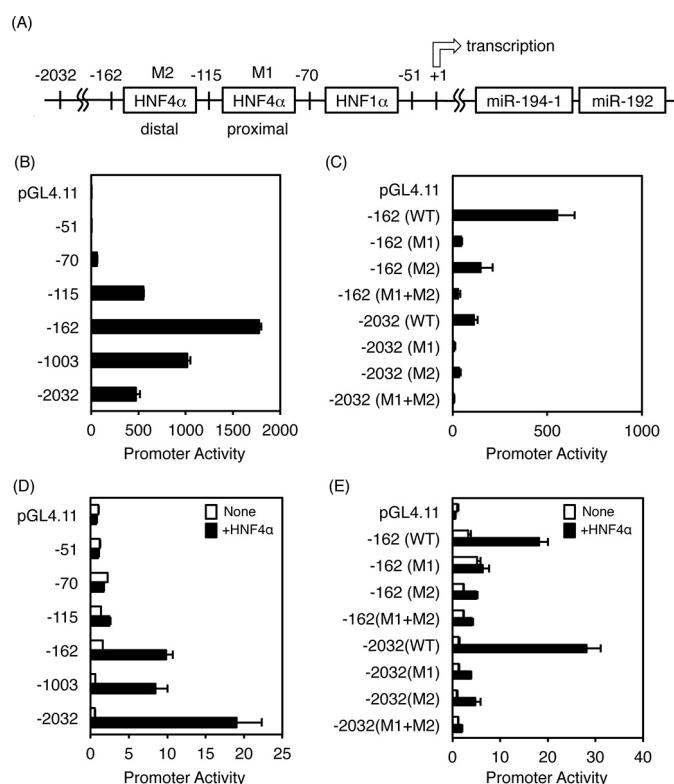
p53 also has the potential to transcriptionally control the miR-194/192 gene (29, 30). However, p53 was up-regulated in *Hnf4a* <sup>$\Delta$ H</sup> mice, whereas it was unchanged in *Hnf1a*-null mice (Fig. 2, A and C). Similarly, the expression of *p21* (*Cdkn1a*) mRNA, encoded by a direct target gene for p53, was also up-regulated in *Hnf4a* <sup>$\Delta$ H</sup> mice and unchanged in *Hnf1a*-null mice (Fig. 2, B and D), indicating that up-regulation of p53 in *Hnf4a* <sup>$\Delta$ H</sup> mice could not overcome repression of miR-194/192 expression caused by loss of HNF4 $\alpha$ .



**Figure 2.** Expression of hepatic *p53* and *Cdkn1a* in *Hnf4a* $\Delta^H$  and *Hnf1a*-null mice. Real-time PCR data for *p53* (A and C) and *Cdkn1a* (B and D) from total liver RNA of liver-specific *Hnf4a* $\Delta^H$  and *Hnf4a*<sup>fl/fl</sup> mice (A and B) and *Hnf1a*-null (*Hnf1a*<sup>-/-</sup>) and control (*Hnf1a*<sup>+/+</sup>) mice (C and D) are shown. Error bars represent S.D. Data are mean  $\pm$  S.D. of four independent mice. \*,  $p < 0.05$ .

### The miR-194/192 gene promoter is transactivated by HNF4 $\alpha$

To investigate the mechanism by which HNF4 $\alpha$  regulates the miR-194/192 gene, the promoter region of the miR-194/192 gene was analyzed. As described above, HNF1 $\alpha$  was already found to transactivate the miR-194/192 gene through an HNF1 $\alpha$ -binding site in colorectal adenocarcinoma-derived Caco-2 cells, and two regions located at -130 to -118 and -110 to -98 upstream of the HNF1 $\alpha$ -binding site were highly conserved among species (28), but the binding proteins were not identified. Because HNF4 $\alpha$  binding to both regions was predicted by JASPAR, an open access database (Fig. 3A), promoter analysis was performed to determine whether HNF4 $\alpha$  has the potential to transactivate the miR-194/192 promoter. In HCC-derived and endogenous HNF4 $\alpha$ -expressing HepG2 cells, the -70/+21 fragment containing an HNF1 $\alpha$ -binding site was transactivated by 50-fold as compared with the promoterless vector pGL4.11 (Fig. 3B). The promoter activity of the -115/+21 fragment containing the HNF1 $\alpha$ -binding site and a proximal HNF4 $\alpha$ -binding site was much higher, and that of the -162/+21 fragment was highest in the presence of both HNF4 $\alpha$ -binding sites, indicating that the minimum promoter of the miR-194/192 gene is located between -162 and +21. Introduction of mutations into the proximal (M1) or distal (M2) HNF4 $\alpha$ -binding site resulted in significant reduction of the activities, and mutations in both binding sites (M1 + M2) did not transactivate the -162 and -2032/+21 regions (Fig. 3C). By co-transfection with the HNF4 $\alpha$  expression vector in HEK293T cells, the promoter activity was slightly induced in the -115/+21 fragment containing the distal HNF4 $\alpha$ -binding site and strongly activated in the -162/+21 fragment in the presence of both HNF4 $\alpha$ -binding sites (Fig. 3D). Furthermore, the induced activities in the -162 and -2032/+21 promoters were markedly suppressed by introduction of mutations into one or both HNF4 $\alpha$ -binding sites (Fig. 3E). These results indi-

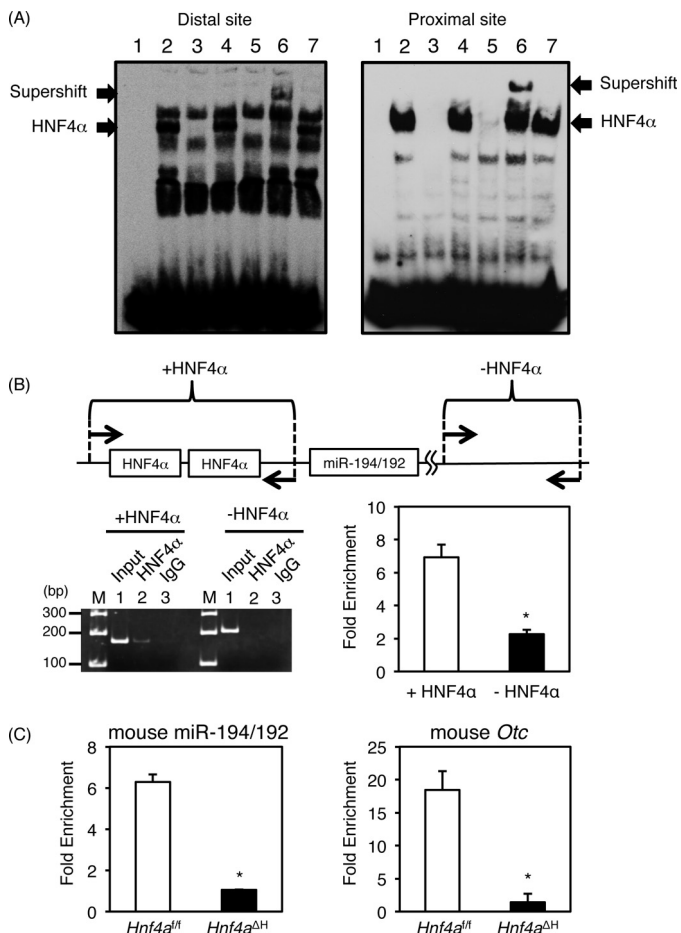


**Figure 3.** Promoter analysis of the human miR-194/192 gene. A, schematic diagram of the promoter region of the human miR-194/192 gene. B, promoter constructs of the miR-194/192 gene (WT) were transfected into HepG2 cells. C, mutations were introduced into the HNF4 $\alpha$ -binding sites (M1 and M2), and the constructs were transfected into HepG2 cells. D and E, promoter constructs of the WT and mutants (M1 and M2) of the miR-194/192 gene were transfected into HEK293T cells with empty vector (*white bar*) or HNF4 $\alpha$  expression vector (*black bar*). The normalized activity is presented as relative activity based on the promoterless vector pGL4.11. Error bars represent S.D. Data are mean  $\pm$  S.D. of three independent experiments.

cate that both HNF4 $\alpha$ -binding sites cooperatively transactivate the miR-194/192 gene.

To determine whether HNF4 $\alpha$  can directly bind to both HNF4 $\alpha$ -binding sites in the miR-194/192 promoter, gel mobility shift analysis was performed (Fig. 4A). Nuclear extracts from HepG2 cells bound to the distal and proximal HNF4 $\alpha$ -binding sites (Fig. 4A, *lane 2*, lower arrows). This complex was diminished by the addition of excess amounts of the unlabeled competitor and OTC competitor that contains an HNF4 $\alpha$ -binding site (Fig. 4A, *lanes 3* and *5*) but not the competitor that contains mutations in the HNF4 $\alpha$ -binding site (Fig. 4A, *lane 4*). Moreover, the complex was supershifted by anti-HNF4 $\alpha$  antibody (Fig. 4A, *lane 6*, upper arrow) but not the anti-C/EBP $\alpha$  antibody (*lane 7*). These results indicate that HNF4 $\alpha$  binds to both HNF4 $\alpha$ -binding sites of the miR-194/192 promoter. Next, chromatin immunoprecipitation (ChIP) was used to determine whether HNF4 $\alpha$  directly binds to the miR-194/192 promoter *in vivo*. We found that HNF4 $\alpha$  was bound to the HNF4 $\alpha$ -binding sites in HepG2 cells (Fig. 4B). In addition, ChIP analysis using the livers of *Hnf4a*<sup>fl/fl</sup> and *Hnf4a* $\Delta^H$  mice indicated that hepatic HNF4 $\alpha$  in *Hnf4a*<sup>fl/fl</sup> mice bound to the promoter region, but the binding was decreased in *Hnf4a* $\Delta^H$  mice (Fig. 4C). Similar results were obtained in the mouse *Otc* promoter that is dependent on an HNF4 $\alpha$ -binding site (8), suggesting that HNF4 $\alpha$

## Regulation of miR-194/192 by hepatic HNF4 $\alpha$

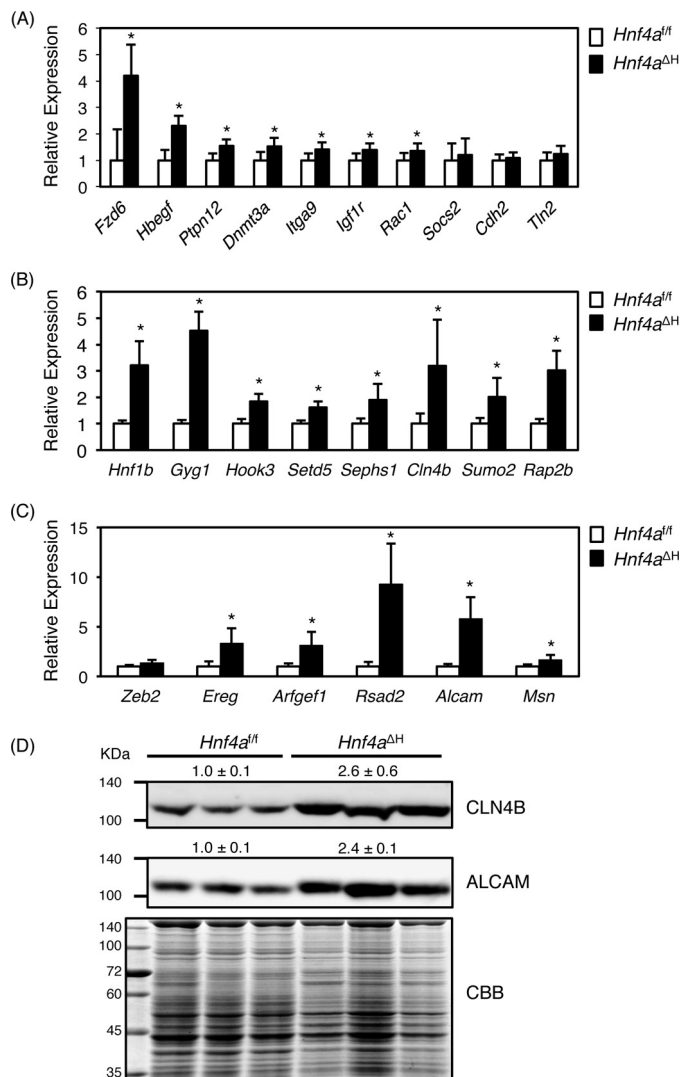


**Figure 4. Identification of HNF4 $\alpha$ -binding sites in the miR-194/192 promoter.** *A*, gel shift analysis. Nuclear extracts from HepG2 cells were incubated with biotin-labeled probe carrying the distal (*left lane*) and proximal (*right lane*) HNF4 $\alpha$ -binding sites in the miR-194/192 promoter in the absence (*lane 2*) or presence of a 50-fold excess of the unlabeled miR-194/192 probe (*lane 3*), the mutated miR-194/192 probe (*lane 4*), and the OTC probe (*lane 5*). For supershift analysis, anti-HNF4 $\alpha$  and anti-C/EBP $\alpha$  antibodies were added (*lanes 6 and 7*). The complex between HNF4 $\alpha$  and the probe and the supershifted complex are indicated by the lower and upper arrows, respectively. *B*, chromatin immunoprecipitation using HepG2 cells was performed with anti-HNF4 $\alpha$  antibody (*lane 2*) and normal goat IgG (*lane 3*) (*left*). Input DNA was used as a positive control (*lane 1*). The regions between -201 and -41 containing both HNF4 $\alpha$ -binding sites (+HNF4 $\alpha$ ) and between +16667 and +16874 without HNF4 $\alpha$ -binding site (-HNF4 $\alpha$ ) in the human miR-194/192 gene were amplified. The data from qPCR were normalized relative to the input and expressed as -fold enrichment (*right*). *C*, chromatin immunoprecipitation using the livers of Hnf4a<sup>H/H</sup> and Hnf4a<sup>f/f</sup> mice with anti-HNF4 $\alpha$  antibody and normal goat IgG. The regions between -164 and -70 containing both HNF4 $\alpha$ -binding sites in the mouse miR-194/192 promoter (*left*), between -264 and -165 containing two HNF4 $\alpha$ -binding sites in the mouse *Otc* promoter (*right*), and between +45820 and +45893 without an HNF4 $\alpha$ -binding site in the mouse *Hmgcs2* gene were amplified, respectively. The data from qPCR were normalized relative to the input and expressed as -fold enrichment over data from IgG control. Error bars represent S.D. Data are mean  $\pm$  S.D. of three independent experiments. \*,  $p < 0.05$ .

physiologically binds to the promoter region of the miR-194/192 gene in humans and mice.

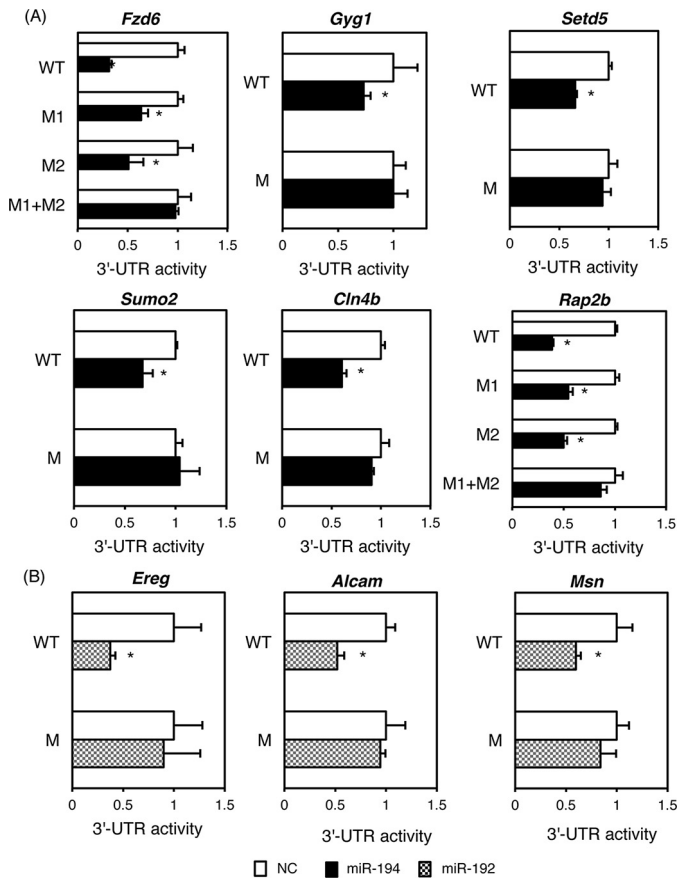
### Identification of miR-194 and miR-192 target mRNAs

It was reported that miR-194 is involved in epithelial-mesenchymal transition (EMT) and HCC metastasis (31). In addition, miR-194 regulates mRNAs encoded by many genes including N-cadherin (*Cdh2*), *Rac1*, heparin-binding epidermal growth factor (*Hbegf*), tyrosine protein phosphatase non-receptor type



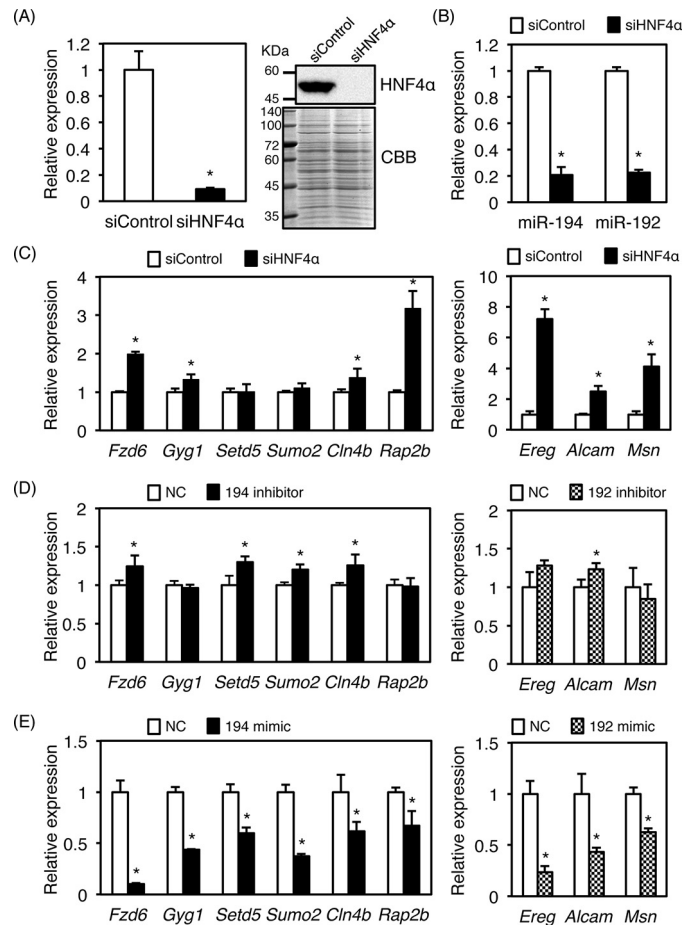
**Figure 5. Hepatic expression of the target genes for miR-194 and miR-192 in Hnf4a<sup>H/H</sup> mice.** *A*, real-time PCR from total liver RNA of Hnf4a<sup>H/H</sup> and Hnf4a<sup>f/f</sup> mice. Known (*A*) and new (*B*) mRNA targets for miR-194 and known and new targets (*C*) for miR-192. Error bars represent S.D. Data are mean  $\pm$  S.D. of five independent mice. \*,  $p < 0.05$ . *D*, Western blot of CLN4B and ALCAM protein from livers of Hnf4a<sup>H/H</sup> and Hnf4a<sup>f/f</sup> mice ( $n = 3$  for each genotype). CLN4B and ALCAM expression normalized by total protein in Hnf4a<sup>H/H</sup> mice is presented as relative to Hnf4a<sup>f/f</sup> mice. CBB, Coomassie Brilliant Blue.

2 (*Ptpn2*), integrin  $\alpha 9$  (*Itna9*), suppressor of cytokine signaling 2 (*Socs2*), DNA methyltransferase 3A (*Dnmt3a*), *Fzd6*, and talin 2 (*Tln2*) that play important roles in tumorigenesis, tumor progression, cell adhesion, cell migration, and EMT (31–33). miR-192 is also known to negatively regulate mRNAs encoding IGF-1, IGF-1R, and ZEB2 that are also involved in EMT and tumor progression and invasion (30, 34). Therefore, qRT-PCR was performed to determine whether hepatic expression of these mRNAs was increased in Hnf4a<sup>H/H</sup> mice. Expression of *Fzd6*, *Hbegf*, *Ptpn2*, *Dnmt3a*, *Itna9*, and *Rac1* mRNAs was significantly increased, whereas expression of *Socs2*, *Cdh2*, *Tln2*, and *Zeb2* mRNAs was unchanged in Hnf4a<sup>H/H</sup> mice (Fig. 5, *A* and *C*). Next, novel target genes for miR-194 and miR-192 were investigated using TargetScan, DIANA-microT, and miRDB databases, and the resulting high-scoring hits that overlapped such as *Hnf1b*, *Gyg1*, hook microtubule-tethering protein 3



**Figure 6. 3'-UTR activities of the target mRNAs for miR-194 and miR-192.** Luciferase vectors harboring the WT 3'-UTR of the target mRNAs for miR-194 (A) and miR-192 (B) were transfected into HEK293T cells with miR-194 (black bar), miR-192 (striped bar), or negative control (NC; white bar). Mutations were introduced into miR-194-binding sites (M). Error bars represent S.D. Data are mean  $\pm$  S.D. of three independent experiments. \*,  $p < 0.05$ .

(Hook3), *Setd5*, selenophosphate synthetase 1 (*Sephs1*), *Sumo2*, *Cln4b*, and *Rap2b* were selected as the target candidates for miR-194 (supplemental Fig. S1, B–F). Regarding the candidates of miR-192 target gene mRNAs, *Ereg*, ADP-ribosylation factor guanine nucleotide-exchange factor 1 (*Arfgef1*), radical S-adenosyl methionine domain-containing 2 (*Rsad2*), *Alcam*, and *Msn* were selected (supplemental Fig. S2, G–I). As expected, the expression of all candidate mRNAs was significantly increased in *Hnf4a*<sup>ΔH</sup> mice (Fig. 5, B and C). The expression of CLN4B protein, a candidate for miR-194 targeting, and ALCAM protein, a candidate for miR-192 targeting, was also increased more than 2-fold in *Hnf4a*<sup>ΔH</sup> mice compared with *Hnf4a*<sup>f/f</sup> mice (Fig. 5D). Therefore, studies were done to determine whether miR-194 and miR-192 could directly interact with the 3'-UTR of these candidate mRNAs and inhibit the protein expression (Fig. 6). miR-194 and miR-192 markedly repressed the 3'-UTR activities in the presence of the complementary sequences of miR-194- and miR-192-binding sites (c-miR-194 and c-miR-192) (supplemental Fig. S2). The 3'-UTR activities of *Fzd6*, *Gyg1*, *Setd5*, *Sumo2*, *Cln4b*, and *Rap2b* using a luciferase reporter system were significantly inhibited by miR-194 (Fig. 6A, wild type (WT)). Because two miR-194-binding sites were predicted within the 3'-UTR of *Fzd6* and *Rap2b*, mutations were introduced to disrupt the



**Figure 7. Conservation of HNF4 $\alpha$ -miR-194/192 cascade in human HCC cell lines.** siRNA (10 nM) was transfected into HepG2 cells. Real-time PCR data for HNF4 $\alpha$  (A), miR-194 and miR-192 (B), and target mRNAs for miR-194 and miR-192 (C) are shown. A Western blot of HNF4 $\alpha$  is shown in A. D, miR-194 and miR-192 inhibitors (100 nM) were transfected into HepG2 cells, and relative expression of the target genes was analyzed by real-time PCR. E, miR-194 and miR-192 mimics (10 nM) were transfected into HLE cells, and relative expression of the target genes was analyzed by real-time PCR. Error bars represent S.D. Data are mean  $\pm$  S.D. of three independent experiments. \*,  $p < 0.05$ . NC, negative control; CBB, Coomassie Brilliant Blue.

binding of miR-194 in both binding sites. Each mutant (M1 and M2) partially rescued the 3'-UTR activity, and both mutants (M1 + M2) completely recovered the activities (Fig. 6A). The same results were also obtained in the mutated 3'-UTR regions of the *Gyg1*, *Setd5*, *Sumo2*, and *Cln4b* (Fig. 6B). Similarly, 3'-UTR activities of *Ereg*, *Alcam*, and *Msn* were suppressed in a miR-192-dependent manner, and the activities were recovered by introduction of mutations into the miR-192-binding sites (Fig. 6B). These results indicate that *Fzd6*, *Gyg1*, *Setd5*, *Sumo2*, *Cln4b*, and *Rap2b* are the target mRNAs for miR-194 and that *Ereg*, *Alcam*, and *Msn* mRNAs are targets for miR-192.

To determine whether the HNF4 $\alpha$ -directed miR-194/192 pathway is also conserved in human HCC-derived cell lines, HNF4 $\alpha$  was knocked down by siRNA. HNF4 $\alpha$  siRNA inhibited mRNA and protein expression of HNF4 $\alpha$  in HepG2 cells, and expression of both miR-194 and miR-192 was largely repressed (Fig. 7, A and B), whereas expression of target mRNAs for miR-194 and miR-192 such as *Fzd6*, *Gyg1*, *Cln4b*, *Rap2b*, *Ereg*, *Alcam*, and *Msn* was increased by HNF4 $\alpha$  knockdown (Fig. 7C). Inhibition of miR-194 and miR-192 also enhanced expression

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of target mRNAs such as *Fzd6*, *Setd5*, *Sumo2*, *Cln4b*, *Ereg*, and *Alcam*. (Fig. 7D). Conversely, miR-194 and miR-192 mimics suppressed expression of all target genes in human HCC-derived HLE cells that hardly express endogenous HNF4 $\alpha$  (Fig. 7E). These results indicate that regulation of miR-194/192 by HNF4 $\alpha$  and the target mRNAs by miR-194/192 may also be conserved in human HCC cell lines.

### Discussion

In this study, HNF4 $\alpha$  was found to directly activate the miR-194/192 gene promoter. Down-regulation of HNF4 $\alpha$  target genes partially could explain the onset of phenotypes in *Hnf4a*<sup>ΔH</sup> mice (7, 8, 26). From microarray analysis using livers of *Hnf4a*<sup>ΔH</sup> mice (13), most mRNAs were markedly down-regulated, probably due to the loss of direct positive regulation of the respective genes by HNF4 $\alpha$ . However, up-regulation of many mRNAs was also observed in *Hnf4a*<sup>ΔH</sup> mice. Thus, HNF4 $\alpha$  might negatively regulate these genes via direct positive regulation of miRNAs that in turn could bind to destabilize mRNAs. HNF4 $\alpha$  was shown to bind to the promoter region of miR-122, the most abundant miRNA in the liver, and transactivate the miR-122 in an HNF4 $\alpha$ -dependent manner (22). However, hepatic expression of miR-122 was unchanged in *Hnf4a*<sup>ΔH</sup> mice. HNF4 $\alpha$  knockdown also inhibited miR-122 expression in mouse liver (22), suggesting that transactivation of miR-122 by HNF4 $\alpha$  would be transient in *Hnf4a*<sup>ΔH</sup> mice as observed, probably due to secondary effects. Likewise, HNF4 $\alpha$  positively regulates miR-7, miR-124, and miR-134 in human HCC and tumor-derived cell lines (23–25). However, unexpectedly, the hepatic expression of these miRNAs was also unchanged in *Hnf4a*<sup>ΔH</sup> mice. Because the expression levels of miR-7, miR-124, and miR-134 were very low or undetectable in normal livers of *Hnf4a*<sup>fl/fl</sup> mice, HNF4 $\alpha$  may not directly regulate these miRNAs as found in normal liver. Thus, HNF4 $\alpha$  might regulate these miRNAs only during the later stages of HCC progression but not the early stages of HCC development because *Hnf4a*<sup>ΔH</sup> mice do not exhibit early spontaneous HCC development.

Unlike miR-7, miR-122, and miR-134, miR-194 and miR-192 were not reported to be down-regulated in human HCC specimens used in these studies, indicating that down-regulation of miR-194 and miR-192 in the liver could be an early event in the onset of liver inflammation and pretumorigenesis. miR-194 is involved in the differentiation of intestinal epithelial cells, and HNF1 $\alpha$  was also reported to transactivate miR-194/192 via an HNF1 $\alpha$ -binding site (28). HNF1 $\alpha$  is a direct target for HNF4 $\alpha$  (2), and hepatic expression of HNF1 $\alpha$  was actually down-regulated in *Hnf4a*<sup>ΔH</sup> mice as expected. Thus, both HNF4 $\alpha$  and HNF1 $\alpha$  might cooperatively control the miR-194/192 gene promoter. However, down-regulation of miR-194 and miR-192 in *Hnf1a*-null mice was not as pronounced when compared with *Hnf4a*<sup>ΔH</sup> mice because sufficient HNF4 $\alpha$  still remains in *Hnf1a*-null mice, indicating that HNF4 $\alpha$  might be a predominant factor in regulation of the miR-194/192 gene as compared with HNF1 $\alpha$ .

p53 was also reported to control expression of the miR-194/192 gene via a p53-binding site (29, 30). The p53-binding site is located upstream of the distal HNF4 $\alpha$ -binding site, and the promoter of -162/+21 fragment including the p53- and

HNF4 $\alpha$ -binding sites was activated in an HNF4 $\alpha$ -dependent manner, suggesting that p53 and HNF4 $\alpha$  might synergistically transactivate the miR-194/192 gene. Expression of miR-194 and miR-192 was markedly down-regulated in a p53-dependent manner in B cell neoplasm multiple myeloma, which rarely expresses endogenous HNF4 $\alpha$  (30). Conversely, the expression of miR-194 and miR-192 was slightly down-regulated in human HCC including mutated or deleted p53 without statistical significance as compared with wild-type p53 (34), indicating that HNF4 $\alpha$  and HNF1 $\alpha$  mainly transactivate the miR-194/192 gene despite p53 status.

EMT is an important process for cells to acquire tumor metastatic potential. Expression of miR-194 was also down-regulated in liver mesenchymal-like cancer cell lines as compared with liver epithelial cancer cell lines, and overexpression of miR-194 suppressed invasion and migration of hepatoma cells (31). Thus, miR-194 and miR-192 expression in liver might retain the differentiated status of hepatocytes and suppress proliferation, migration, and malignant transformation. Re-expression of HNF4 $\alpha$  in dedifferentiated hepatoma cells resulted in reversion to differentiated phenotypes (4). Moreover, reduced expression of HNF4 $\alpha$  correlated with human HCC progression (25), and forced expression of HNF4 $\alpha$  reduced the proliferation and induced epithelial formation (25, 35, 36). These results strongly suggest that HNF4 $\alpha$  is one of the most important factors to induce hepatocyte differentiation by direct transactivation of liver-specific genes and to inhibit dedifferentiation of hepatocytes by indirect negative regulation of oncogenes and EMT-related genes via direct up-regulation of miR-194 and miR-192. However, hepatic expression of EMT-activating genes including *Cdh2* as an miR-194 target and *Zeb2* as an miR-192 target mRNA was not increased in mice, showing that down-regulation of miR-194 and miR-192 in inflammatory and non-/pretransformed hepatocytes in *Hnf4a*<sup>ΔH</sup> mice could not yet activate EMT-related genes because *Hnf4a*<sup>ΔH</sup> mice die at about 8 weeks of age before showing tumorigenesis (7).

Others have reported that HNF1 $\alpha$ -regulated miR-194 repressed the 3'-UTR activity of FZD6 (32). In this study, two functional miR-194-binding sites were identified in the 3'-UTR of *Fzd6* mRNA. FZD6 is a membrane receptor for Wnt ligand signaling and controls cell differentiation (37). Because overexpression of FZD6 was confirmed in human HCC (38) and hepatic expression of FZD6 was not significantly increased in HNF1 $\alpha$ -null mice (32), repression of FZD6 expression through negative regulation by miR-194 via HNF4 $\alpha$  could be important for suppression of tumorigenesis.

There are no reports that GYG1 is involved in HCC and metastasis. Glycogen synthesis starts from self-glycosylation of GYG1 followed by elongation of glucose chains by glycogen synthase (39). Hepatic expression of glycogen synthase 2, a limited enzyme for glycogen synthesis in the liver and a direct target for HNF4 $\alpha$ , was markedly decreased in *Hnf4a*<sup>ΔH</sup> mice (40). Eventually, expression of GYG1 might be increased to compensate for reduced production of *de novo* glycogen synthesis in *Hnf4a*<sup>ΔH</sup> mice.

CUL4B is the main component of Cullin4B-Ring ubiquitin ligase complex and promotes tumorigenesis (41) and SETD5 is a histone methyltransferase on the histone H4 tail at lysines 5, 8,

and 12 in yeast (42). Loss of function mutations in the *SETD5* gene were found in patients with intellectual disability (43); however, the physiological significance of H4 methylation by SETD5 remains unsolved. Histone H4 lysines 5, 8, and 12 are also acetylated by histone acetyltransferases (44), and methylation of these sites by SETD5 might change chromatin structure and influence transcription. HNF4 $\alpha$ -dependent suppression of *Cul4B* and *Setd5* could partially control the epigenetic program and be important to retain the differentiated status in normal hepatocytes.

SUMO2 has an important role to regulate a variety of biological functions such as transcription, DNA repair, cell cycle, and subcellular transport by SUMOylation to the target proteins (45). Similar to an ES cell-based model of hepatocyte differentiation in which SUMO2-modified proteins were decreased during hepatocyte differentiation (46), increased activity of SUMOylation by SUMO2 could induce hepatocyte dedifferentiation in *Hnf4a*<sup>ΔH</sup> mice.

RAP2B functions as a p53-mediated prosurvival factor. RAP2B was also found to be up-regulated in many types of tumors, but RAP2B had very weak transformation activity, indicating that RAP2B itself is not an oncogene but maintains the tumorigenic status (47). Because hepatic expression of p53 and *Cdkn1a* was increased in *Hnf4a*<sup>ΔH</sup> mice, induced p53 might be responsible for additional induction of *Rap2b* mRNA.

In addition, *Ereg*, *Alcam*, and *Msn* mRNAs are targets for miR-192 in the liver. EREG is one of the ligands for epidermal growth factor receptor and is involved in inflammation and tumorigenesis (48). Knockdown of *EREG* suppressed cell growth in human hepatoma cell lines (49), and *Ereg*-null mice exhibited a significant reduction of tumor number and size in HCC model experiments (50), indicating that EREG may contribute to HCC progression. Unlike other target genes for miR-194 and miR-192 examined in this study, EREG is more highly expressed in hepatic stellate cells than in hepatocytes (50), suggesting that miR-192 synthesized in hepatocytes might be secreted into hepatic stellate cells followed by repression of EREG in normal liver. ALCAM, a cell adhesion molecule, is a marker of colorectal cancer stem cells (51). Although ALCAM expression was inhibited by miR-192 in a gastric cell line, the 3'-UTR of *Alcam* mRNA failed to respond to miR-192 (52). By contrast, the 3'-UTR activity of *Alcam* mRNA was dependent on a miR-192-binding site. ALCAM was overexpressed in human hepatoma cell lines, and silencing of ALCAM suppressed cell growth and promoted apoptosis (53), suggesting that ALCAM could accelerate tumor cell survival by an antiapoptotic role in HCC.

MSN functions as a cross-linker between the plasma membrane and actin filament. High expression of moesin was observed in breast cancers and implicated in EMT (54, 55). However, expression of *Msn* mRNA was induced only 1.6-fold in *Hnf4a*<sup>ΔH</sup> mice. Because other EMT-related mRNAs such as *Cdh2* and *Zeb2* were not induced in *Hnf4a*<sup>ΔH</sup> mice, these EMT-related mRNAs might not be induced sufficiently in inflammatory and non-transformed hepatocytes of *Hnf4a*<sup>ΔH</sup> mice.

In conclusion, HNF4 $\alpha$  was found to repress the expression of a variety of mRNAs through positive regulation of miR-194 and

miR-192 in non-transformed liver. Most of these mRNAs encode proteins involved in tumorigenesis, prosurvival, epigenetic change, and EMT. These observations revealed an HNF4 $\alpha$  cascade that inhibits hepatocyte dedifferentiation. Further studies may contribute to the development of novel diagnostic markers for the early detection of HCC using the HNF4 $\alpha$ -miR-194/192 axis, and inhibition of the miR-194/192 targets by induced expression and/or activation of miR-194/192 could be useful for HCC therapy.

## Experimental procedures

### Animals

*Hnf4a*<sup>ΔH</sup> mice carrying a mixed SvJ129, FVB, and C57BL/6 background were generated as described previously (7). All experiments were performed with 45-day-old male *Hnf4a*<sup>fl/fl</sup> and *Hnf4a*<sup>ΔH</sup> mice. *Hnf1a*-null mice were also used (56). The mice were housed in a pathogen-free animal facility under a standard 12-h light/12-h dark cycle with *ad libitum* water and chow. All experiments with mice were carried out under Association for Assessment and Accreditation of Laboratory Animal Care guidelines with approval of the National Cancer Institute Animal Care and Use Committee and Gunma University Animal Care and Experimentation Committee.

### miRNA microarrays

Total RNA from the livers of *Hnf4a*<sup>fl/fl</sup> and *Hnf4a*<sup>ΔH</sup> mice was purified using the miRNeasy mini kit (Qiagen, Tokyo, Japan). Extracted RNA was labeled with Cy5 using the Label IT miRNA labeling kit (Takara Bio, Otsu, Japan). Labeled RNAs were hybridized onto 3D-Gene Mouse miRNA Oligo chips (v.11.1; Toray Industries, Tokyo, Japan). The annotation and oligonucleotide sequences of the probes conformed to the miRBase miRNA database (<http://www.mirbase.org/>).<sup>3</sup> After stringent washes, fluorescence signals were scanned with the ScanArray Express Scanner (PerkinElmer Life Sciences) and analyzed using GenePix Pro version 5.0 (Molecular Devices, Sunnyvale, CA). The raw data of each spot were normalized by subtraction of the mean intensity of the background signal determined by all blank spots' signal intensities of 95% confidence intervals. Measurements of both duplicate spots with signal intensities greater than two standard deviations (S.D.) of the background signal intensity were considered to be valid. A relative expression level of a given miRNA was calculated by comparing the signal intensities of the averaged valid spots with their mean value throughout the microarray experiments after normalization by their median values adjusted equivalently. miRNA microarray data are described in supplemental Table S1. The data are also available at the Gene Expression Omnibus under accession number GSE70516.

### Reverse transcription and real-time PCR

Total RNA was extracted using TriPure Isolation Reagent (Roche Applied Science). Reverse transcription for mature miRNA was performed using a Mir-X miRNA First-Strand Synthesis kit (Clontech). Real-time PCR was performed on a

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LightCycler 480 system II (Roche Applied Science). cDNAs encoding mature miRNA and U6 RNA were amplified using a Mir-X miRNA qRT-PCR SYBR kit (Clontech). The levels of mature miRNA were expressed relative to U6 RNA as an internal control using the  $\Delta\Delta C_t$  method. For detection of two transcripts for pri-miR-194 and mRNAs, cDNA from the livers of *Hnf4a<sup>fl/fl</sup>* and *Hnf4a<sup>ΔH</sup>* mice was transcribed using ReverTraAce qPCR RT Master with gDNA Remover (Toyobo, Osaka, Japan), and real-time PCR was performed using FastStart SYBR Green Master (Roche Applied Science). The levels of expression were normalized relative to *Gapdh* mRNA as an internal control using the  $\Delta\Delta C_t$  method. Sequences for the primers are shown in [supplemental Table S2](#).

### Cloning of the human miR-194/192 gene promoter region

The -2032, -1003, -162, -115, -70, and -51/+21 fragments of the human miR-194/192 promoter containing KpnI and XhoI sites were amplified with genomic DNA from HepG2 cells by PCR and cloned into the luciferase reporter vector pGL4.11 (Promega, Madison, WI). Mutations were introduced into the HNF4 $\alpha$ -binding sites in the miR-194/192 reporter vector by PCR-based site-directed mutagenesis. Sequences for the primers are shown in [supplemental Table S3](#).

### Transient transfection and luciferase assay

HepG2 and HEK293T cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (HyClone, Logan, UT) and 1,000 units of penicillin and 0.1 mg of streptomycin/ml (Sigma-Aldrich). For suspension transfection, miR-194 promoters cloned into pGL4.11 and pGL4.74 encoding *Renilla* luciferase regulated under herpes simplex virus thymidine kinase promoter (Promega) were transfected into HepG2 cells with polyethylenimine Max (Polyscience, Warrington, PA) as a transfection reagent. For co-transfection using HEK293T cells, HNF4 $\alpha$  expression plasmid was used. After 48 h of transfection, the cells were washed with phosphate-buffered saline (PBS), and promoter activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

### Transfection of siRNA and miRNA mimics and miRNA inhibitors

siRNA (10 nM) against *Hnf4a* mRNA (Sigma-Aldrich), 10 nM miR-192 and miR-194 mimics (miRIDIAN microRNA mimics, GE Healthcare), and 100 nM miR-192 and 194 inhibitors (miScript miRNA inhibitors, Qiagen) were transfected into HepG2 and HLE cells with Lipofectamine RNAiMAX (Life Technologies). After 48 h of transfection, total RNA and protein were extracted from the transfected cells.

### Western blotting

Liver samples from *Hnf4a<sup>fl/fl</sup>* and *Hnf4a<sup>ΔH</sup>* mice and HepG2 cells treated with siRNA against *Hnf4a* mRNA were homogenized in lysis buffer (7 M urea, 2 M thiourea, and 1% Triton X-100) and allowed to sit on ice for 30 min. The homogenate was centrifuged at 12,000  $\times g$  for 30 min at 4 °C, and the supernatants were used as whole-cell lysates. Total protein (40  $\mu$ g) with Laemmli sample buffer was incubated at 65 °C for 15 min

and fractionated by 10% SDS-polyacrylamide gel electrophoresis. The gels were stained with Coomassie Brilliant Blue R-250 or blotted onto a PVDF membrane (GE Healthcare). The membrane was incubated for 1 h with mouse monoclonal antibodies against human HNF4 $\alpha$  (Perceus Proteomics, Tokyo, Japan) and rabbit polyclonal antibodies against human CLN4B (GeneTex, Irvine, CA) and human ALCAM (Sigma-Aldrich). After washing, the membrane was incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling Technology, Tokyo, Japan), and the reaction product was visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce) on an ImageQuant LAS4000 (GE Healthcare). Expression of CLN4B and ALCAM proteins was quantified by densitometric analysis using ImageJ software, and the expression normalized by total protein in *Hnf4a<sup>ΔH</sup>* was presented as expression differences relative to the *Hnf4a<sup>fl/fl</sup>* mice.

### Gel mobility shift analysis

Nuclear extracts from HepG2 cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific), and gel shift analysis carried out using the LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific). The following double-stranded probes were used: wild-type and mutants of distal and proximal HNF4 $\alpha$ -binding sites for the human miR-194/192 promoter and the HNF4 $\alpha$ -binding site for the mouse *Otc* promoter as a positive control. Nuclear extracts and the 5'-biotin-labeled probe of the HNF4 $\alpha$ -binding sites for the miR-194/192 promoter (wild type) were added, and the reaction mixture was incubated on ice for 10 min. For competition experiments, a 50-fold excess of unlabeled probe was added to the reaction mixture, and the mixture was incubated on ice for 10 min prior to the addition of the 5'-biotin-labeled probe. For supershift analysis, anti-HNF4 $\alpha$  or anti-C/EBP $\alpha$  antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the mixture, and the mixture was incubated on ice for 10 min after the addition of the 5'-biotin-labeled probe. DNA-protein complexes were fractionated by 7% PAGE and blotted onto a Biotodyne B nylon membrane (Pall, Tokyo, Japan). After washing, complexes were visualized using the detection module in the kit on an ImageQuant LAS4000. Sequences for the primers are shown in [supplemental Table S4](#).

### Chromatin immunoprecipitation

HepG2 cells were fixed in 0.5% formaldehyde and quenched by 125 mM glycine at room temperature. After washing, cells were resuspended in 3 ml of lysis buffer 1 (50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 0.25% Triton X-100, and protease inhibitor (Roche Applied Science)) on ice for 10 min and then centrifuged at 1,400  $\times g$  for 5 min. The cell pellet was resuspended in 3 ml of lysis buffer 2 (10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, and 5 mM EGTA) for 10 min at room temperature and then centrifuged at 1,400  $\times g$  for 5 min. The cell pellet was resuspended in 1 ml of lysis buffer 3 (10 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 0.1% sodium deoxycholate). Liver samples were ground into pieces with a

pestle and mortar under liquid nitrogen and fixed in PBS containing 20 mM sodium butyrate, 1% formaldehyde, and protease inhibitor mixture for 10 min at room temperature. After centrifugation, the pellet was resuspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% sodium dodecylsulfate, 20 mM sodium butyrate, and protease inhibitor). The cell lysate from HepG2 cells and liver samples was disrupted by sonication (UR-20P, Tomy, Tokyo, Japan) for 5 min on ice and then diluted 10-fold with RIPA ChIP buffer (10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 20 mM sodium butyrate, and protease inhibitor) followed by centrifugation at  $20,000 \times g$  for 10 min. A small volume of the supernatant was stored at 4 °C as the input sample. The remaining supernatant was precleared by adding of 15  $\mu$ l of a 50% slurry of protein G-Sepharose 4 Fast Flow (GE Healthcare) with sonicated salmon sperm DNA and rotated for 4 h at 4 °C followed by centrifugation at  $1,900 \times g$  for 5 min. The supernatant was divided into two aliquots and incubated with 4  $\mu$ g of anti-HNF4 $\alpha$  antibody or control normal goat IgG (Santa Cruz Biotechnology) for 16 h at 4 °C and incubated with 40  $\mu$ l of a 50% slurry of protein G-Sepharose 4 Fast Flow. The reaction mixture was centrifuged at  $1,900 \times g$  for 5 min at 4 °C, and the pellet was collected. The pellet was washed for 5 min at 4 °C with 1 ml of RIPA-1 buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate), RIPA-1 buffer containing 300 mM NaCl, LiCl wash solution (10 mM Tris-HCl (pH 8.0), 0.25 M LiCl, 1 mM EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate), and Tris-EDTA. Then ChIP direct elution buffer (10 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA, and 0.5% SDS) was added to the pellet and incubated for 16 h at 65 °C for decross-linking. After treatment with RNase A for 30 min at 37 °C and proteinase K for 2 h at 55 °C, DNA was purified and amplified by PCR and real-time PCR using the  $\Delta\Delta$ Ct method. Enrichment of HNF4 $\alpha$  binding was normalized to the input samples and expressed as -fold enrichment as compared with the control normal IgG antibody. Sequences for the primers are shown in [supplemental Table S4](#).

### 3'-UTR assays

3'-UTRs of the mouse candidate genes for the miR-194 and miR-192 were amplified by PCR and cloned downstream of firefly luciferase gene in pGL3-control (Promega). As positive controls, complementary sequences for miR-194 and miR-192 (c-miR-194 and c-miR-192) were self-annealed and then cloned into the XbaI site of the pGL3-control. For 3'-UTR assay, pGL3-control/3'-UTR plasmids and pGL4.74 with 20 nM miR-194 or miR-192 mimic were co-transfected into HEK293T cells using HilyMax (Dojindo, Kumamoto, Japan) as a transfection reagent and assayed by the Dual-Glo Luciferase Assay System (Promega). Sequences for the primers of the 3'-UTRs are shown in [supplemental Table S5](#).

### Statistical analysis

All values are expressed as the mean  $\pm$  S.D. All data were analyzed by unpaired Student's *t* test for significant differences between the mean values of each group.

**Author contributions**—A. M., M. K., and Y. T. conducted most of the experiments and analyzed the results. T. N. conducted experiments of microarray analysis. S. S. and T. Maeda conducted experiments of promoter analysis. C. S., T. Matsuta, M. S., M. A., and N. N. conducted experiments of real-time PCR and ChIP analysis. Y. I. conceived the idea for the project and wrote the paper with F. J. G.

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