

Hepatocyte Nuclear Factor 4 α Suppresses the Development of Hepatocellular Carcinoma

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Abstract

Hepatocyte nuclear factor 4 α (HNF4 α) is a transcription factor that plays a key role in hepatocyte differentiation and the maintenance of hepatic function, but its role in hepatocarcinogenesis has yet to be examined. Here, we report evidence of a suppressor role for HNF4 α in liver cancer. HNF4 α expression was progressively decreased in the diethylnitrosamine-induced rat model of liver carcinogenesis. In human liver tissues, HNF4 α expression was decreased in cirrhotic tissue and further decreased in hepatocarcinoma relative to healthy tissue. Notably, an inverse correlation existed with epithelial-mesenchymal transition (EMT). Enforced expression of HNF4 α attenuated hepatocyte EMT during hepatocarcinogenesis, alleviated hepatic fibrosis, and blocked hepatocellular carcinoma (HCC) occurrence. In parallel, stem cell marker gene expression was inhibited along with cancer stem/progenitor cell generation. Further, enforced expression of HNF4 α inhibited activation of β -catenin, which is closely associated with EMT and hepatocarcinogenesis. Taken together, our results suggest that the inhibitory effect of HNF4 α on HCC development might be attributed to suppression of hepatocyte EMT and cancer stem cell generation through an inhibition of β -catenin signaling pathways. More generally, our findings broaden knowledge on the biological significance of HNF4 α in HCC development, and they imply novel strategies for HCC prevention through the manipulation of differentiation-determining transcription factors in various types of carcinomas. *Cancer Res*; 70(19); 7640–51.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most frequent cause of cancer death globally (1). It is one of the few cancers with well-defined risk factors, which include cirrhosis, hepatitis virus infection, and alcohol consumption. An epidemiologic study showed that hepatitis B virus infection was up to 10% in China, and hepatitis C virus prevalence was ~3% in the general population (2). Additionally, it has been well documented that the vast majority of HCC occurs in cirrhotic

livers where chronic inflammation usually presents, and the annual HCC incidence in cirrhotic patients (2.0–6.6%) is much higher than that in noncirrhotic patients (0.4%; ref. 1). Therefore, prevention of HCC in high-risk cases, particularly in those with established cirrhosis, would be highly desirable. Unfortunately, effective strategies for HCC prevention in patients remain scarce to date. Antiviral therapies with IFN and nucleotide analogues were reported to be effective in prevention of HCC associated with hepatitis virus infection (3, 4); as for HCC patients independent of hepatitis, the effect still needs to be confirmed. A randomized controlled study indicated that acyclic retinoid was able to reduce the recurrence of HCC in patients who had undergone surgical treatment (5). Moreover, IFN was also reported to prevent HCC recurrence and improve the survival of the HCC patients (6). Additionally, anticancer drugs including imatinib mesylate and gefitinib were able to prevent HCC occurrence in mice or rats (7, 8), but the intolerable side effects of these agents hamper their clinical application in cancer prevention. Thus, strategies to improve HCC prevention are urgently needed.

Hepatocyte nuclear factor 4 (HNF4), a liver-enriched transcription factor, belongs to the nuclear hormone receptor superfamily. As a key member of the HNF4 family, HNF4 α is indispensable for the hepatic epithelium formation during embryonic development and for epithelial phenotype maintenance of hepatocytes in mature liver (9, 10). Substantial evidence has revealed that HNF4 α is suppressed in human

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HCC tissues compared with the adjacent noncancerous tissues (11–13). Among the members of HNF family, HNF4 α is most closely associated with the differentiation status of HCC (11). Forced reexpression of HNF4 α inhibited the proliferation of HCC cells and reduced the tumor formation in congenic mice. Our previous study has indicated that upregulation of HNF4 α could induce the differentiation of hepatoma cells into hepatocytes, which implies that HNF4 α might present as a novel therapeutic agent for HCC (13). More recently, we also showed that HNF4 α could block the activation of myofibroblasts, and forced expression of HNF4 α in livers of two distinct fibrotic rat models remarkably ameliorated hepatic fibrosis (14). Nevertheless, the preventive effect of HNF4 α on hepatocarcinogenesis has not been determined thus far. It is therefore important to investigate whether HNF4 α could inhibit HCC occurrence and, if that is the case, what will be the molecular mechanism.

With this report, we clarified that HNF4 α introduction could not only attenuate the liver fibrosis and cirrhosis but also completely block the hepatocarcinogenesis in rats. These results indicate that the gene delivery of HNF4 α could be a promising approach for HCC prevention in HCC high-risk population.

Materials and Methods

Cells and culture

Human hepatoma cell line HepG2, human embryonic kidney cell line 293T, and rat hepatocytes BRL 3A were obtained from the American Type Culture Collection in 2009. Human normal liver cell line L02 was obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Human HCC cell line SMMC-7721 was obtained from the Department of Pathology of the Second Military Medical University (Shanghai, China). All the cells had been authenticated and were cultured in DMEM (Calbiochem) containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂.

Experimental HCC model in rats

Male Wistar rats, weighting about 120 to 150 g each, were purchased from Shanghai Experimental Center of Chinese Science Academy and housed under standard animal laboratory conditions in the experimental animal center of Second Military Medical University. This study was approved by the local Ethical Committee of the University. Rats were subjected to i.p. injections of diethylnitrosamine (DEN) at 70 mg/kg body weight once a week (Sigma-Aldrich). After 10-week DEN administration, 36 rats were randomly divided into model group, AdGFP group, and AdHNF4 α group (12 rats in each group), which were infused with PBS, 5 × 10⁹ plaque-forming units of AdGFP, or the same amount of AdHNF4 α via the tail vein every other week, respectively (Supplementary Fig. S1). The recombinant adenoviruses of AdHNF4 α and AdGFP (control) were prepared as described previously (13). To assess tumor development during the course of DEN administration, two animals from each group

were sacrificed at the indicated time point after gene delivery. All of the remaining rats were sacrificed 22 weeks after the first DEN administration. Five age-matched normal rats were used as normal controls.

Histologic examination and immunohistologic staining

Human liver tissues were obtained from surgical resection specimens of HCC patients who had not undergone radiotherapy or chemotherapy in Eastern Hepatobiliary Surgery Hospital (Shanghai, China). Diagnosis of all cases of HCC was made by an experienced pathologist. The procedure of human sample collection was approved by China Ethical Review Committee. The tissue sections of formalin-fixed, paraffin-embedded liver sample from rat or human were subjected to H&E for standard histologic examination. Masson's trichrome staining and Sirius red staining were used to determine the collagen deposition according to the manufacturers' protocol. Immunohistochemical examinations were performed to detect the expression of HNF4 α , epithelial-mesenchymal transition (EMT)-related proteins, fibrosis, and tumor markers. The primary antibodies used were listed in Supplementary Table S2. For the semiquantitative analysis, the blue-stained connective area in Masson's trichrome-stained section was measured on an image analyzer by a technician blinded to the samples.

Tissue microarray analysis

A tissue microarray block containing 210 tissue samples consisting of 136 HCCs and 74 noncancerous liver samples (18 cirrhotic liver samples and 56 normal liver samples) was constructed using a tissue microarrayer (Beecher Instruments). Immunostaining for HNF4 α was performed on the consecutive tissue microarray slides. Assessment of the nuclear HNF4 α staining was based on the percentage of positively stained cells and the nuclear staining intensity on a four-point scale (0, absence of staining; 1+, faint staining; 2+, moderate staining; 3+, strong staining). A nuclear immunostaining score was derived by multiplying the percentage of positively stained cells and the nuclear staining intensity.

Primary hepatocyte isolation and immunofluorescence staining

Primary hepatocytes were isolated from male Sprague-Dawley rats as described previously (14). Forty-eight hours after isolation, the hepatocytes were infected by AdGFP or AdHNF4 α at a multiplicity of infection of 10 and then exposed to medium containing 2 ng/mL transforming growth factor- β 1 (TGF- β 1; R&D Systems) for 72 hours. The hepatocytes were stained by HNF4 α antibody together with one of the primary antibodies (E-cadherin, vimentin, and β -catenin) and then visualized under a laser scanning confocal fluorescent microscope (Carl Zeiss, Inc.).

Quantitative real-time PCR analysis

Total RNA was extracted from the cells or tissues using Trizol reagent (Invitrogen), and cDNA was synthesized with random primers and Moloney murine leukemia virus reverse

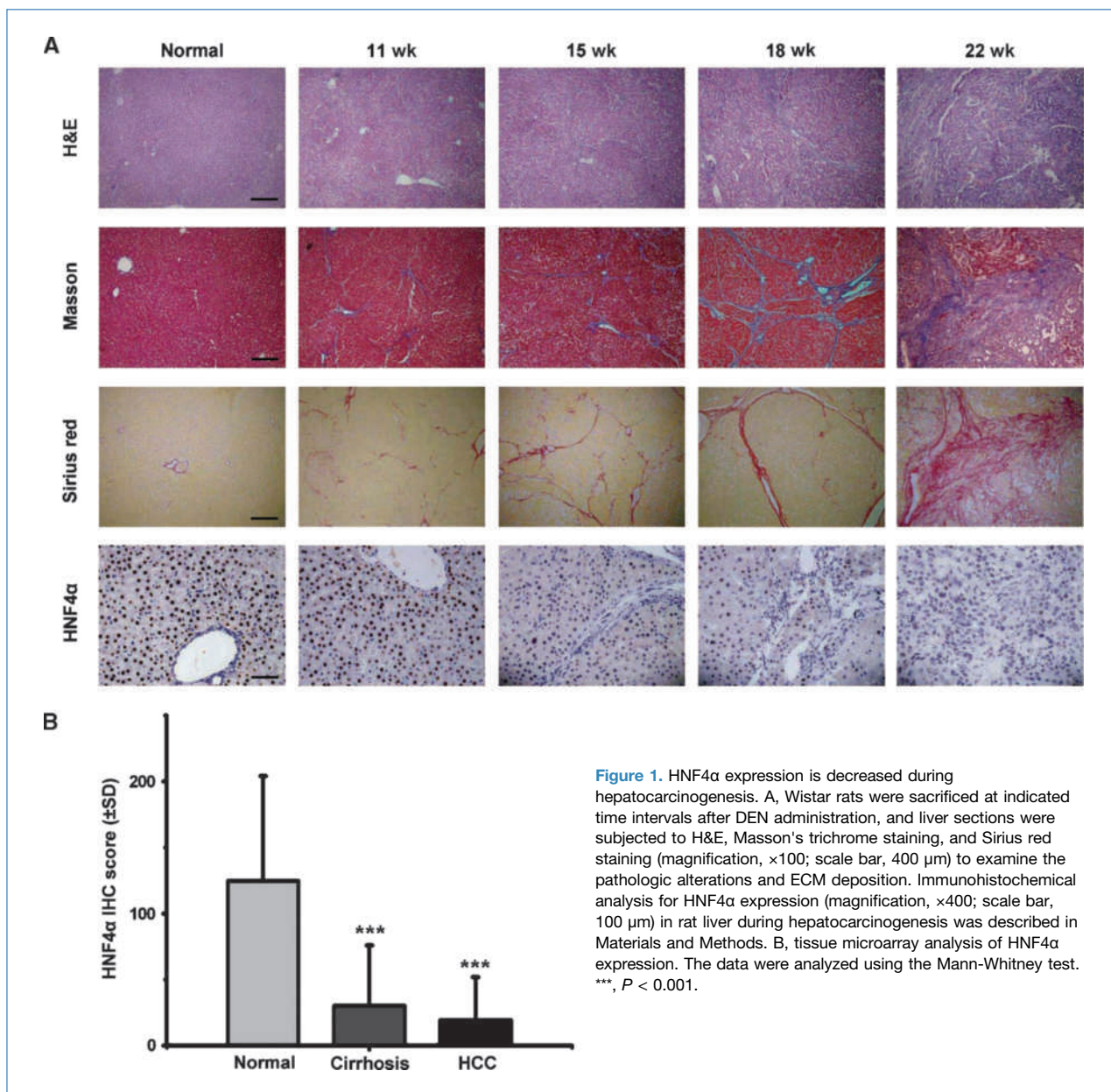


Figure 1. HNF4 α expression is decreased during hepatocarcinogenesis. A, Wistar rats were sacrificed at indicated time intervals after DEN administration, and liver sections were subjected to H&E, Masson's trichrome staining, and Sirius red staining (magnification, $\times 100$; scale bar, $400\ \mu\text{m}$) to examine the pathologic alterations and ECM deposition. Immunohistochemical analysis for HNF4 α expression (magnification, $\times 400$; scale bar, $100\ \mu\text{m}$) in rat liver during hepatocarcinogenesis was described in Materials and Methods. B, tissue microarray analysis of HNF4 α expression. The data were analyzed using the Mann-Whitney test. ***, $P < 0.001$.

transcriptase in accordance with the manufacturer's protocol. The original amount of the specific transcripts was measured by real-time PCR with a SYBR Green PCR kit (Applied Biosystems) using the ABI Prism 7900 sequence detector (Applied Biosystems). The expression of specific transcripts was normalized against β -actin. Primers for these transcripts are listed in Supplementary Table S3.

Magnetic-activated cell sorting

SMMC-7721 cells were labeled with primary OV6 or CD133 antibody, subsequently magnetically labeled with rat anti-mouse IgG1 microbeads, and separated on MACS

LS column (Miltenyi Biotec), respectively. All the procedures were carried out according to the manufacturer's instructions.

Luciferase assay

To assess the effect of HNF4 α on β -catenin activity, a dual-luciferase reporter assay was carried out according to the manufacturer's instructions (Promega) using wild-type Tcf-luciferase construct (pGL3-OT) and the mutant Tcf-luciferase reporter construct (pGL3-OF), which were kindly provided by Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). pRL-TK, which contains the

herpes simplex virus thymidine kinase promoter to provide low to moderate levels of *Renilla* luciferase expression, was cotransfected with each reporter plasmid to normalize the transfection efficiency. The luciferase activity was measured using the Promega luciferase assay reagent and Synergy 2 Multi-Detection Microplate Reader (BioTek). Relative β -catenin activation was determined by the OT/OF ratio from at least three independent experiments.

Nuclear protein extraction and Western blotting assay

Extraction of nuclear protein was performed according to the protocol from Upstate Biotechnology, and the protein extracted was separated by SDS-PAGE and transferred to nitrocellulose membrane. Lamin A and α -tubulin were used as nuclear and cytosolic fractionation controls. The membrane was probed with specific primary antibody, followed by incubation with an IRDye680-conjugated anti-mouse secondary antibody (Rockland, Inc.). Detection was performed using an Odyssey IR imaging system (LI-COR Biotechnology).

Statistical analysis

The ANOVA and Student's *t* test were used for comparison among the groups and between paired data, respectively. Mann-Whitney tests were used for the continuous data of tissue microarray. $P < 0.05$ was considered statistically significant.

Results

Expression of HNF4 α is decreased during hepatocarcinogenesis

To investigate the changes of HNF4 α expression during hepatocarcinogenesis, liver samples were collected from Wistar rats subjected to DEN administration. As illustrated by H&E, Masson's trichrome, and Sirius red staining, the livers of rats exhibited severe fibrosis and cirrhosis on DEN treatment, and all the animals developed HCC at the 22nd week after DEN administration (Fig. 1A). Intriguingly, immunohistochemistry showed that HNF4 α expression was progressively decreased during the process of hepatocarcinogenesis

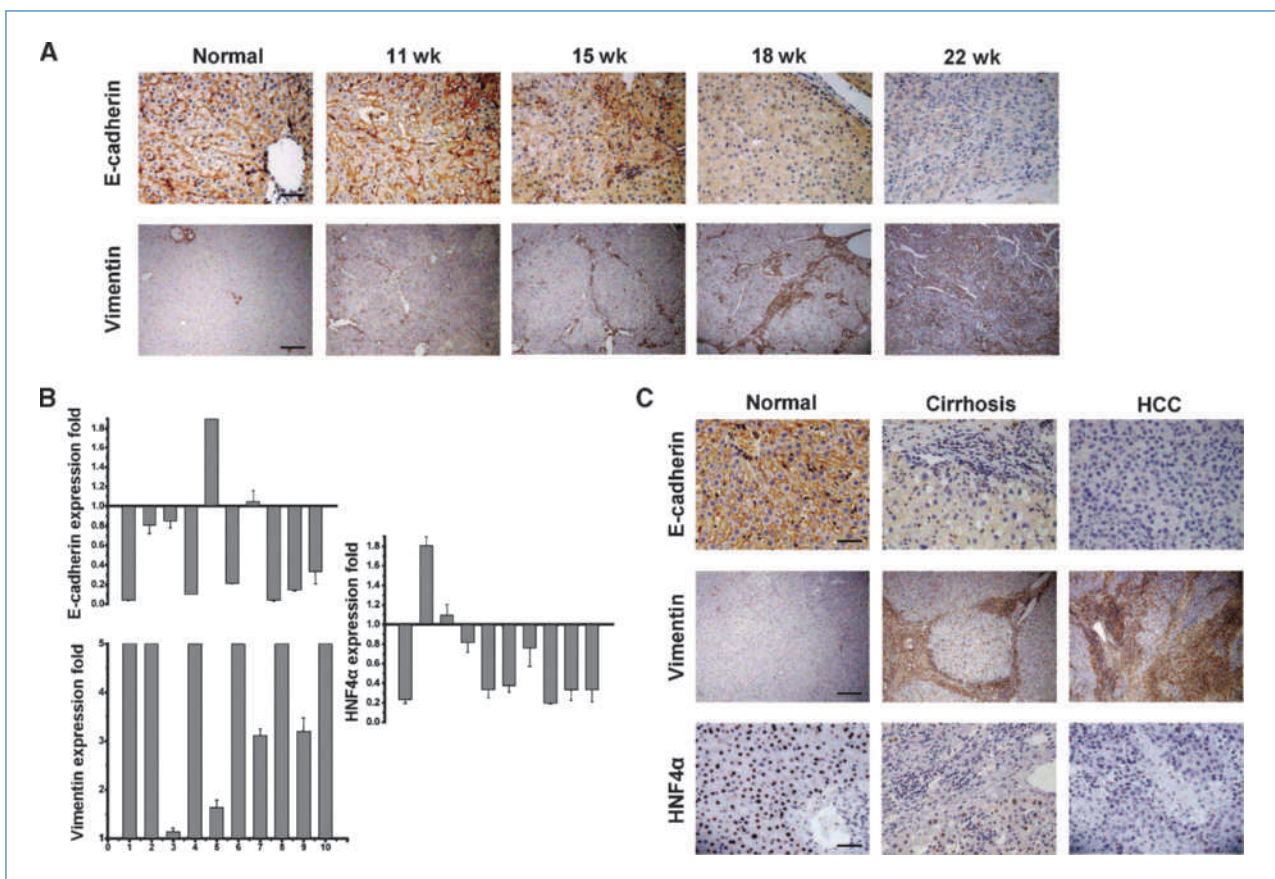


Figure 2. Expression of HNF4 α inversely correlates with hepatocyte EMT. A, immunohistochemical analysis of E-cadherin (magnification, $\times 400$; scale bar, 100 μm) and vimentin (magnification, $\times 100$; scale bar, 400 μm) during DEN-induced hepatocarcinogenesis in rats. B, the mRNA levels of HNF4 α , E-cadherin, and vimentin in HCC or paracancerous tissue of patients were detected by real-time PCR. The result showed the gene expression folds of HCCs versus paracancerous tissues. C, representative images for immunohistochemical assay of human HNF4 α , E-cadherin (magnification, $\times 400$; scale bar, 100 μm), and vimentin (magnification, $\times 100$; scale bar, 400 μm).

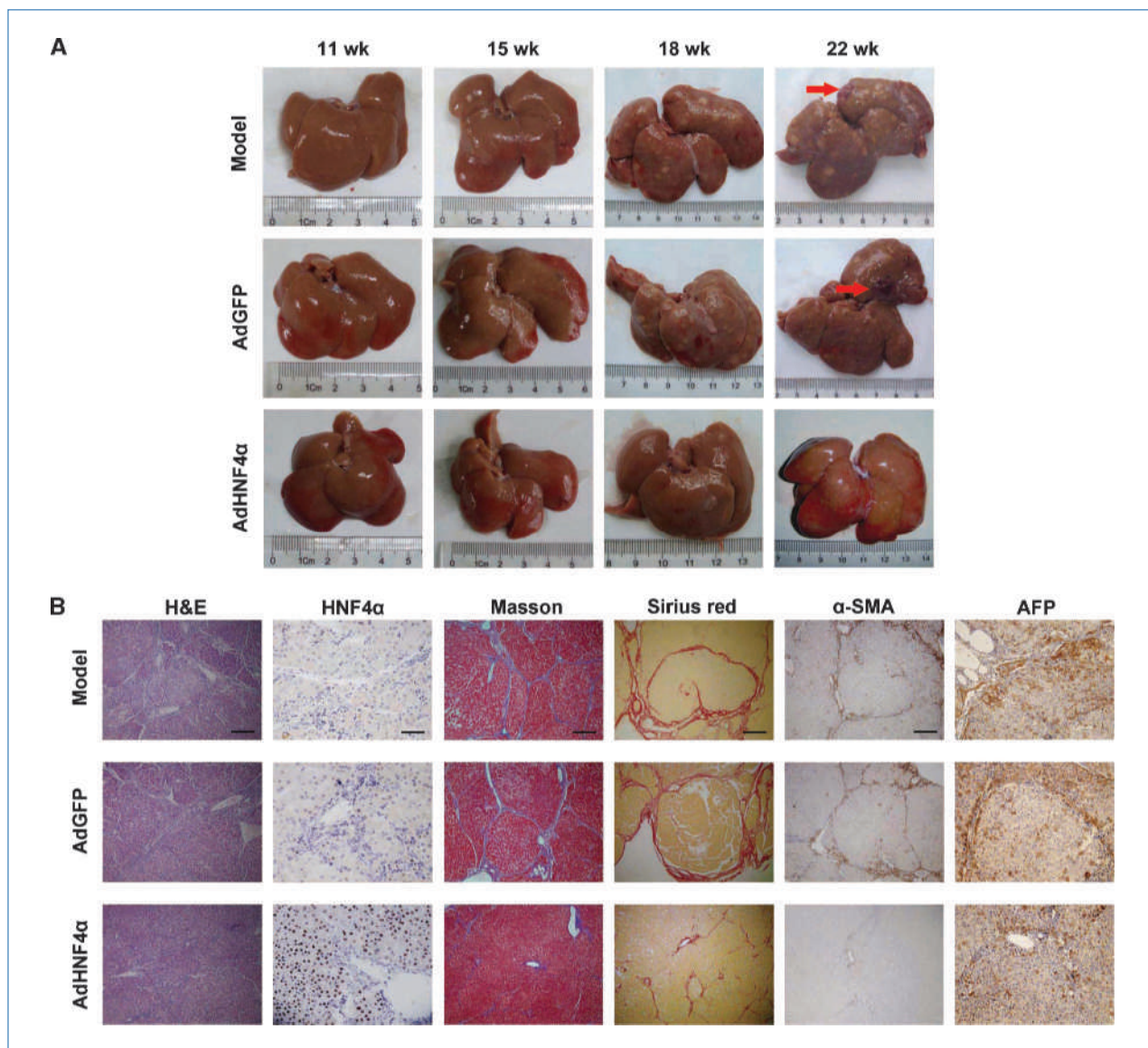


Figure 3. HNF4 α ameliorates DEN-induced hepatic fibrosis and blocks hepatocarcinogenesis. A, adenovirus-administered rats were sacrificed at various time points, and the livers were removed for analysis. Arrows indicate HCC nodules. Representative results from six independent animals in the 22nd week are shown. B, livers of rats in the 18th week were assessed by H&E staining, Masson's trichrome staining, and Sirius Red staining (magnification, $\times 100$; scale bar, 400 μm). Expression of HNF4 α (magnification, $\times 400$; scale bar, 100 μm), α -SMA, and α -fetoprotein (AFP; magnification, $\times 100$; scale bar, 400 μm) in rat liver was assessed by immunohistochemistry.

in rats. In a further study, we also performed human tissue microarray to assess the expression of HNF4 α in human hepatocarcinogenesis. The result showed that HNF4 α expression was significantly decreased in cirrhotic liver and even less in HCC tissues compared with that in the normal liver (Fig. 1B).

HNF4 α expression inversely correlates with hepatocyte EMT during carcinogenesis

Accumulating evidence indicates that downregulation of HNFs, in particular HNF4 α , closely correlates with hepatocyte EMT. Therefore, it is important to explore the relevance

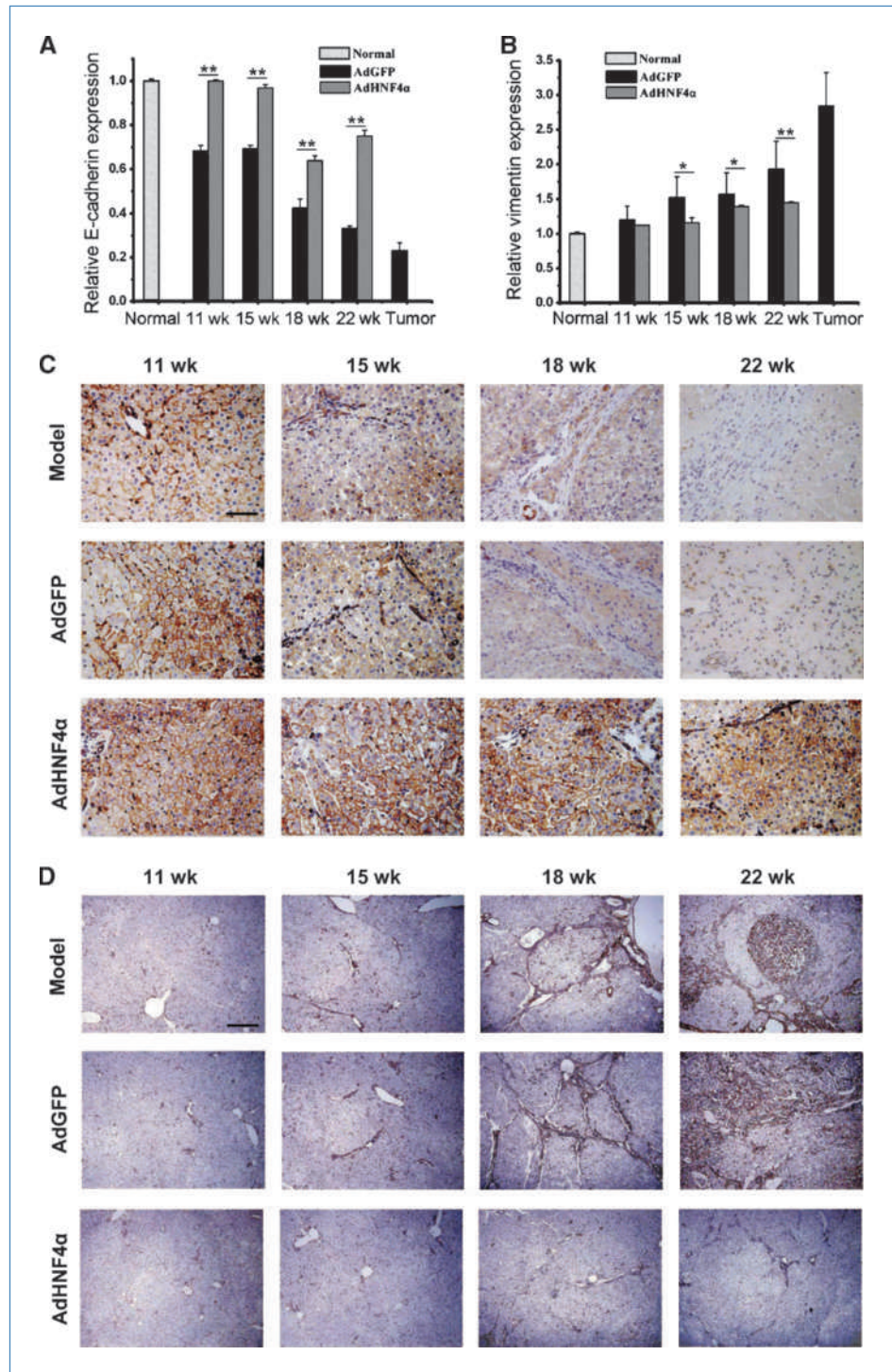
of HNF4 α reduction with EMT in hepatocarcinogenesis. As shown in Fig. 2A, concomitant with the reduction of HNF4 α , epithelial cell marker E-cadherin was significantly decreased during DEN-induced hepatocarcinogenesis, whereas the expression of mesenchymal cell marker vimentin was notably increased. Real-time reverse transcription-PCR clearly revealed the decreased HNF4 α expression and enhanced hepatocyte EMT in human HCC tissues compared with that in the paracancerous tissues (Fig. 2B). Moreover, immunohistochemistry revealed the hepatocyte EMT during human hepatocarcinogenesis, which was accompanied by HNF4 α reduction (Fig. 2C).

HNF4 α attenuates DEN-triggered hepatic fibrosis and blocks carcinogenesis

To assess the preventive effect of HNF4 α on development of HCC, AdHNF4 α or AdGFP was delivered into Wistar rats exposed to DEN. As shown in Fig. 3A and Supplementary Table S1, morphologic examination revealed that cirrhosis

was significantly alleviated in AdHNF4 α group at the 18th week compared with that in the control groups. All livers in the model group and green fluorescent protein (GFP) control group exhibited neoplastic nodules at the 22nd week, whereas there was no HCC nodule in the HNF4 α group. Masson's trichrome and Sirius red staining showed reduced collagen

Figure 4. HNF4 α attenuates hepatocyte EMT during DEN-triggered hepatocarcinogenesis. The mRNA level of E-cadherin (A) and vimentin (B) in rat liver during hepatocarcinogenesis was detected by real-time PCR. The result showed the relative gene expression folds of AdHNF4 α - or AdGFP-treated group versus the normal rat control. *, $P < 0.05$; **, $P < 0.01$. Immunohistochemical staining was performed to assess the expression of (C) E-cadherin (magnification, $\times 400$; scale bar, 100 μm) and (D) vimentin (magnification, $\times 100$; scale bar, 400 μm) in the process of DEN-induced carcinogenesis at different time points after adenovirus injection.



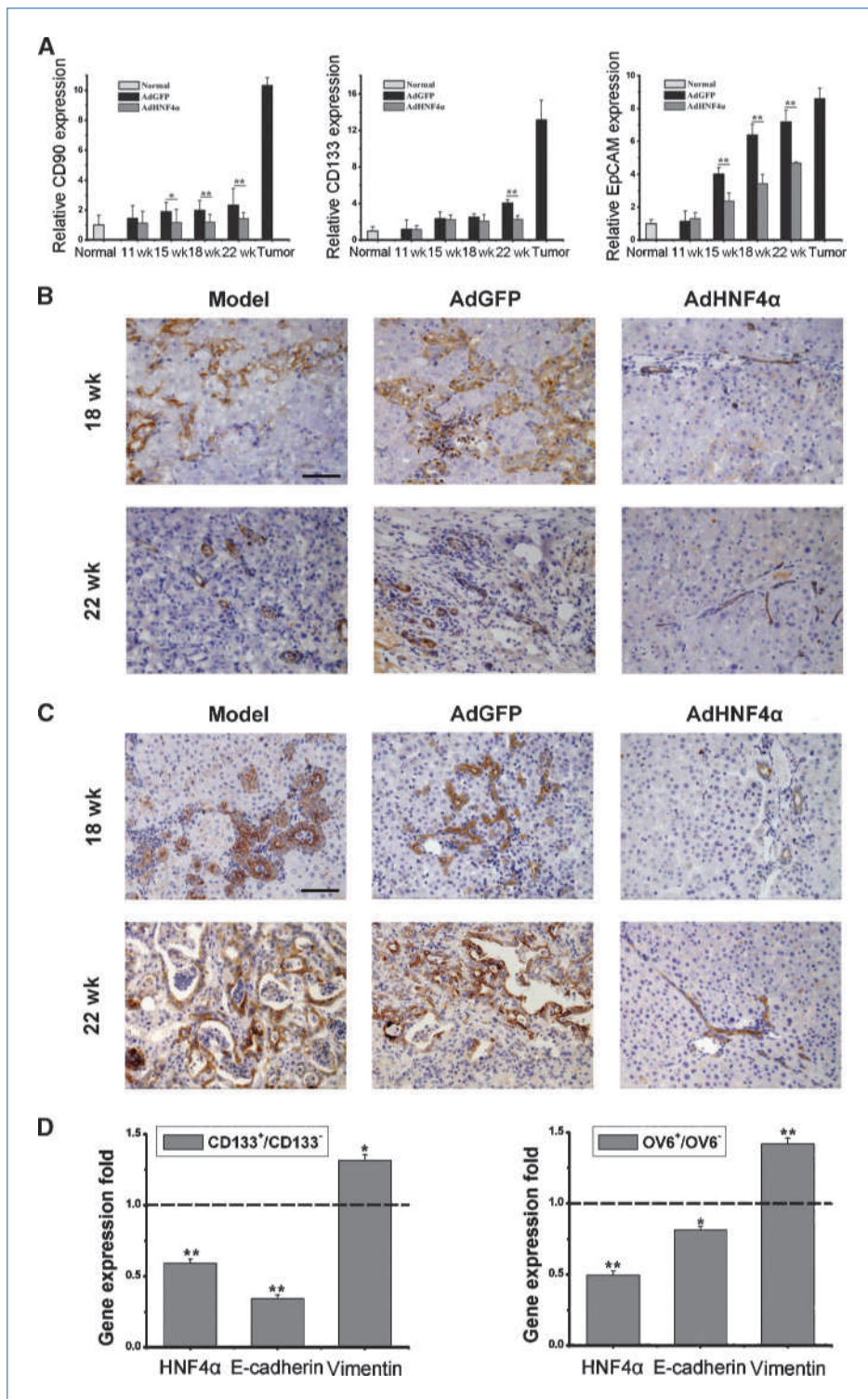


Figure 5. HNF4α diminishes hepatoma stem/progenitor cell generation. **A**, the mRNA level of EpCAM, CD133, and CD90 in the liver of rats during carcinogenesis was measured by real-time PCR. Gene expression folds were normalized against β-actin. Columns, mean; bars, SD. *, $P < 0.05$; **, $P < 0.01$, Student's *t* test. **B** and **C**, immunohistochemical staining was carried out to detect the EpCAM⁺ and OV6⁺ cells (magnification, ×400; scale bar, 100 μm) in rat cirrhotic liver or HCC tissue. **D**, MACS-sorted CD133⁺ or OV6⁺ cells from SMMC-7721 population were subjected to real-time PCR. The difference in HNF4α expression between CD133⁺ and CD133⁻ cells or OV6⁺ and OV6⁻ cells was compared, respectively. **D**, the results showed the relative expression folds of indicated genes in CD133⁺ versus CD133⁻ cells or OV6⁺ versus OV6⁻ cells. *, $P < 0.05$; **, $P < 0.01$.

deposition in the AdHNF4α group than in control groups (Fig. 3B). Semiquantitative analysis of the extracellular matrix (ECM) area revealed that AdHNF4α injection reduced the ECM area by 59% ($P < 0.01$ versus AdGFP controls). Immunohisto-

chemistry showed that HNF4α dramatically blocked the expression of not only α-smooth muscle actin (SMA) but also proliferating cell nuclear antigen and AFP in livers of rats exposed to DEN (Fig. 3B; Supplementary Fig. S2).

HNF4 α blocks hepatocyte EMT in DEN-induced hepatocarcinogenesis

Considering the important role of HNF4 α in maintaining the epithelial cell morphology, we investigated the effect of HNF4 α on hepatocyte EMT in DEN-induced carcinogenesis. Real-time PCR revealed that expression of E-cadherin was downregulated in a time-dependent manner during hepatocarcinogenesis, and HNF4 α delivery significantly blocked the reduction of E-cadherin in rat liver (Fig. 4A). Concomitantly, expression of vimentin, which was notably increased in the process of HCC development, was robustly suppressed by HNF4 α introduction (Fig. 4B). Consistent results were also achieved by immunohistochemistry (Fig. 4C and D). Expression of Snail in fibrotic areas and cancerous tissues was notably enhanced in DEN-triggered carcinogenesis, and

it was significantly reduced by HNF4 α delivery (Supplementary Fig. S3A). As illustrated in Supplementary Fig. S3B, HNF4 α introduction remarkably increased E-cadherin expression and decreased the expression of a cluster of mesenchymal genes in L02 cells treated with TGF- β 1, a conventional EMT inducer. Moreover, ectopic HNF4 α expression was able to enhance E-cadherin promoter activation in a time- and dose-dependent manner, suggesting that increase of E-cadherin by HNF4 α contributed to HNF4 α -mediated EMT inhibition (Supplementary Fig. S3C and D).

HNF4 α suppresses the generation of hepatoma stem/progenitor cells

To date, CD133 (15–17), CD90 (18), EpCAM (19), and OV6 (20) are considered as potential markers to distinguish cancer

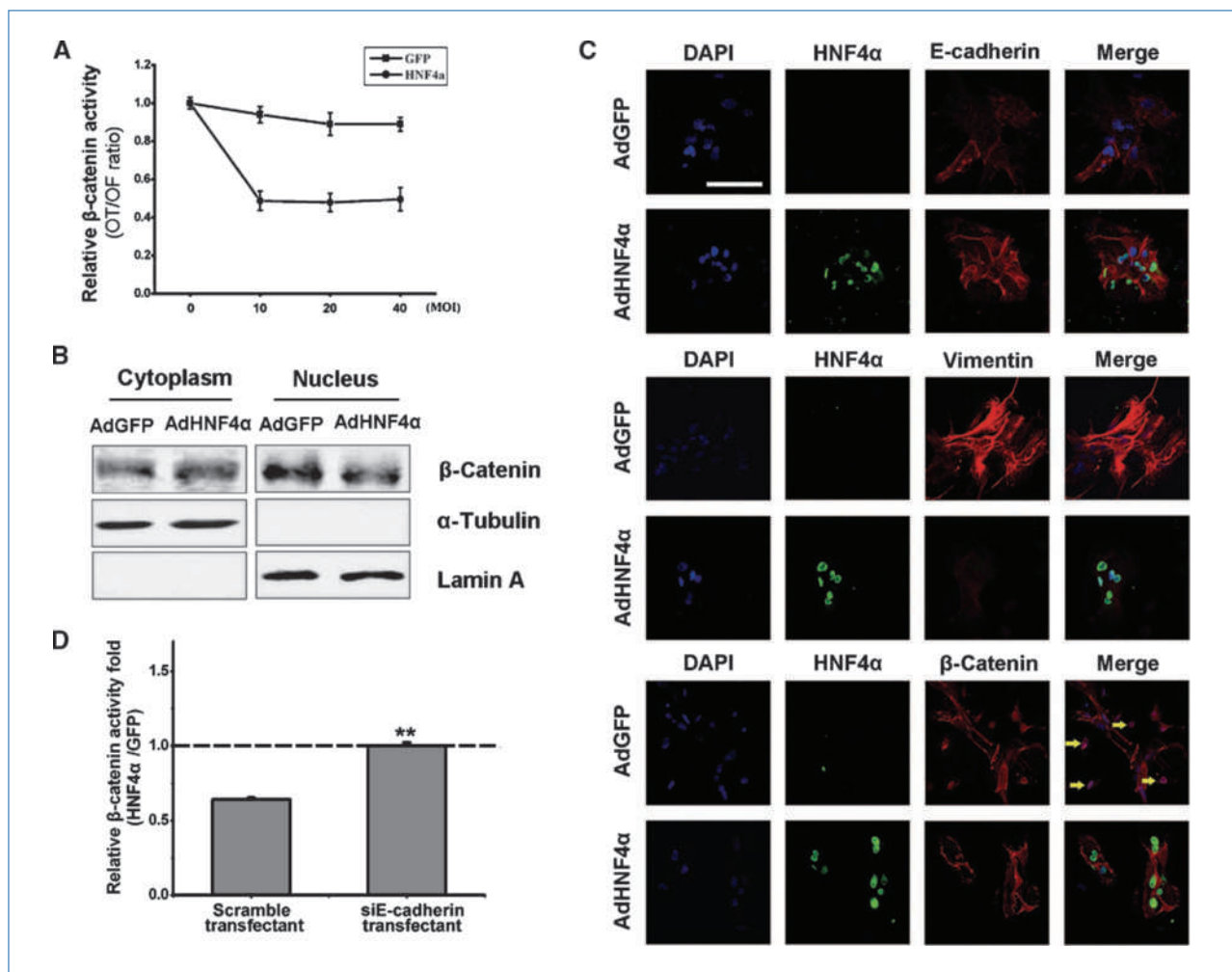


Figure 6. HNF4 α suppresses Wnt/ β -catenin signaling. **A**, Luciferase reporter activity of β -catenin in HepG2 cells was measured 48 h after adenovirus infection. The β -catenin-mediated transcription activity was assessed by the OT/OF ratio. MOI, multiplicity of infection. **B**, Western blot analysis of nuclear extraction from AdHNF4 α - or AdGFP-infected L02 cells underwent TGF- β 1 treatment for 24 h. **C**, The rat primary hepatocytes were double immunostained for HNF4 α (green) together with E-cadherin (red), vimentin (red), or β -catenin, respectively. 4',6-Diamidino-2-phenylindole (DAPI) was used for the counterstaining of DNA (blue). Magnification, \times 400; scale bar, 100 μ m. **D**, siE-cadherin-transfected 293T cells and the control cells were infected by AdGFP and AdHNF4 α , respectively. Luciferase reporter activity of β -catenin was analyzed as described above. The result showed the relative β -catenin activation folds of AdHNF4 α -infected cells versus AdGFP-infected cells in siE-cadherin transfectant and scramble transfectant, respectively. **, $P < 0.01$.

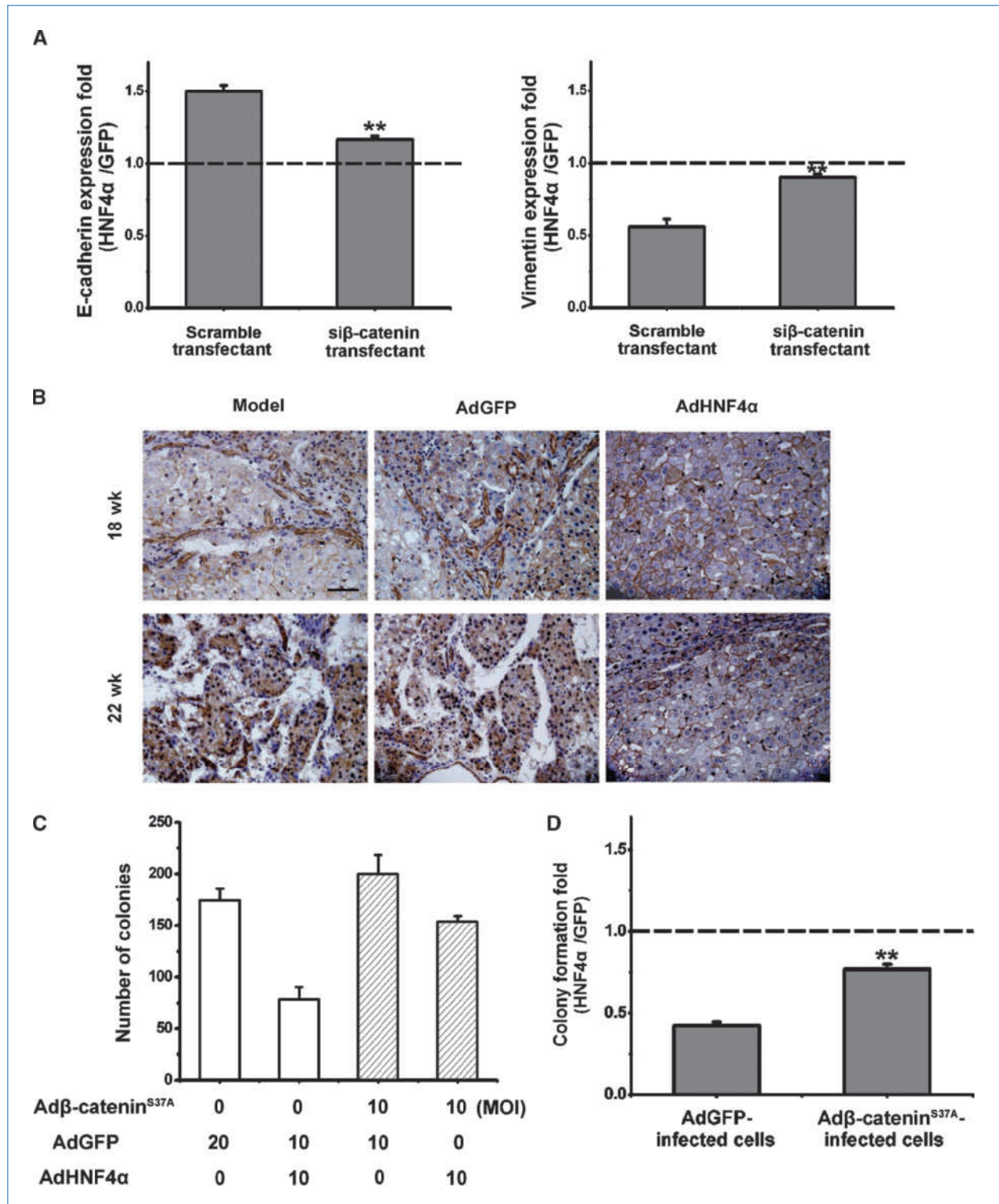


Figure 7. β -Catenin was required in HNF4 α -mediated suppression of EMT and carcinogenesis. A, si β -catenin–transfected 293T cells and the control cells were infected by AdGFP and AdHNF4 α , respectively. Expression of E-cadherin or vimentin was analyzed by real-time PCR. The result showed the gene expression folds of AdHNF4 α -infected cells versus AdGFP-infected cells in si β -catenin transfectant and scramble transfectant, respectively. B, immunohistochemical staining of β -catenin (magnification, $\times 400$; scale bar, 100 μ m) in the liver of rats in the 18th or 22nd week. C, 1×10^3 SMMC-7721 cells infected by indicated adenovirus for 48 h and then cultured in 25-cm² dish for 2 wk. The colonies formed were counted after crystal violet staining. D, colony formation folds of AdHNF4 α -infected cells versus AdGFP-infected cells in the presence of Ad β -catenin^{S37A} or AdGFP control. **, $P < 0.01$.

stem/progenitor cells in a portion of HCC. As elucidated in Fig. 5A, expression of CD133, CD90, and EpCAM was notably increased during hepatocarcinogenesis, and it could be dramatically blocked by HNF4 α introduction. Immunohistochemistry revealed that the proportion of OV6⁺ and EpCAM⁺ cells was markedly increased in the process of DEN-induced HCC, and it could be significantly reduced by HNF4 α delivery (Fig. 5B and C). In addition, we clarified a small portion of SMMC-7721 cells exhibiting CD133 or OV6 antigenic phenotype and presenting depressed HNF4 α expression and enhanced EMT state compared with the majority of CD133⁻ or OV6⁻ cells (Fig. 5D). These data indicate that HNF4 α -mediated suppression of EMT might decrease the generation of the cancer stem/progenitor cells.

HNF4 α impairs β -catenin signaling via suppression of β -catenin nuclear translocation

Luciferase assay of β -catenin reporter unraveled that HNF4 α was able to downregulate β -catenin transcriptional activity in HepG2 cells (Fig. 6A). Consistent results were also achieved in 293T cells and rat BRL hepatocytes (Supplementary Fig. S4). As illustrated in Fig. 6B, TGF- β 1-triggered β -catenin nuclear translocation was strikingly attenuated by ectopic expression of HNF4 α , and the expression of β -catenin target genes was suppressed as well (Supplementary Fig. S5). Distinct nucleus translocation of β -catenin with concomitant induction of EMT was observed in primary liver cells exposed to TGF- β 1 (data not shown). As expected, HNF4 α introduction not only blocked TGF- β 1-triggered hepatocyte EMT but also repressed the nuclear translocation of β -catenin (Fig. 6C). Moreover, inhibition of glycogen synthase kinase (GSK) by its specific inhibitor or dominant-negative mutant remarkably enhanced β -catenin activation, but the suppression of β -catenin by HNF4 α was not affected, implicating that the downregulation of β -catenin activity by HNF4 α was through noncanonical Wnt/ β -catenin pathway (Supplementary Fig. S6A and B). HNF4 α was not able to decrease the activation of β -catenin in the absence of E-cadherin, implying that E-cadherin is required in the suppression of β -catenin by HNF4 α (Fig. 6D and Supplementary Fig. S6C).

β -Catenin is involved in HNF4 α -mediated suppression of EMT and carcinogenesis

The Wnt/ β -catenin pathway plays an essential role in EMT and tumorigenesis. Therefore, it is important to investigate whether β -catenin is involved in HNF4 α -mediated suppression of hepatocyte EMT and hepatocarcinogenesis. HNF4 α -mediated increase of E-cadherin and decrease of vimentin were significantly impaired in si β -catenin (small interference RNA of β -catenin)-transfected cells, indicating that β -catenin was also involved in HNF4 α -mediated EMT inhibition (Fig. 7A). Immunohistochemistry revealed the accumulation of β -catenin in cytoplasm and nucleus of hepatocytes during hepatocarcinogenesis, and it could be significantly impaired by HNF4 α introduction (Fig. 7B). HNF4 α -mediated suppression of tumorigenesis was significantly impaired in hepatoma cells infected with adenovirus

expressing β -catenin^{S37A}, a constitutively active mutant of β -catenin, suggesting that inhibition of β -catenin was involved in HNF4 α -mediated suppression of tumorigenesis (Fig. 7C and D).

Discussion

The maintenance of liver architecture and function is cross-regulated by a set of hepato-specific transcription factors. One of the central mediators in this regulatory network is HNF4 α , which regulates not only the hepatocyte proliferation and differentiation but also the hepatic epithelial morphology (12). Previous studies clarified that the expression of HNF4 α was decreased in rodent and human HCC in comparison with the adjacent noncancerous tissues (21–23). With this study, we reported the progressive reduction of HNF4 α during hepatocarcinogenesis. The pathologic process and gene expression profile of DEN-elicited rodent-HCC are similar to those of human HCC (24). Therefore, the preventive effect of HNF4 α against hepatic carcinogenesis was further studied using this rat experiment model. Surprisingly, introduction of HNF4 α dramatically blocked the development of HCC in rats subjected to DEN administration. This finding strongly implies that upregulation of HNF4 α is an effective strategy for HCC prevention.

Activated fibroblasts, which have been accepted as the key mediators of hepatic fibrosis, are traditionally considered to derive via activation and proliferation of resident stellate cells or periportal fibroblasts, whereas increasing evidence has implicated that the hepatocytes in fibrotic liver may transform into activated fibroblasts via EMT and contribute to hepatic fibrogenesis (25). EMT is defined as the process through which epithelial cells progressively lose their epithelial signatures while acquiring the characteristics of mesenchymal cells, including morphology, cellular structure, and biological functions (26, 27). Our latest study also elucidated the strikingly suppressive effect of HNF4 α on hepatic fibrosis with inhibited EMT of both hepatocytes and hepatic satellite cells (14). Here, we observed that upregulation of HNF4 α markedly attenuated the hepatic fibrosis/cirrhosis during DEN-induced hepatocarcinogenesis. Because hepatic fibrosis/cirrhosis is considered as a unique stage of preneoplastic lesion in most HCC cases (28), the inhibition of hepatic fibrosis and cirrhosis should also account, at least partially, for the preventive effect of HNF4 α on HCC occurrence. To date, there is evidence showing that the induction of EMT in immortalized cells results in the acquisition of stem cell characteristics (29). Moreover, stem cell-like cells isolated from mouse or human carcinomas expressed EMT markers, and transformed human cells that have undergone the Snail/ Twist-mediated EMT exhibit the phenotype of cancer stem cells (CSC; refs. 29 and 30). These findings implicate the close association between EMT and tumorigenesis. Although the direct evidence supporting this notion remains inadequate, extension of EMT concepts in carcinogenesis is believed to shed new light on cancer prevention and therapy. In this study, we clarified that HNF4 α delivery could significantly

antagonize the hepatocyte EMT in hepatocarcinogenesis, which may also contribute to the inhibitory effect of HNF4 α on HCC occurrence.

The “cancer stem cell hypothesis” proposes the significance of a minor proportion of cells with self-renewal ability in the pathogenesis of cancer (31). Increasing evidence indicates that the HCC development is attributed to the propagation of hepatic cancer stem/progenitor cells, which display distinct surface marker pattern (15–20). However, the precise cell(s) of origin, molecular genetics, and specific markers of HCC stem cells remain poorly understood. CD90⁺ cells in human liver cancer have been reported to present CSC property (18). Furthermore, CD133⁺ HCC cells isolated from cultured HCC cell lines or primary hepatoma tissue possess high capacity of tumorigenesis (17). Moreover, OV6 or EpCAM may serve as cancer stem/progenitor cell marker of HCC on account of its significant upregulation in premalignant hepatic tissues (20). In the present study, we observed that HNF4 α delivery significantly reduced the expression of CD133, CD90, and EpCAM during hepatocarcinogenesis, and the proportion of OV6⁺ and EpCAM⁺ cells, which have been predicted as hepatoma stem/progenitor cells, was also markedly decreased. These findings implicate that the preventive effect of HNF4 α on HCC onset is likely due to the suppression of HCC stem/progenitor cell generation.

The Wnt/ β -catenin pathway plays an essential role in the development of multiple tissues through the regulation of cell proliferation, differentiation, and migration (32). Aberrant activation of Wnt/ β -catenin signaling is frequently detected in malignant tumors, especially in HCC (33). In the current study, we elucidated the suppression of β -catenin nuclear translocation and transcriptional activity by HNF4 α in hepatocytes. It has been well documented that β -catenin-mediated expression of Slug (34) and Twist (35) presents a predominant effect on the EMT process. Thus, it is rational to deduce that the inhibitory effect of HNF4 α on hepatocyte EMT is attributed, at least in part, to the depression of β -catenin. Moreover, activation of β -catenin in stem cells could enhance their self-renewal capacity, and excessive self-renewal is one of the key cellular events in the initial stage of tumorigenesis (20, 36). We previously sorted a subpopulation of OV6⁺ cancer stem/progenitor cells from both HCC cell lines and hepatoma tissues, which were endowed with endogenously active β -catenin. Moreover, inhibition of β -catenin signaling significantly reduced the proportion of these OV6⁺ cells, implicating the importance of the β -catenin activation in hepatic CSC generation (20). Hereby, HNF4 α -mediated suppression of β -catenin might be responsible for the suppressive effect of HNF4 α on cancer stem/progenitor cell generation and hepatocarcinogenesis. Enhanced β -catenin accumulation and

nuclear translocation are usually due to the suppression of GSK-3 β by canonical Wnt/ β -catenin pathway activation. Alternatively, downregulation of E-cadherin, which decreases the stability of E-cadherin/ β -catenin complex, leads to the disassociation and release of β -catenin from plasma membrane to cytosol. Our results showed that the suppression of β -catenin by HNF4 α was not influenced by GSK inhibition, which suggests that the canonical Wnt/ β -catenin pathway was not involved in the repression of β -catenin by HNF4 α . HNF4 α has been reported to function as a major regulator of E-cadherin transcription in hepatocytes (10). Here, our results clarified that HNF4 α introduction not only enhanced E-cadherin expression but also reduced the nuclear translocation of β -catenin. Furthermore, suppression of β -catenin by HNF4 α was notably compromised in siE-cadherin-transfected cells. Taken together, we conclude that suppression of β -catenin by HNF4 α is due to the increase of E-cadherin/ β -catenin complex at the plasma membrane and the subsequent decrease of β -catenin nuclear translocation. Moreover, downregulation of β -catenin activity by HNF4 α is responsible, at least partially, for HNF4 α -mediated inhibition of hepatocyte EMT and carcinogenesis.

The majority of chronic hepatitis and cirrhosis patients are at high risk of liver cancer, which renders the prevention of HCC extremely important globally. Nevertheless, effective strategy for HCC prevention is scarce to date. In the current study, our results clearly show that HNF4 α delivery presents a potent inhibitory effect on hepatocarcinogenesis. Furthermore, we clarified that the preventive effect of HNF4 α on HCC development is due to the suppression of hepatocyte EMT and CSC generation through the inhibition of β -catenin signaling. These findings not only deepen our understanding of the biological significance of HNF4 α but also provide a promising way for HCC prevention in clinical trials. Furthermore, this strategy of cancer prevention using differentiation-determining transcription factors might be extended to other diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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