Gut Online First, published on August 10, 2009 as 10.1136/gut.2008.174904

Hepatocyte nuclear factor 4α attenuates hepatic fibrosis in rats

A running title: HNF4α attenuates hepatic fibrosis in rats

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Keywords: transcriptional factor; transforming growth factor-β1; hepatocyte differentiation; hepatic

stellate cells; deactivation.

Word count: 3746 words

Abbreviations:

HNF4α, hepatocyte nuclear factor 4α; EMT, epithelial-mesenchymal transition; MET, mesenchymal to epithelial transition; ECM, extracellular matrix; HSCs, hepatic stellate cells; FSP-1, fibroblast-specific protein-1; DMN, dimethylnitrosamine; BDL, bile duct ligation; MOI; multiplicity of infection; HE, hematoxylin-eosin; VG, Van Gieson; α-SMA, α-smooth muscle actin; TIMP-1, tissue inhibitor of metalloproteinase-1; ALB, albumin; GS, glutamine synthetase; CYP1α2, cytochrome P4501α2; PCNA, proliferating cell nuclear antigen; DAPI, 4', 6-diamidino-2-phenylindole; NC, negative control; pfu, plaque forming unit

Abstract

Background & Aims: Hepatocyte nuclear factor 4α (HNF4α) is a central transcriptional regulator of hepatocyte differentiation and function. The aim of this study is to evaluate the effect of HNF4α on attenuation of hepatic fibrosis. ***Methods:*** The adenoviruses carrying HNF4α gene or containing siRNA targeting HNF4α were injected through tail vein on two distinct hepatic fibrosis models either induced by dimethylnitrosamine or by bile duct ligation in rats. Moreover, HNF4α, epithelial-mesenchymal transition (EMT)-related and fibrotic markers in hepatocytes, hepatic stellate cells (HSCs) and liver tissues were detected by real time PCR, immunofluorescence or immunohistochemistry. ***Results:*** We demonstrated that decreased expression of HNF4α and epithelial markers accompanied by enhanced expression of mesenchymal markers occurred in fibrotic liver. More importantly, forced expression of HNF4α remarkably alleviated hepatic fibrosis and improved liver function with suppression of EMT in both fibrosis models. In contrast, down-regulation of HNF4α by siRNA aggravated hepatic fibrosis and decreased the expression of E-cadherin in company with the enhanced expression of vimentin and fibroblast-specific protein-1. *In vitro* study revealed that HNF4α could suppress the EMT process of hepatocytes induced by transforming growth factor-β1 and increase the expression of liver-specific genes. Similar phenomenon of EMT process was observed during the activation of HSCs, which was abrogated by HNF4α. Additionally, HNF4α deactivated the myofibroblasts through inducing the mesenchymal to epithelial transition and inhibited their proliferation. ***Conclusions:*** Our study suggests that HNF4α is critical for hepatic fibrogenesis and up-regulation of HNF4α might present as an ideal option for the treatment of hepatic fibrosis.

Introduction

Hepatic fibrosis, a wound-healing response to chronic liver injury, is characterized by excess production and deposition of extracellular matrix (ECM), which leads to loss of liver function and disruption of liver structure.[1, 2] The activation and proliferation of resident hepatic stellate cells (HSCs) has been well established as the central event in the development of hepatic fibrosis and activated HSC is an attractive target for anti-fibrotic therapy based on their key role in ECM accumulation during liver injury in the past decades.[2-5]

Recently, a series of studies strongly suggested that a new pathogenetic mechanism would complement the "canonical principle" of hepatic fibrogenesis, which indicated that epithelial-mesenchymal transition (EMT) is an important contributor to the progression of hepatic fibrosis.[6, 7] During EMT, epithelial cells gradually lose their epithelial signatures while acquiring the characteristics of mesenchymal cells.[8, 9] It has been proven that EMT is a general pathogenetic principle of chronic cholestatic liver diseases [10] and an initiating event for the recurrence of primary biliary cirrhosis in the allograft after liver transplantation.[11] In addition, a study by Kaimori *et al*. has revealed that transforming growth factor-β1 (TGF-β1) induces EMT state in mouse mature hepatocytes by activating the snail transcription factor and the Smad2/3 pathway. The EMT state is marked by significant up-regulation of α1 (I) collagen mRNA expression and type I collagen deposition.[12] Moreover, hepatocytes which undergo EMT contribute substantially to the population of fibroblast-specific protein-1 (FSP-1) -positive fibroblasts in CCl_4-induced liver fibrosis.[13] A very recent study by Dooley *et al.* demonstrates that hepatocytes undergo TGF-β–dependent EMT-like phenotypic changes and actively participate in fibrogenesis. Ablation of

TGF-β signaling specifically in this cell type is sufficient to blunt the fibrogenic response.[14] Additionally, it has been confirmed that HSCs express E-cadherin in the quiescent state and E-cadherin switches to N-cadherin during HSCs activation, suggesting that HSCs activation represents the transdifferentiation from an epithelial to a mesenchymal phenotype.[15] Taken together, all of these studies suggest that both HSCs and the parenchymal epithelial cells of the liver participate in hepatic fibrogenesis through EMT.

Hepatocyte nuclear factor 4α (HNF4α), an important transcriptional factor of the nuclear hormone receptor family, is essential for normal liver architecture, morphological and functional differentiation of hepatocytes, and generation of a hepatic epithelium.[16, 17] Overproduction of HNF4α in cultured rat hepatocytes could result in the maintenance of the cell viability.[18] Our recent study demonstrates that up-regulation of HNF4α expression could induce the differentiation of hepatoma cells into hepatocytes with re-expression of characteristic hepatocyte markers.[19] Additionally, it has been proven that EMT in hepatocytes induced by TGF-β correlates with the down-regulation of HNFs, in particular of HNF4α.[20] Most interestingly, ectopic HNF4α expression in fibroblasts is sufficient to induce a mesenchymal to epithelial transition (MET).[17] Based on these findings, we hypothesize that overexpression of HNF4α might ameliorate function in fibrotic liver and attenuate hepatic fibrogenesis with the inhibition of EMT.

In the present study, we demonstrated that up-regulation of HNF4α could ameliorate liver fibrosis and function with the inhibition of EMT in both hepatocytes and activated HSCs in rats. Conversely, HNF4α siRNA deteriorated the development of hepatic fibrosis. The results suggest the "transcriptional factor therapy" with HNF4α might present as a promising novel strategy in the

treatment of hepatic fibrosis in future clinical application.

Materials and methods

See supplementary materials for detailed experimental materials and methods.

Construction of adenoviral vectors. The recombinant adenoviruses of AdHNF4α expressing HNF4α efficiently and AdGFP (control) were prepared as described previously.[19] To address the effect of endogenous HNF4α on hepatic fibrogenesis, three siRNAs targeting rat HNF4α mRNA and a scrambled siRNA used as a negative control (NC) were designed with software on www.ambion.com and synthesized by GenePharma (Shanghai GenePharma Co., Ltd, Shanghai, China). The selected siRNA sequence with highest inhibitive effect and the scrambled siRNA were listed in Supplementary table 1. The construction of the adenoviral vector containing the siRNA targeting HNF4α (AdshHNF4α) and the control adenovirus (AdshNC) was carried out as previously described.[21]

Animal fibrosis models and adenoviral delivery in vivo. Male Sprague–Dawley rats, weighing about 200g each, were housed in cages 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. To evaluate the effect of AdHNF4α on normal rats, 4×10^9 plaque forming unit (pfu) AdGFP or AdHNF4α adenoviruses or PBS were injected into normal rats through tail vein and three animals in each group were sacrificed at different time points of 3 days, 10 days and 1 month after injection, respectively.

To investigate the effect of HNF4α on liver fibrosis, two distinct models of hepatic fibrosis were induced in rats either by dimethylnitrosamine (DMN) injection or by bile duct ligation (BDL) (Fig 1). For DMN model, rats were injected intraperitoneally with 1% DMN (10 μg/kg) for 3 consecutive

days per week up to 5 weeks. After DMN injection for 12 times, rats were infused with PBS, 4×10^9 pfu AdGFP or AdHNF4α via tail vein, respectively (10 rats in each group). Two weeks after gene delivery, the animals were sacrificed (Fig 1A). BDL fibrosis model was induced as described previously (5). The rats were infused with PBS, 4×10^9 pfu AdGFP or AdHNF4α via tail vein, 2 days after BDL respectively (10 rats in each group) and sacrificed 3 weeks after BDL (Fig 1B).

In order to demonstrate whether endogenous HNF4α could regulate liver fibrogenesis, PBS, 4×10^9 pfu AdshHNF4α or AdshNC were injected via rat tail vein 2 days before DMN injection or BDL and the animals were sacrificed 3 or 2 weeks later respectively (10 rats in each group) (Fig 1C-D).

Biochemical assays and measurement of hydroxyproline content. Serum biochemical parameters were determined by an automated analyzer at the clinical immunology center in Shanghai Changzheng hospital. The content of hydroxyproline was determined according to the protocol of Hydroxyproline Testing Kit (Jiancheng, Nanjing, China) as previously described.[21]

Histology and immunohistochemistry. Liver tissue sections were stained with Hematoxylin-Eosin (HE) for histopathological examination and Van Gieson (VG), Sirius red staining or Masson's trichrome staining were used for collagen determination. Immunohistochemical examinations were carried out to detect the expression of HNF4α, vimentin, TGF-β1, α-smooth muscle actin (α-SMA), proliferating cell nuclear antigen (PCNA), E-cadherin and FSP-1.

Hepatocytes culture and treatment. Rat hepatocytes were primarily prepared from the male Sprague–Dawley rats as previously described.[22] In order to show an EMT state in mature hepatocytes *in vitro*, the culture medium was replaced with medium containing 2 ng/ml of TGF-β1 (R & D Systems, Minneapolis, MN) 48 hours after isolation.[12] To determine the block effect of

HNF4α on TGF-β1-induced EMT, the hepatocytes were infected by AdGFP or AdHNF4α at multiplicity of infection (MOI) 10 simultaneously with treatment of TGF-β1.

HSCs culture and treatment. HSC-T6 was kindly provided by Dr. Scott L Friedman.[23] Primary HSCs were freshly isolated and cultured as described.[24] The primary HSCs were cultured in plastic cell culture dishes, or treated with TGF-β1 (1 ng/ml, beginning on day 2 of culture) to mimic the activation process *in vitro*. The cells were infected by AdGFP or AdHNF4α at different culture time points.

Quantitative real time PCR analysis. The original amount of the specific transcripts was detected via real time PCR with a SYBR Green PCR Kit (Applied Biosystems, Foster City, CA). Primers for these transcripts were listed in Supplementary table 2.

Immunofluorescence staining. The cells were stained with one or different combinations of the following primary antibodies: HNF4α, α-SMA, vimentin, E-cadherin and snail, and were visualized under a laser scanning confocal fluorescent microscope (Carl Zeiss, Inc., Germany).

Cell proliferation assay. At different time point after virus infection, the number of viable cells was determined colorimetrically at 450 nm using the Cell Counting Kit-8 (Dojindo, Tokyo, Japan).

Patients. Liver tissues were obtained from Liver tissue bank of Eastern Hepatobiliary Surgery Hospital by State key infection disease project of China. The liver tissues were from two groups of subjects (1) control individuals (n =7) with normal liver or minimal changes in the liver such as angeioma or liparomphalus. (2) individuals with fibrosis or cirrhosis (n =16).

Statistical analysis. The analysis of variance (ANOVA) and Student's *t* test were used for comparison of normally distributed data among the groups and between paired data respectively.

Data not normally distributed were compared using the Mann-Whitney tests. All the tests were 2-sided and a P value < 0.05 was considered statistically significant.

Results

Hepatic fibrogenesis is associated with decreased expression of HNF4α. As expected, both of DMN injection and BDL induced the prominent hepatic fibrosis in rats as shown by Masson's trichrome staining (Fig 2). In the fibrotic livers the hepatocytes showed faint HNF4α staining and apparently decreased expression of E-cadherin was also observed on the membranes of the parenchymal and nonparenchymal cells. Meanwhile, the immunohistochemical studies showed the enhanced expression of α-SMA and vimentin in fibrous areas. Interestingly, in the fibrotic livers, the intense distribution of FSP-1 staining was not only seen around fibrotic septa in correlation with that of α-SMA, but also localized in the hepatocytes (Fig 2). Furthermore, the levels of HNF4α mRNA were significantly reduced in patients with fibrosis or cirrhosis compared with control livers (P=0.0056) (Fig 3A). Immunohistochemical studies also showed intense HNF4α staining in normal control liver tissue. By contrast, only faint HNF4α staining was observed in the fibrotic or cirrhotic livers (Fig 3B).

HNF4α ameliorates ECM deposition and liver function in fibrotic liver. In normal rats, AdHNF4α injection did not have obvious adverse effect on liver function parameters, the body and liver weight (Supplementary table 3-4). Histological analysis with HE staining revealed that AdHNF4α infusion did not result in apparent damage in the organs including liver, kidney, lung, spleen and heart (data not shown). Similarly, AdHNF4α administration did not have significant effect on body and liver weight in BDL model (Supplementary table 5) and DMN model (data not shown), compared with AdGFP groups.

We then examined the effect of AdHNF4α on hepatic fibrosis. AdHNF4α injection greatly increased

HNF4α expression which was mainly located in hepatocytes, HSCs, and fibroblasts in the periportal regions (Fig 4). Intriguingly, a single administration of AdHNF4α inhibited the development of hepatic fibrosis as confirmed by HE, VG and Masson's trichrome staining (Fig 4). Semiquantitative analyses in the ECM area of VG staining revealed no significant histological difference between rats receiving AdGFP and PBS (data not shown). AdHNF4α injection reduced the ECM area by 69% and 71% in the DMN and BDL fibrotic models, respectively (P<0.01, in comparison to the AdGFP controls). A quantitative analysis in the DMN model revealed a significantly lower hydroxyproline content in rats receiving AdHNF4α (217.16±42.5 vs. 361.37±112.8 μg/g in the AdGFP controls; P<0.05). AdHNF4α administration also reduced the amount of hydroxyproline content in BDL treated rats (data not shown). Moreover, AdHNF4α injection significantly improved serum levels of biochemical parameters indicative of hepatic function in both models (Supplementary table 6).

HNF4α suppresses EMT process in hepatocyte induced by TGF-β1. Treatment of hepatocytes with TGF-β1 significantly decreased the mRNAs of HNF4α and E-cadherin (Fig. 5A). Expression of markers for mesenchymal cells (vimentin, snail) and collagen type I, in contrast, was significantly increased by TGF-β1. In parallel with increased expression of mesenchymal markers in hepatocytes, liver-specific genes, such as albumin (ALB) and glutamine synthetase (GS), were down-regulated (Fig 5A). Immunofluorescence results showed the intense snail staining in the TGF-β1 treated hepatocytes, which was accompanied by decreased expression of HNF4α and E-cadherin compared with those of the controls (Fig 5B).

To investigate the role of HNF4α in restoring biological characteristics of primary hepatocytes treated by TGF-β1, the expression of a cluster of hepatocyte marker genes and cell morphology were

detected after AdHNF4α treatment for 48 and 72 hours. The hepatocytes treated by TGF-β1+AdHNF4α preserved a more cuboidal/hexagonal shape compared with TGF-β1+AdGFP treated ones (Fig 5C). In addition, forced expression of HNF4α repressed the mRNA levels of mesenchymal phenotype genes (e.g. vimentin, snail, slug, twist1), as well as collagen types I and III (P<0.05). In contrast, the expression of liver-specific genes and epithelial phenotype genes such as HNF4α, GS, ALB, cytochrome P4501α2 (CYP1α2), occludin and E-cadherin was up-regulated significantly (P<0.05) (Fig 5D). Moreover, double immunostaining further confirmed that HNF4α induced the expression of E-cadherin, while decreased snail expression (Fig 5E, Supplementary fig 1).

HNF4α blocks the EMT process of HSCs activation. To observe the EMT process during the activation of HSCs, we detected the gene expression of epithelial and mesenchymal markers in both quiescent and activated HSCs. In contrast to the freshly isolated HSCs, the expression of fibrotic and mesenchymal markers was markedly increased in the HSCs cultured on plastic surfaces of the dishes from day 5 to day 12. However, the expression of E-cadherin mRNA decreased significantly (Supplementary fig 2A). In addition, immunofluorescence staining demonstrated that down-regulation of E-cadherin occurred in parallel with the increased expression of snail and α-SMA proteins during the activation of HSCs (Supplementary fig 2B).

Subsequently, to address the effect of HNF4α on HSCs activation, AdHNF4α infection was used to deliver the exogenous gene into the primary activated HSCs. Overexpression of HNF4α mRNA accompanied by enhanced expressions of smad7 and epithelial phenotype genes, including E-cadherin, occludin, plakoglobin and desmoplakin, was detected at different culture time points

after HNF4α transfection for 72 hours in both culture-activated HSCs (Fig 6A-i) and TGF-β1-activated HSCs (Fig 6A-ii). Meanwhile, the expression of some profibrogenic markers such as α-SMA, tissue inhibitor of metalloproteinase-1 (TIMP-1), as well as mesenchymal markers was repressed significantly by AdHNF4α (Fig 6A). Moreover, forced expression of HNF4α in both of the activated HSCs could promote expression of E-cadherin with simultaneous reduction of α-SMA protein level (Fig 6B-C, Supplementary fig 3).

HNF4α deactivates the myofibroblasts through inducing the MET process. HSC-T6 cells are immortalized cells which exhibit the typical features of myofibroblasts. To further address whether up-regulation of HNF4α could result in deactivation of myofibroblasts through inducing the MET process, HSC-T6 was treated by AdHNF4α up to 72 hours. Real time PCR revealed that the expression of vimentin, snail, slug, twist1, collagen types I and III and α-SMA significantly decreased in AdHNF4α-treated HSC-T6, accompanied by increasing expression of epithelial phenotype genes (E-cadherin, plakoglobin and desmoplakin) (Fig 7A.). Additionally, immunofluorescence staining revealed the enhanced expression of HNF4α and E-cadherin, accompanied by decreased protein levels of α-SMA, vimentin and snail after AdHNF4α infection compared with their control counterparts (Fig 7B). Furthermore, overexpression of HNF4α inhibited the proliferation of these cells ($P<0.05$) (Fig 7C).

HNF4α inhibits EMT in fibrotic liver in rats. To provide further proof that the reduction of ECM production by HNF4α gene delivery is due to the inhibition of EMT in experimental hepatic fibrosis, epithelial or mesenchymal mRNAs and proteins in the liver tissue were analyzed by real time PCR and immunohistochemistry. AdHNF4α injection in fibrotic rats up-regulated expression of

liver-specific genes, and down-regulated the expression of mesenchymal markers and α-SMA (Supplementary fig 4). Delivery of HNF4α enhanced the expression of E-cadherin and PCNA, and reduced the expression of vimentin, with concomitant decreased expression of TGF-β1 and α-SMA in fibrotic areas. In addition, FSP-1 expression in hepatocytes and around fibrotic septa was also significantly suppressed (Fig 8).

HNF4α siRNA aggravates experimental hepatic fibrosis. AdshHNF4α injection greatly decreased HNF4α expression in liver as demonstrated by immunohistochemistry (Fig 9). As shown by HE, Sirius red staining and Masson's trichrome staining, the control livers showed some proliferating bile ducts and small amount of ECM deposition 2 weeks after BDL, while the livers treated with AdshHNF4α exhibited overt ECM deposition around the proliferating bile ducts and a continuous meshwork of connective tissue infiltrating the hepatic parenchyma. Similarly, AdshHNF4α also led to more ECM deposition in the fibrotic livers inducd by DMN injection (Fig 9). As shown by Sirius red staining, AdshHNF4α injection increased the ECM area by 55% and 85% in the BDL and DMN fibrotic models, respectively, compared with that in AdshNC controls (P<0.05). Moreover, hydroxyproline content was increased in the AdshHNF4α-treated fibrotic livers either induced by DMN injection (391.31 ± 104.6 vs. 229.40 ± 73.8 μg/g in the AdshNC controls; P<0.05) or by BDL (275.35 ± 44.8 vs. 191.96 ± 18.5 μg/g in the AdshNC controls; P<0.05). Meanwhile, immunohistochemistry showed that AdshHNF4α decreased the expression of E-cadherin in company with the enhanced expression of vimentin and FSP-1 in both fibrosis models (Fig 10). Furthermore, the expression of PCNA was almost absent in AdshHNF4α treated group, while was evident in AdshNC group (Fig 10). However, there was no statistically significant difference

concerning liver and body weight among the three groups of BDL model (Supplementary table 7) and DMN model (data not shown).

Discussion

HNF4α is a dominant transcriptional regulator of the hepatocyte function and epithelial phenotype. Down-regulation of HNF4α decreases expression of the genes involved in epithelial phenotype and cytoskeletal organization, and therefore compromises hepatocyte function.[17, 25] In contrast, ectopic expression of HNF4α could induce MET in fibroblasts and restore epithelial phenotype in hepatocytes under EMT.[17] Nevertheless, there is no study regarding the HNF4α therapy of hepatic fibrosis to date. In the current study, we found significantly decreased expression of HNF4α in fibrotic liver. Our results also revealed that forced expression of HNF4α attenuated the deposition of ECM, while down-regulation of the HNF4α expression exacerbated the development of hepatic fibrosis in two independent rat models. Additionally, treatment with AdHNF4α significantly improved biochemical measures indicative of hepatic function. These data suggest that HNF4α has the potential to effectively treat hepatic fibrosis.

In the past decades, little attention has been given to the direct contribution of mature hepatocytes to liver fibrogenesis. It was believed that hepatocytes had an insignificant role in the genesis of liver fibrosis and progression to cirrhosis.[26] Nevertheless, recent reports have revealed that the hepatocytes in fibrotic liver can also contribute to the accumulation of activated fibroblasts via EMT, and EMT involving hepatocytes plays a significant role in hepatic fibrogenesis.[13] It is well recognized that TGF-β1 is a key contributor to the initiation and progression of hepatic fibrosis, and also plays an important role in inhibition of hepatocytes proliferation and induction of apoptosis.[27, 28] Furthermore, TGF-β1 is recently considered as the most powerful inducer of EMT to promote the genetic and phenotypic programming of hepotocytes to mesenchymal cells (fibroblasts).[12]

Using primary hepatocyte culture as a model, we demonstrated that forced expression of HNF4α preserved the epithelial phenotype against EMT induced by TGF-β1. Cells expressing HNF4α had a more cuboidal/hexagonal shape. These cells had lower expression of mesenchymal markers, and significantly increased expression of liver-specific genes and epithelial phenotype genes such as E-cadherin. The up-regulation of these reliable indicators for hepatocyte phenotype and function demonstrates that HNF4α is an inhibitor of TGF-β1 induced EMT and sufficient to confer enhancing expression of a set of functional genes and epithelial marker genes of hepatocytes.

It is suggested that the activation of HSCs might be considered as an EMT phenomenon.[15] Our present study confirmed that the expression of vimentin, snail and α-SMA was increased during HSCs activation, accompanying the down-regulation of E-cadherin which was a universal epithelial marker to maintain cellular integrity. More importantly, this study has clearly demonstrated that forced expression of HNF4α could increase the expression of epithelial markers and down-regulate the mesenchymal marker expression in both culture-activated HSCs and TGF-β1-activated HSCs, implying that HNF4α could abrogate the activation of HSCs. In addition, we also demonstrated that HNF4α deactivated the myofibroblasts through inducing the MET process and inhibited their proliferation. These data suggest that HNF4α might block hepatic fibrogenesis at least partly through the inhibition of the EMT process of HSCs activation.

It has been demonstrated that TGF-β1 mediates EMT by the induction of snail, a repressor of E-cadherin transcription.[12] The snail gene is recognized as a key regulator of EMT, and its expression has shown an inverse correlation with expression of the HNFs. Snail could both control epithelial polarity and influence the liver specific gene expression through binding to the E-boxes

presenting in HNF4α promoter in hepatocytes.[29] Meanwhile, a dominant antagonism by the ectopic expression of HNF4α on snail regulation of EMT was also reported.[29] Interestingly, the present study clearly revealed that forced expression of HNF4α decreased snail expression remarkably in EMT involving hepatocytes and activated HSCs. We also found reduced expression of TGF-β1 and snail in parallel with increased expression of epithelial phenotype markers in fibrotic liver after AdHNF4α injection. These findings further supported the role of HNF4α in inhibiting TGF-β1-induced EMT.

It is recognized that hepatic fibrosis is associated with reduced volume and functional loss of hepatocytes.[30] Intriguingly, we witnessed that forced expression of HNF4α led to elevated expression of a cluster of liver-specific genes both *in vitro* and *in vivo*. Additionally, enhanced PCNA staining in hepatocytes was clearly detected after AdHNF4α delivery, while less PCNA-positive hepatocytes were observed after AdshHNF4α injection, suggesting the pro-proliferating effect of HNF4α on hepatocytes *in vivo*. Thus, the improvement of liver function with HNF4α treatment might be ascribed to a direct up-regulation of liver-specific genes and proliferation of hepatocytes.

It has generally been accepted that activation of HSCs plays the pivotal role in hepatic fibrogenesis.[31] Consequently, it is a major strategy for hepatic fibrosis therapy by repressing activation, proliferation and migration of HSCs as well as inducing their apoptosis in the past decades.[1, 3] However, the available anti-fibrotic therapy has not achieved satisfactory efficacy in clinical practice. Recently, the contribution of the HSCs to liver development and regeneration has been highlighted.[23, 32-34] Thus, inhibition of HSCs activation for the treatment of hepatic fibrosis

may exert a negative action on liver regeneration. Unlike the previous studies, our current work offers a novel strategy to attenuate hepatic fibrosis using HNF4α, a key transcriptional factor for hepatocyte differentiation and function, which exerts dual effects on blocking the EMT process of both hepatocytes and HSCs, and simultaneously stimulating the proliferation of hepatocytes and improving the liver function. Our recent study reveals that HNF4α has the much more potent therapeutic effect on hepatocellular carcinoma than other known differentiation inducers.[19] We believe that, as a meaningful approach, the strategy using transcriptional factor HNF4α might present as an ideal option for the therapy of hepatic fibrosis.

In summary, the present investigation provides strong evidence for the suppression effect of HNF4α on liver fibrosis with inhibiting EMT of both hepatocytes and HSCs. A key advantage of using HNF4α in comparison to previous approaches is the preservation of hepatocyte function. These results suggest that increasing the expression of HNF4α could be potentially used to control liver fibrosis in patients. Our findings also encourage the evaluation of “transcriptional factor therapy” for fibrosis of organs other than the liver.

This work was supported by grants from the National Science Fund for Distinguished Young Scholars (No. 30825020), the National Natural Science Foundation of China (No. 30570838), the Natural Science Foundation of Shanghai (No. 08ZR1404800) and Chenguang Project Foundation of Shanghai (No. 2007CG50). The authors have no conflicting financial interests.

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Figure legends

Figure 1. Schematic representation of induction of experimental hepatic fibrosis in rats. Hepatic fibrosis was induced by either injection of dimethylnitrosamine (DMN) (A, C) or bile duct ligation (BDL) (B, D). AdGFP or AdHNF4α were injected via rat tail vein 4 weeks after DMN injection (A) or 2 days after BDL (B), and AdshHNF4α or AdshNC were injected 2 days before DMN injection (C) or BDL (D).

Figure 2. The expression of hepatocyte nuclear factor 4α (HNF4α), epithelial and mesenchymal markers during the development of hepatic fibrosis. Immunochemistry analysis for the protein expression of HNF4α, epithelial and mesenchymal markers in the liver tissue treated by DMN or BDL as described in Methods. At different time points of hepatic fibrogenesis, rats were sacrificed, and Masson's trichrome staining (×100) of liver sections was performed to quantify extracellular matrix (ECM) deposition. Immunochemistry results showed the protein expression of α-smooth muscle actin (α-SMA) (×100), HNF4α (×400) and epithelial and mesenchymal markers including E-cadherin (×400), vimentin (×200) and fibroblast-specific protein-1 (FSP-1) (×200).

Figure 3. Decreased expression of HNF4α in livers from patients with fibrosis or cirrhosis. The mRNA levels of HNF4α in the liver from the patients were detected by real time PCR (A) and the protein level by immunohistochemistry as well (B). The mRNA expression was normalized against β-actin. The data were compared using the Mann-Whitney tests. Horizontal lines in Fig 3A indicate median values. Fig 3B is the representative images for HNF4α staining by immunohistochemistry (×400).

Figure 4. HNF4α ameliorates hepatic fibrosis in rats treated by either DMN or BDL. After

adenoviruses-treated rats were sacrificed, HNF4α expression in liver sections was assessed by immunohistochemistry. More obvious staining for HNF4α (×400) was mainly located in hepatocytes, hepatic stellate cells (HSCs) and fibroblasts of periportal regions than controls. The significantly decreased amount of ECM was confirmed by HE (×100), VG (×40) and Masson's trichrome staining (×100) after HNF4α gene delivery. Data shown are the representative of 10 animals.

Figure 5. HNF4α suppresses epithelial-mesenchymal transition (EMT) of primary hepatocytes induced by transforming growth factor-β1 (TGF-β1). (**A**) The mRNA levels of HNF4α, epithelial and mesenchymal genes, and some liver-specific genes of hepatocytes in the presence or absence of TGF-β1 (2 ng/ml treated for 48 hours). The mRNA expression was normalized against β-actin. The result showed the gene expression folds of TGF-β1-treated hepatocytes versus TGF-β1 (-) control. (**B**) The hepatocytes were immunostained for HNF4α, E-cadherin or snail (red) with 4', 6-diamidino-2-phenylindole (DAPI) counterstaining for DNA (blue). TGF-β1 decreased the expression of HNF4α, E-cadherin, while enhanced snail expression. (**C**) After infection by adenoviruses in TGF-β1 treated hepatocytes for 48 and 72 hours, the GFP expression showed the cellular morphology of the hepatocytes. AdHNF4α-treated hepatocytes preserved a more cuboidal/hexagonal shape than controls. (**D**) Real time PCR compared the expression of HNF4α, epithelial and mesenchymal and some liver-specific genes in these hepatocytes. The mRNA expression levels were normalized against β-actin. The result showed the gene expression folds of AdHNF4α-infected hepatocytes versus AdGFP-infected control. (**E**) The cells were double-immunostained for HNF4α (yellow) and E-cadherin (red) or HNF4α (yellow) and snail (red) with DAPI counterstaining for DNA (blue) 48 hours after infection. Original magnification, ×400.

Each value represents the mean ± SD for triplicate samples. (* P<0.05, ** P<0.01, ***P<0.001, Student's t test)

Figure 6. HNF4α inhibits EMT during the activation process of primary HSCs. **(A)** The gene expression in culture-activated HSCs (**i**) or TGF-β1-activated (1 ng/ml) HSCs (**ii**) were detected by real time PCR. Cells were collected at indicated time points after adenoviral infection for 72 hours. The mRNA expression levels were normalized against β-actin. Gene expression folds in AdHNF4α group were normalized by that of AdGFP group. Each value represents the mean ± SD for triplicate samples. Culture-activated HSCs **(B)** or TGF-β1-activated HSCs **(C)** were treated by AdHNF4α or AdGFP. Immunostaining was performed to examine the expression of E-cadherin, HNF4α and α-SMA (red) counterstained for DNA (blue) 72 hours after infection on day 5. Significantly increased expression of E-cadherin and decreased expression of α-SMA protein were detected after AdHNF4α infection. The insets showed the higher resolution images. Original magnification, ×400. (* P<0.05, ** P<0.01, ***P<0.001, Student's t test)

Figure 7. HNF4α deactivates the myofibroblasts through inducing the epithelial-mesenchymal transition process of activated HSCs. (**A**) Real time PCR was carried out to determine the expression of fibtrotic markers (α-SMA, TIMP-1 and collagen types I and III) and EMT related genes (vimentin, snail, slug, twist1, E-cadherin, plakoglobin and desmoplakin). The mRNA expression levels were normalized against β-actin. Gene expression folds in AdHNF4α group were normalized by that of AdGFP group. Each value represents the mean ± SD for triplicate samples. (* P<0.05, ** P<0.01, ***P<0.001, Student's t test) (**B**) HSC-T6 cells treated by AdHNF4α or AdGFP were immunostained for HNF4α, E-cadherin, vimentin, snail or α-SMA (red) plus DAPI

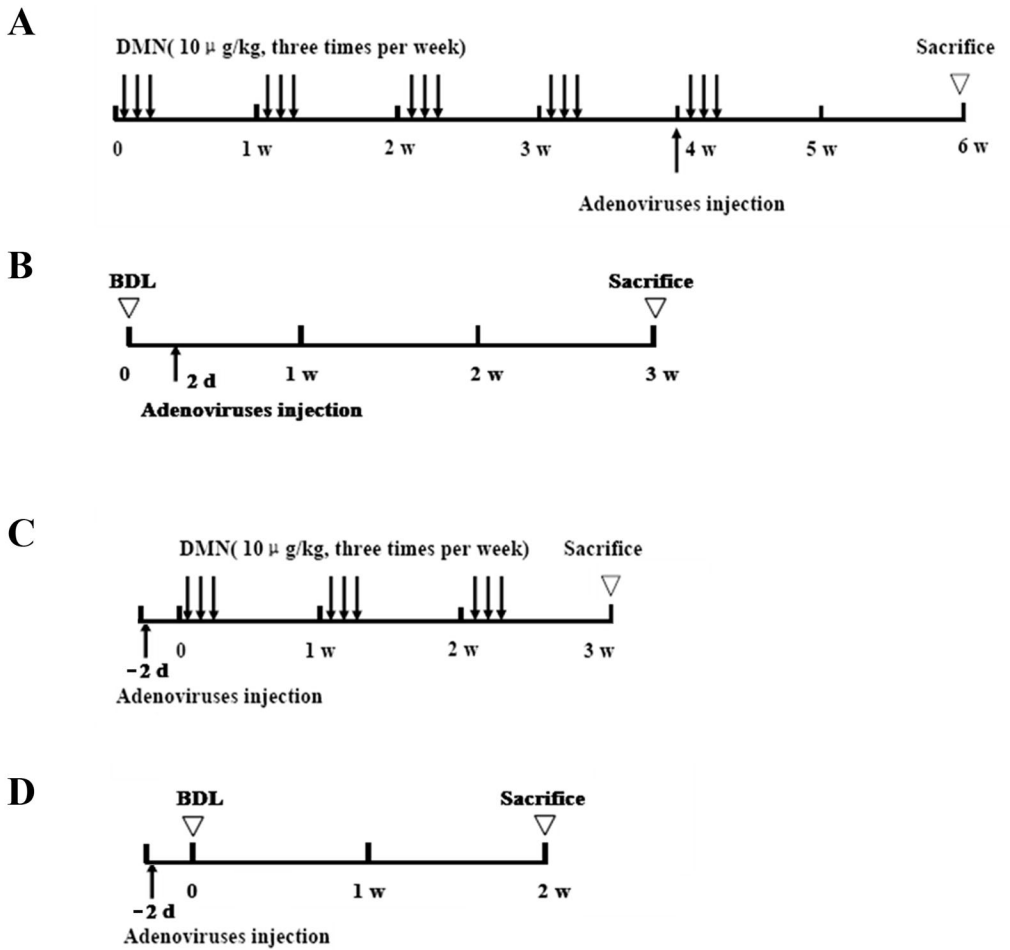
counterstaining for DNA (blue). Immunofluorescence staining showed the enhanced expression of E-cadherin, accompanied by decreased α-SMA, vimentin and snail protein expression after AdHNF4α infection for 72 hours. The insets showed the higher resolution images. (**C**) The proliferation of HSC-T6 was inhibited by HNF4α gene delivery ($P<0.05$, one-way analysis of variance). Each value represents the mean ± SD for triplicate samples.

Figure 8. HNF4α inhibits EMT in fibrotic liver in rats. Immunohistochemical staining was carried out to detect the expression of α-SMA (×100), E-cadherin (×400), vimentin (×200), FSP-1 (×200), proliferating cell nuclear antigen (PCNA) (×200) and TGF-β1 (×100) in fibrotic livers after adenovirus injection. The protein levels of vimentin and FSP-1 decreased significantly in correlation with that of α-SMA and TGF-β1 mainly around fibrous septa. Enhanced PCNA staining was observed in hepatocytes and up-regulation of E-cadherin was found on the membranes of the parenchymal and nonparenchymal cells after AdHNF4α delivery.

Figure 9. HNF4α siRNA aggravates experimental hepatic fibrosis. Immunohistochemistry was carried out to assess HNF4α expression in liver sections from DMN treatment and BDL. AdshHNF4α injection greatly decreased HNF4α expression. The significantly increased amount of ECM deposition was confirmed by HE (×100), Sirius red staining (×40) and Masson's trichrome staining (×100) after AdshHNF4α gene delivery compared with that in AdshNC controls .

Figure 10. HNF4α siRNA promotes EMT in fibrotic liver in rats. Immunohistochemical staining was carried out to detect the expression of E-cadherin (×400), vimentin (×200), FSP-1 (×200), PCNA (×200) in fibrotic livers. The protein levels of vimentin and FSP-1 were enhanced significantly in company with the decreased expression of E-cadherin in AdshHNF4α treated liver.

In addition, the PCNA positive staining hepatocytes were almost absent in AdshHNF4α treated group, while were evident in AdshNC group.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3 w
4 w
5 w
6 w
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B
BDL
Sacrifice
0
2 d
1 w
2 w
3 w
Adenoviruses injection
C
DMN(10 μ g/kg, three times per week)
Sacrifice
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Adenoviruses injection
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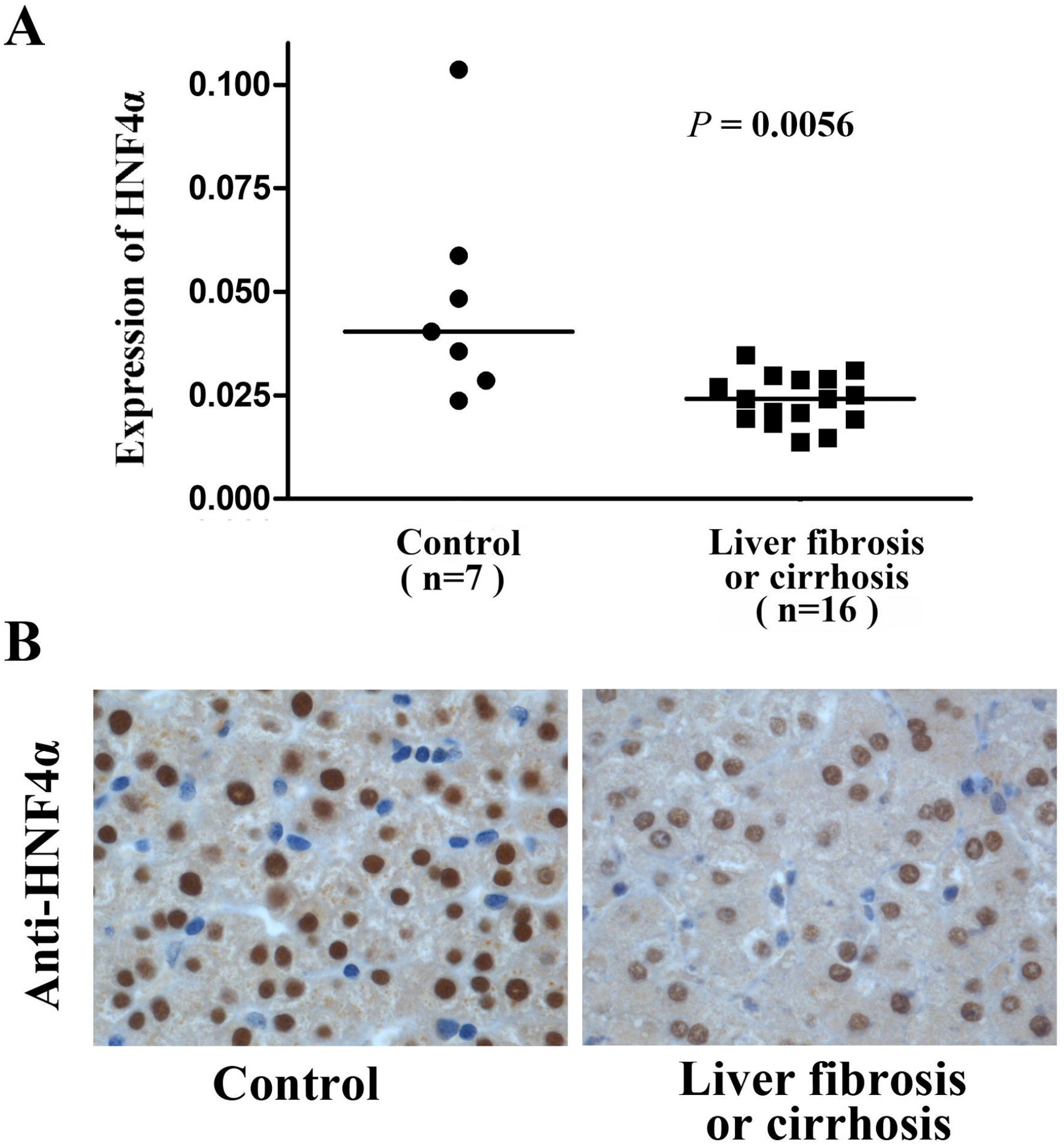
Adenoviruses injection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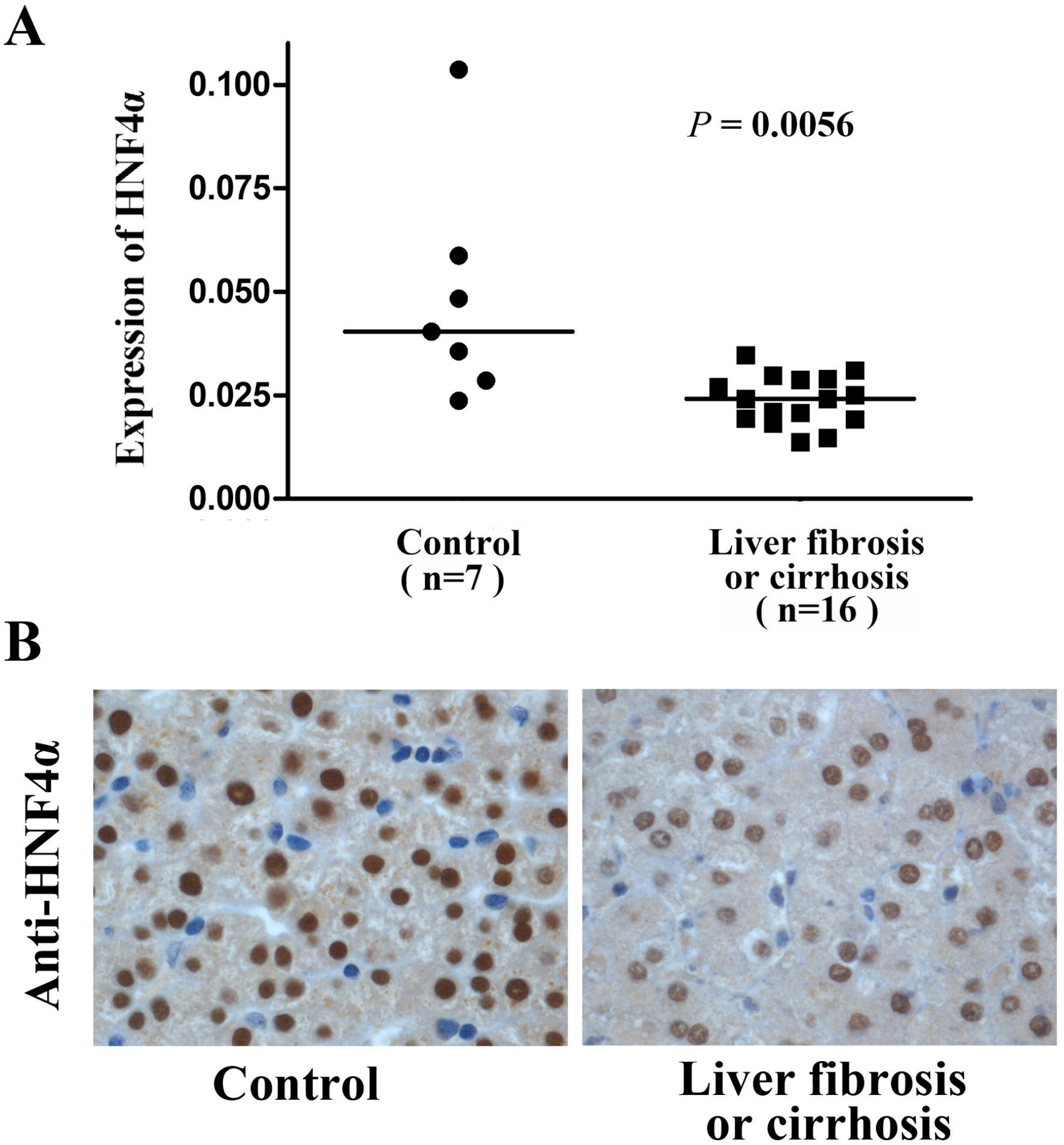
or cirrhosis
(n=16)
B
Anti-HNF4α
Control
Liver fibrosis
or cirrhosis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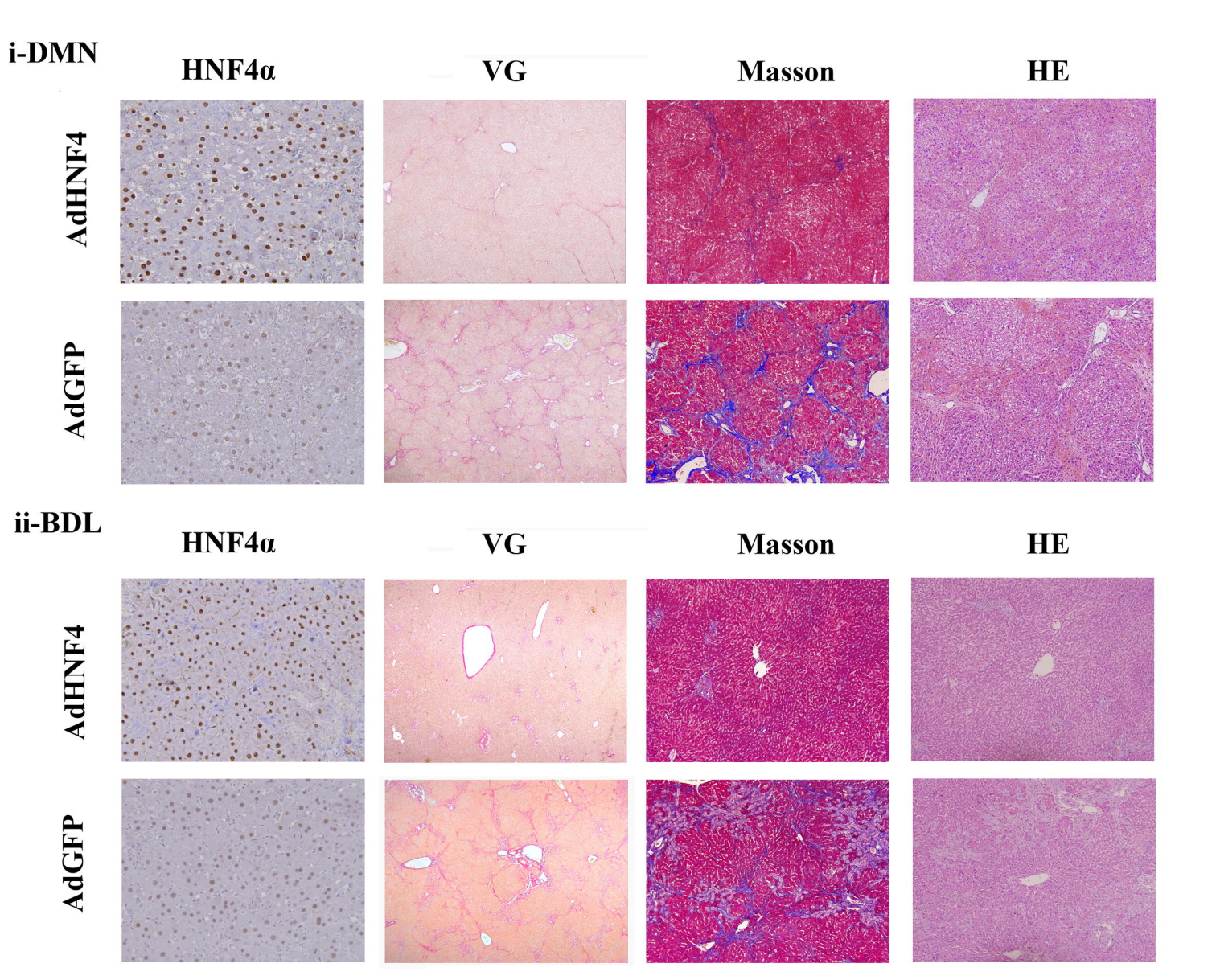
Control
Liver fibrosis
or cirrhosis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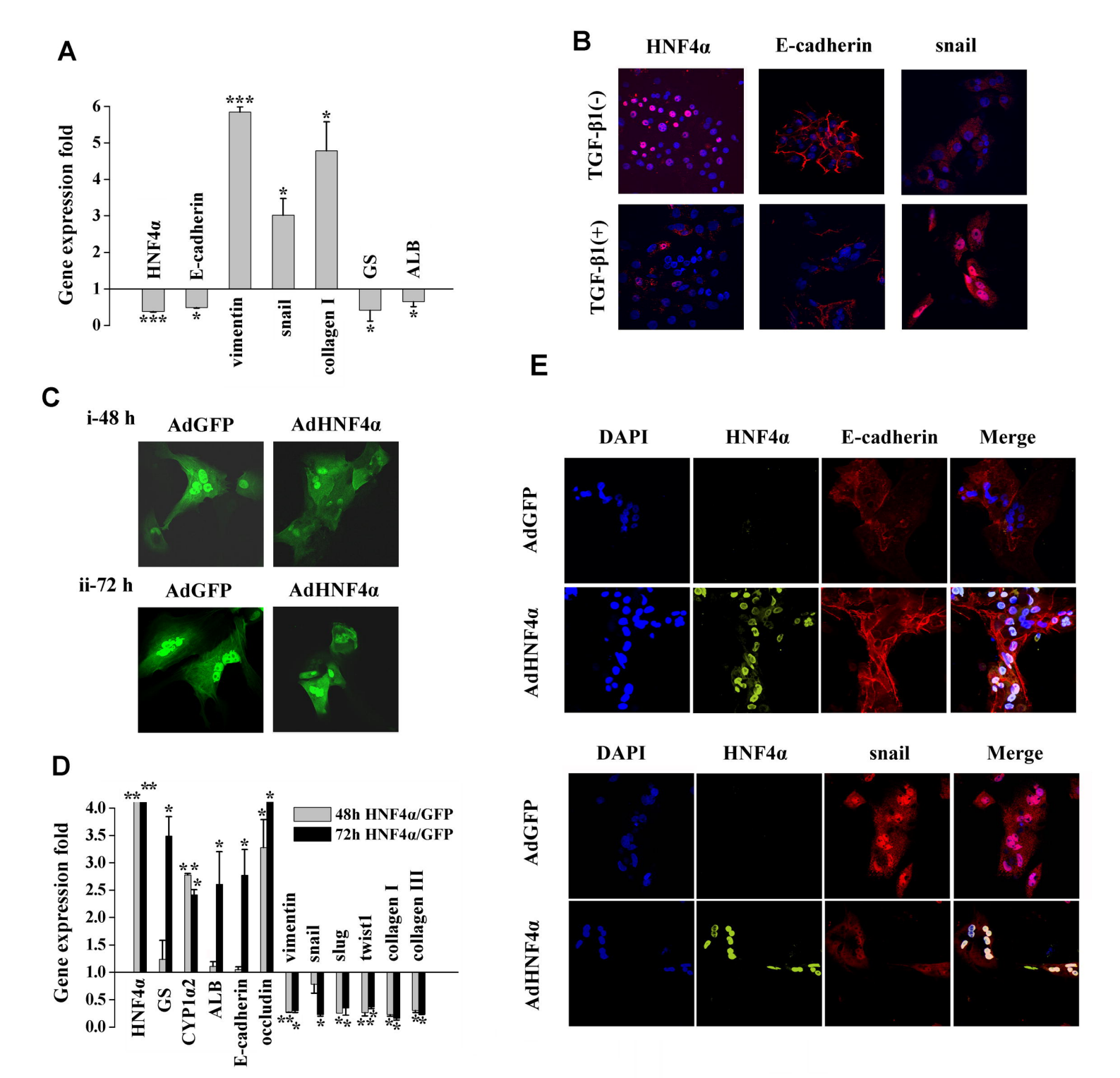
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TGF-β1(+)
C
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AdGFP
AdHNF4α
ii-72 h
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collagen III
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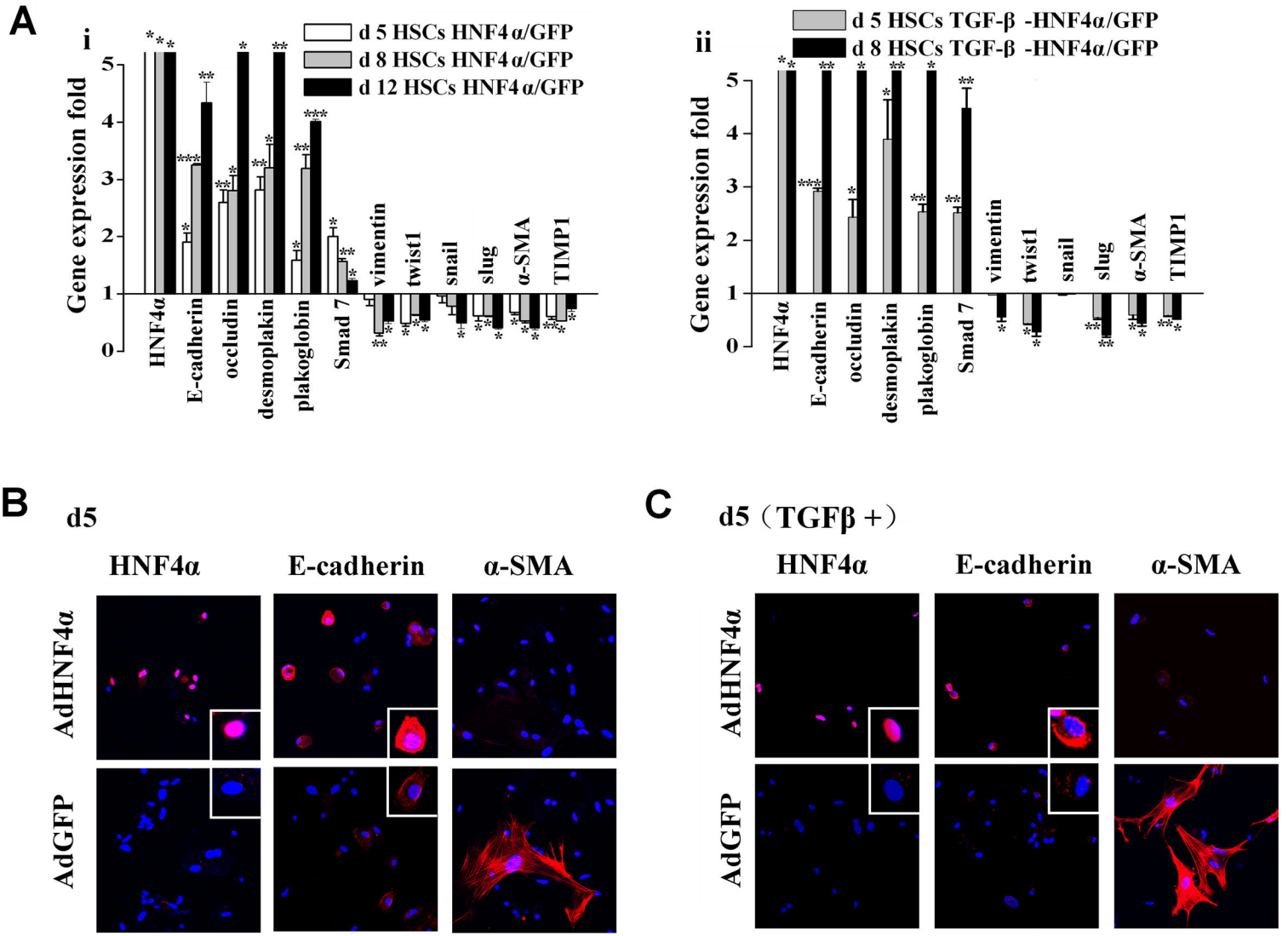
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plakoglobin
Smad 7
vimentin
twist1
snail
slug
α-SMA
TIMP1
ii
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d 8 HSCs TGF-β -HNF4α/GFP
B
d5
HNF4α
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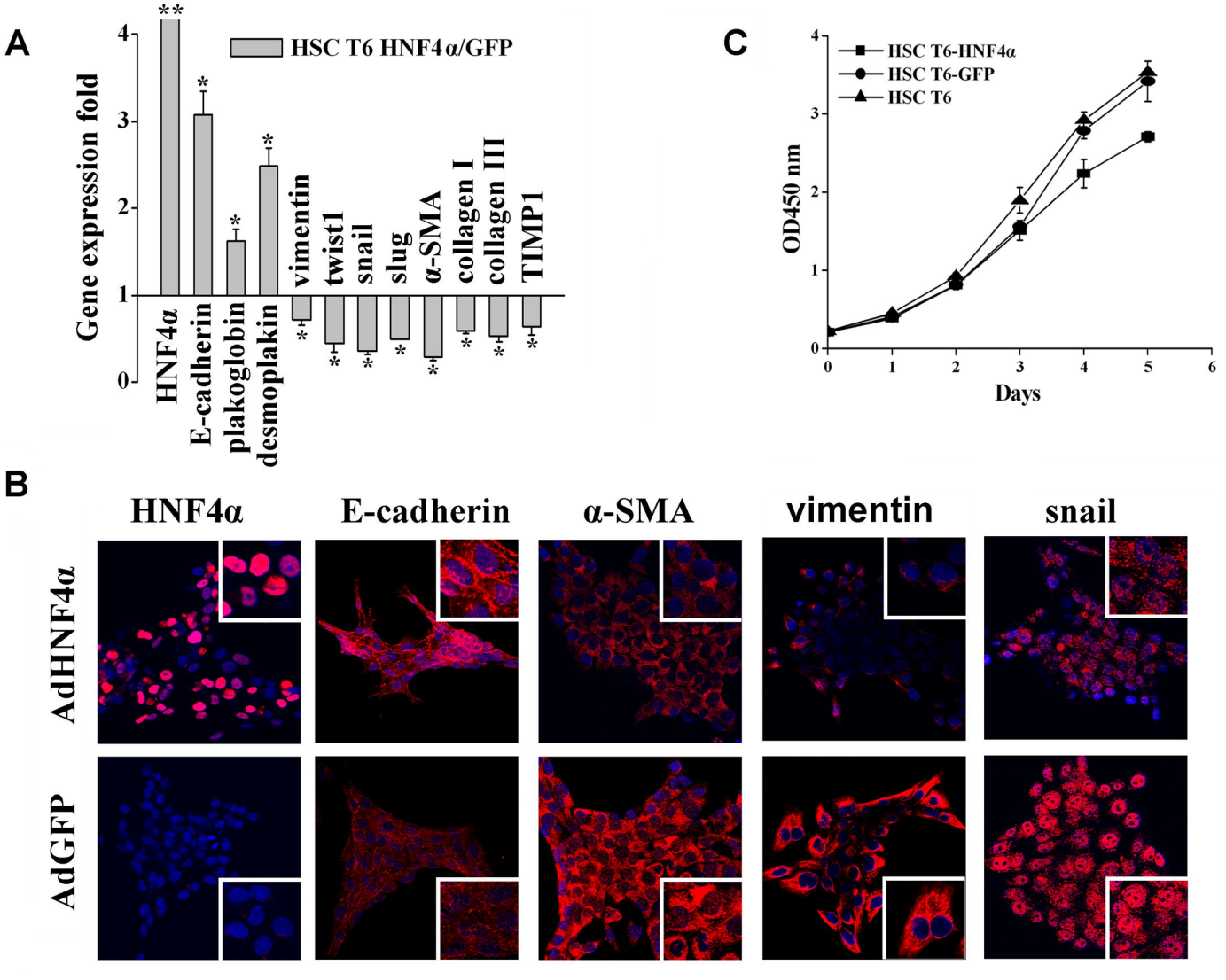
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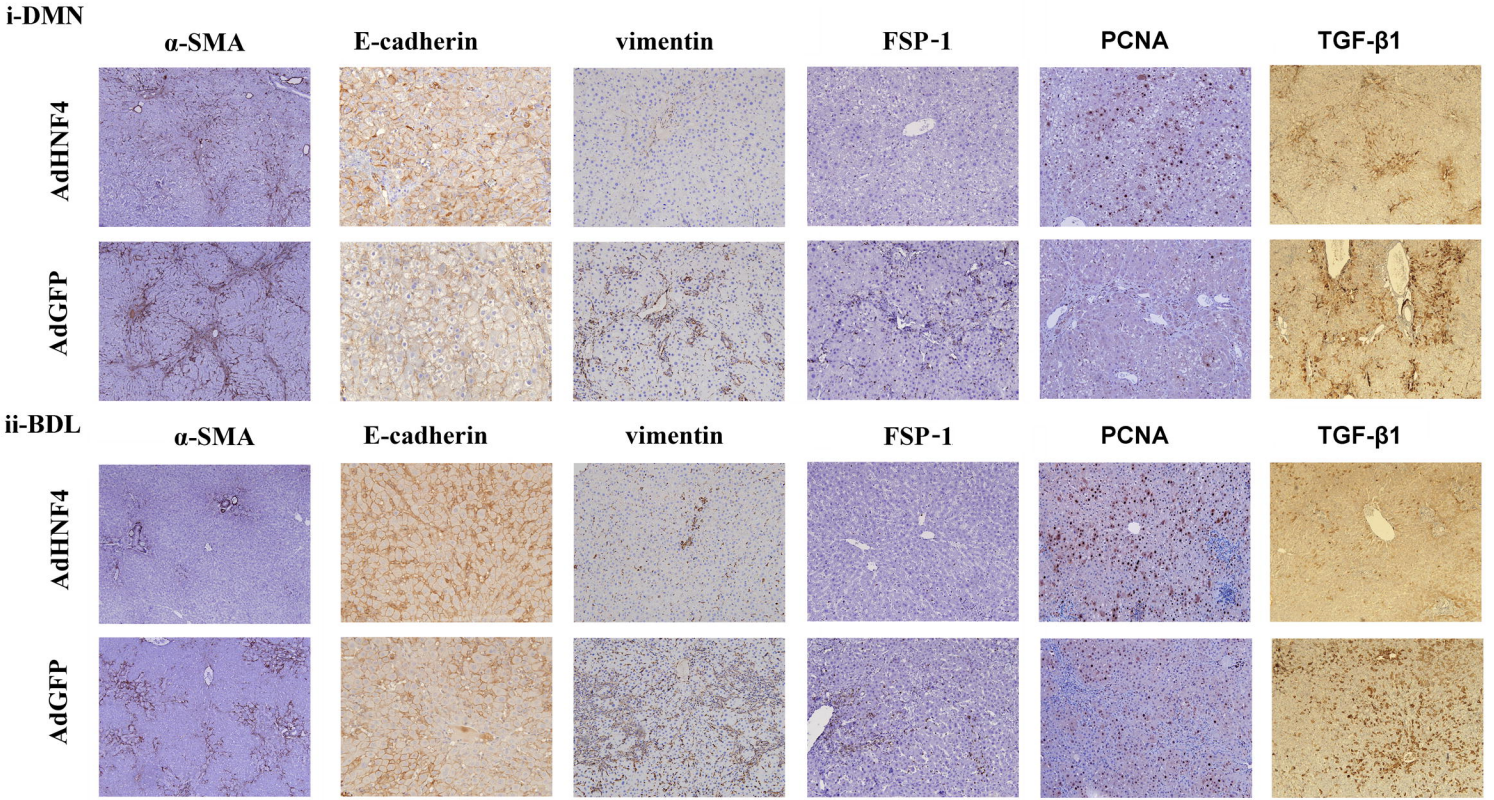
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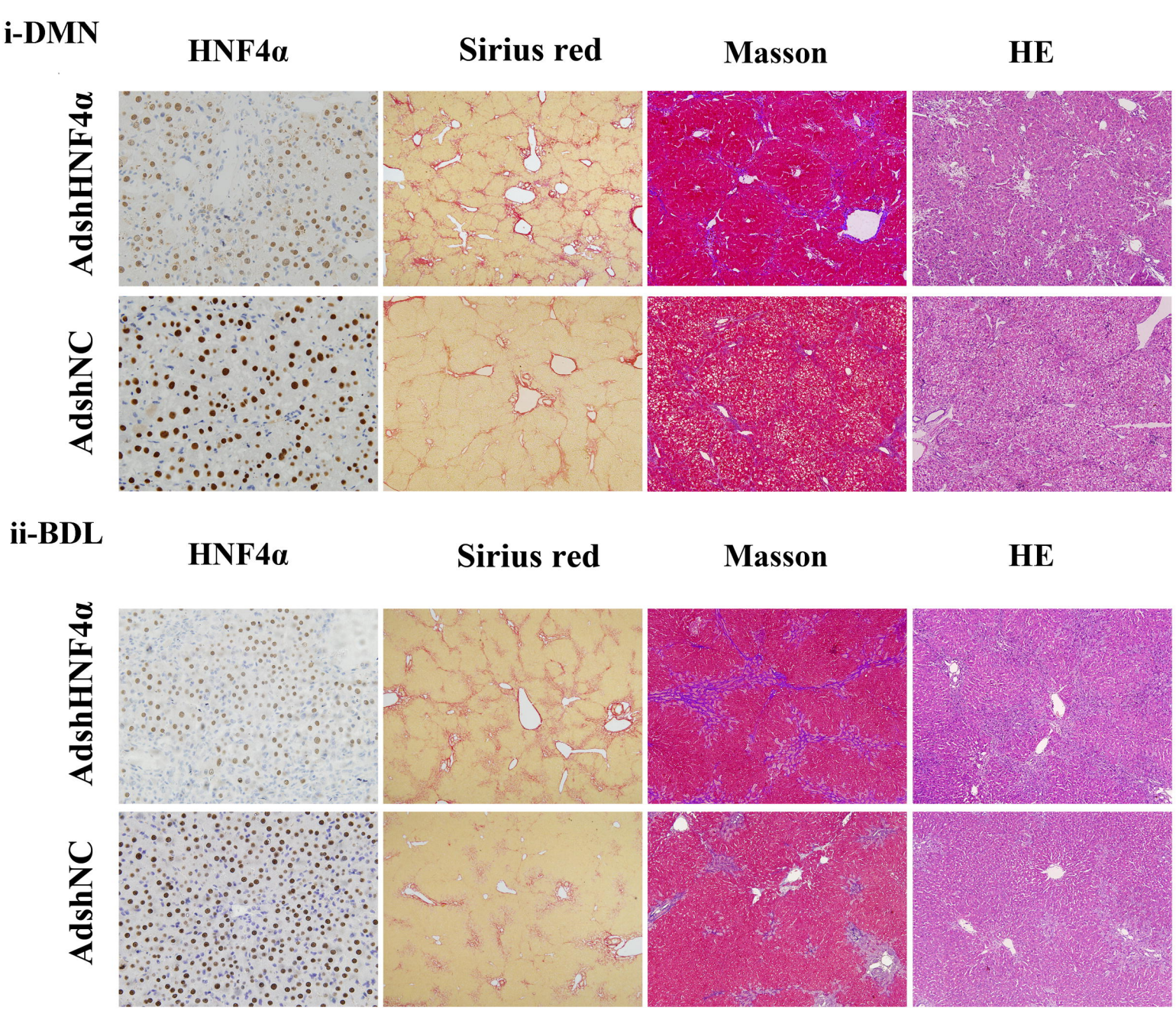
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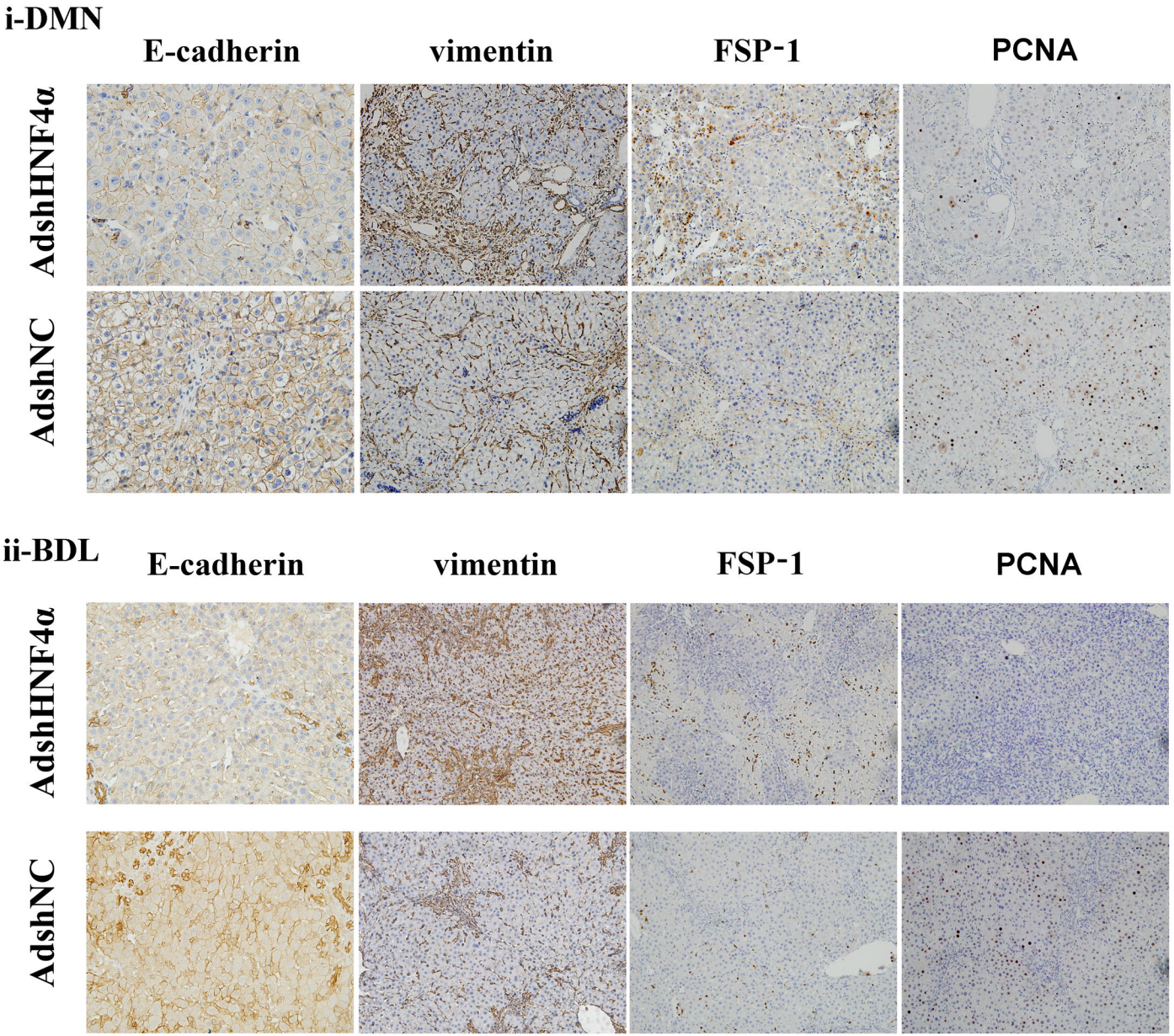
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E-cadherin
vimentin
FSP-1
PCNA
AdshHNF4α
AdshNC

Supplementary Materials

Immunohistochemistry. Immunohistochemical examinations were carried out to detect the expression of hepatocyte nuclear factor 4α (HNF4α), epithelial-mesenchymal transition (EMT) related proteins and fibrotic markers. The primary antibodies used for staining were as follows: HNF4α (1:50; R & D Systems, Minneapolis, MN); fibroblast-specific protein-1 (FSP-1, 1:100; Vision Lab, Belgium); E-cadherin (1:100; Becton Dickinson, Franklin Lakes, NJ); proliferating cell nuclear antigen (PCNA, 1:100; Santa Crutz Biotechnology, Inc., Santa Crutz, CA), α-smooth muscle actin (α-SMA, 1:50; Sigma Chemicals, St. Louis, MO), transforming growth factor-β1 (TGF-β1, 1:100; Santa Crutz Biotechnology, Inc., Santa Crutz, CA) and vimentin (1:100, BioGenex, San Ramon, CA). The paraffin sections of left median hepatic lobes were incubated with 3% H_2O_2 in methanol at 37℃ for 10 minutes to quench endogenous peoxidase activity. After blocked at room temperature for 1 hour, the sections of tissue were incubated with primary antibodies overnight at 4℃, followed by incubation with HRP-conjugated secondary antibody (DACO, Kyoto, Japan) at 37℃ for 20 minutes. The signals were detected using Diaminobenzidine Substrate Kit (Vector Laboratories, Inc. Burlingame, CA). For the semiquantitative analysis of collagen expression, the red-stained areas in the VG or Sirius red stained sections were measured on an image analyzer by a technician blinded to the samples.

Quantitative real time PCR analysis. Total RNA was extracted from the cells or tissues with Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized with an oligo (dT) primer and M-MLV reverse transcriptase according to the manufacturer's instructions. The original amount of the specific transcripts was detected via real time PCR with a SYBR Green PCR Kit (Applied Biosystems, Foster City, CA). The expression of specific transcripts was normalized

against that of the housekeeping gene β-actin.

Immunofluorescence staining. The cells were incubated overnight at 4°C with one or with different combinations of the following primary antibodies: HNF4α (1:200; Santa Crutz Biotechnology, Inc., Santa Crutz, CA), α-SMA (1:100), vimentin (1:200); E-cadherin (1:200), and snail (1:400; Abcam Inc, Cambridge, MA), followed by 1 hour incubation of cy3-coupled or 633-coupled specific secondary antibody (1:500; Invitrogen, Carlsbad, CA). The cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and the staining was visualized under a laser scanning confocal fluorescent microscope (Carl Zeiss, Inc., Germany).

Cell proliferation assay. To test the inhibitory effect of HNF4α on hepatic stellate cells (HSCs) -T6 proliferation, cells were plated in triplicate wells into a 96-well plate at 2×10^3/well and cultured for 24 hours. The cells were then infected with adenovirus. The number of metabolically active mitochondria and viable cells was determined colorimetrically at 450 nm using the Cell Counting Kit-8 (Dojindo, Tokyo, Japan).

Supplementary figure legends

Supplementary figure 1. Hepatocyte nuclear factor 4α (HNF4α) suppresses epithelial-mesenchymal transition (EMT) of primary hepatocytes induced by transforming growth factor-β1 (TGF-β1). (A) Double-immunostained for HNF4α (yellow) and E-cadherin (red) with 4', 6-diamidino-2-phenylindole (DAPI) counterstaining for DNA (blue). (B) Double-immunostained for HNF4α (yellow) and snail (red) with DAPI counterstaining for DNA (blue). Primary hepatocytes were double-immunostained after treated with TGF-β1 and adenoviruses for 72 hours. The differential interference contrast (DIC) images showed that AdHNF4α-treated hepatocytes preserved a more cuboidal/hexagonal shape than controls. Original magnification, ×400.

Supplementary figure 2. EMT changes during the activation of primary hepatic stellate cells (HSCs). (A) Real time PCR was performed to analyze changes of gene expression of E-cadherin, fibrotic and mesenchymal markers in culture-activated HSCs on cultured day 2, 5, 8 and 12. The mRNA expression levels were normalized against β-actin. Gene expression folds in activated HSCs were normalized by that of HSCs cultured on day 2. (B) Cells were immunostained for E-cadherin, α-smooth muscle actin (α-SMA) and snail (red) with DAPI counterstaining for DNA (blue) during culture-activation of HSCs. The insets showed the higher resolution images. Original magnification, ×400. Each value represents the mean ± SD for triplicate samples. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, Student's *t* test)

Supplementary figure 3. HNF4α inhibits EMT during the activation process of primary HSCs. TGF-β1-activated HSCs (A) or culture-activated HSCs (B) were treated by AdHNF4α or AdGFP. Immunostaining was performed to examine the expression of E-cadherin, HNF4α and α-SMA (red)

counterstained for DNA (blue) after 72 hours' infection. Significantly increased expression of E-cadherin and decreased expression of α-SMA protein were detected after AdHNF4α infection at different indicated time points. The insets showed the higher resolution images. Original magnification, ×400.

Supplementary figure 4. HNF4α inhibits EMT process and up-regulates liver-specific genes expression at mRNA level. Real time PCR was employed to determine the mRNA expression of fibrotic and EMT related genes as well as liver-specific genes in the livers of experimental hepatic fibrosis (dimethylnitrosamine model) treated by AdHNF4α or AdGFP injection. The mRNA expression levels were normalized against β-actin. Genes expression folds in AdHNF4α group were normalized by that of AdGFP group. Each value represents the mean ± SD for triplicate samples. (* $P<0.05$, ** $P<0.01$, ***$P<0.001$, Student's *t* test)

Supplementary table 1. The sequences of siRNA against HNF4α (413) and a scrambled siRNA (NC)

siRNA	***sense (5′- 3′)***	***Antisense (5′- 3′)***
413 siRNA	GCAGCCTACCCTCCATTAA	TTAATGGAGGGTAGGCTGC
NC siRNA	TTCTCCGAACGTGTCACG	ACGTGACACGTTCGGAGAA

Supplementary table 2. PCR primers used in this study

Rattus norvegicus gene	***Forward primer (5'- 3')***	***Reverse primer (5'- 3')***	***Product size (bp)***
β-actin	ACCGTGAAAAGATGACCCAGAT	AACCCTCATAGATGGGCACAGT	162
HNF4α	AAATGTGCAGGTGTTGACCA	CACGCTCCTCCTGAAGAATC	178
GS	AAGAGGGCATAGCCCAGACT	TTGGAAGCTTCGTTGGTCTT	199
CYP1α2	CAGGTTCCCAAAGGTCTGAA	TTCACTAGGGCCTGCTTGAT	200
ALB	TGCAGGCTTGCTGTGATAAG	AGTAATCGGGGTGCCTTCTT	201
E-cadherin	GGGTTGTCTCAGCCAATGTT	CACCAACACACCCAGCATAG	185
vimentin	CTTCGCCAACTACATCGACAAG	GAGCATCTCCTCCTGCAATTTT	244
snail	GTTCACCTTCCAGCAGCCCTAC	TCCTCATCGGACAGAGAAGTCA	201
desmoplakin	ACGGACTGAGGAAGAAGGTGAC	GAAGCATGACTGTGGATTCTGG	240
occludin	GATCATAGTCAGCGCAATCCTG	CCATCTTTCTTCGGGTTTTCAC	290
plakoglobin	AACTCTGTGCGCCTCAACTATG	ATCTCCTCCATCCTCACACCAT	257
slug	AAGAAGCCCAACTACAGCGAAC	CCCCAAAGATGAGGAGTATCCA	210
twist1	CGCTGAACGAGGCATTTG	AGACGGAGAAGGCGTAGCTG	201
smad7	CCTTACTCCAGATACCCGATGG	CTTGTTGTCCGAATTGAGCTGT	291
α-SMA	CCGAGATCTCACCGACTACC	TCCAGAGCGACATAGCACAG	120
TIMP-1	TCCCCAGAAATCATCGAGAC	TCAGATTATGCCAGGGAACC	250
Collagen type I	AGCTGCATACACAATGGCCTAA	CCTATGACTTCTGCGTCTGGTG	209
Collagen type III	CCTGAACTCAAGAGCGGAGAAT	CAGGATTGCCATAGCTGAACTG	229

Supplementary table 3. Effect on body and liver weight by up-regulation of HNF4α in normal rats

	Group	Body weight (g)	Liver weight (g)	Liver index
d3	PBS	196.1 ± 11.1	8.5 ± 0.4	0.0439 ± 0.0013
	AdGFP	194.6 ± 16.3	8.8 ± 1.3	0.0447 ± 0.0049
	AdHNF4α	205.8 ± 8.4	9.6 ± 1.3	0.0454 ± 0.0044
d31	PBS	351.7 ± 29.3	13.5 ± 2.2	0.0382 ± 0.0030
	AdGFP	339.3 ± 8.0	13.2 ± 1.1	0.0389 ± 0.0023
	AdHNF4α	350.7 ± 27.5	14.0 ± 1.7	0.0399 ± 0.0024

There is no statistical difference among AdHNF4α, AdGFP and PBS groups ($P>0.05$).

Supplementary table 4. Effect on serum biochemical parameters by up-regulation of HNF4α in normal rats

	Group	TB(umol/L)	ALB(g/L)	γ-GT(U/L)	ALT(U/L)	AST(U/L)
	PBS	1.7 ± 0.6	37.3 ± 2.5	1.0 ± 0.0	46.3 ± 3.8	127.3 ± 6.7
d3	AdGFP	1.7 ± 0.6	33.0 ± 1.0	0.0	55.3 ± 3.1 [a]	153.7 ± 9.0 [a]
	AdHNF4α	1.0 ± 0.0	34.3 ± 0.6	0.0	50.0 ± 6.1	118.0 ± 15.4 [b]
	PBS	1.3 ± 0.6	35.7 ± 2.1	1.0 ± 0.0	48.7 ± 7.1	148.3 ± 10.8
d10	AdGFP	1.3 ± 0.6	32.3 ± 2.5	1.0 ± 0.0	43.3 ± 9.3	154.7 ± 4.2
	AdHNF4α	2.0 ± 0.0	36.3 ± 6.0	1.0 ± 0.0	47.3 ± 4.0	138.7 ± 24.4
	PBS	1.0 ± 0.0	36.5 ± 3.4	0.0	42.8 ± 3.4	113.5 ± 29.7
d31	AdGFP	1.0 ± 0.0	39.0 ± 1.8	0.0	47.3 ± 6.2	123.5 ± 6.2
	AdHNF4α	1.3 ± 0.6	37.0 ± 4.4	0.0	45.3 ± 2.5	114.7 ± 4.2

[a] compared with PBS group, $P<0.05$; [b] compared with AdGFP group, $P<0.05$

Supplementary table 5. Effect on body and liver weight by up-regulation of HNF4α in BDL rats

Group	Body weight (g)	Liver weight (g)	Liver index
Sham	320.2±14.8	14.8±1.9	0.0461±0.0043
PBS	293.1±29.4	28.5±4.6	0.0969±0.0075
AdGFP	292.7±23.9	27.9±4.7	0.0947±0.0090
AdHNF4α	285.7±11.7	25.3±1.4	0.0885±0.0026

There is no statistical difference among AdHNF4α, AdGFP and PBS groups (P>0.05).

Supplementary table 6. Effect on improvement of serum levels by up-regulation of HNF4α in both fibrosis models induced by DMN and BDL

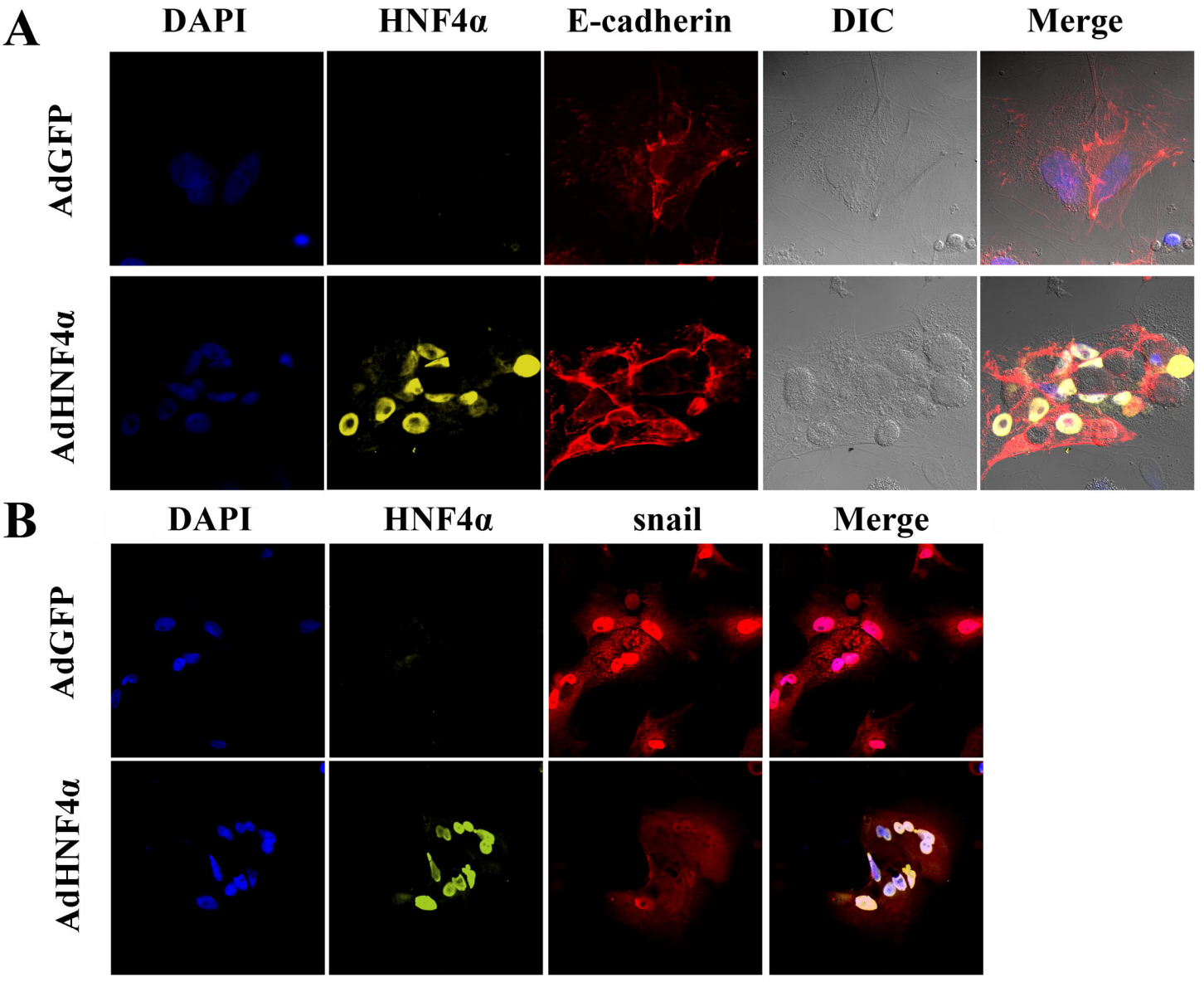
Group	PT（s）	ALT（U/L）	AST (U/L)
DMN Model			
Normal	18.4±1.2	40.8±4.8	105.3±10.5
PBS	29.4±1.6	163.8±19.4	228.5±44.2
AdGFP	27.4±1.9	165.2±27.1	235.2±70.8
AdHNF4α	23.8±1.4 [a, b]	113.2±21.6 [a, b]	187.6±30.6 [a, b]
BDL Model			
Sham	18.9±1.0	58.0±11.2	145.4±30.8
PBS	20.2±1.4	109.2±19.6	696.4±110.0
AdGFP	21.0±0.8	127.8±13.9	726.8±129.7
AdHNF4α	20.0±0.9	86.4±13.9 [a, b]	523.6±90.0 [a, b]

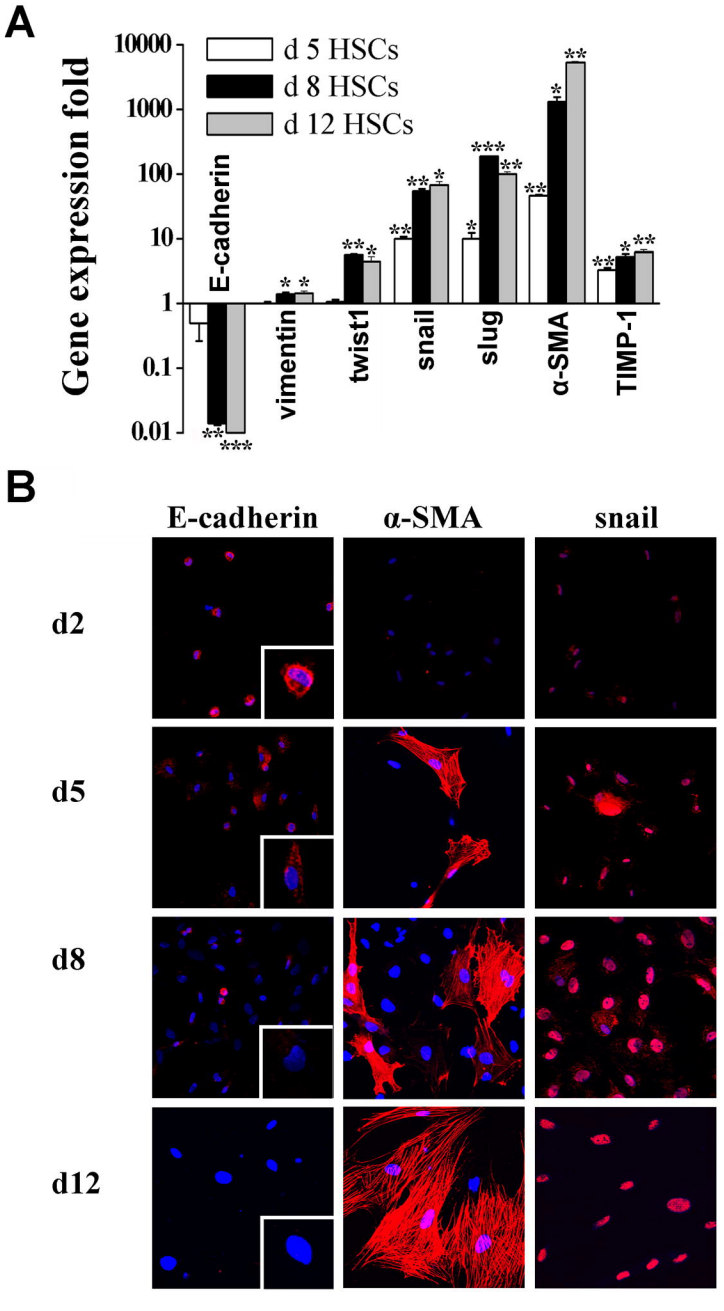
[a] compared with PBS group, $P<0.05$; [b] compared with AdGFP group, $P<0.05$

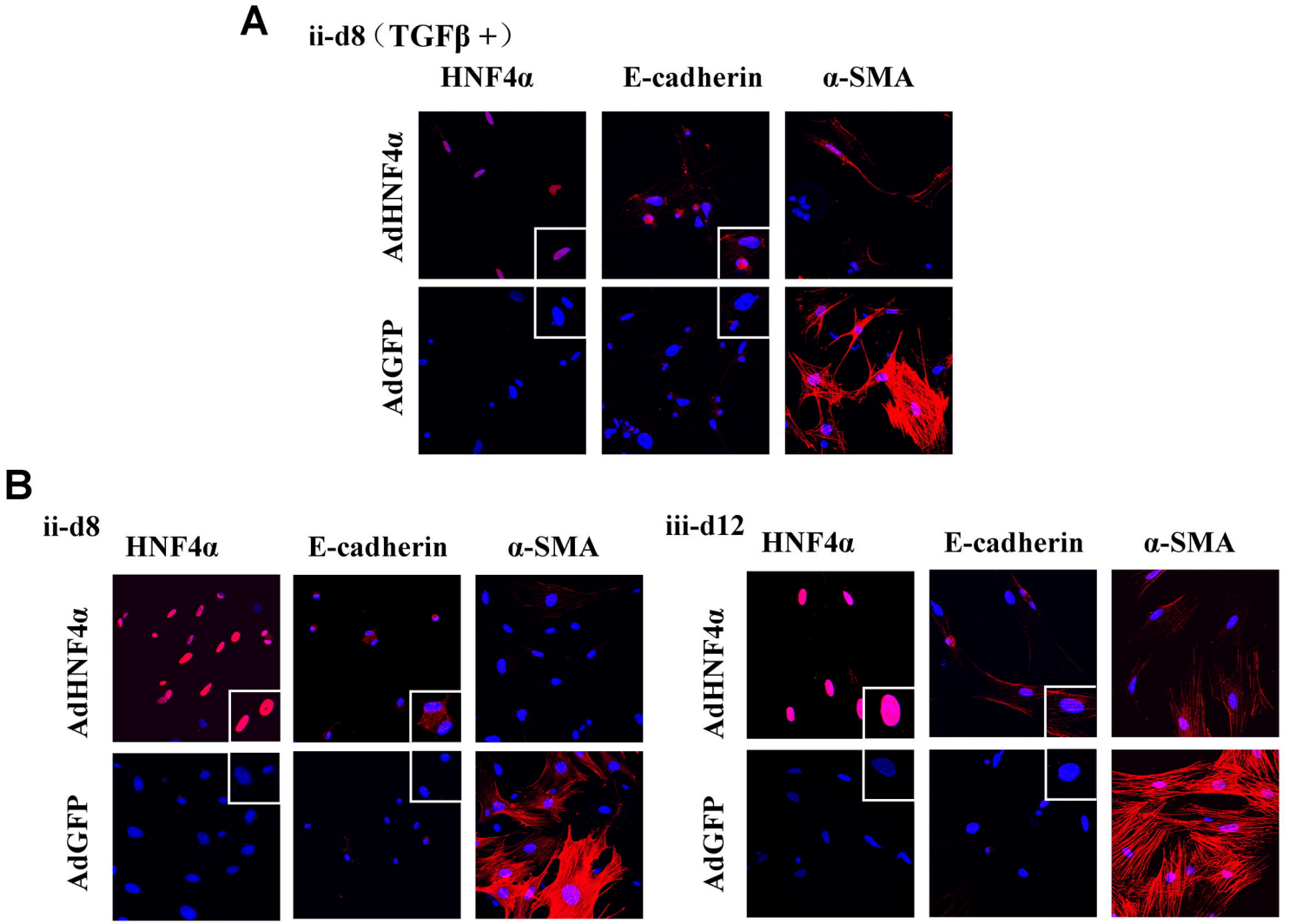
Supplementary table 7. Effect on body and liver weight by down-regulation of HNF4α in BDL rats

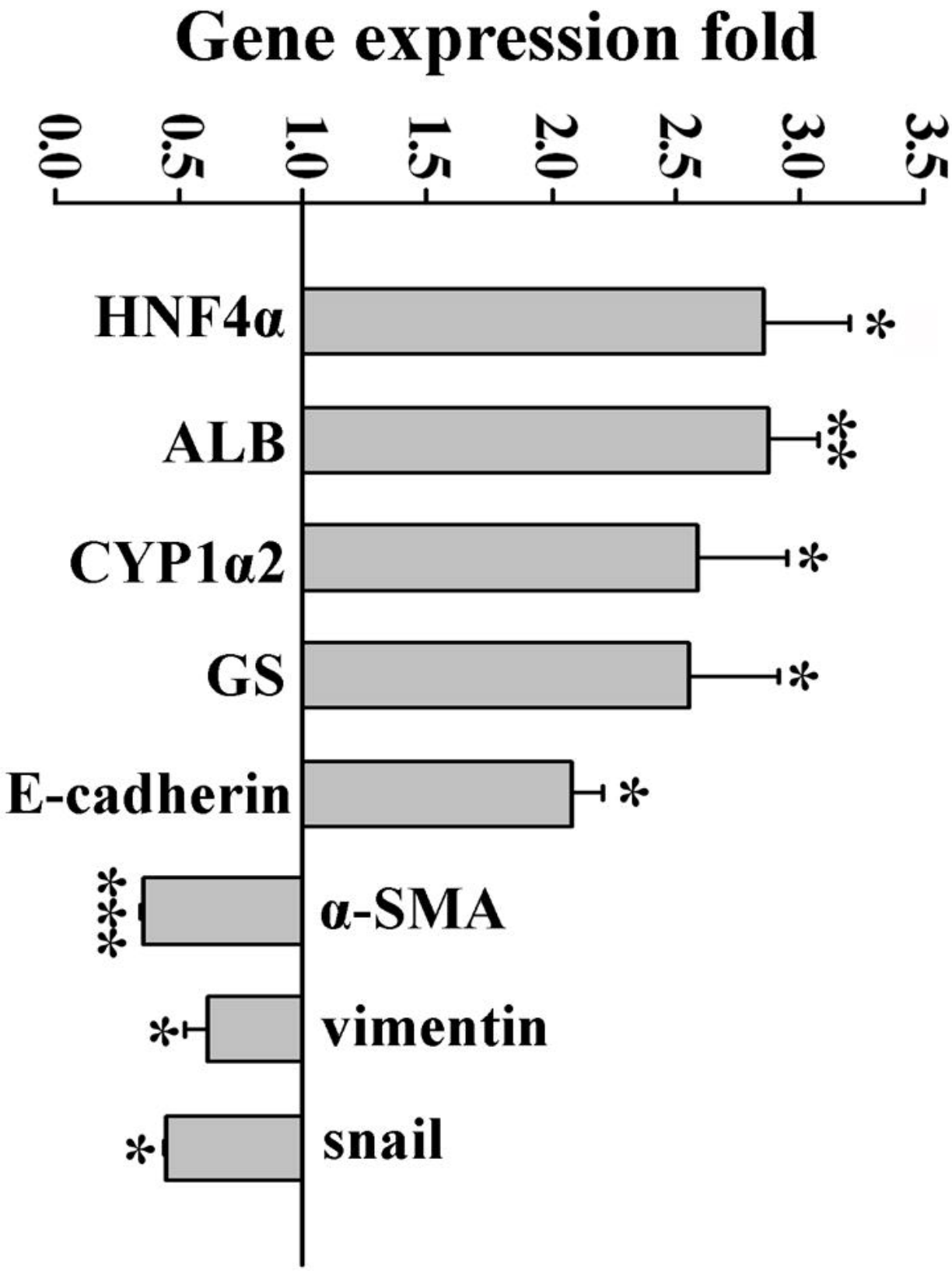
Group	Body weight (g)	Liver weight (g)	Liver index
Sham	263.2 ± 23.7	9.5 ± 1.9	0.0359 ± 0.0036
PBS	230.2 ± 16.4	15.9 ± 3.1	0.0689 ± 0.0122
AdshNC	236 ± 11.8	15.5 ± 2.9	0.0657 ± 0.0119
AdshHNF4α	211.9 ± 33.7	15.0 ± 2.9	0.0708±0.0067

There is no statistical difference among AdHNF4α, AdGFP and PBS groups (P>0.05).


A
DAPI
HNF4α
E-cadherin
DIC
Merge
AdGFP
AdHNF4α
B
DAPI
HNF4α
snail
Merge
AdGFP
AdHNF4α


A
Gene expression fold
10000
1000
100
10
1
0.1
0.01
d 5 HSCs
d 8 HSCs
d 12 HSCs
E-cadherin
vimentin
twist1
snail
slug
α-SMA
TIMP-1
B
E-cadherin
α-SMA
snail
d2
d5
d8
d12


A
ii-d8（TGFβ +）
HNF4α
E-cadherin
α-SMA
AdHNF4α
AdGFP
B
ii-d8
HNF4α
E-cadherin
α-SMA
AdHNF4α
AdGFP
iii-d12
HNF4α
E-cadherin
α-SMA
AdHNF4α
AdGFP


Gene expression fold
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
HNF4α
ALB
CYP1α2
GS
E-cadherin
α-SMA
vimentin
snail
*
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Hepatocyte nuclear factor 4α attenuates hepatic fibrosis in rats

Hai-Yan Yue, Chuan Yin, Jun-Liang Hou, Xin Zeng, Yue-Xiang Chen, Wei Zhong, Ping-Fang Hu, Xing Deng, Jun-Ping Zhang, Bei-Fang Ning, Jian Shi, Xin Zhang, Yong Lin and Wei-Fen Xie

Gut published online August 10, 2009

Notes

approved clinical trial and registry. However, it is a fact and needs to be pointed out, that only one third of our identified IAR (80 of 205) participated in the recommended screening programme. A pilot study on 32 of these IAR using standard questionnaires and interviews (Beck Depression Inventory (BDI) and Brief Symptom Inventory (BSI)) around counselling (days −7, 0, +30) conducted by a psychiatrist revealed, that these IAR were critically biased by cognitive coping strategies (unpublished data). Pancreatic cancer (PC) screening is clearly different from other cancer screening programmes, given the disastrous prognosis of PC, the unknown true penetrance in the different settings of hereditary PC, the lack of a major gene defect, the lack of reliable imaging or biomarkers, the lack of evidence to improve prognosis or to save lives by any screening, and the high risk of morbidity and mortality of potential preventive surgery. Some authors even advocate that at present 'doing nothing' provides the greatest remaining quality of life-adjusted years and the lowest costs.[6]

We fully agree that we need to gain much more knowledge about hereditary PC to draw a definite conclusion about the true value of PC screening in IAR. However, based on our data, we strongly believe, in accordance with the recommendations of the Fourth International Symposium of Inherited Diseases of the Pancreas,[5] that all screening procedures should be performed as part of peer-reviewed protocols combined with a scientific appraisal of the screening methods and human subject protection. At present there is no data, that would justify a general PC screening even of high risk individuals outside of such protocols as suggested by Harinck *et al*. In contrast, it has to be feared that uncritical use and interpretation of screening results obtained with the presently available tools on a healthcare basis may cause unnecessary physical harm and psychological distress. On the other hand over-estimation of the power of our present screening tools may lead to a deceptive, unjustified and potentially dangerous level of safety, if done uncritically and uncontrolled. The message of our paper thus is not 'to do nothing', but to carefully evaluate screening methods for IAR from familial pancreatic cancer (FPC) families in the setting of board approved clinical trials, to continuously improve our knowledge and strategies.

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Competing interests None.

Provenance and peer review Not commissioned; not externally peer reviewed.

Gut 2010;**59**:1006–1007. doi:10.1136/gut.2010.214163

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CORRECTIONS

doi:10.1136/gut.2009.208975ecorr1

Safety and comfort for colonoscopy in the over seventies-time to achieve a balance? *Gut* 2010;**59**(Suppl 1):A23. The correct author list should have been Hancock J, Ali F, Sarkar A, Parr J.

doi:10.1136/gut.2009.190439corr1

Ibeakanma C, Vanner S. *Gut* 2010;**59**:612–21. TNFα is a key mediator of the pronociceptive effects of mucosal supernatant from human ulcerative colitis on colonic DRG neurons. The figures were ordered incorrectly in the print version of the paper. The latest online pdf and full text have been corrected. The journal apologises for the error.

doi:10.1136/gut.2009.180182corr1

Long-term outcome of endoscopic dilatation in patients with Crohn's disease is not affected by disease activity or medical therapy. *Gut* 2010;**59**:320–4. The correct author list should have been Thienpont C, D'Hoore A, Vermeire S, Demedts I, Bisschops R, Coremans G, Rutgeerts P, Van Assche G. The latest online pdf and full text have been corrected. The journal apologises for the error.

doi:10.1136/gut.2008.155226corr1

De-Xin Zhang, Peng-Tao Zhao, Lin Xia, *et al*. *Gut* 2010;**59**:292–9. Potent inhibition of human gastric cancer by HER2-directed induction of apoptosis with anti-HER2 antibody and caspase-3 fusion protein. Panels E–H were missing in figure 5. The latest online pdf and full text have been corrected. The journal apologises for this error.

doi:10.1136/gut.2008.174904corr1

Yue H-Y, Yin C, Hou J-L, *et al*. *Gut* 2010;**59**;236–46. Hepatocyte nuclear factor 4α attenuates hepatic fibrosis in rats. Professor Lin Yong and Dr Weifen Xie should have been co-corresponding authors on this paper. The latest online pdf and full text have been corrected.

doi:10.1136/gut.2008.155853corr1

Park DH, Kim M-H, Chari S T. *Gut* 2009;**58**:1680–9. Recent advances in autoimmune pancreatitis. M-H Kim and S T Chari should have been co-corresponding authors on this paper. The latest online pdf and full text have been corrected.

doi:10.1136/gut.2009.179606corr1

Mangia A, Andriulli A. Tailoring the length of antiviral treatment for Hepatitis C. *Gut* 2010;**59**:1–5. There were several errors in table 1 which have now been corrected online.

doi:10.1136/gut.2008.174607corr1

Cahill RA, Lindsey I, Cunningham C. *Gut* 2009;**58**:1168–9. NOTES for colorectal neoplasia—surgery through the looking glass. The surname of the third author should be spelt Cunningham, not Cunnigham. The latest online pdf and full text have been corrected.

doi:10.1136/gut.2009.183608corr1

Lysosomal accumulation of gliadin p31e43 peptide induces oxidative stress and tissue transglutaminase-mediated PPARγ down-regulation in intestinal epithelial cells and coeliac mucosa. *Gut* 2010;**59**:311–9. The correct author list should have been Luciani A, Rachela Villella V, Vasaturo A, Giardino I, Pettoello-Mantovani M, Guido S, Cexus O N, Peake N, Londei M, Quaratino S, Maiuri L as it appeared in the online first version. We have replaced the latest online pdf and full text. The journal apologises for the error.