A double-negative feedback loop between Wnt–β-catenin signaling and HNF4α regulates epithelial–mesenchymal transition in hepatocellular carcinoma

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Accepted 7 September 2013
Journal of Cell Science 126, 5692–5703

doi: 10.1242/jcs.135053

Summary

Wnt–β-catenin signaling participates in the epithelial–mesenchymal transition (EMT) in a variety of cancers; however, its involvement in hepatocellular carcinoma (HCC) and downstream molecular events is largely undefined. HNF4α is the most prominent and specific factor maintaining the differentiation of hepatic lineage cells and a potential EMT regulator in HCC cells. However, the molecular mechanisms by which HNF4α maintains the differentiated liver epithelium and inhibits EMT have not been completely defined. In this study, we systematically explored the relationship between Wnt–β-catenin signaling and HNF4α in the EMT process of HCC cells. Our results indicated that HNF4α expression was negatively regulated during Wnt–β-catenin signaling-induced EMT through Snail and Slug in HCC cells. In contrast, HNF4α was found to directly associate with TCF4 to compete with β-catenin but facilitate transcription co-repressor activities, thus inhibiting expression of EMT-related Wnt–β-catenin targets. Moreover, HNF4α may control the switch between the transcriptional and adhesion functions of β-catenin. Overexpression of HNF4α was found to completely compromise the Wnt–β-catenin-signaling-induced EMT phenotype. Finally, we determined the regulation pattern between Wnt–β-catenin signaling and HNF4α in rat tumor models. Our studies have identified a double-negative feedback mechanism controlling Wnt–β-catenin signaling and HNF4α expression *in vitro* and *in vivo*, which sheds new light on the regulation of EMT in HCC. The modulation of these molecular processes may be a method of inhibiting HCC invasion by blocking Wnt–β-catenin signaling or restoring HNF4α expression to prevent EMT.

Key words: Hepatocellular carcinoma, Epithelial-mesenchymal transition, Wnt–β-catenin signaling, HNF4α

Introduction

Hepatocellular carcinoma (HCC) accounts for more than five percent of all cancer cases and is the third leading cause of cancer mortality worldwide (El-Serag and Rudolph, 2007; Bruix et al., 2004). The extremely poor prognosis of patients with HCC is primarily because of the high frequency of early tumor metastasis, which leads to the loss of opportunity for surgical resection (Portolani et al., 2006). Metastasis is a complex process that ultimately causes cancer-related death (Liotta, 1985). Transformation to a fibroblastic phenotype is the first step for the cancer cells in successful metastasis (Stracke and Liotta, 1992). Accumulating evidence suggests that the induction of epithelial to mesenchymal transition (EMT) plays a crucial role in cancer cell transformation and progression (Nieto, 2011; Thiery, 2002). In contrast, the process of mesenchymal to epithelial transition (MET) may promote the growth of metastatic cancer cells in secondary sites (Chaffer et al., 2006). The central hallmarks of EMT include the downregulation of E-cadherin, which is essential for cell–cell adhesion, and upregulation of vimentin, which represents the mesenchymal phenotype (Huber et al., 2005). Multiple signaling pathways, including TGF-β, Wnt–β-catenin, Notch, EGF, HGF, FGF and HIF, among others, have been documented to trigger EMT in both embryonic development and normal and transformed cells (Thiery et al., 2009). These signaling pathways activate several EMT-related transcription factors, such as Snail (Snai1), Slug (Snai2), Twist, EF1/ZEB1, SIP1/ZEB2 and E47, which directly or indirectly inhibit E-cadherin production (Yang and Weinberg, 2008).

The Wnt–β-catenin signaling pathway is crucial in both normal development and tumorigenesis (Moon and Miller, 1997; Polakis, 2000; Wodarz and Nusse, 1998). β-catenin plays a pivotal role as a transcriptional co-activator in this process. In the absence of Wnt stimulation, cytoplasmic β-catenin becomes phosphorylated by glycogen synthase kinase-3β (GSK-3β) in a complex containing Axin and the tumor suppressor adenomatous polyposis coli (APC), and is targeted for ubiquitin-mediated proteasomal degradation (Lustig and Behrens, 2003). Stimulation by Wnt leads to the inhibition of the phosphorylation and degradation of β-catenin, which then enters the nucleus and binds to a member of the lymphoid-enhancing factor 1/T-cell factor (LEF1/TCF) family of transcription factors to regulate the expression of target genes involved in diverse cellular

processes (Liu et al., 2002; MacDonald et al., 2007). In the absence of Wnt–β-catenin signaling, TCF acts as a repressor of Wnt–β-catenin target genes by forming a complex with the Groucho/TLE transcriptional co-repressor. The repressing effect of Groucho/TLE is mediated by its interaction with histone deacetylase (HDAC), another transcriptional co-repressor (Sekiya and Zaret, 2007). β-catenin can convert TCF into a transcriptional activator of the same genes that are repressed by TCF alone (Daniels and Weis, 2005). Among the EMT-related transcription factors, the *Slug* gene has been demonstrated to be a direct Wnt–β-catenin target (Sakai et al., 2005), whereas Snail protein is upregulated by another Wnt–β-catenin target gene, *Axin2*, and in turn, Axin2 acts as a nuclear–cytoplasmic chaperone for GSK3β, the dominant kinase responsible for controlling Snail protein turnover and activity (Yook et al., 2006). Twist is also activated by Wnt–β-catenin signaling, although the regulation is indirect (Howe et al., 2003). In addition to being a nuclear transcription factor, β-catenin also functions in cell adhesion at the plasma membrane, where it connects cadherins to α-catenin and the cytoskeleton (Takeichi, 1995). The molecular mechanism of the switch between the transcriptional and adhesion functions of β-catenin remains largely unclear. Wnt–β-catenin signaling participates in EMT process in several cancer types (Chen et al., 2008; Chung et al., 2009; DiMeo et al., 2009; Guo et al., 2007; Jiang et al., 2007); however, this relationship and the underlying molecular mechanism in HCC has not been completely defined. Moreover, the regulation of Wnt–β-catenin signaling during the EMT process in HCC also remains largely unknown.

The nuclear receptor HNF4α is a key regulator of both hepatocyte differentiation during embryonic development and the maintenance of the differentiated phenotype of the mature liver (Hayhurst et al., 2001; Li et al., 2000; Parviz et al., 2003). As a transcription factor, the activities of HNF4α are usually mediated by its binding as a homodimer to specific promoter sequences (Sladek et al., 1990). Moreover, HNF4α probably acts as the furthest upstream mediator functioning as a master gene to control the transcription factor cascade that drives the differentiation of the hepatic lineage (Naiki et al., 2002). It has been shown that the majority of de-differentiated hepatoma cell lines and tissues fail to express HNF4α, but forced expression of this factor is sufficient to re-express a set of hepatocyte marker genes and restore the epithelial morphology of these cells (Späth and Weiss, 1998; Tanaka et al., 2006), which implies that it plays a role in the regulation of EMT. Moreover, forced expression of HNF4α completely induces the differentiation of hepatoma cells into hepatocytes in mice and blocks hepatocarcinogenesis in rats (Ning et al., 2010; Yin et al., 2008). However, the molecular mechanisms by which HNF4α maintains the differentiated liver epithelium and inhibits EMT have not been completely explored.

In this study, we provide evidence of a double-negative feedback loop between Wnt–β-catenin signaling and HNF4α in HCC cells and rat tumor tissues during EMT. Our study reveals a complex association between HNF4α and the central EMT signaling pathway, which highlights the role of Wnt–β-catenin signaling and HNF4α in regulation of HCC malignant phenotypes.

Results

HNF4α expression is negatively regulated during Wnt–β-catenin signaling-induced EMT in HCC cells

Wnt–β-catenin signaling has been demonstrated to participate in the EMT process in many cancer types; however, the involvement of Wnt–β-catenin signaling in HCC cells and the downstream molecular events has not been completely defined. To address this question, we examined whether the inhibition of Wnt–β-catenin signaling in an invasive HCC cell line is able to convert the mesenchymal phenotypes. We inhibited Wnt–β-catenin activity by forced expression of a FLAG-tagged dominant-negative TCF4 (dnTCF4), which lacks the β-catenin binding domain. We chose this approach instead of knocking down β-catenin reasoning that β-catenin is also required for cell–cell adhesion. Notably, Wnt–β-catenin inhibition induced a change in MHCC-97H cells from the mesenchymal phenotype into an epithelial phenotype as manifested by increased expression of the epithelial marker E-cadherin concomitant with a downregulation of the mesenchymal marker vimentin, determined by confocal immunofluorescence analysis (Fig. 1A). In addition, Wnt–β-catenin inhibition resulted in reduced cell migration and invasion, as assessed by a wound-healing assay (Fig. 1B) and Transwell invasion assay (Fig. 1C). These changes in EMT markers were also verified by quantitative real-time PCR (qPCR) and western blotting (Fig. 1D,E).

To determine the downstream molecular events, we detected the EMT-related Wnt–β-catenin target genes upon Wnt–β-catenin inhibition in MHCC-97H cells. As shown in Fig. 1D, the mRNA levels of *AXIN2* and *SLUG* were significantly reduced in dnTCF4-expressing cells, but *TWIST* mRNA expression was not detectable in MHCC-97H cells. The EMT-related Wnt–β-catenin target proteins were also examined, and similar results were observed, including reduced Snail and Slug protein levels (Fig. 1E). Twist protein was not detectable when using a Twist-positive MDA-MB231 breast cancer cell line as a control (supplementary material Fig. S1). These results suggest that Wnt–β-catenin signaling may be involved in the EMT process in HCC cells through activation of the downstream targets Snail and Slug. To further identify the hepatic cell-specific factor(s) regulated by Wnt–β-catenin signaling, we examined the expression of HNF4α. To our surprise, HNF4α expression showed an obvious induction at both the mRNA (Fig. 1D) and protein levels (Fig. 1E) in Wnt–β-catenin-inhibited cells. To confirm this result in the opposite direction, we further detected HNF4α expression in Wnt–β-catenin-activated cells and found a marked reduction of this protein in β-catenin-overexpressing Hep3B cells (Fig. 1F). From the results above, we concluded that HNF4α expression is negatively regulated during Wnt–β-catenin signaling-induced EMT in HCC cells.

Wnt–β-catenin signaling negatively regulates HNF4α through Snail and Slug

To define the underlying molecular mechanism by which Wnt–β-catenin signaling induces HNF4α suppression, we examined the human genomic sequence around the transcriptional start site (TSS) of *HNF4A* and found one consensus Snail/Slug/Twist binding site (E-box: CAGGTG) at −1387 bp. We deduced that *HNF4A* is probably a direct target of Snail/Slug/Twist. To confirm this hypothesis, we examined the expression of HNF4α in Hep3B cells overexpressing either Snail, Slug or Twist. As shown in Fig. 2A, Snail and Slug were observed to dramatically inhibit *HNF4A* mRNA expression, whereas Twist showed a slight but significant enhancement of *HNF4A* mRNA levels. Similar results were observed in the protein levels by western blotting, although the Twist-treated cells exhibited HNF4α expression equal to that of the control. The results suggest that Snail and Slug but not Twist may suppress HNF4α expression in HCC cells.

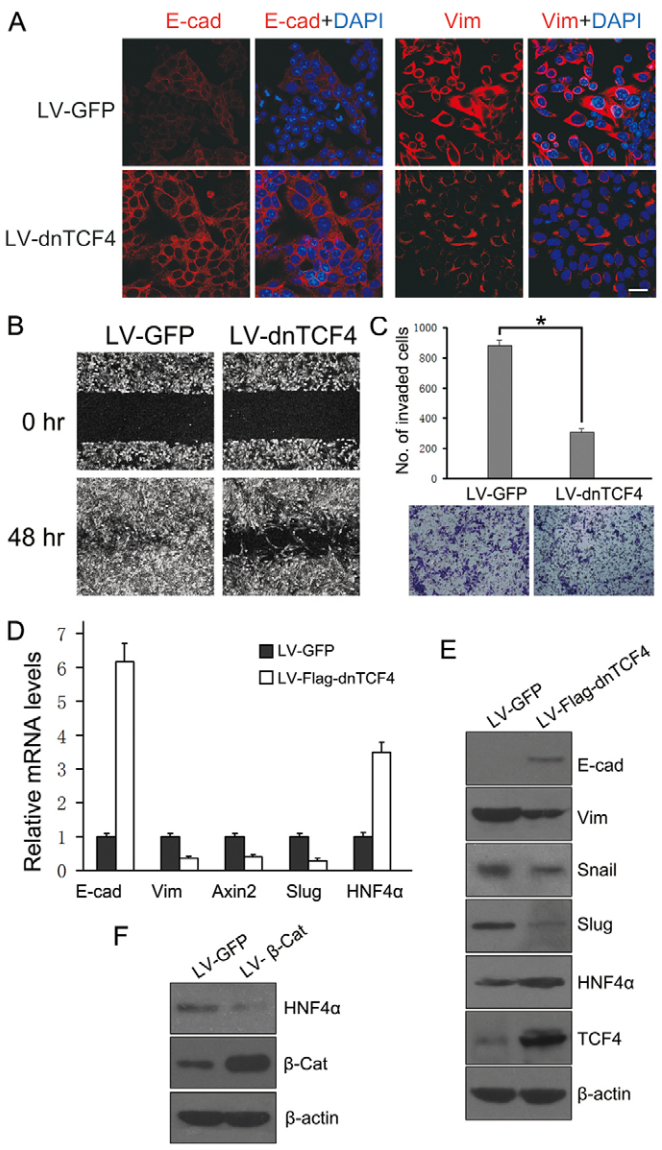

Fig. 1. HNF4α expression is negatively regulated during Wnt–β-catenin signaling-induced EMT in HCC cells. (A) MHCC-97H cells were infected with lentivirus expressing GFP (LV-GFP) or dnTCF4 (LV-dnTCF4). The expression of E-cadherin (E-cad) and vimentin (Vim) were determined by confocal immunofluorescence analysis. (B) The migration ability of the above cells was assessed using a wound-healing assay. The experiment was performed in triplicate. Representative images from one experiment are shown. (C) The invasive ability of the above cells is presented as the total number of cells that entered the bottom invasion chamber, counted across eight fields. Each sample was measured in triplicate, and each experiment was repeated three times. Representative images from one experiment are shown, and the bar graph shows the mean values for the three different experiments. (D,E) Relative expression levels of EMT marker and EMT-related Wnt–β-catenin target genes and proteins and HNF4α after Wnt–β-catenin inhibition in MHCC-97H cells, using qPCR (D) and western blotting (E). (F) Western blots of the relative expression of HNF4α protein in Hep3B cells stably expressing LV-GFP (control) and LV-β-catenin. In C and D data are the means ± s.d. of three independent experiments (*$P<0.001$). Scale bar: 50 μm.

Next, we performed a luciferase assay by cloning the fragment extending from 1467 bp upstream to 50 bp downstream of the *HNF4a* TSS into a PGL3-basic promoterless luciferase reporter cassette. The luciferase reporter activity was inhibited in a dose-dependent manner when stimulated with either Snail or Slug in 293T cells. In contrast, mutation of the E-box resulted in a significantly compromised inhibitory effect by Snail or Slug (Fig. 2B). We further cloned the 165 bp fragment flanking the E-box (−1467 bp to −1302 bp) into the TATA box-containing PGL4.26 reporter cassette. The results indicated that this fragment was also sufficient to produce an inhibitory effect upon Snail or Slug stimulation. Moreover, mutation of the E-box in this fragment resulted in a complete loss of inhibition by Snail or Slug (Fig. 2C). In a chromatin immunoprecipitation (ChIP) assay, both Snail and Slug were found to occupy the endogenous *HNF4A* promoter flanking the E-box (Fig. 2D). Together, our findings establish that Snail and Slug suppress HNF4α expression by direct binding to the *HNF4A* promoter, which provides compelling evidence that *HNF4A* is a direct negative target gene of Snail and Slug. Finally, we examined whether Wnt–β-catenin signaling-induced HNF4α repression occurs through Snail and Slug. The results indicated that β-catenin overexpression no longer induced HNF4α repression in Snail and Slug knockdown Hep3B cells (Fig. 2E), which underscores the importance of Snail and Slug in mediating Wnt–β-catenin signaling-induced HNF4α repression during the EMT process in HCC cells.

HNF4α inhibits Wnt–β-catenin signaling in HCC cells

Because Wnt–β-catenin signaling regulates HNF4α expression in HCC cells, we asked whether HNF4α also affects Wnt–β-catenin signaling activity. To test this idea, we performed TCF reporter assays in 293T cells. Overexpression of HNF4α repressed the transcriptional activation of the Wnt–β-catenin-dependent TCF reporter TOPFLASH by either LiCl or β-catenin in a dose-dependent manner (supplementary material Fig. S2A). Consistent with this result, when we depleted endogenous HNF4α protein using a pool of siRNAs against human *HNF4A* in LO2 cells, the TOPFLASH assay showed greatly increased Wnt–β-catenin activity (supplementary material Fig. S2B). As a control, we showed that co-transfection with a rat *Hnf4a* expression construct lacking the human siRNA target sequence could rescue the TOPFLASH activity (supplementary material Fig. S2B).

To map the position of HNF4α action along the Wnt–β-catenin signaling pathway, we performed several experiments for an epistasis analysis. First, we overexpressed HNF4α in Hep3B cells and checked the β-catenin expression. As shown in supplementary material Fig. S2C, β-catenin mRNA levels were not changed upon HNF4α overexpression. Moreover, β-catenin protein remained stable in the presence of high levels of HNF4α, indicating that HNF4α does not promote β-catenin degradation. The above data suggest that HNF4α does not act upstream of β-catenin in the cytoplasm because any effect on those components would promote β-catenin degradation. To further locate the point of HNF4α action, we examined whether the constitutive signaling activity of N-terminally mutated β-catenin-S33Y responds to HNF4α action. β-catenin-S33Y-induced TOPFLASH activity was also greatly inhibited by HNF4α (supplementary material Fig. S2D). Collectively, these results suggest that HNF4α negatively regulates β-catenin-mediated transactivation and acts at the very bottom of the Wnt–β-catenin signaling transduction cascade in parallel with or downstream of nuclear β-catenin.

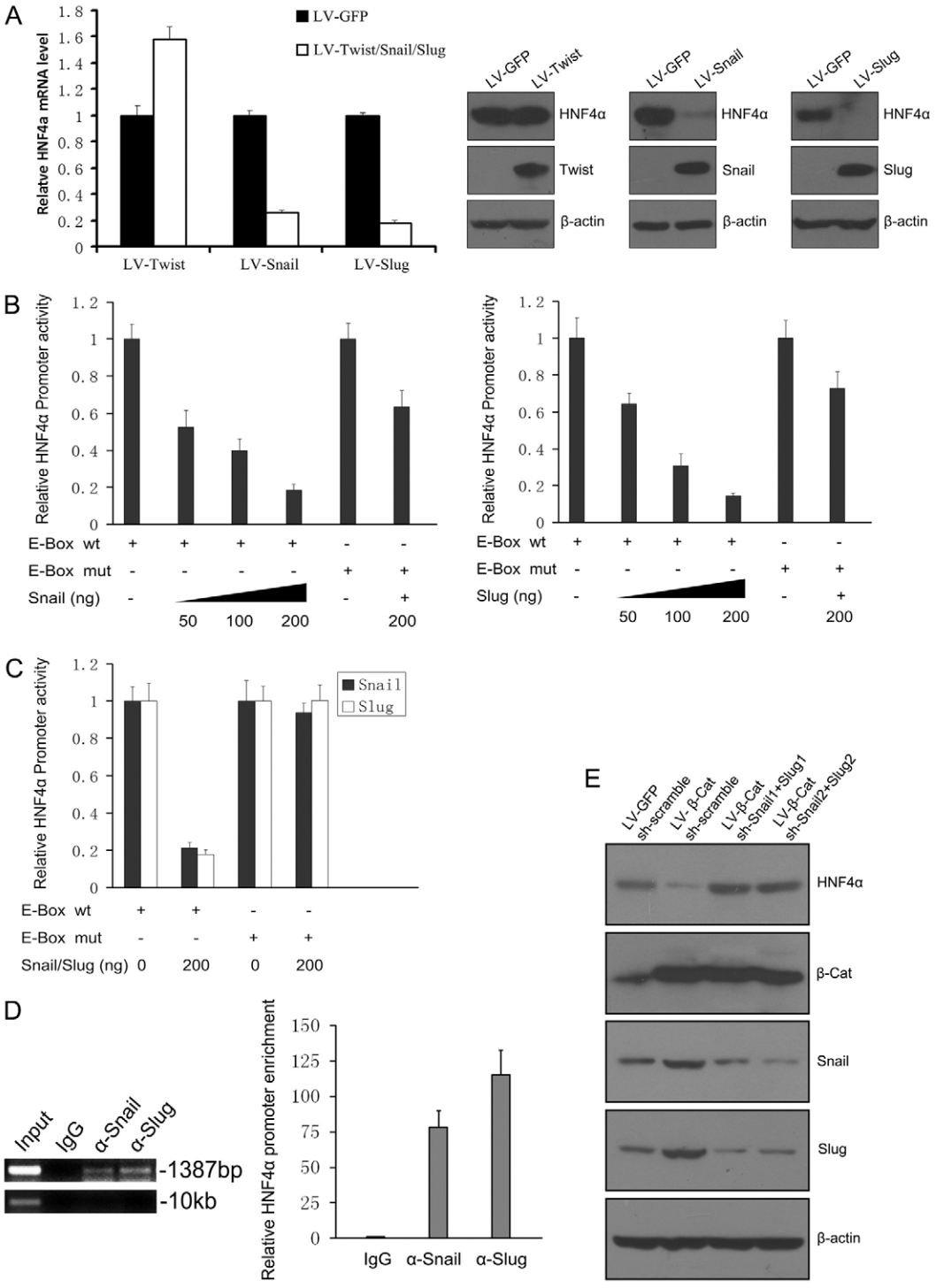

Fig. 2. Wnt–β-catenin signaling negatively regulates HNF4α through Snail and Slug. (A) Relative mRNA and protein expression levels of HNF4α in Hep3B cells stably expressing LV-GFP and LV-Twist, -Snail or -Slug, using qPCR (left) and western blotting (right). (B) The luciferase activity of the pGL3-HNF4α promoter (−1467 bp to +50 bp) vector containing the putative E-box binding site was inhibited in a dose-dependent manner upon Snail (left) or Slug (right) treatment in 293T cells. Mutation of the E-box resulted in a significantly compromised inhibitory effect. (C) The luciferase activity of the pGL4.26-HNF4α promoter (−1467 bp to −1302 bp) vector containing the putative E-box binding site was inhibited upon Snail or Slug treatment in 293T cells. Mutation of the E-box in this fragment resulted in a complete loss of inhibition by Snail or Slug. (D) Chromatin immunoprecipitation (ChIP) assay of Snail and Slug for the HNF4α promoter using semi-quantitative RT-PCR (left) and qPCR (right) analyses. Left, primers amplifying a region 10 kbp upstream of the HNF4α promoter were used as a negative control. Shown is a representative experiment of two independent experiments. Right, data are the means ± s.d. of three independent experiments. (E) Repression of HNF4α by Wnt–β-catenin signaling is Snail and Slug dependent. Hep3B cells were infected with LV-β-catenin and treated with Sh-Snail and Sh-Slug simultaneously. Western blotting was used to measure the levels of the indicated proteins. In A–C data are the means ± s.d. of three independent experiments.

HNF4α directly associates with TCF4 in HCC cells

Owing to the HNF4α action point, we reasoned that HNF4α might physically interact with β-catenin or TCF4. To examine this possibility, we co-transfected HA- or FLAG-tagged HNF4α and β-catenin or TCF4 to perform co-immunoprecipitation (Co-IP) experiments. As shown in Fig. 3A, TCF4 but not β-catenin reciprocally interacted with HNF4α. TCF proteins contain an N-terminal β-catenin-binding domain and central HMG DNA-binding domain. A Co-IP experiment revealed that a β-catenin-binding-domain deletion mutant (dnTCF4) failed to immunoprecipitate HNF4α, indicating that this domain is essential for the interaction (Fig. 3B). Similarly, we also mapped the HNF4α interaction domain to its DNA-binding-domain-containing N-terminal region (HNF4α-N; Fig. 3C). Next, we examined whether HNF4α directly associates with TCF4. An *in vitro* pull-down assay was performed, and it demonstrated direct binding between TCF4 and HNF4α-N (Fig. 3D). Finally, we investigated whether the endogenous proteins interact by performing immunoprecipitation experiments using nuclear extracts from non-transfected LO2 cells. As shown in Fig. 3E, immunoprecipitation of HNF4α pulled down TCF4 but not β-catenin. We conclude that HNF4α is normally present in a complex with TCF4 *in vivo*.

HNF4α competes with β-catenin for binding to TCF4 but facilitates transcriptional co-repressors to inhibit the expression of EMT-related Wnt–β-catenin target genes

The interaction capability of HNF4α implies that HNF4α might compete with β-catenin for binding to TCF4. To confirm this, we performed competitive Co-IP assay in 293T cells and found that the amount of β-catenin co-precipitation with TCF4 decreased as the amount of HNF4α associated with TCF4 increased (Fig. 4A). In contrast, knockdown of HNF4α expression in LO2 cells resulted in significantly increased co-precipitation of endogenous β-catenin with TCF4 (Fig. 4B).

To address whether HNF4α also affects the binding of β-catenin to the target chromatin at the Axin2 or Slug loci, we performed a ChIP assay using Hep3B cells. As shown in

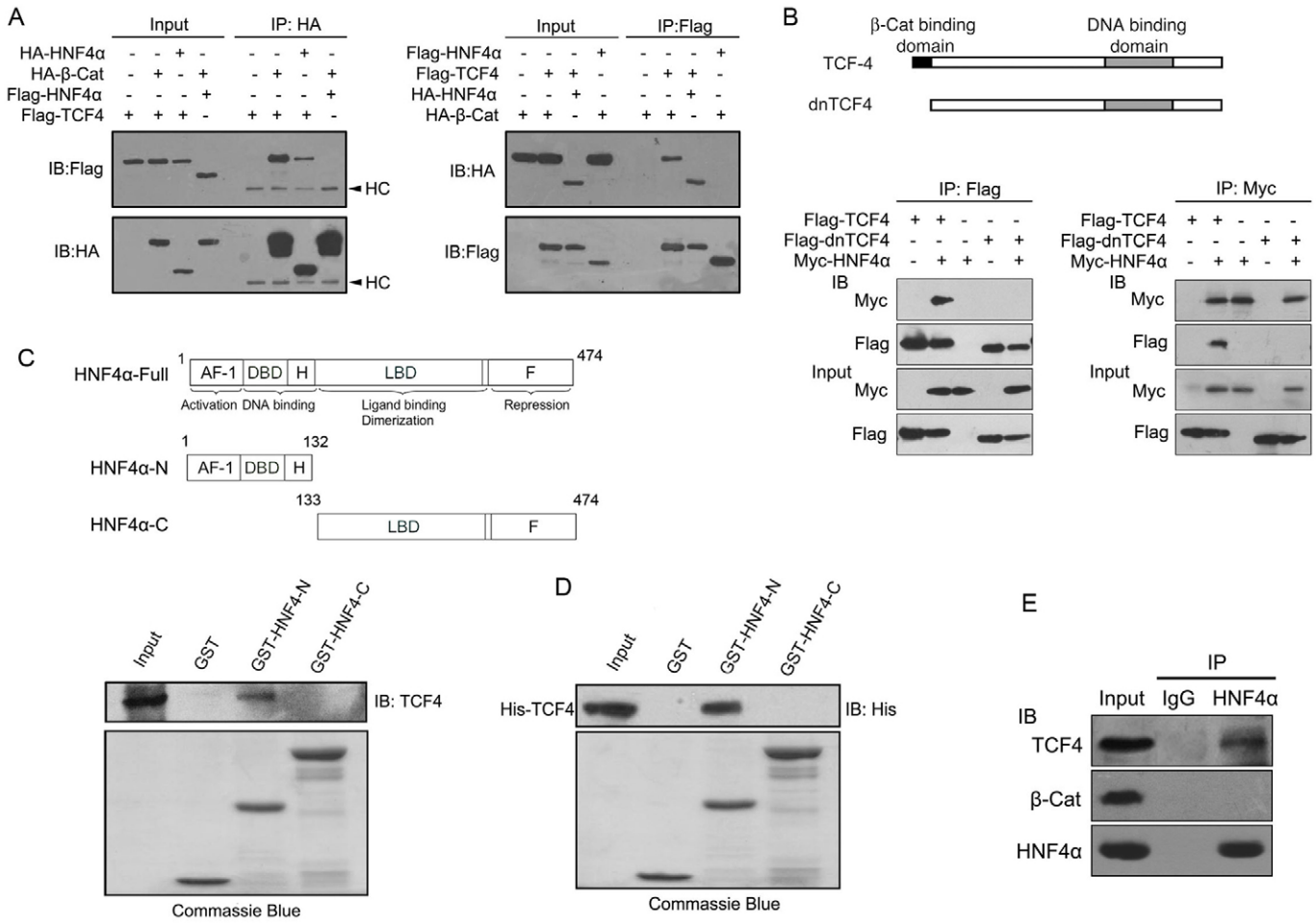

Fig. 3. HNF4α directly associates with TCF4 in HCC cells. (A) TCF4 but not β-catenin was shown to reciprocally interact with HNF4α. Shown are the results of Co-IP experiments using 293T cells co-transfected with HA- or FLAG-tagged HNF4α and β-catenin or TCF4, respectively. (B) The β-catenin binding domain of TCF4 (shown in the schematic) is essential for the interaction with HNF4α. A Co-IP experiment between Myc-tagged HNF4α and FLAG-tagged TCF4 or dnTCF4 was performed. (C) Top: Schematic of full length HNF4α and its N- and C-terminal regions. Bottom: GST pull-down of nuclear extracts from LO2 cells showing HNF4α interaction with TCF4 through its N-terminal region. (D) An *in vitro* pull-down assay demonstrated direct binding between TCF4 and the HNF4α N-terminal region. (E) HNF4α is normally present in a complex with TCF4 *in vivo*. Endogenous Co-IP experiments between HNF4α and TCF4 or β-catenin from nuclear extracts of non-transfected LO2 cells revealed that HNF4α pulled down TCF4 but not β-catenin.

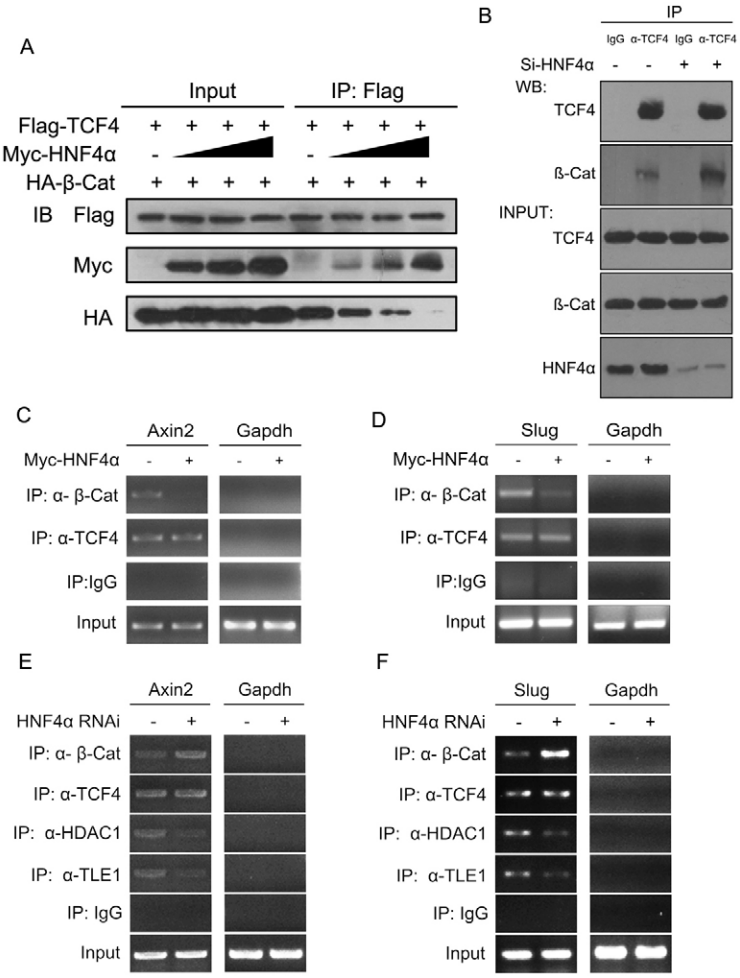

Fig. 4. HNF4α competes with β-catenin for binding to TCF4 but facilitates transcriptional co-repressors activities. (A) HNF4α competes with β-catenin for binding to TCF4. 293T cells were co-transfected with FLAG-tagged TCF4, HA-tagged β-catenin and increasing amounts of Myc-tagged HNF4α and then immunoprecipitated with an anti-FLAG antibody. (B) Knockdown of HNF4α expression using siRNA in LO2 cells resulted in significantly increased co-precipitation of endogenous β-catenin with TCF4. (C,D) A ChIP assay of Hep3B cells indicated that transient overexpression of HNF4α led to reduced occupancy by β-catenin but not by TCF4 at the Axin2 (C) or Slug (D) promoters. (E,F) A ChIP assay of Hep3B cells indicated that knockdown of HNF4α expression using siRNA resulted in increased occupancy by β-catenin but decreased occupancy by TLE1 or HDAC1 at the Axin2 (E) or Slug (F) promoters.

Fig. 4C,D, overexpression of HNF4α led to reduced occupancy by β-catenin but not TCF4 at the Axin2 or Slug promoters. In contrast, knockdown of HNF4α expression in Hep3B cells resulted in increased occupancy by β-catenin. Moreover, the occupancy by TLE1 or HDAC1 in these cells was decreased (Fig. 4E,F). Together, these results provide evidence that HNF4α competes with β-catenin for binding to TCF but facilitates transcriptional co-repressors.

To explore the biological relevance of the HNF4α–TCF4 interaction, we next examined whether this interaction is required for the involvement of HNF4α in the repression of endogenous Wnt–β-catenin target genes. Overexpression of HNF4α resulted in significantly reduced levels of *MYC, ID2, MMP7, AXIN2* and *SLUG* mRNAs in Hep3B cells, as revealed by qPCR assay (Fig. 5A). Consequently, the EMT-related Wnt–β-catenin target proteins Snail and Slug also showed a remarkable reduction (Fig. 5B), providing direct evidence that the HNF4α–TCF4 interaction is required for the repression of HNF4α-mediated Wnt–β-catenin downstream targets.

HNF4α regulates the switch between the transcriptional and adhesion functions of β-catenin

TCF4 has been demonstrated to be a dominant nuclear retention factor of β-catenin (Krieghoff et al., 2006). The finding in this study that HNF4α is a competitor of β-catenin and thus limits the binding of β-catenin to TCF4 led us to postulate that HNF4α might modulate the nuclear–cytoplasmic distribution of β-catenin. To verify this hypothesis, we checked the relative protein levels of β-catenin in the nucleus and cytoplasm after HNF4α treatment. As shown in Fig. 5C, the nuclear distribution of β-catenin was dramatically reduced in HNF4α-overexpressing MHCC-97H cells, as determined by western blotting, whereas no significant change was observed in the cytoplasmic distribution. Given the result in supplementary material Fig. S2C that the total β-catenin level is not changed in HNF4α-treated cells and that β-catenin has a well-known adhesion function at the plasma membrane, it is reasonable to deduce that HNF4α-driven cytoplasmic β-catenin undergoes membrane localization. Western blotting analysis indicated that when MHCC-97H cells were infected with a lentivirus expressing HNF4α, the membranous β-catenin was greatly increased compared with the control cells (Fig. 5D). Confocal immunofluorescence analysis of the same cells revealed that the nuclear β-catenin level was remarkably decreased, while the membranous distribution of β-catenin was greatly increased, which is consistent with the western blotting results (Fig. 5E). Collectively, these data suggest that HNF4α may control the switch between the transcriptional and adhesion functions of β-catenin, which indicates it has an important role in regulating the EMT process.

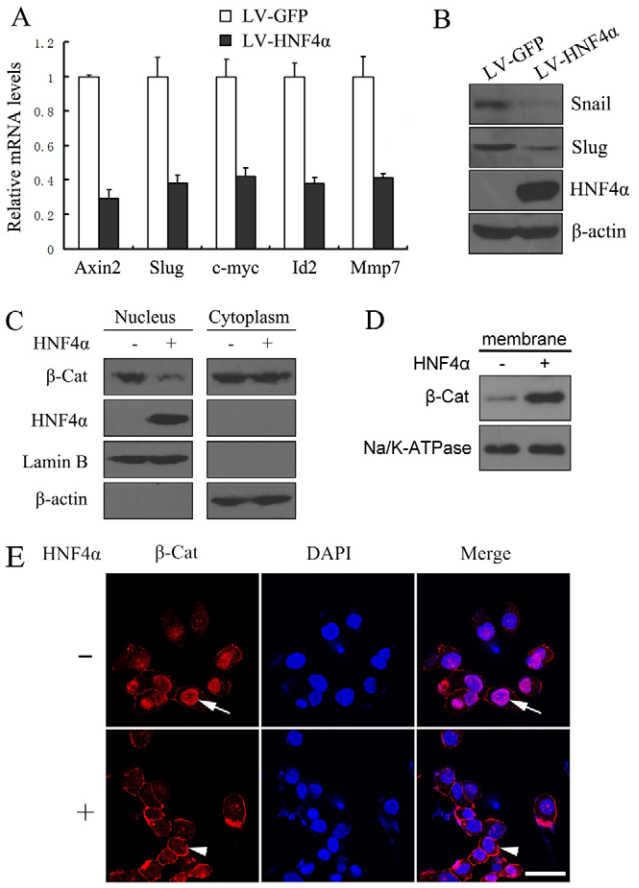

Fig. 5. HNF4α inhibits the expression of EMT-related Wnt–β-catenin target genes and regulates the switch between the transcriptional and adhesion functions of β-catenin. (A) Hep3B cells were infected with LV-GFP or LV-HNF4α. qPCR analysis indicated the relative mRNA expression levels of Wnt–β-catenin target genes in these cells. (B) Western blotting indicated the relative expression levels of EMT-related Wnt–β-catenin target proteins in above cells. (C) The relative nuclear–cytoplasmic distribution of β-catenin in MHCC-97H cells stably expressing LV-GFP and LV-HNF4α, using western blotting. (D) The relative membranous distribution of β-catenin in the above cells, using western blotting. Na^+/K^+ ATPase was used as a internal control. (E) The relative nuclear–membranous distribution of β-catenin in the above cells using confocal immunofluorescence analysis. Arrows indicated nuclear staining and arrowheads indicated membrane staining. Scale bar: 50 μm.

Functional interaction between Wnt–β-catenin signaling and HNF4α during EMT in HCC cells

To elucidate the functional interaction between Wnt–β-catenin signaling and HNF4α, we investigated how these two factors affect each other in Wnt–β-catenin signaling-induced EMT in HCC cells. As illustrated in Fig. 6A,B, forced expression of β-catenin in Hep3B cells resulted in substantially increased Axin2/Snail and Slug mRNA and protein levels, as observed by qPCR and western blotting. The simultaneous overexpression of HNF4α in these cells was observed to completely inhibit expression of β-catenin-activated downstream targets. Moreover, the results from different experiments revealed that overexpression of HNF4α also completely compromised β-catenin-driven E-cadherin downregulation and vimentin upregulation (Fig. 6A–C). Next, we checked other EMT phenotypes in Wnt–β-catenin-activated cells. Forced expression of β-catenin in Hep3B cells resulted in enhanced cell migration (Fig. 6D) and invasion (Fig. 6E). However, simultaneous overexpression of HNF4α in these cells was observed to completely inhibit β-catenin-activated EMT phenotypes. Taken together, these results suggest a functional interaction between Wnt–β-catenin signaling and HNF4α during EMT in HCC cells.

The double-negative feedback loop between Wnt–β-catenin signaling and HNF4α *in vivo*

To investigate the regulatory features between Wnt–β-catenin signaling and HNF4α *in vivo*, a diethylnitrosamine (DEN)-induced rat hepatoma model was used. When the level of β-catenin mRNA was constant, the protein level progressively increased during the progression of rat liver carcinogenesis, as detected by semi-quantitative PCR and western blotting. In contrast, HNF4α showed gradually decreasing expression of both mRNA and protein in this process (supplementary material Fig. S3). Immunostaining results revealed that the nuclear β-catenin progressively increased but the membrane β-catenin progressively decreased. Nuclear staining of HNF4α gradually decreased (Fig. 7A). To assess the functional role of both Wnt–β-catenin signaling and HNF4α in driving this feedback loop *in vivo*, adenovirus expressing dnTCF4 or HNF4α was injected into the tail vein of the rat tumor models. As illustrated in Fig. 7B, administration of Ad-dnTCF4 resulted in substantially increased HNF4α expression in hepatoma tissue as revealed by qPCR, western blotting and immunostaining. However, administration of Ad-HNF4α was observed to inhibit expression of the Wnt–β-catenin targets Axin2/Snail and Slug. Moreover, Ad-HNF4α remarkably changed the nuclear–plasma membrane distribution of β-catenin (Fig. 7C). Finally, both Ad-dnTCF4 and Ad-HNF4α were observed to enhance E-cadherin and inhibit vimentin expression (Fig. 7D). Collectively, these data provide *in vivo* evidence supporting the existence of a double-negative feedback loop between Wnt–β-catenin signaling and HNF4α during EMT in HCC cells.

Discussion

Wnt–β-catenin signaling plays a pivotal role in the development of normal tissues through the regulation of cell proliferation, differentiation and migration (Polakis, 2000). Aberrant activation of Wnt–β-catenin signaling has been found in a wide range of cancers, especially HCC (Thompson and Monga, 2007). Moreover, the activation of this pathway may induce EMT through its downstream targets Twist, Snail and Slug. Previous studies have shown that Wnt–β-catenin signaling participates in EMT in numerous cancers; however, the phenotypes and downstream molecular events are somewhat different, which reflects the dependence on cellular context and tissue specificity. In this study, we detected a typical EMT process induced by Wnt–β-catenin signaling in HCC cells. The HNF4α expression status was also investigated because it is the most prominent and specific factor in maintaining the differentiation of hepatic lineage cells and a potential EMT regulator in HCC cells. Our experiments showed that HNF4α expression was negatively regulated by Wnt–β-catenin signaling, implying the involvement of this liver-specific factor in the Wnt–β-catenin signaling-induced EMT process. Furthermore, we identified *HNF4A* as a direct downstream negative target gene of Snail and Slug. A previous study using mouse hepatocytes indicated that Snail inhibits *Hnf4a* expression (Cicchini et al., 2006). We found in our

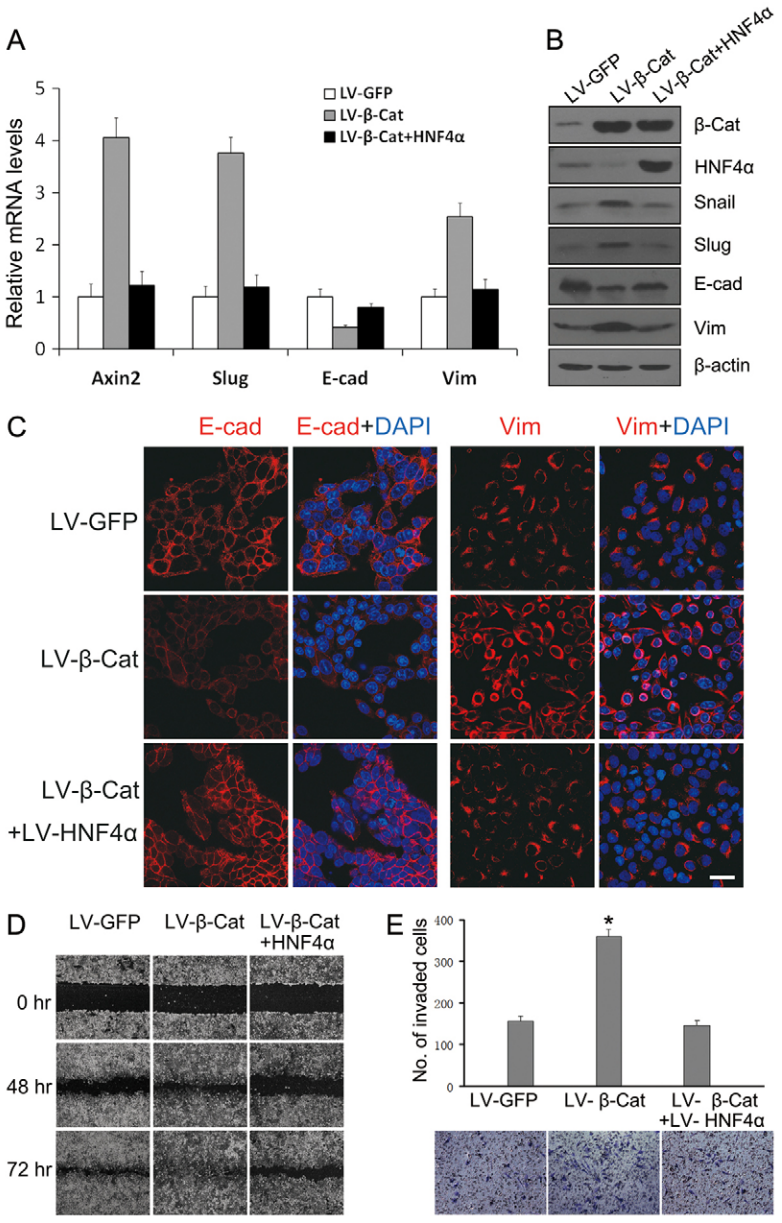

Fig. 6. The regulatory interaction between Wnt–β-catenin signaling and HNF4α affects EMT in HCC cells. (A,B) HNF4α completely reversed the changes in EMT-related Wnt–β-catenin targets and EMT markers induced by β-catenin. Hep3B cells were infected with LV-β-catenin and treated with LV-HNF4α simultaneously. (A) qPCR was used to measure the mRNA expression levels. (B) Western blotting was used to measure the levels of the indicated proteins. (C) Confocal immunofluorescence analysis of E-cadherin and vimentin expression of the above cell lines. (D) The migration ability of the above cells was assessed using a wound-healing assay. The experiment was performed in triplicate. Representative images from one experiment are shown. (E) The invasive ability of the above cells is presented as the total number of cells that entered the bottom invasion chamber, counted across eight fields. Each sample was measured in triplicate, and each experiment was repeated three times. Representative images from one experiment are shown, and the bar graph shows the mean values for three different experiments. In A and E data are the means ± s.d. of three independent experiments (*P<0.001). Scale bar: 50 μm.

approach that Snail also inhibits *HNF4A* expression in human and rat hepatoma cells, which suggests the conservation among species. Moreover, in our study Slug but not Twist was also found to suppress *HNF4A* expression, which has added a new regulator of HNF4α. Wnt–β-catenin signaling inhibits HNF4α through Snail and Slug, two 'EMT master genes', which highlights the molecular mechanism for Wnt–β-catenin signaling in regulating HNF4α expression during EMT in HCC cells.

Studies on the transcriptional regulatory elements of genes expressed in hepatocytes identified a number of hepatic transcription factors capable of modulating hepatocyte gene expression in hepatoma cells (Cereghini, 1996). One of the most crucial hepatic transcription factors in this regulatory network is HNF4α, which not only controls the expression of functional liver genes but is also involved in regulating proliferation, differentiation and morphogenesis (Lazarevich and Fleishman, 2008). Downregulation of the *HNF4A* gene is associated with progression of rodent and human HCC and contributes to increased proliferation, loss of epithelial morphology, and de-differentiation (Lazarevich et al., 2010). HNF4α was originally identified as a member of the nuclear hormone receptor family of transcription factors, which bind DNA as a homodimer to regulate downstream gene expression (Sladek et al., 1990). Aberrantly activated Wnt–β-catenin signaling is also well known to contribute to increased proliferation, loss of epithelial morphology, and de-differentiation, which is the direct opposite of the effects of HNF4α. Therefore, we deduced that HNF4α might modulate the Wnt–β-catenin signaling pathway in both hepatic and HCC cells. A previous study indicated that HNF4α regulates the intestinal balance between proliferation and differentiation through regulating the Wnt–β-catenin signaling pathway (Cattin et al., 2009). However, the detailed and precise mechanisms are not known. In another study, it was reported that HNF4α interacts with LEF1 to affect liver zonation. In this

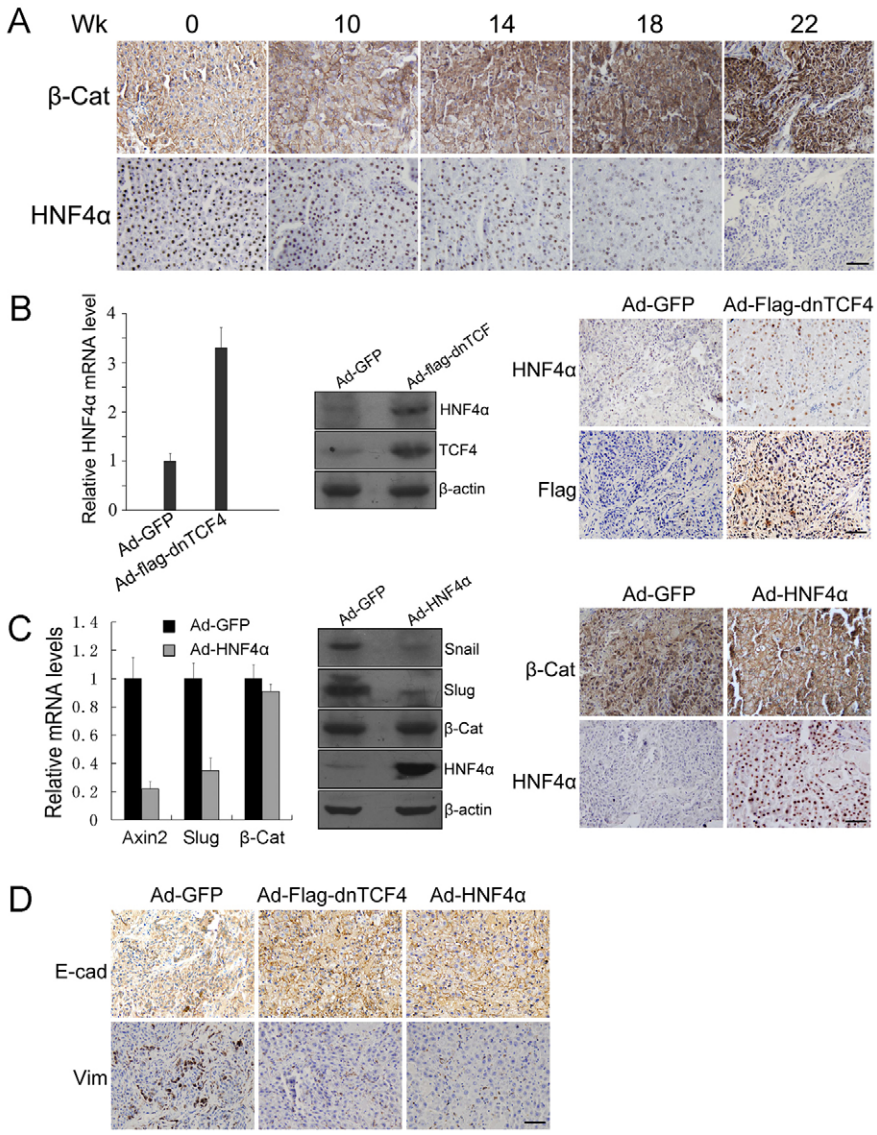

Fig. 7. The double-negative feedback loop between Wnt–β-catenin signaling and HNF4α *in vivo*. (A) Immunostaining showed the expression trend and localization of β-catenin and HNF4α in rat hepatoma samples throughout the progression of DEN-induced carcinogenesis. (B) Inhibition of Wnt–β-catenin signaling resulted in increased HNF4α expression in rat hepatoma models. GFP-expressing adenovirus (Ad-GFP) and FLAG-dnTCF4-expressing adenovirus (Ad-FLAG-dnTCF4) were injected into the tail vein of rat tumor models at 22 weeks after DEN induction. Two weeks later, the hepatoma samples were harvested for experimentation. qPCR (left) and western blotting (middle) indicated the mRNA and protein levels of HNF4α and dnTCF4. An immunostaining experiment (right) showed the expression and localization of HNF4α and dnTCF4. (C) Overexpression of HNF4α inhibits the expression of the Wnt–β-catenin targets Axin2/Snail and Slug and changes the nuclear–plasma membrane distribution of β-catenin in rat hepatoma models. Ad-GFP and Ad-HNF4α were injected into the rats as described for B. qPCR (left) and western blotting (middle) revealed the mRNA and protein expression levels of the indicated genes and proteins. Immunostaining (right) showed the expression and localization of HNF4α and β-catenin. (D) Immunostaining of the samples as in B and C revealed that both Ad-dnTCF4 and Ad-HNF4α enhance E-cadherin expression but inhibit vimentin expression. In A–D a representative experiment from three independent experiments is shown; in B and C data are the means ± s.d. of three independent experiments. Scale bars: 50 μm.

context, both HNF4α and LEF1 directly bind the DNA sequence of HNF4α consensus site to activate the expression of a zonation-specific gene *Cyp1a1* (Colletti et al., 2009), which is different from the repression mechanism in our study and reflects the context and gene dependence. Moreover, the underlying activation mechanism was not further investigated in the study by Colletti et al. In our approach, HNF4α was found not only to associate with TCF4 to compete with β-catenin, but also to facilitate transcriptional co-repressor activities, which may contribute to the gene repression. We speculated that three potential mechanisms could be involved in the recruitment of transcriptional co-repressors to the TCF4–HNF4α complex on chromatin: (1) HNF4α inhibits the association between β-catenin and TCF4, so that TCF4 can convert to recruit the co-repressors; (2) TCF4–HNF4α complex recruits the co-repressors through HNF4α; (3) the TCF4–HNF4α complex recruits the co-repressors through both HNF4α and TCF4. In support of the first mechanism, it is well-known that TCF can bind either the co-repressors or β-catenin depending on whether Wnt is turned off or turned on. In the TCF4–HNF4α complex, HNF4α might mimic the Wnt turned-off status. In support of the last two mechanisms, published reports indicated that HNF4α associates with HDAC-containing SMRT complex to repress gene expression (Ruse et al., 2002; Ungaro et al., 2010). In this scenario, the TCF4–HNF4α complex may recruit the co-repressors through HNF4α or HNF4α and TCF4 together. The detailed mechanism needs further investigation. As a transcription factor, HNF4α either activates or suppresses gene expression by direct binding to the gene promoter region. In a few cases, protein–protein interaction mechanisms are also involved in the regulation of gene expression (Hanse et al., 2012), which is consistent with our observation and reflects the multiple functions of HNF4α.

Previous studies involving inducible HNF4α deletion support of our findings (Bonzo et al., 2012; Walesky et al., 2013). When re-analyzing the microarray and RNA sequencing data we found that hepatocyte-specific deletion of HNF4α in adult mice resulted in upregulation of many Wnt target genes including *Myc*, *Ccnd1* (cyclin D1), *Vegf*, *Sox9*, *Egfr* and *Jun*. Moreover, the epithelial markers claudin-1, claudin-2 and occludin were found to be

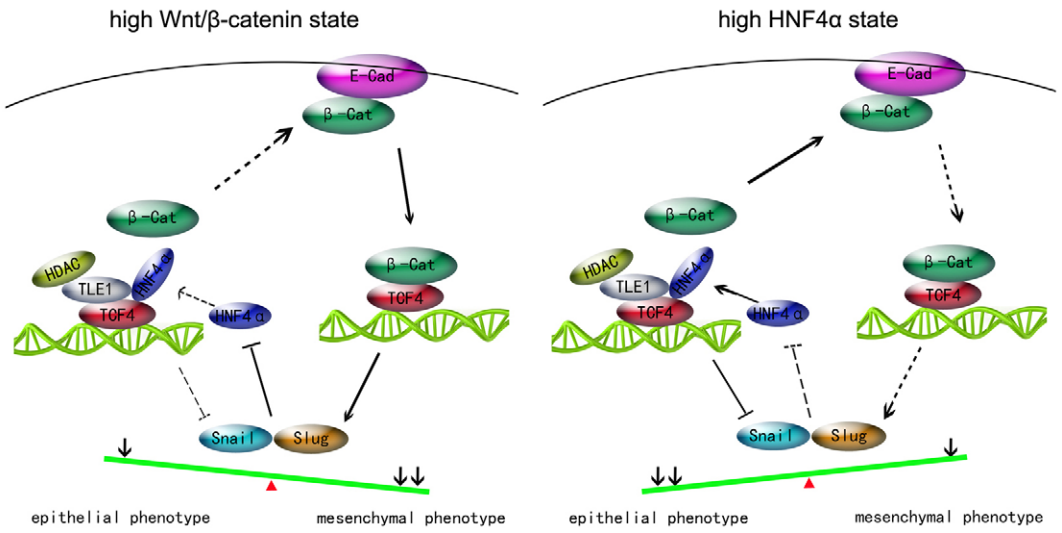

Fig. 8. A schematic diagram summarizing the working model of the double-negative feedback loop between Wnt–β-catenin signaling and HNF4α. Left: the high Wnt–β-catenin state. Right: the high HNF4α state. There are two potential complexes on the TCF4 locus, TCF4-HNF4α or TCF4-β-catenin. Solid lines indicate the activated signaling events. Dotted lines indicate the inhibited signaling events.

downregulated, which could be considered as a supplement of the EMT marker changes of our experiments. These results have strengthened our conclusion that HNF4α inhibits Wnt–β-catenin-signaling-induced EMT.

As a key EMT regulator, β-catenin plays a dual role in mediating the EMT process, in which it activates Snail/Slug/Twist expression in the nucleus to drive mesenchymal features but connects cadherins to α-catenin and the cytoskeleton in cell adhesion at the plasma membrane to maintain the epithelial phenotype. However, the regulation of the switch between the transcriptional and adhesion functions of β-catenin was still largely unknown. A recent study revealed a retention mechanism that mediates the nuclear–cytoplasmic distribution of β-catenin, in which TCF4 and BCL9/Pygo recruit β-catenin to the nucleus and APC and Axin enrich β-catenin in the cytoplasm (Krieghoff et al., 2006). The fact that HNF4α prevents β-catenin from binding to TCF4 suggests that HNF4α may regulate the nuclear export of β-catenin, and we did observe this phenomenon in our experiment. Two fates could be adopted by β-catenin after it is exported to the cytoplasm: either it undergoes degradation or connects to the cell membrane. E-cadherin has been demonstrated to increase the membrane association of β-catenin (Takeichi, 1995). Our study indicated that the inhibition of Wnt–β-catenin activity by HNF4α results in upregulation of E-cadherin. As a result, the increase of E-cadherin could subsequently enhance the membrane association of β-catenin, and our experiment confirmed this hypothesis. Collectively, these data indicate that HNF4α regulates EMT in HCC cells by both inhibiting β-catenin transcription and enhancing β-catenin cell membrane localization.

The regulatory properties mentioned above lead us to propose a double-negative feedback loop between Wnt–β-catenin signaling and HNF4α, in which the expression of either factor is mutually exclusive to the other. Our results showed that Wnt–β-catenin signaling represses the expression of HNF4α, and by binding to TCF4, HNF4α prevents Wnt–β-catenin signaling from repressing HNF4α. Thus, Wnt–β-catenin signaling indirectly de-represses its own activity, creating a self-reinforcing loop. HNF4α also enhances its own expression through an identical mechanism. Once activated, the loop remains locked in either a high Wnt–β-catenin or high HNF4α state, which causes the cells to adopt either a mesenchymal or epithelial phenotype. We also predict that the triggering of the EMT process is determined by the Wnt–β-catenin signaling activation but not HNF4α inhibition because the β-catenin protein level is progressively increased during the progression of rat liver carcinogenesis. From all the data in this study, we suggest a model, as shown in Fig. 8. There are two potential complexes on the TCF4 locus, TCF4–β-catenin or TCF4–HNF4α. In the high Wnt–β-catenin state, TCF4 mainly recruits β-catenin. The TCF4–β-catenin complex activates Snail and Slug expression; simultaneously, the TCF4–β-catenin complex also removes its own repression by HNF4α, which creates a self-reinforcing loop. In this context, HCC cells adopt a mesenchymal phenotype. In the high HNF4α state, HNF4α competes with β-catenin for binding to TCF4 to inhibit the expression of Snail and Slug, thus removes its own repression by Wnt–β-catenin signaling, which also creates a self-reinforcing loop. In this context, HCC cells adopt an epithelial phenotype.

In summary, we have identified a feedback mechanism controlling Wnt–β-catenin signaling and HNF4α expression *in vitro* and *in vivo*, which sheds new light on the regulation of the epithelial to mesenchymal transition. The modulation of these molecular processes may be a method of inhibiting HCC invasion through blocking Wnt–β-catenin signaling or restoring HNF4α expression to prevent EMT.

Materials and Methods

Cell culture, infection and transfection

The human normal hepatic cell line LO2 and human HCC cell lines Hep3B and MHCC-97H were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. LO2 cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Hep3B and MHCC-97H cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin and 10 μg/ml streptomycin. Cells were infected with lentivirus or transfected with plasmids using Turbo transfection reagent (Fermentas).

Plasmids

Plasmids were constructed using standard procedures. See supplementary material Table S1 for detailed primer information.

Lentivirus- and adenovirus-mediated gene transfer or RNA interference

The lentiviral pLV-CS2.0 vector was used to express Snail, Slug and Twist; the pBobi vector was used to express HNF4α, β-catenin and dnTCF4. See supplementary material Table S1 for detailed information on the primers.

Lentiviral Plko.1 vector was used to express short hairpin RNA (shRNA) directed against *Slug* (sh-Slug) *Snail* (sh-Snail) or a non-silencing scrambled control sequence. The sequences are as follows: Snail1, 5′-CCGGCCA-ATCGGAAGCCTAACTACACTCGAGTGTAGTTAGGCTTCCGATTGGTTT-TTG-3′; Snail2, 5′-CCGGCCACTCAGATGTCAAGAAGTACTCGAGTACT-TCTTGACATCTGAGTGGTTTTTG-3′; Slug1, 5′-CCGGGTGCTGACCAAC-CAAATAATACTCGAGTATTATTTGGTTGGTCAGCACTTTTTG-3′; Slug2, 5′-CCGGCCCATTCTGATGTAAAGAAATCTCGAGATTTCTTTACATCAG-AATGGGTTTTTG-3′; Control, 5′-CCGGCCTAAGGTTAAGTCGCCCTCGC-TCGAGCGAGGGCGACTTAACCTTAGGTTTTTG-3′.

The genes for HNF4α or dnTCF4 were cloned into the shuttle vector PAdtrack-CMV using the following paired primers: HNF4α, 5′-GGGGTACCGCGT-GGAGGCAGGGAGAATG-3′ and 5′-CCCAAGCTTCCCCAGCGGCTTGCTA-GATAAC-3′; dnTCF4, 5′-GGGGTACCGCGTGGAGGCAGGGAGAATGCGA-CTCTCCATGGACTACAAAGACCATGAC-3′ and 5′-CCGCTCGAGCTATTC-TAAAGACTTGGTGACG-3′. The resultant plasmid was linearized by digestion with the restriction endonuclease *Pme*I and subsequently co-transformed into *E. coli* BJ5183 cells with the adenoviral backbone plasmid pAdEasy-1. Recombinants were selected for kanamycin resistance, and recombination was confirmed by restriction endonuclease analysis. Finally, the linearized recombinant plasmid was transfected into adenovirus-packaging 293 cells. Recombinant adenoviruses were typically generated within 5–7 days. A high titer stock could be made with 2×10^8 293 cells. The adenoviruses were enriched through cesium chloride gradient centrifugation. The titer of the generated adenovirus was $\sim1\times10^{11}$ PFU/ml. The purified adenoviruses were injected into rats (1×10^{10} PFU/kg) via tail vein injection.

Real-time quantitative PCR (qPCR)

Total RNA (2 μg) from cells or liver tissues was used for the first-strand synthesis of cDNA (Invitrogen). Platinum SYBR Green qPCR SuperMix (Invitrogen) was used for the qPCR reaction, and quantification was determined using the ΔΔCt method. Specific primer sequences are available in supplementary material Table S2.

Luciferase assays

HEK 293T cells in 24-well plates were transfected at 50–60% confluency using a calcium-phosphate method. The pGL3-HNF4α promoter (50 ng), pGL4.26-HNF4α promoter (50 ng), TOPFLASH (50 ng) and cytomegalovirus (CMV)-β-galactosidase (25 ng) reporter plasmids were co-transfected with the following expression plasmids: Snail, Slug, β-catenin or HNF4α. The total amount of plasmid DNA transfected was normalized by adding empty vector. Cells were harvested after 24 hours and processed for luciferase and β-galactosidase assays, and the data were normalized to β-galactosidase levels.

Chromatin immunoprecipitation assays

ChIP was performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit following the manufacturer's instructions (Upstate). Briefly, chromatin samples were prepared from 1×10^7 fixed cells and immunoprecipitated with IgG, anti-HNF4α, anti-TCF4, anti-β-catenin, anti-TLE1 or anti-HDAC1 antibody. Purified DNA was subjected to PCR with primers flanking the potential E-box consensus sequences of the HNF4α- or TCF4-binding sites of Axin2 and Slug. Supplementary material Table S3 lists the primer sequences. Supplementary material Table S4 lists the information on the antibodies. The data presented is from three independent experiments.

Co-immunoprecipitation, GST-pulldown and western blotting

Transfected cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH 8.0, 1% Triton X-100) for subsequent co-immunoprecipitation (Co-IP). The cell lysates were precleared with protein A/G beads for 1 hour at 4℃ with agitation. Specific or control IgG antibodies were added to the precleared samples and they were incubated with rotation at 4℃ for 4 hours or overnight. The immune complexes were captured with 20 μl protein A/G beads at 4℃ for 1 hour, washed three times with washing buffer and subjected to SDS-PAGE for subsequent western blotting.

Glutathione *S*-transferase fusion proteins were expressed in the *E. coli* strain BL21. To purify the GST fusion proteins, cells were lysed by sonication in lysis buffer [phosphate-buffered saline (PBS), 1% Triton X-100, 2% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride], and the resulting lysates were incubated for 1 hour at 4℃ with glutathione–Sepharose beads. The beads were pelleted by centrifugation and washed with dialysis buffer for subsequent experiments. Nuclear extracts were then incubated with resin-bound proteins by rotating at 4℃ for 3 hours, washed four times in washing buffer (20 mM HEPES, pH 7.9, 0.2 mM EDTA, pH 8.0, 20% glycerol, 0.15 M KCl, 0.2% NP-40), and analyzed by western blotting using appropriate antibodies. Western blotting was performed according to standard procedures.

Supplementary material Tables S4 and S5 summarize the information on the antibodies used in the above experiments.

Cell invasion and migration

The *in vitro* invasion assay was performed using BioCoat Matrigel invasion chambers (BD Bioscience). MHCC-97H or Hep3B cell suspensions (5×10^5 cells/ml) were placed into the upper chamber in 0.5 ml of serum-free medium. The lower compartment was filled with complete medium. After incubation for 24 or 48 hours, cells that had migrated to the lower surface of the filters were fixed in ethanol for 5 minutes at room temperature and visualized with a Crystal Violet staining method. Cells were counted in eight fields of triplicate membranes.

For the migration assay, a confluent monolayer was scratched with a pipette tip, washed with PBS, and incubated in culture medium supplemented with 10% FBS. The cultures were photographed using phase-contrast microscopy at 0, 48 and 72 hours. All experiments were performed in triplicate.

Experimental HCC model in rats

Male Wistar rats weighing ~120 g received 10 mg/kg per day of diethylnitrosamine (DEN; Sigma) for 22 weeks. Rats were given the weekly dose of DEN in a volume of drinking water (0.01% vol/vol) corresponding with the estimated water consumption over 6 days. Once the animals consumed the administered DEN solution, they were given DEN-free water for the remainder of the week. To assess the gene and protein expression levels during the progression of DEN-induced carcinogenesis, two animals from each group were euthanized at the indicated time points. After the 22-week DEN administration, nine rats were randomly divided into Ad-GFP, Ad-dnTCF4 and Ad-HNF4α groups (three rats in each group) and infused with 1×10^{10} PFU/kg of the selected adenovirus by tail vein injection. Two weeks later, all rats were euthanized, and tumor samples were taken for examination. This study was approved by the local Ethical Committee of Xiamen University.

Immunofluorescence and immunostaining

MHCC-97H or Hep3B cells were seeded at a density of 1.5×10^5 cells/well on 8 mm coverslips in 12-well plates. After 48 hours, coverslips were fixed using ice-cold methanol and probed with primary anti-E-cadherin (R&D) or anti-vimentin antibodies at dilutions between 1/100 and 1/1000. The slides were then incubated with the appropriate secondary antibody conjugated to Alexa Fluor 594 (Invitrogen). Nuclear DNA was stained with 4-,6-diamidino-2-phenylindole (DAPI), and the coverslips were mounted with FluorSave reagent (Calbiochem). Confocal images were taken using an LSM780 (Carl Zeiss) confocal microscope at 63× magnification.

Rat liver specimens were fixed in 10% formalin, embedded in paraffin, and sectioned. After dewaxing and rehydration, the sections were pretreated with peroxidase blocking buffer (Maxim, Fuzhou, China) for 20 minutes at room temperature. Antigen retrieval was performed by boiling in Tris-EDTA (pH 6.0) for 20 minutes. After treatment with blocking buffer (5% normal goat serum in PBS) for 1 hour at room temperature, sections were incubated with antibodies against HNF4α, β-catenin, vimentin, E-cadherin and TCF4 in blocking buffer. Secondary antibody reagents were from the SABC kit (Boster Biological Technology, Wuhan, China) or DAB kit (Maxim Biological Technology, Fuzhou, China).

Supplementary material Table S6 lists the information for the antibodies used in the above experiments.

Statistical analysis

All experiments were repeated at least three times, and a representative result is shown for each experiment. The results are presented as the means ± s.d. Statistical significance was determined by one-way ANOVA (when there were more than two groups) or a Student's *t*-test (two groups only). A value of $P<0.05$ was considered significant.

Author contributions

M.Y., S.-N.L., K.-M.A. and L.-X.G. performed the experiments and analyzed the data. S.-S.Z., J.L., J.-K.C., Q.-F.L., G.-D.Y., W.-J.W., J.-F.W., W.-Y.C., G.-B.S. and Y.-J.L., R.-F.L. performed the experiments. Z.-M.Z. designed the experiments. B.-A.L. designed the experiments and wrote the manuscript.

Funding

This work was supported by grants from the '973' Project of the Ministry of Science and Technology [grant numbers 2013CB530600, 2009CB52220 to B.-A.L.]; the National Natural Science Foundation of China [grant numbers U1205023, 81201616, 81272384 to B.-A.L., Q.-F.L.]; the major project of science and technology from the

Department of Education [grant number 313051 to B.-A.L.]; the Natural Science Foundation of Fujian Province [grant number 13111125 to Z.-M.Z.]; key Projects of Fujian Province [grant number 2011Y01010460 to Z.-M.Z.]; project of the Fujian Health Department [grant number 2011-CXB-34 to Z.-M.Z.]; project of the Xiamen Technique Bureau [grant number 3502Z20114003 to Z.-M.Z.]; and 'Project 111' sponsored by the State Bureau of Foreign Experts and Ministry of Education [grant number B06016 to B.-A.L.].

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.135053/-/DC1

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