Activation of Hepatic Stem Cell Marker EpCAM by Wnt–β-Catenin Signaling in Hepatocellular Carcinoma

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Abstract

The heterogeneous nature of hepatocellular carcinoma (HCC) and the lack of appropriate biomarkers have hampered patient prognosis and treatment stratification. Using a gene expression profiling approach, we recently identified a novel prognostic HCC subtype that resembles hepatic progenitor cells with the activation of stem cell markers and Wnt–β-catenin signaling, based on EpCAM (epithelial cell adhesion molecule, a hepatic stem cell marker) expression. In this study, we investigated whether the activation of the Wnt–β-catenin pathway regulates EpCAM expression. We found that nuclear accumulation of β-catenin induced, whereas the degradation of β-catenin or inhibition of Tcf/β-catenin complex formation reduced EpCAM gene expression in cultured normal human hepatocytes and HCC cell lines. We identified two Tcf binding elements in the EpCAM promoter that specifically bound to Tcf-4 in an electrophoretic mobility shift assay. EpCAM promoter luciferase activity was down-regulated by the degradation of β-catenin or inhibition of Tcf/β-catenin complex formation. Furthermore, we found that EpCAM-positive HCC is much more sensitive to Tcf/β-catenin binding inhibitors than EpCAM-negative HCC *in vitro*. Taken together, our data indicate that EpCAM is a Wnt–β-catenin signaling target gene and may be used to facilitate HCC prognosis by enabling effective stratification of patients with predicted pharmacologic responses to Wnt–β-catenin signaling antagonists. [Cancer Res 2007;67(22):10831–9]

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide with a dismal outcome (1). A rising incidence and mortality from HCC have recently been observed in most industrialized countries including the United States. The prevalence of HCC is largely attributed to hepatitis B and hepatitis C virus infection. HCC seems to be a heterogeneous disease because its clinical outcome can greatly vary. Although the underlying molecular mechanisms of HCC pathogenesis remains largely unknown, multiple epigenetic and genetic changes have been associated with HCC, including the activation of oncogenes (e.g., *N-ras, c-myc, c-fos*) and inactivation of tumor suppressor genes (e.g., *p53, p16, Rb*; refs. 2, 3). Two signal transduction cascades that have been proposed to be critical in HCC are Wnt/Frizzled/β-catenin and insulin/insulin-like growth factor-I (IGF-I)/insulin receptor substrate-1/mitogen-activated protein kinase pathways (4). In colorectal tumors, mutations in the Wnt/β-catenin signaling pathway such as in the adenomatous polyposis coli (APC) gene seem dominant (5). In contrast, various mutations in the Wnt components, including APC, AXIN1, TCF4, and β-catenin, have been found in only a small subset of HCC (6). To address the heterogeneity of HCC, new technologies such as microarray-based gene expression profiling and proteomic analyses have been applied to HCC classification (7–11), and several HCC subtypes have recently been revealed (12, 13). One study suggested that the Wnt/β-catenin signaling pathway may be involved in a subtype of HCC with good prognosis (12). Functional analyses of these various signaling pathways offer promise for improving our understanding of HCC.

Epithelial cell adhesion/activating molecule (EpCAM; also known as CD326, CO17-1A, EGP, EGP40, GA733-2, KSA, Ly74, M1S2, M4S1, MIC18, MK-1, TROP1, hEGP-2), one of the first tumor-associated antigens identified, is encoded by the *TACSTD1* gene (14). EpCAM is highly expressed in a large variety of human adenocarcinomas and squamous cell carcinomas (15). Although the function of EpCAM and its regulatory mechanism are largely unknown, it has been implicated in cell-cell adhesion (16) and may act as an oncogenic signaling protein (17). Our recent gene expression profiling study indicates that EpCAM may serve as an early biomarker of HCC because its expression is highly elevated in premalignant hepatic tissues and in a subset of HCC (18). Similar results were observed by others (19–21), reiterating the significance of EpCAM in HCC development. In addition, using gene expression profiling and pathway analysis, we also showed that EpCAM-positive HCC displayed a distinct molecular signature with features of hepatic progenitor cells (HPCs), including the presence of known stem/progenitor markers (e.g., c-Kit, CK19, vimentin, CD133, and α-fetoprotein) and the activation of Wnt–β-catenin signaling.[1] It seems that there is a close functional link between EpCAM expression and the activation of Wnt–β-catenin signaling. It is known that mature hepatocytes are negative for EpCAM expression. In contrast, the majority of hepatocytes in the embryonic liver show EpCAM expression (19). Several recent studies indicate that EpCAM may serve as a hepatic stem cell marker (22, 23). Moreover, it is known that Wnt–β-catenin signaling plays a pivotal role in embryogenesis and the maintenance of stem cell growth (24) and is known to be activated during liver development/regeneration (25, 26). Taken together, these results suggest that EpCAM and Wnt–β-catenin signaling

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[1] T. Yamashita et al., submitted for publication.

are functionally connected, and both may play a role in the maintenance of hepatic cancer stem cells.

In this study, we aimed to determine whether EpCAM and Wnt–β-catenin act in the same signaling pathway. We found that EpCAM is one of the direct transcriptional targets of Wnt–β-catenin signaling in normal human hepatocytes and HCC cell lines. In addition, there was a concordant elevated EpCAM expression and the activation of Wnt–β-catenin in HCC cells, and EpCAM-positive HCC cells were sensitive to Tcf/β-catenin antagonists. Similarly, silencing of EpCAM by RNA interference led to cell growth inhibition. We suggest that EpCAM induction by Wnt–β-catenin signaling may be critical in maintaining hepatic cancer stem cell growth, and that HCC classification based on EpCAM expression may provide a selection criterion to identify HCC patients who may benefit from adjuvant therapies by targeting β-catenin or EpCAM.

Materials and Methods

Quantitative real-time reverse transcription-PCR (qRT-PCR). Total RNA was extracted using TRIzol (Invitrogen). *TACSTD1, BAMBI, DKK1, CCND1, CTNNB1*, and *MYC* expression were measured in triplicate using the Applied Biosystems 7700 Sequence Detection System. Probes used for the analyses were as follows: *TACSTD1*, Hs00158980_m1; *BAMBI*, Hs00180818_m1; *DKK1*, Hs00183740_m1; *CCND1*, Hs00277039_m1; *CTNNB1*, Hs00170025_m1; *MYC*, Hs00153408_m1; *18S*, Hs99999901_s1 (Applied Biosystems). All procedures were done according to the manufacturer's instructions.

Immunohistochemical analysis. Immunohistochemical (IHC) analysis was done using Envision+ kits (DAKO) according to the manufacturer's instructions. The primary antibodies used were as follows: anti–β-catenin monoclonal antibody clone 14 (BD Transduction Laboratories) and anti-EpCAM monoclonal antibody clone VU-1D9 (Oncogene Research Products).

Immunofluorescence. An indirect immunofluorescence assay was done as previously described (27). Briefly, cells were cultured on chamber slides and treated with the indicated chemicals for 48 h. Cells were then fixed with 4% paraformaldehyde for 10 min and methanol for 20 min and incubated in PBS. Samples were blocked with 10% normal donkey serum for 1 h at room temperature and stained with primary antibodies for 1 h at 37°C, followed by Alexa 568 Texas Red–conjugated anti-mouse antibodies (Molecular Probes).

Electrophoretic mobility shift assay. Recombinant Tcf-4 was expressed in *Escherichia coli* as a glutathione *S*-transferase (GST) fusion protein and extracted as previously described (28). Electrophoretic mobility shift assay (EMSA) was done using a LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's instructions. Double-stranded DNA oligonucleotides containing the putative Tcf binding sites of the EpCAM promoter (TBE1; -TTCAAAG-, TBE2; -CTTTGAT-, shown in Fig. 3*A*) and 10 adjacent nucleotides upstream and 10 downstream were constructed and used as probes. Mutant TBE1 and TBE2 probes (mut-TBE1; -GCCAAAG-, mut-TBE2; -CTTTGGC-) were also constructed to assess binding specificity. HepG2 nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Anti-human TCF-4 monoclonal antibody clone MAB3755 (Chemicon) was used for the supershift assays.

Cell lines, plasmids, and chemicals. Four human liver cancer cell lines (Hep3B, HepG2, MHCC97, and SK-Hep-1) were routinely cultured as previously described (7). HT29-APC and HT29-GAL were generous gifts from Bert Vogelstein (The Johns Hopkins University Medical Institutions, Baltimore, MD; ref. 29). For culture of the HT29-APC cells, 0.6 mg/mL of hygromycin (Roche) was added. To induce full-length APC, HT29-APC cells were incubated with 100 μmol/L of zinc chloride (Sigma). Normal human primary hepatocytes were provided by the University of Pittsburgh, managed through the Liver Tissue Procurement and Distribution System (LTPADS; NIH contract N01-DK-9-2310), and cultured as previously described (30). Wnt10B-conditioned media were prepared using a HuH7 clone stably expressing Wnt10B (WNT10B clone R8).[2] The pTOP-FLASH and pFOP-FLASH luciferase constructs used in the β-catenin/Tcf-4 transcriptional assay, mutant β-catenin expression vector (pCI-NEO-βcateninXL), wild-type-Tcf4 expression vector (pcDNA/myc-hTcf4), and dominant-negative Tcf4 expression vector (pcDNA/myc-ΔN-hTcf4) were kindly provided by Bert Vogelstein (31). The pGL3-EpCAM luciferase plasmid was constructed by inserting a 2.2-kb EpCAM promoter/enhancer fragment into a pGL3 luciferase reporter vector. Lithium chloride and sodium chloride were purchased from Sigma. Three small-molecule antagonists of the Tcf/β-catenin protein complex (PKF118-310, PKF115-584, and CGP049090) were generous gifts from Alexander Wood (Novartis Institutes for Biomedical Research, Cambridge, MA).

Plasmid transfection. Hep3B cells were nucleofected (Amaxa Inc.) with pcDNA3.1 (3 μg), pCI-NEO-βcateninXL (3 μg), pcDNA/myc-hTcf4 (3 μg), pCI-NEO-βcateninXL with pcDNA/myc-hTcf4 (1.5 + 1.5 μg), and pCI-NEO-βcateninXL with pcDNA/myc-ΔN-hTcf4 (1.5 + 1.5 μg), in combination with pmaxGFP (1 μg). Green fluorescent protein–positive cells were selected by fluorescence-activated cell sorting 48 h after transfection, re-seeded in six-well plates, cultured for an additional 24 h, followed by total RNA extraction.

RNA interference. Small interfering RNAs (siRNA) with two thymidine residues (dTdT) at the 3′-end of the sequence were constructed for targeting *CTNNB1* (β-catenin-1, 5′-AGCTGATATTGATGGACAG-3′; β-catenin-2, 5′-CAGTTGTGGTTAAGCTCTT-3′) by Qiagen. The sense and antisense strands of *TACSTD1* were designed as sense, 5′-GUUUGCGGACUGCACUUCAdTdT-3′; antisense, 5′-UGAAGUGCAGUCCGCAAACdTdT-3′. Negative control siRNAs were also purchased from Qiagen. Transfection was done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Total RNA was extracted from each sample using TRIzol (Invitrogen) 96 h after transfection.

Chromatin immunoprecipitation. HepG2 cells (1×10^6) were cultured on a 10-cm dish, and the chromatin immunoprecipitation (ChIP) assay was done using the Chromatin Immunoprecipitation Assay Kit (Upstate) and anti-human TCF-4 monoclonal antibody (Chemicon) according to the manufacturer's protocol. PCR was done using TaKaRa LA PCR Kit Ver.2.1 (TAKARA BIO INC.). PCR reactions contained 2 μL of immunoprecipitate or diluted total input, 25 μL of GC Buffer I, 400 nmol/L of deoxynucleotide triphosphates (dNTP), 200 nmol/L of each primer, and 2.5 units of TAKARA LA Taq™ in a total volume of 50 μL. The primers for detecting the EpCAM promoter sequence were designed as follows: forward 5′-CACT-CATTTTCTTCCCAAGAG-3′ and reverse 5′-GAACTGGATAGAGGAACGTG-3′. After 32 cycles of amplification (94°C for 30 s, 56°C for 30 s, and 72°C for 40 s), PCR products were run on a 1.5% agarose gel and analyzed by ethidium bromide staining.

Western blotting. Nuclear and cytoplasmic proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Proteins were electrophoresed on SDS-PAGE gels and transferred to a nitrocellulose membrane (Invitrogen). Protein detection was done using the following antibodies: anti-EpCAM monoclonal antibody clone 158210 (R&D Systems, Inc.), anti–β-catenin monoclonal antibody clone 14 (BD Transduction Laboratories), and anti–β-actin monoclonal antibody (Sigma). Bound antibodies were detected using enhanced chemiluminescence detection reagents (Amersham Biosciences).

Cell proliferation assay. HCC cell lines were seeded in 96-well plates at 3×10^3 cells per well, maintained overnight at 37°C, and incubated in the presence of small molecules at various concentrations (0, 0.125, 0.25, 0.5, 1, 2, and 4 μmol/L). Cell viability was monitored after 72 h using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega) according to the manufacturer's instructions. The effect of each compound is expressed as the concentration required to reduce the A_{490} by 50% (IC_{50}) relative to DMSO-treated cells.

[2] Yoshikawa et al., Molecular Biology of the Cell, in press.

Luciferase reporter gene assay. Lipofectamine 2000 (Invitrogen) was used to cotransfect cell lines with each of the reporter constructs (1 μg of pTOP-FLASH, 1 μg of pFOP-FLASH, or 1 μg of pGL3-EpCAM). pRL-null *Renilla* luciferase plasmid (100 ng) was used as an internal control. Firefly and *Renilla* luciferase activities were determined in triplicate by a dual-luciferase reporter assay system (Promega). Relative luciferase activities were expressed as a fraction of luciferase activity for each sample.

Statistical analysis. The comparison of gene expression data or luciferase reporter data among different test groups was examined by Student's *t* tests using GraphPad Prism software 4.0.

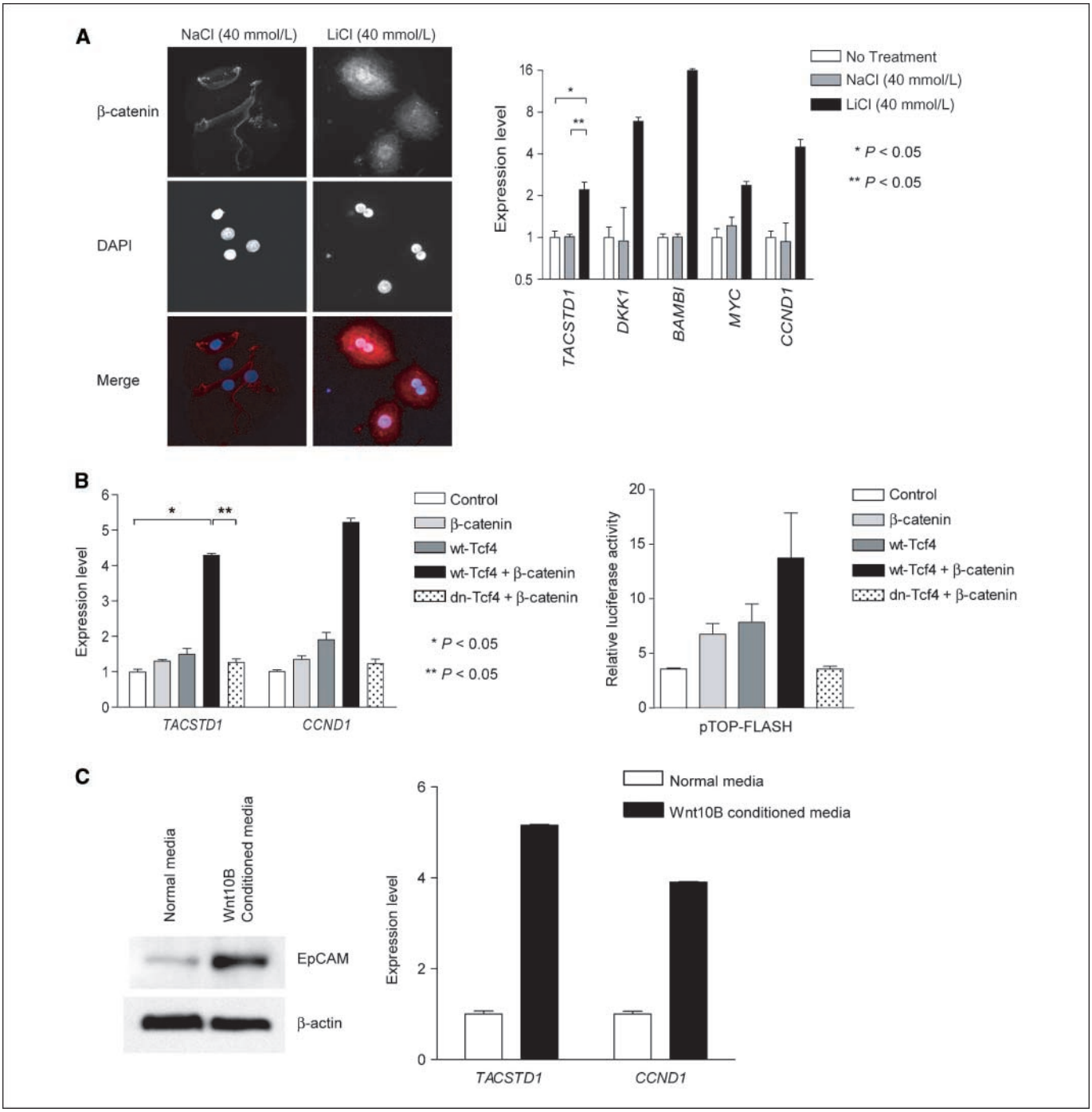

Figure 1. Activation of EpCAM gene expression by Wnt–β-catenin signaling. *A, left,* immunofluorescence of normal human hepatocytes treated with sodium chloride (*NaCl*, 40 mmol/L) or lithium chloride (*LiCl*, 40 mmol/L) and stained with anti–β-catenin antibody (*top*). 4′,6-Diamidino-2-phenylindole (*DAPI, middle*) and merged (*bottom*) images are also shown. *A, right,* qRT-PCR analysis of normal human hepatocytes treated with sodium chloride (40 mmol/L), lithium chloride (40 mmol/L), or nontreated. Gene expression was measured in triplicate and is shown as mean ± SD. *, $P < 0.05$; **, $P < 0.05$, statistical significance as calculated by Student's *t* tests. *B, left,* qRT-PCR analysis of Hep3B cells overexpressing β-catenin, wt-Tcf4, and dn-Tcf4. Gene expression was measured in triplicate and is shown as mean ± SD. *, $P < 0.05$; **, $P < 0.05$, statistical significance as calculated by Student's *t* tests. *B, right,* the TOP-FLASH reporter assay on Hep3B cells overexpressing β-catenin, wt-Tcf4, and dn-Tcf4. The assay was done in triplicate. *C,* parental HuH7 cells were incubated in conditioned media from Wnt10B-overexpressing R8 cells and were analyzed by Western blotting (*left*) and qRT-PCR (*right*).

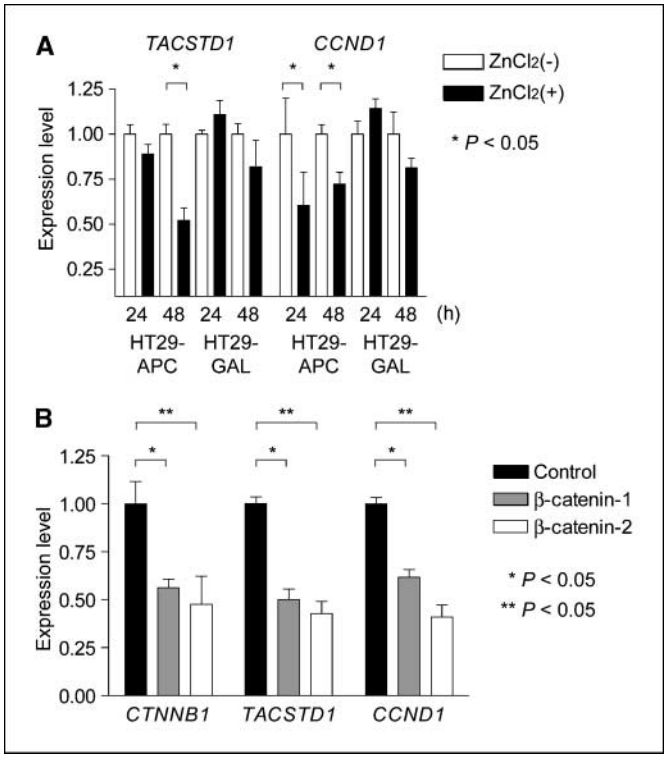

Figure 2. Suppression of EpCAM gene expression by β-catenin degradation. *A,* qRT-PCR analysis of HT29-APC cells and HT29-GAL cells treated with or without zinc chloride (100 μmol/L). *, $P < 0.05$, statistical significance as calculated by Student's *t* tests. Gene expression was measured in triplicate and is shown as mean ± SD. *B,* qRT-PCR analysis of HepG2 cells treated with a control siRNA or siRNAs targeting two different exons of *CTNNB1* (*β-catenin-1* and *β-catenin-2*). Gene expression was measured in triplicate and is shown as mean ± SD.

Results

Induction of EpCAM by the activation of Wnt–β-catenin signaling. Our recent studies indicate that many cellular genes are coexpressed with EpCAM.[1] Notably, a significant elevation in the expression of Wnt–β-catenin signaling genes, e.g., *BAMBI* and *DKK1* (32, 33), was correlated with EpCAM expression. To determine whether Wnt–β-catenin signaling activates EpCAM, we first treated freshly cultured normal primary human hepatocytes with various concentrations (i.e., 10, 20, 40 mmol/L) of lithium chloride (LiCl), which is known to inhibit glycogen synthase kinase-3β activity, thereby activating β-catenin (34). LiCl at only a 40 mmol/L concentration, but not 10 or 20 mmol/L (data not shown), induced an accumulation of β-catenin in both the nucleus and cytoplasm, whereas a sodium chloride (NaCl) control had no effect (Fig. 1*A, left*). Consequently, LiCl at 40 mmol/L, but not 10 or 20 mmol/L (data not shown), also induced 2.2-, 15.9-, and 6.9-fold increases in the mRNA expression of *TACSTD1, BAMBI*, and *DKK1*, respectively (Fig. 1*A, right*). Other classic β-catenin target genes such as *MYC* and *CCND1* were also induced by LiCl.

The effect of β-catenin activation on EpCAM expression was also investigated in Hep3B cells by assaying the activity of the Tcf4/β-catenin transcription complex. Using plasmids encoding constitutively active β-catenin (mutated at S33Y) or wild-type Tcf4 (wt-Tcf4), we found that EpCAM expression was slightly increased by β-catenin or wt-Tcf4 alone, but was significantly elevated by the coexpression of β-catenin and wt-Tcf4 (Fig. 1*B, left*). The specificity of this response was shown by a blockage of EpCAM expression by coexpressing a dominant-negative Tcf4 (dn-Tcf4). Similar results were obtained using the TOP-FLASH reporter assay (Fig. 1*B, right*; ref. 35). To further substantiate this finding, we determined the EpCAM protein level in the presence of Wnt

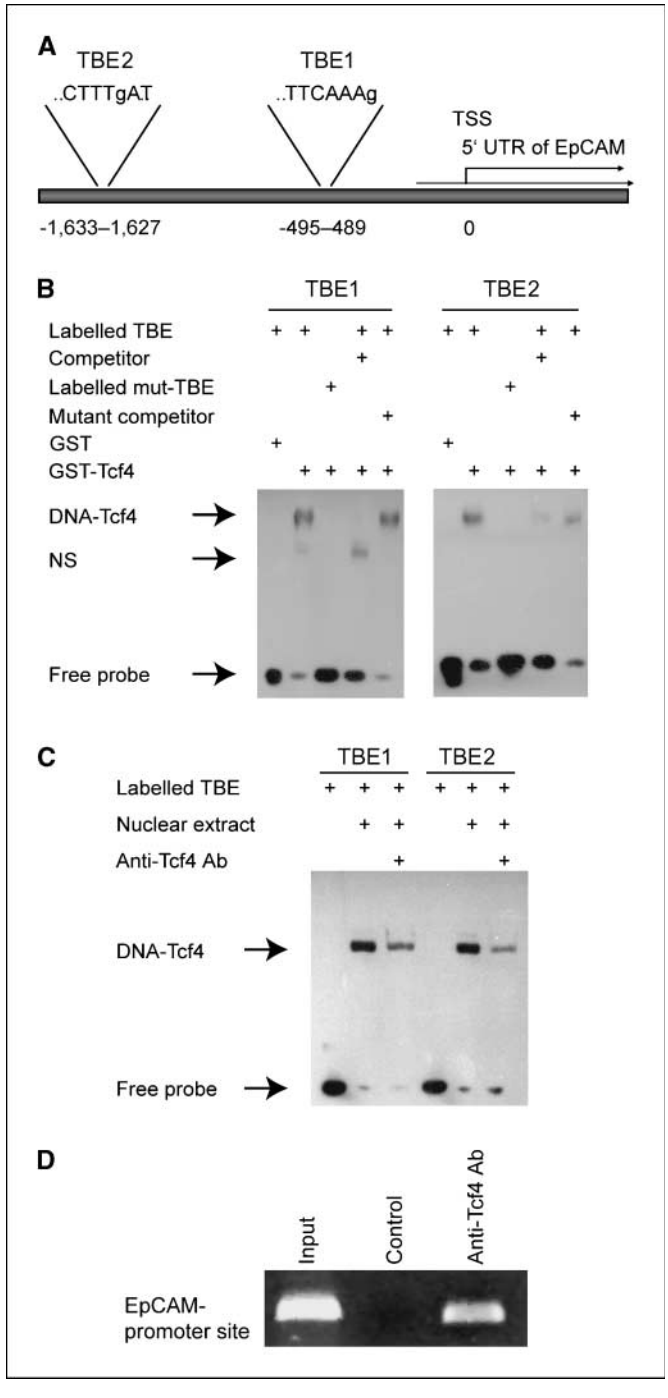

Figure 3. EpCAM promoter and Tcf/β-catenin transcription complex. *A,* schematic of the EpCAM promoter region. Two Tcf/β-catenin binding elements (*TBE1* and *TBE2*) are located in the promoter region of EpCAM. *B,* EMSA of TBE1 and TBE2 with recombinant GST-Tcf4. Both TBE1 and TBE2 specifically bound to the GST-Tcf4 fusion protein. *NS,* nonspecific. *C,* EMSA of TBE1 and TBE2 with nuclear extracts of HepG2 cells. *Lanes 2* and *5,* normal mouse immunoglobulin G (IgG) was used as controls. *D,* ChIP assay of endogenous EpCAM promoter sequence in Hep3B cells following immunoprecipitation by control mouse IgG or anti-Tcf4 antibody.

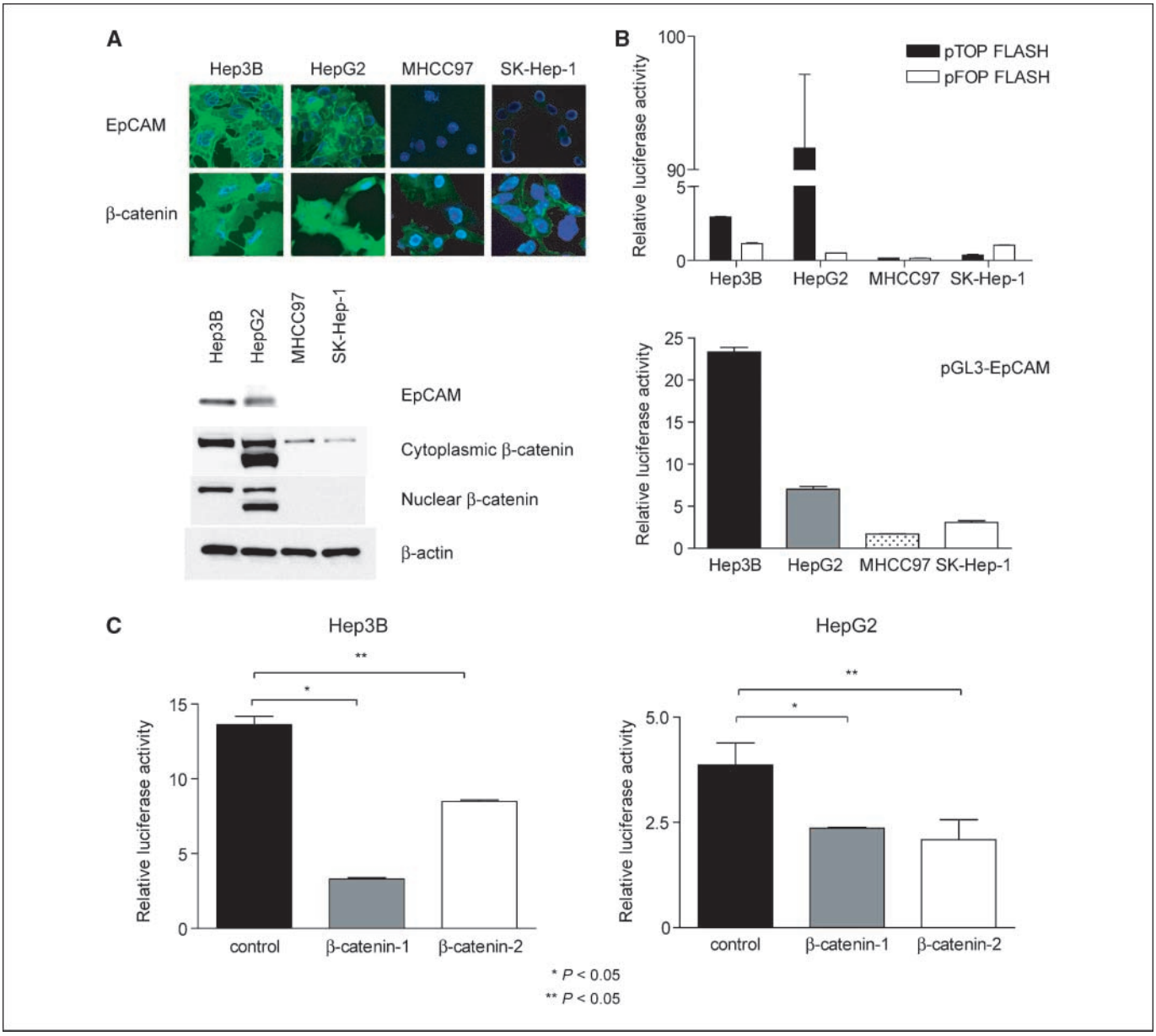

Figure 4. EpCAM luciferase activities in HCC cell lines. *A,* immunofluorescence of four HCC cell lines (*HepG2, Hep3B, MHCC97*, and *SK-Hep-1*) stained with anti-EpCAM or anti–β-catenin antibodies (*top*). EpCAM-positive HCC cells exhibited cellular and nuclear accumulation of β-catenin. Cell lysates were prepared from these cells and subjected to Western blot with anti-EpCAM and anti–β-catenin antibody (*bottom*). *B,* luciferase activities of pTOP-FLASH/pFOP-FLASH and pGL3-EpCAM in four HCC cell lines. The pTOP-FLASH/pFOP-FLASH or pGL3-EpCAM plasmid (1 μg each) were transiently transfected into four HCC cell lines with the pRL-null renilla plasmid (100 ng) as an internal control for transfection efficiency. Luciferase activity was measured in triplicate 24 h after transfection and is shown as mean ± SD. *C,* effect of RNAi targeting *CTNNB1* on EpCAM luciferase activity. Hep3B and HepG2 cells were transfected with a control siRNA (2 μg) or siRNAs targeting two different exons of *CTNNB1* (*β-catenin-1* and *β-catenin-2*; 2 μg each). The pGL3-EpCAM plasmid (1 μg) and the pRL-null plasmid (100 ng) were cotransfected 48 h after each siRNA treatment. Luciferase activity was measured in triplicate 72 h after siRNA treatment and is shown as mean ± SD. *, $P < 0.05$; **, $P < 0.05$, statistical significance as calculated by Student's *t* tests.

conditioned media. For this purpose, we used a HuH7 clone, i.e., WNT10B clone R8, stably expressing Wnt10B[2] and found that the Wnt10B conditioned media induced both EpCAM and cyclin D1 expression in parental HuH7 cells that expressed a low level of EpCAM (Fig. 1*C*).

Inactivation of EpCAM by Wnt–β-catenin signaling blockage. APC induction is known to accelerate the degradation of β-catenin by the ubiquitin-proteasome system (29). To determine the effect of APC on EpCAM expression, we used the HT29-APC cell line, which contains an *APC* gene under the control of a zinc-activated metallothionein promoter. We found that both *TACSTD1* and *CCND1* were down-regulated in HT29-APC cells, but not in control HT29-GAL cells, 48 h after $ZnCl_2$ treatment (Fig. 2*A*). We also used the siRNA silencing approach to down-regulate the expression of β-catenin by targeting two different sites of the *CTNNB1* gene (i.e., β-catenin-1 and β-catenin-2) in a hepatoma cell line (HepG2). Both siRNA oligos were able to inhibit the expression of *CTNNB1* as well as *CCND1* and *TACSTD1* when transfected into HepG2 cells (Fig. 2*B*). Taken together, we concluded that EpCAM expression can be up-regulated by the activated β-catenin

canonical pathway, suggesting that EpCAM might be a transcriptional target of Tcf4/β-catenin.

The EpCAM gene as a transcriptional target of Tcf/β-catenin. To identify whether *TACSTD1* could be a direct transcriptional target of the Tcf/β-catenin complex, we examined the genomic sequence of the human BAC clone (RP11-295P2 from 2, AC079775) and identified two classic consensus Tcf binding elements at 489 bp and 1,627 bp upstream of the *TACSTD1* transcription start site (designated TBE1 and TBE2, respectively; Fig. 3*A*). Using EMSA, we found that both TBE1 and TBE2 specifically bound to the GST-Tcf4 fusion protein, whereas mut-TBE1 and mut-TBE2 did not (Fig. 3*B*). This interaction was inhibited by an excess of unlabeled TBE1 or TBE2, but not mut-TBE1 or mut-TBE2. Similarly, a HepG2 nuclear extract induced shifts of both TBE1 and TBE2 probes under the same EMSA condition, which could be inhibited by anti-Tcf4 antibody (Fig. 3*C*). We also did an *in vivo* ChIP assay and found that Tcf4 bound to an EpCAM promoter sequence containing the TBE1 site (Fig. 3*D*). Therefore, it seemed that Tcf4 can bind specifically to TBE in the EpCAM promoter *in vitro* and *in vivo*.

Next, we characterized the expression of EpCAM and β-catenin in four HCC cell lines (i.e., Hep3B, HepG2, MHCC-97, and SK-Hep-1). By immunofluorescence analysis, we found that Hep3B and HepG2 cells were positive, whereas MHCC-97 and SK-Hep-1 cells were negative for EpCAM staining (Fig. 4*A* and data not shown). Noticeably, cytoplasmic as well as nuclear accumulation of β-catenin was prominent in EpCAM-positive HCC cells, whereas nuclear and cytoplasmic accumulation was not detected in EpCAM-negative cells (Fig. 4*A*). Similar results were obtained by Western blot analysis (Fig. 4*A, bottom*) and IHC analysis (data not shown). Thus, EpCAM and β-catenin expression was concordant in cultured HCC cell lines. We also transfected a luciferase reporter construct containing an EpCAM promoter (pGL3-EpCAM) or the pTOP-FLASH/pFOP-FLASH reporters into these cells. We found that the luciferase activities of both the EpCAM promoter and the β-catenin responsive promoter were high in both EpCAM-positive Hep3B and HepG2 cells, but very low in EpCAM-negative SK-Hep-1 cells, and nondetectable in EpCAM-negative MHCC97 cells (Fig. 4*B*). Furthermore, we investigated the effect of inhibiting Wnt–β-catenin signaling on EpCAM reporter activity by transfecting two siRNAs

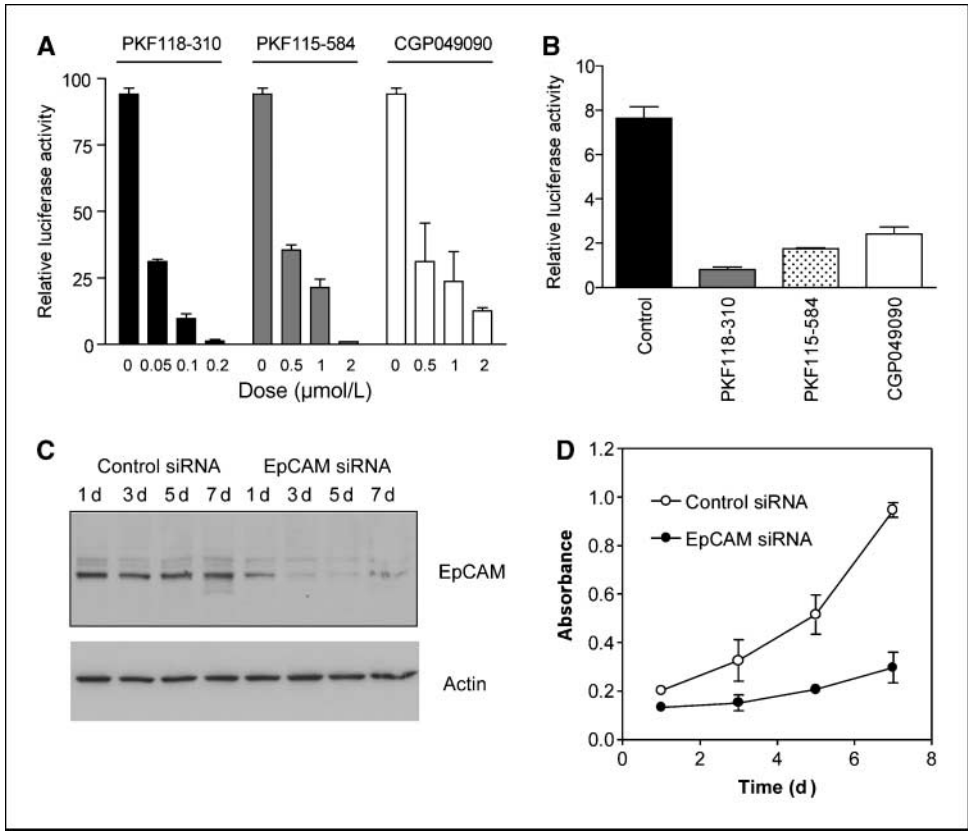

Figure 5. Functional consequences of inhibiting EpCAM or β-catenin in HCC cells. *A,* TOP-FLASH reporter assay on HepG2 cells treated with Tcf/β-catenin binding inhibitors (mean ± SD). The pTOP-FLASH plasmid (1 μg) and the pRL-null plasmid (100 ng) were transiently transfected into HepG2 cells. Small molecules (*PKF118-310, PKF115-584*, and *CGP049090*) were added to the media at the concentration indicated. *B,* effect of Tcf/β-catenin binding inhibitors on EpCAM luciferase activity. The pGL3-EpCAM plasmid (1 μg) and the pRL-null plasmid (100 ng) were cotransfected into HepG2 cells. PKF118-310 (0.05 μmol/L), PKF115-584 (0.5 μmol/L), and CGP049090 (0.5 μmol/L) were added to the media immediately after transfection. Luciferase activity was measured in triplicate 24 h after transfection and is shown as mean ± SD. *C,* EpCAM expression in Hep3B cells after transfection with a control siRNA or a siRNA specific to EpCAM (*EpCAM siRNA*) and incubation for up to 7 d was analyzed by Western blotting with a monoclonal antibody against EpCAM (clone 158210; R&D Systems, Inc.). A monoclonal antibody against β-actin was used as an internal control. All of the siRNA were synthesized by Qiagen Inc. The sequences of EpCAM siRNA were sense, 5′-GUUUGCGGACUGCACUUCAdTdT-3′; antisense, 5′-UGAAGUGCAGUCCGCAAACdTdT-3′. The sequences of control nonsilencing siRNA were sense, 5′-UUCUCCGAACGUGUCACGUdTdT-3′; antisense, 5′-ACGUGACACGUUCGGAGAAdTdT-3′. Transfection of siRNA was carried out using TransIT-TKO transfection reagent (Mirus Corp.) according to the manufacturer's protocol, with a total of 200 nmol/L siRNA duplex per transfection. *D,* cell proliferation of Hep3B was determined by the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. Data are an average of three independent experiments (mean ± SD).

Table 1. Pharmacologic responses to Tcf/β-catenin binding inhibitors in EpCAM-positive and EpCAM-negative HCC

Tcf/β-catenin binding inhibitors	IC_{50} in MTS assay*				Average IC_{50} ratio
	EpCAM-positive		EpCAM-negative		MHCC97/HepG2
	HepG2	Hep3B	MHCC97	SK-Hep-1	
PKF118-310	<0.125	1.06 ± 0.09	3.53 ± 0.61	3.62 ± 1.09	>28
PKF115-584	0.21 ± 0.06	0.80 ± 0.08	1.45 ± 0.17	3.07 ± 1.19	6.9
CGP049090	0.68 ± 0.06	1.48 ± 0.28	1.71 ± 0.13	2.98 ± 1.40	2.5

*Cytotoxicity assays (MTS) of three Tcf/β-catenin binding inhibitors were done on EpCAM-positive HCC and EpCAM-negative HCC cell lines. Four independent experiments were done in triplicate, and results are expressed as the average inhibitor concentration (mean ± SD) that gives 50% maximum inhibition (IC_{50}).

oligos specific to β-catenin in Hep3B and HepG2 cells. We found that both siRNA oligos were able to inhibit luciferase activity (Fig. 4*C*). Taken together, these results corroborate the immunofluorescence and Western blot data and indicate that EpCAM is a direct transcriptional target of the Wnt–β-catenin canonical signaling pathway.

Blockage of β-catenin or EpCAM affects HCC cell growth. Next, we examined the functional consequence of a β-catenin/EpCAM interaction by assaying their role in HCC cell growth. A recent study identified three natural compounds (PKF118-310, PKF115-584, and CGP049090) that are high-specificity inhibitors of the Tcf/β-catenin protein complex from a screen of about 7,000 natural compounds (36). Thus, we first examined the inhibitory effect of these three small molecules on Tcf/β-catenin transcription activity in HepG2 cells. We found that each of these three different compounds successfully inhibited the transcriptional activation of the Tcf/β-catenin complex in HepG2 cells in a dose-dependent manner (Fig. 5*A*), as evaluated by the TOP-FLASH or FOP-FLASH reporter assay (35). Consistently, these compounds also effectively suppressed EpCAM luciferase activity (Fig. 5*B*).

Next, we determined the growth-inhibitory activity of these Tcf/β-catenin binding inhibitors on HCC cells. Lethality of these compounds was assessed on the four HCC cell lines used above, and their IC_{50} doses were calculated (Table 1). It seemed that HepG2 and Hep3B cells were more sensitive to these compounds than MHCC97 and SK-Hep-1 cells. In particular, PKF118-310, PKF115-584, and CGP049090 showed >28-, 6.9-, and 2.5-fold lower IC_{50} for HepG2 cells than MHCC97 cells. Thus, it seemed that EpCAM-positive HCC cells were much more sensitive to Tcf/β-catenin antagonists than EpCAM-negative HCC cells *in vitro*. We also tested the effect of EpCAM expression on HCC cell growth. Silencing of EpCAM by RNA interference in Hep3B cells resulted in growth inhibition (Fig. 5*C* and *D*). Therefore, blockage of both β-catenin and EpCAM can lead to EpCAM-positive HCC cell growth inhibition.

Discussion

Cancer heterogeneity in biological behavior and treatment response can significantly compromise treatment options (37–39). The accurate classification of cancer according to its biological nature is required to provide the best qualified therapy for each cancer patient. Accordingly, identification of potential cancer biomarkers to provide both appropriate stratification of patients and discovery of therapeutic targets is of great interest for advancing personalized therapies (40). In addition, new classifications according to identified cancer-related molecular pathways may provide clues for novel and patient-specific therapeutic approaches. This concept is illustrated by the effective use of trastuzumab (Herceptin), gefitinib (Iressa), and imatinib (Gleevec) in the treatment of breast cancer, lung cancer, and leukemia, respectively (41–43). We therefore expect that molecular-targeted therapy for appropriately selected patients using biomarkers that are associated with compromised molecular pathways will be applied to other types of cancer (44).

In a recent study, we showed that EpCAM-positive HCCs resemble HPCs at molecular levels with activation of Wnt–β-catenin signaling, although the precise molecular interaction between EpCAM and Wnt–β-catenin signaling remained unclear.[1] In this study, we have shown that EpCAM is a novel transcriptional target of Tcf/β-catenin. Moreover, both EpCAM and β-catenin seem to be critical in maintaining the growth of EpCAM-positive HCC cells. Wnt–β-catenin signaling has been largely studied in developing embryos and has been proposed to participate in self-renewal, proliferation, or differentiation of stem cells (45). EpCAM has also recently been shown to be activated in embryogenesis and liver development (16). Thus, it is possible that Wnt–β-catenin signaling may act upstream of EpCAM to maintain HPC function, and that EpCAM may serve as a biosensor for activated stem cell signaling in HCC. Curiously, induction of EpCAM in Hep3B cells is weak by β-catenin alone but can be enhanced significantly by coexpressing TCF4 in our transient transfection assay. In addition, there is a discrepancy in correlation between the total amounts of endogenous EpCAM and β-catenin in Hep3B and HepG2 cells. Whether this is a unique feature of EpCAM–β-catenin signaling remains to be further determined using other cellular models. It should also be noted that our study does not currently include a site-directed mutagenesis approach to alter the two TBE sites in the EpCAM promoter as our repeated attempts in constructing these reporters have not been successful due to the high GC-rich contents of these regions. Thus, these studies will be the subject of further investigation.

Aberrant signaling by the Wnt–β-catenin pathway is linked to a range of diseases including many human cancers (24, 45). Although the impact of this signaling pathway in hepatocarcinogenesis is well established, a method to detect its activity remains controversial (4, 46, 47). In fact, reliable evaluation of nuclear β-catenin

accumulation by IHC is technically difficult in clinical specimens potentially due to the differential proportion of HCCs with activated Wnt–β-catenin signaling (46). Therefore, a reliable detection method for the canonical Wnt–β-catenin pathway activation is required. In a recent study using HCC clinical specimens, we showed that EpCAM may serve as an excellent biomarker for the activation of the canonical β-catenin pathway.[1] In this study, we further investigated the expression of EpCAM in HCC cell lines and found that we could easily separate EpCAM-positive and EpCAM-negative HCC that are correlated with the status of β-catenin by IHC. Using several approaches, we have shown that the activation of Wnt–β-catenin signaling transcriptionally induces EpCAM expression. Thus, the EpCAM detection assay seems to be a superior tool for a confident assignment of both EpCAM status and activation of the canonical β-catenin pathway in HCC.

The results from our cell culture experiments indicate that both EpCAM and β-catenin may be required for maintaining the growth of HCC cells with features of hepatic stem cells, including the activation of the β-catenin signaling, an attractive molecular target for personalized therapy (48). We found that small-molecule antagonists specific to the oncogenic Tcf/β-catenin protein complex had a selective growth-inhibitory effect on EpCAM-positive HCC cells. Similarly, silencing of EpCAM by RNA interference also resulted in growth inhibition of EpCAM-positive HCC cells. It seems that Wnt–β-catenin signaling may be indispensable for cell growth in EpCAM-positive HCC, whereby cells in this subtype are addicted to the activation of Wnt–β-catenin signaling (49). Our recent study indicated that a subtype of HCC that is positive for both EpCAM and α-fetoprotein had a poor prognostic outcome, including a high rate of recurrence after resection. It will be of interest to extend this study using EpCAM as a marker to stratify HCC patients for the development of personalized targeted therapy employing antagonists of either Wnt–β-catenin signaling or EpCAM. It should be noted that EpCAM-negative HCC cell lines are not sensitive to Tcf/β-catenin binding inhibitors, suggesting that Wnt–β-catenin signaling inhibitors may have a selective effect on EpCAM-positive HCC. It will therefore be of interest to determine key molecular pathways that are activated in EpCAM-negative HCC and conduct comprehensive mechanistic analyses to examine the biological differences between EpCAM-positive and EpCAM-negative HCC. We also wish to point out that our findings are limited by the examination of only four HCC cell lines in a cell culture system. Future studies require the examination of more HCC cell lines for the expression and drug sensitivity concordance and for an establishment of a preclinical model to evaluate their *in vivo* relevance.

In summary, our data suggest that EpCAM, a liver stem cell marker, is a novel Wnt–β-catenin signaling target gene, which may serve as a biomarker for Wnt–β-catenin activation. Furthermore, these findings are potentially clinically useful in that HCC classification based on EpCAM expression could predict the pharmacologic response to Wnt–β-catenin signaling inhibitors. This predictive capacity may allow for the development of personalized molecular targeted therapy for HCC patients, a hypothesis that remains to be tested using an *in vivo* animal model.

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