

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide.¹⁶ Although the cellular origin of HCC is unclear,^{17,18} HCC has heterogeneous pathologies and genetic/genomic profiles,¹⁹ suggesting that HCC can initiate in different cell lineages.²⁰ The liver is considered as a maturational lineage system similar to that in the bone marrow.²¹ Experimental evidence indicates that certain forms of hepatic stem cells (HpSCs), present in human livers of all donor ages, are multipotent and can give rise to hepatoblasts,^{22,23} which are, in turn, bipotent progenitor cells that can progress either into hepatocytic or biliary lineages.^{22,24} α -fetoprotein (AFP) is one of the earliest markers detected in the liver bud specified from the ventral foregut,^{25,26} but its expression has been found only in hepatoblasts and to a lesser extent in committed hepatocytic progenitors, not in later lineages or in normal human HpSC.²² Recent studies also have indicated that EpCAM is a biomarker for HpSC because it is expressed in HpSCs and hepatoblasts.²²⁻²⁴

We recently identified a novel HCC classification system based on EpCAM and AFP status.²⁷ Gene expression profiles revealed that EpCAM⁺ AFP⁺ HCC (referred to as *HpSC-HCC*) has progenitor features with poor prognosis, whereas EpCAM⁻ AFP⁻ HCC (referred to as *mature hepatocyte-like HCC*; MH-HCC) have adult hepatocyte features with good prognosis. Wnt/ β -catenin signaling, a critical player for maintaining embryonic stem cells,²⁸ is activated in EpCAM⁺ AFP⁺ HCC, and EpCAM is a direct transcriptional target of Wnt/ β -catenin signaling.²⁹ Moreover, EpCAM⁺ AFP⁺ HCC cells are more sensitive to β -catenin inhibitors than EpCAM⁻ HCC cells *in vitro*.²⁹ Interestingly, a heterogeneous expression of EpCAM and AFP was observed in clinical tissues, a feature that may be attributed to the presence of a subset of CSCs. In this study, we have confirmed that EpCAM⁺ HCC cells are highly invasive and tumorigenic, and have activated Wnt/ β -catenin signaling. We also show a crucial role of EpCAM in the maintenance of hepatic CSCs. Our data shed new light on the pathogenesis of HCC and may open new avenues for therapeutic interventions for targeting hepatic CSCs.

Materials and Methods

Clinical Specimens

HCC samples were obtained with informed consent from patients who underwent radical resection at the Liver Cancer Institute of Fudan University, Eastern Hepatobiliary Surgery Institute, and the Liver Disease Center of Kanazawa University Hospital, and the study was approved by the institutional review boards of the respective institutes. The microarray data from clinical specimens are available publicly (GEO accession number, GSE5975).²⁷ Array data from a total of 156 HCC cases (155 hepatitis B virus [HBV]-positive) corresponding to 2 subtypes of HCC (ie, HpSC-HCC and MH-HCC), were

used to search for HpSC-HCC-associated genes (Supplementary Table 1; see supplementary material online at www.gastrojournal.org). A total of 79 formalin-fixed and paraffin-embedded HCC samples were used for immunohistochemistry (IHC) analyses (Supplementary Table 2; see supplementary material online at www.gastrojournal.org), 56 of which also were used in a recent study.³⁰ The classification of HpSC-HCC and MH-HCC was based on previously described criteria.²⁷

Cell Cultures and Sorting

Human liver cancer cell lines (HuH1 and HuH7) were derived from Health Science Research Resources Bank (JCRB0199 and JCRB0403, respectively) and routinely cultured as previously described.³¹ Normal human MHs, provided by the University of Pittsburgh through Liver Tissue Cell Distribution System, were cultured as previously described.³² Human HpSCs were isolated from fetal livers and cultured in Kubota and Reid's³³ medium as previously described. Wnt10B conditioned medium was prepared as described.³⁴ Embryonic stem cell culture medium was prepared using Knockout Dulbecco's modified Eagle medium supplemented with 18% of Serum Replacement (Invitrogen, Carlsbad, CA). The pTOP-FLASH and pFOP-FLASH luciferase constructs were described previously.²⁹ BIO and MeBIO were generous gifts from Ali Brivanlou (The Rockefeller University, New York, NY). For isolating single cell-derived colonies to determine whether heterogeneity is an intrinsic property of EpCAM⁺ cells, HuH1 and HuH7 cells were resuspended and plated as a single cell per well in 96-well plates. A total of 192 single cells were plated successfully. The clones that grew well were selected 2 weeks after seeding and used for immunofluorescence (IF) analysis. The 5-fluorouracil (5-FU) stock (2 mg/mL; Sigma, St Louis, MO), was prepared in distilled water. Fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) analyses were used to isolate EpCAM⁺ HCC cells (Supplementary materials; see supplementary Materials and Methods online at www.gastrojournal.org).

Clonogenicity, Spheroid Formation, Invasion, Quantitative Reverse Transcription-Polymerase Chain Reaction, and IHC Assays

For colony formation assays, 2000 EpCAM⁺ or EpCAM⁻ cells were seeded in 6-well plates after FACS. After 10 days of culture, cells were fixed by 100% methanol and stained with methylene blue. For spheroid assays, single-cell suspensions of 1000 EpCAM⁺ or EpCAM⁻ cells were seeded in 6-well Ultra-Low Attachment Microplates (Corning, Corning, NY) after FACS. The number of spheroids was measured 14 days after seeding. Invasion assays were performed using BD Bio-Coat Matrigel Matrix Cell Culture Inserts and Control Inserts (BD Biosciences, San Jose, CA) essentially as pre-

viously described.³¹ Reverse transcription-polymerase chain reaction and IHC assays are described in detail in the supplementary materials (see supplementary material online at www.gastrojournal.org).

Tumorigenicity in NOD/SCID Mice

Six-week-old NOD/SCID mice (NOD/NCrCrl-*Prkdc^{scid}*) were purchased from Charles River (Charles River Laboratories, Inc, Wilmington, MA). The protocol was approved by the National Cancer Institute-Bethesda Animal Care and Use Committee. Cells were suspended in 200 μ L of Dulbecco's modified Eagle medium and Matrigel (1:1), and a subcutaneous injection was performed. The size and incidence of subcutaneous tumors were recorded. For histologic evaluation, tumors were formalin-fixed, paraffin-embedded or embedded directly in OCT compound (Sakura Finetek, Torrance, CA) and stored at -80° C.

RNA Interference

A small interfering RNA (siRNA) specific to *TACSTD1* (SI03019667) and a control siRNA (1022076) were designed and synthesized by Qiagen (Qiagen, Valencia, CA). Transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. A total of 200 nmol/L of siRNA duplex was used for each transfection.

Statistical Analyses

The class comparison and gene clustering analyses were performed as previously described.³⁰ The canonic pathway analysis was performed using Ingenuity Pathways Analysis (v5.5; Ingenuity Systems, Redwood City, CA). The association of HCC subtypes and clinicopathologic characteristics was examined using either the Mann-Whitney *U* test or the chi-square test. Student *t* tests were used to compare various test groups assayed by colony formation, spheroid formation, or invasion assays. The Kaplan-Meier survival analysis was performed to compare patient survival or tumorigenicity.

Results

A Poor Prognostic HCC Subtype With Molecular Features of HpSC

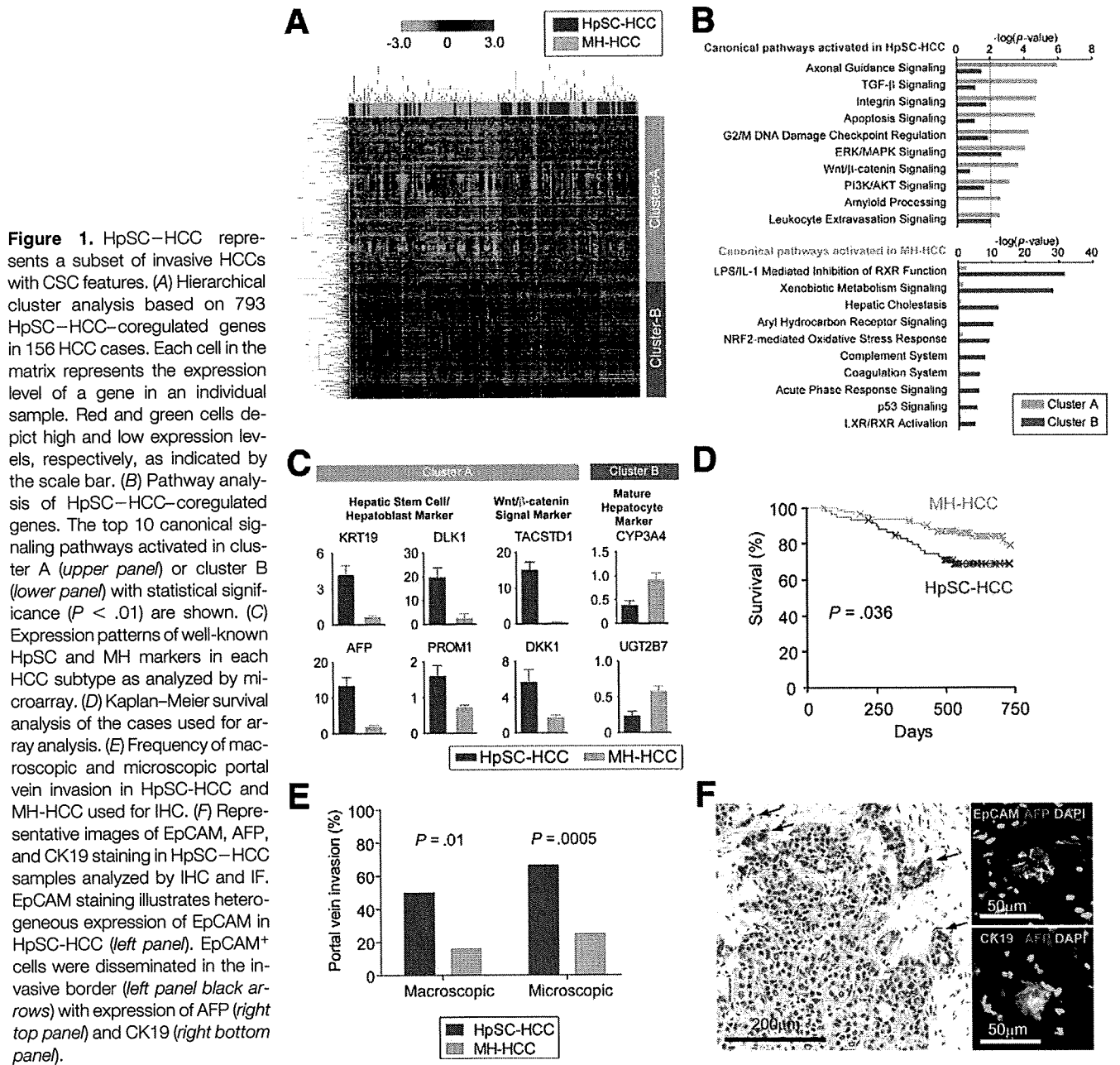
We re-evaluated the gene expression profiles that were uniquely associated with 2 recently identified prognostic subtypes of HCC (ie, HpSC-HCC and MH-HCC), using a publicly available microarray dataset of 156 HCC cases (GEO accession number: GSE5975). Sixty cases were defined as HpSC-HCC with a poor prognosis and 96 cases were defined as MH-HCC with a good prognosis, based on EpCAM and AFP status.²⁷ A class-comparison analysis with univariate *t* tests and a global permutation test (1000 \times) yielded 793 genes that were expressed differentially between HpSC-HCC and MH-HCC ($P < .001$). Hierarchical cluster analyses revealed 2 main gene clus-

ters that were up-regulated (cluster A; 455 genes) or down-regulated (cluster B; 338 genes) in HpSC-HCC (Figure 1A). Pathway analysis indicated that the enriched genes in cluster A were associated significantly with known stem cell signaling pathways such as transforming growth factor- β , Wnt/ β -catenin, PI3K/Akt, and integrin ($P < .01$) (Figure 1B). In contrast, genes in cluster B were associated significantly with mature hepatocyte functions such as xenobiotic metabolism, complement system, and coagulation system ($P < .01$). Noticeably, known HpSC markers such as *KRT19* (CK19), *TACSTD1* (EpCAM), *AFP*, *DKK1*, *DLK1*, and *PROM1* (CD133) were up-regulated significantly in HpSC-HCC, whereas known liver maturation markers such as *UGT2B7* and *CYP3A4* were expressed more abundantly in MH-HCC (Figure 1C and Supplementary Tables 3 and 4; see supplementary material online at www.gastrojournal.org). Kaplan-Meier survival analysis revealed that HpSC-HCC patients had a significantly shorter survival than MH-HCC patients ($P = .036$) (Figure 1D). Consistently, HpSC-HCC patients had a high frequency of macroscopic and microscopic portal vein invasion (Figure 1E).

However, IHC analyses of an additional 79 HCC cases revealed that among 24 HpSC-HCC cases, EpCAM staining was very heterogeneous with a mixture of EpCAM⁺ and EpCAM⁻ tumor cells in each tumor (Figure 1F, *left panel*). Noticeably, many of the EpCAM⁺ tumor cells were located at the invasion border zones and often were disseminated at the invasive front (*black arrows*). IF analysis revealed that HCC cells located at the invasive front co-expressed EpCAM, CK19, and AFP (Figure 1F, *right panels*). Noticeably, HpSC-HCC patients were significantly younger than MH-HCC patients (Supplementary Tables 1 and 2; see supplementary material online at www.gastrojournal.org). Enrichment of EpCAM⁺ AFP⁺ tumor cells at the tumor-invasive front suggested their involvement in HCC invasion and metastasis.

Isolation and Characterization of EpCAM⁺ Cells in HCC

The results described earlier suggest that HpSC-HCC may be organized in a hierarchical fashion in which EpCAM⁺ tumor cells act as stem-like cells with an ability to differentiate into EpCAM⁻ tumor cells. To test this hypothesis, we first evaluated the expression pattern of 7 hepatic stem/maturation markers (EpCAM, CD133, CD90, CK19, Vimentin, Hep-Par1, and β -catenin) in 6 HCC cell lines (Figure 2A). All 3 AFP⁺ cell lines (HuH1, HuH7, and Hep3B) expressed EpCAM, CD133, and cytoplasmic/nuclear β -catenin, whereas the other 3 AFP⁻ cell lines (SK-Hep-1, HLE, and HLF) did not, consistent with the microarray data. Interestingly, AFP⁺ cell lines had no CD90⁺ cell population, which recently was identified as hepatic CSCs,³⁵ whereas AFP⁻ cell lines had such a population. Consistent with the IF data, FACS analysis showed that AFP⁺ cell lines had a subpopulation of



EpCAM⁺ and CD133⁺, but no CD90⁺ cells, whereas AFP⁻ cell lines had a subpopulation of CD90⁺ cells but no EpCAM⁺ or CD133⁺ cells (Figure 2B). These data indicate that HpSC-HCC and MH-HCC cell lines have distinct stem cell marker expression patterns, and EpCAM as well as CD133 may be hepatic CSC markers specifically in HpSC-HCC.

We selected 2 human HCC cell lines (HuH1 and HuH7) to isolate EpCAM⁺ cells because both lines were heterogeneous in EpCAM, AFP, CK19, and β -catenin expression (Figure 2A and B and Supplementary Figure 1A; see supplementary material online at www.gastrojournal.org).²⁹ We successfully enriched EpCAM⁺ and EpCAM⁻ populations from HuH7 cells by FACS, with more than 80%

purity in EpCAM⁺ cells and more than 90% purity in EpCAM⁻ cells 1 day after sorting (Figure 3A). Similar results were obtained when the purity check was performed immediately after sorting (data not shown). EpCAM⁺ cells also were positive for CK19 and β -catenin (Figure 3B and Supplementary Figure 1B; see supplementary material online at www.gastrojournal.org) and most were AFP⁺ (data not shown). In contrast, EpCAM⁻ cells were negative for these markers but positive for HepPar1, a monoclonal antibody specific to hepatocytes (Figure 3B). Consistent with the microarray data described earlier, the levels of *TACSTD1*, *MYC*, and *hTERT* (known HpSC markers) were increased significantly in EpCAM⁺ HuH7 cells, whereas the levels of *UGT2B7* and *CYP3A4*

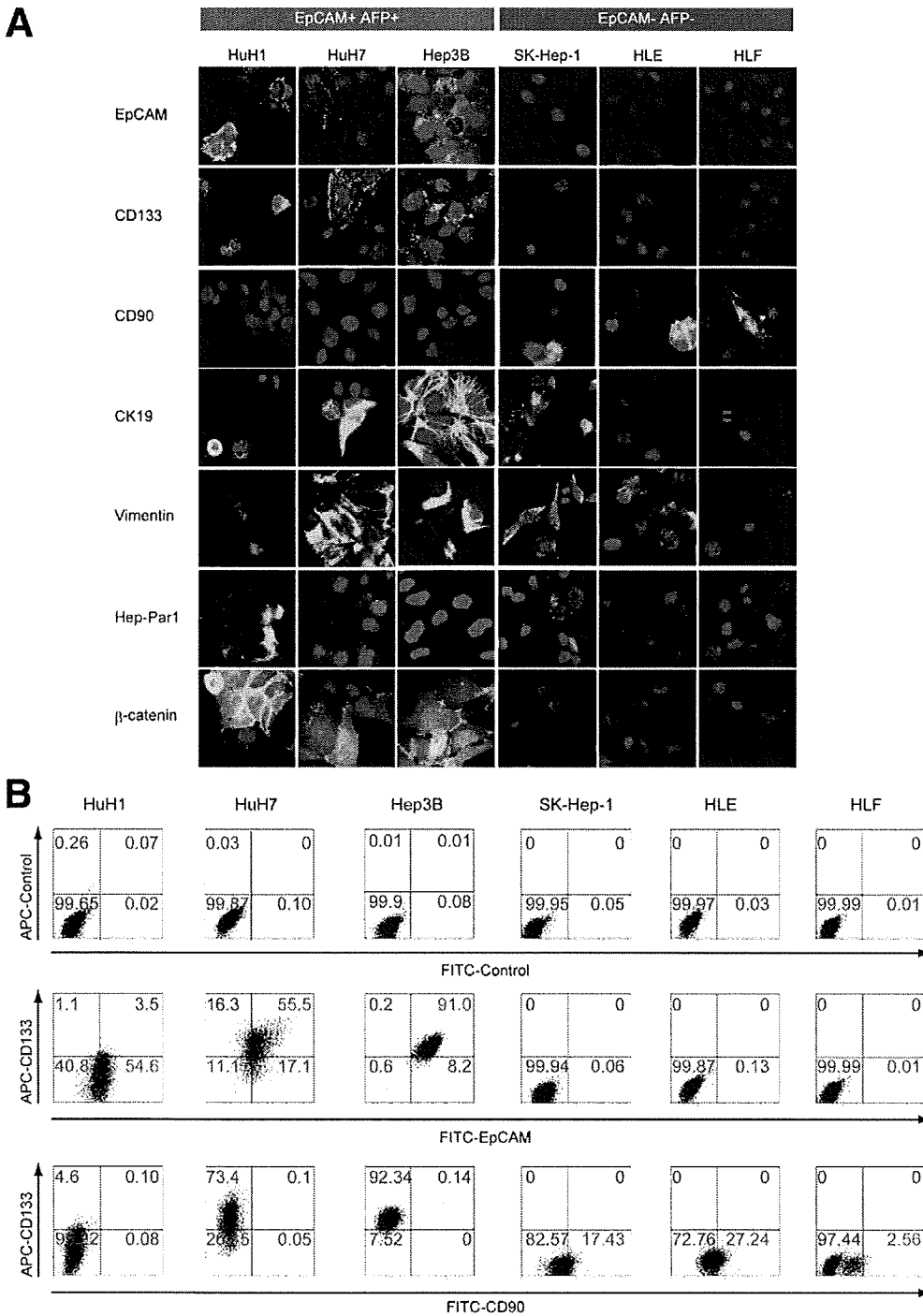
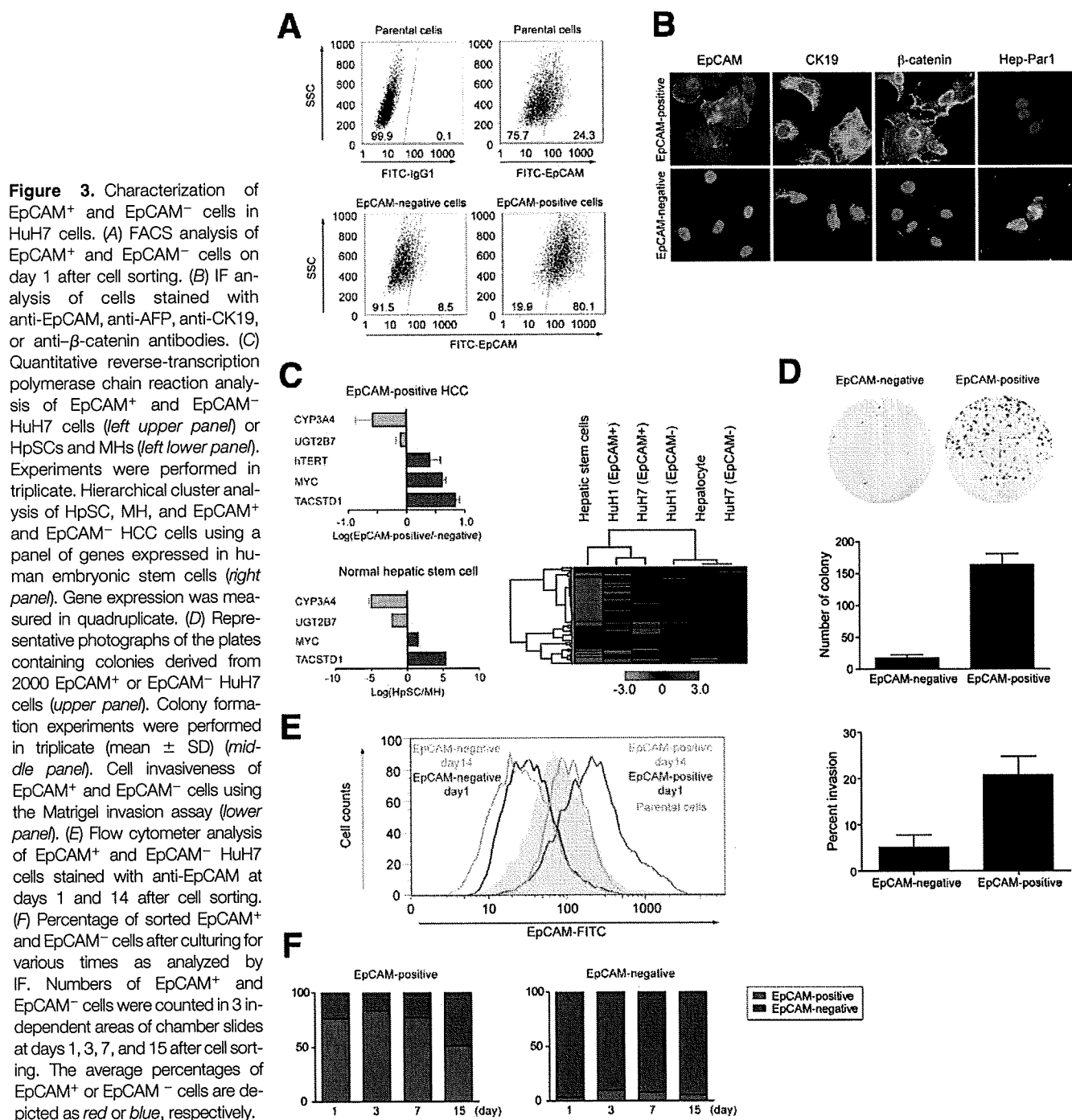


Figure 2. Characterization of hepatic stem cell marker expression in HCC cell lines. (A) IF analysis of 6 HCC cell lines (EpCAM⁺ AFP⁺ cell lines: HuH1, HuH7, and Hep3B; EpCAM⁻ AFP⁻ cell lines: SK-Hep-1, HLE, and HLF) stained with anti-EpCAM, anti-CD133, anti-CD90, anti-CK19, anti-Vimentin, anti-Hep-Par1, and anti-β-catenin antibodies. (B) FACS analysis of 6 HCC cell lines stained with anti-EpCAM, anti-CD133, and anti-CD90 antibodies.

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(known mature hepatocyte markers) were significantly higher in EpCAM⁻ HuH7 cells (Figure 3C, left upper panel). This expression pattern was reminiscent of human HpSC cells (Figure 3C, left lower panel). Similar results were obtained from HuH1 cells (data not shown). We also compared gene expression patterns of isolated HuH1, HuH7, MH, and HpSC cells using the TaqMan Human Stem Cell Pluripotency Array (Applied Biosystems, Foster City, CA) containing 96 selected human stem cell-related genes. Although a differential expres-

sion pattern of stem cell-related genes was evident among HpSC, EpCAM⁺ HuH1, and EpCAM⁺ HuH7 cells, the EpCAM⁺ HCC cells were related more closely to HpSC cells whereas EpCAM⁻ HCC cells were related more closely to diploid adult mature hepatocytes (Figure 3C, right panel; and Supplementary Figure 1C; see supplementary material online at www.gastrojournal.org). Thus, it appeared that EpCAM⁺ HCC cells had a gene expression pattern that is related more closely to HpSC than EpCAM⁻ HCC cells.



The isolated EpCAM⁺ HuH7 cells formed colonies efficiently whereas EpCAM⁻ cells failed to do so (Figure 3D, upper and middle panels; and Supplementary Figure 2A for HuH1 cells; see supplementary material online at www.gastrojournal.org). In addition, EpCAM⁺ HuH7 cells were much more invasive than EpCAM⁻ cells ($P < .03$) (Figure 3D, lower panel; and Supplementary Figure 2B for HuH1 cells; see supplementary material online at www.gastrojournal.org). The EpCAM⁺ fraction decreased with time in sorted EpCAM⁺ HuH7 cells from greater than 80% to 50% (Figure 3E). However, a small percentage

of EpCAM⁺ cells remained constant in sorted EpCAM⁻ HuH7 cells. FACS analysis confirmed the results of IF analysis (Figure 3F and Supplementary Figure 2C for HuH7 and HuH1 cells, respectively; see supplementary material online at www.gastrojournal.org), suggesting that EpCAM⁺ cells could differentiate into EpCAM⁻ cells, eventually allowing an enriched EpCAM⁺ fraction to revert back to parental cells after 14 days of culture. In contrast, EpCAM⁻ cells maintained their EpCAM⁻ status. In addition, we successfully isolated 12 HuH1 and 2 HuH7 colonies from 192 single-cell-plated culture wells.

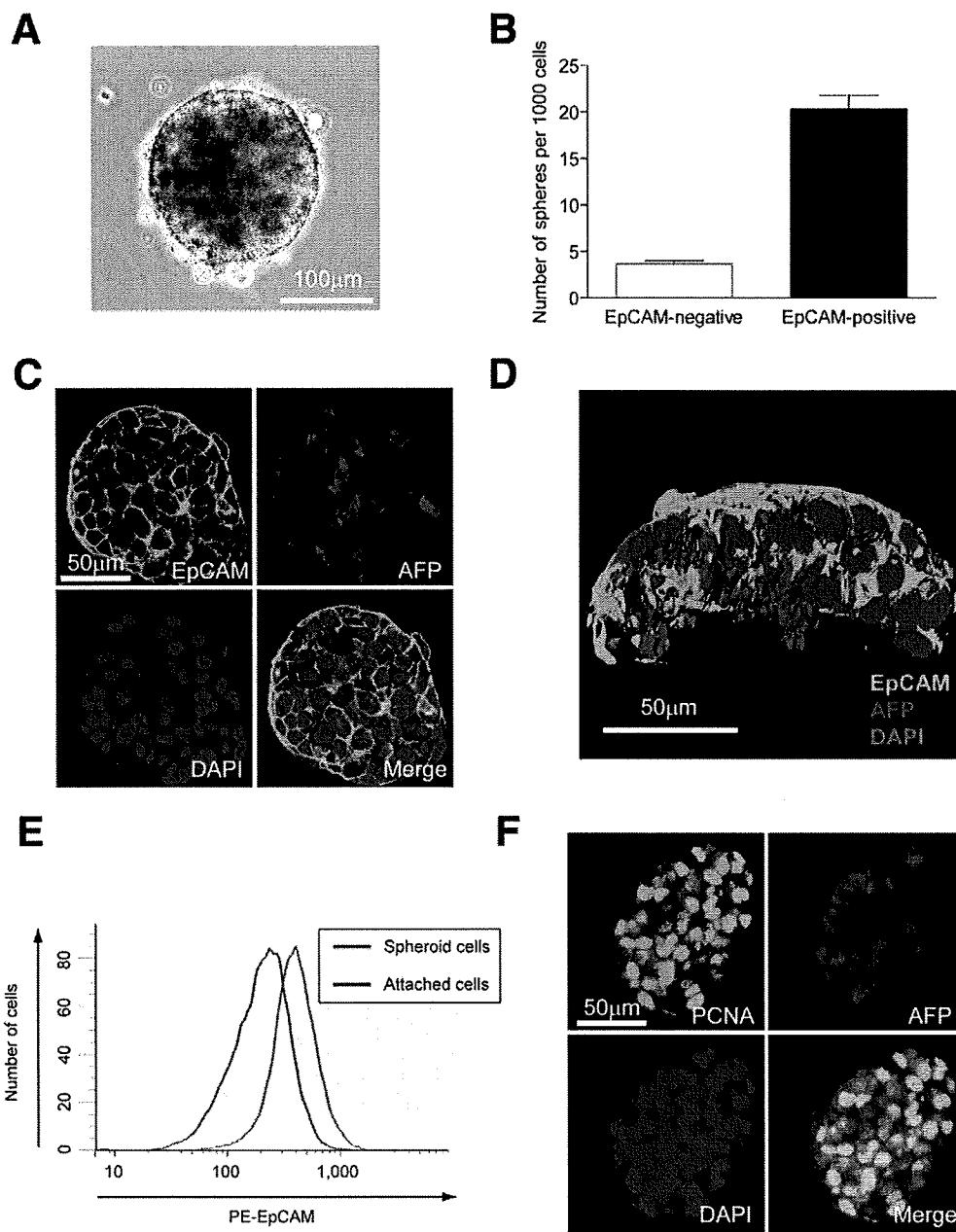


Figure 4. Spheroid formation of EpCAM⁺ HuH1 HCC cells. (A) A representative phase-contrast image of an HCC spheroid derived from an EpCAM⁺ cell (scale bar, 100 μ m) and (B) total numbers of spheroids from 1000 sorted cells are shown. Experiments were performed in triplicate and data are shown as mean \pm SD. (C) Representative confocal images of an HCC spheroid co-stained with anti-EpCAM, anti-AFP, and 4',6-diamidino-2-phenylindole (DAPI) (scale bar, 50 μ m). (D) A 3-dimensional image of an HCC spheroid co-stained with anti-EpCAM, anti-AFP, and DAPI (scale bar, 50 μ m) reconstructed from confocal images using surface rendering. (E) FACS analysis of EpCAM⁺ cells cultured as spheroid cells (red) or attached cells (blue) for 14 days after cell sorting. (F) Confocal images of an HCC spheroid co-stained with anti-PCNA, anti-AFP, and DAPI (scale bar, 50 μ m).

However, all colonies were heterogeneous in EpCAM and AFP expression and no colony was completely EpCAM⁻ (data not shown). Taken together, these results indicate that EpCAM⁺ HCC cells resemble HpSC features. It appears that EpCAM⁺ cells, but not EpCAM⁻ cells, have self-renewal and differentiation capabilities with the ability to form colonies from a single cell, and produce both EpCAM⁺ and EpCAM⁻ cells.

It has been shown previously that stem/progenitor cells and cancer stem/progenitor cells can form spheroids in vitro in a nonattached condition.^{36,37} Consistently, EpCAM⁺ cells could form spheroids efficiently, reaching to about 150 to approximately 200 μ m in diameter after 14 days of culture (Figure 4A and B). Interestingly, all cells in a spheroid were EpCAM⁺, whereas AFP expres-

sion was relatively heterogeneous (Figure 4C and D, and Supplementary movie 1; see supplementary material online at www.gastrojournal.org). Rarely, a few spheroids derived from an EpCAM⁻ cell fraction were positive for EpCAM (data not shown), suggesting that these spheroids were derived from contaminated residual EpCAM⁺ cells by FACS sorting. All spheroid cells maintained EpCAM expression while half of the attached cells lost EpCAM expression when the EpCAM⁺ fraction was cultured for 14 days (Figure 4E). Most spheroid cells also abundantly expressed proliferating cell nuclear antigen (PCNA), implying active cell proliferation (Figure 4F and Supplementary movie 2; see supplementary material online at www.gastrojournal.org). Thus, a subset of EpCAM⁺ cells, but not EpCAM⁻ cells, can form spheroids.

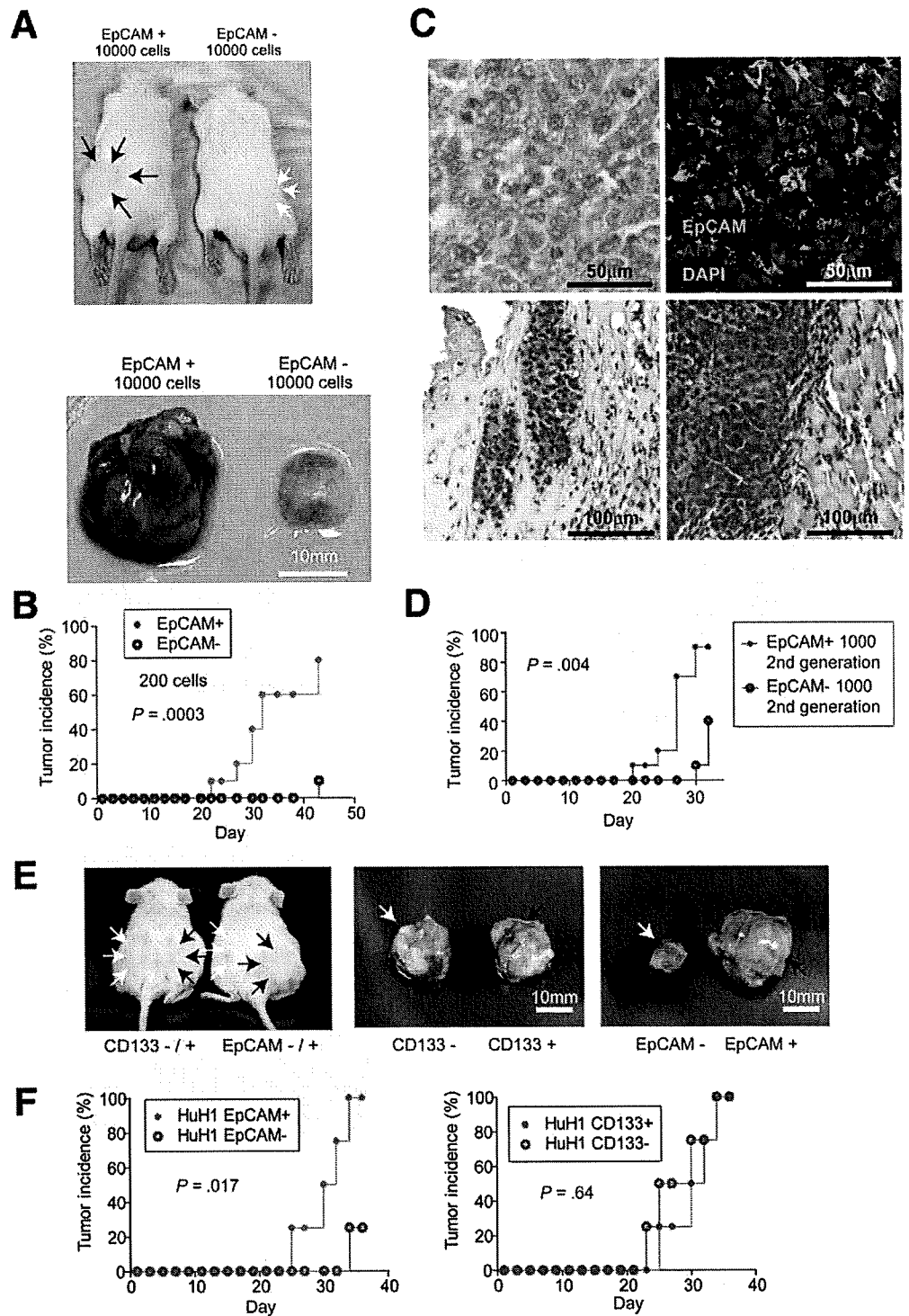


Figure 5. Tumorigenic and invasive potential of EpCAM⁺ HCC cells. (A) Representative NOD/SCID mice (upper panel) with subcutaneous tumors (lower panel) from EpCAM⁺ (black arrows) or EpCAM⁻ (white arrows) HuH1 cells. (B) Tumorigenicity of 200 sorted HuH1 cells. (C) Histologic analysis of EpCAM⁺ HuH1-derived xenografts. H&E staining of a subcutaneous tumor (left upper panel) with capsular invasion (left lower panel) and muscular invasion (right lower panel) and IF of the tumor stained with anti-EpCAM, anti-AFP, and 4',6-diamidino-2-phenylindole (DAPI) (right upper panel) (scale bar, 50 µm). (D) Tumorigenicity of 1000 sorted cells derived from an EpCAM⁺ HuH1 xenograft. Data are generated from 10 mice in each group. (E) Representative NOD/SCID mice (left panel) with subcutaneous tumors from CD133⁺ (black arrows) or CD133⁻ (white arrows) (middle panel) and EpCAM⁺ (black arrows) or EpCAM⁻ (white arrows) (right panel) HuH1 cells. (F) Tumorigenicity of 1000 HuH1 cells sorted by anti-EpCAM (left panel) or anti-CD133 (right panel) antibodies.

EpCAM⁺ HCC Cells as Tumor-Initiating Cells

EpCAM⁺ HCC cells, but not EpCAM⁻ HCC cells, could efficiently initiate invasive tumors in NOD/SCID mice (Figure 5). For example, 10,000 EpCAM⁺ HuH1 cells produced large hypervascular tumors in 100% of mice whereas EpCAM⁻ cell fractions produced only small and pale-looking tumors in 30% of mice 4 weeks after injection (Figure 5A and Supplementary Figure 3A; see supplement-

ary material online at www.gastrojournal.org). Similar results were obtained with HuH7 cells (Supplementary Figure 3B-D; see supplementary material online at www.gastrojournal.org). As little as 200 EpCAM⁺ cells could initiate tumors in 8 of 10 injected mice, whereas 200 EpCAM⁻ cells produced only 1 tumor among 10 injected mice at 6 weeks after transplantation, and the tumor sizes were much larger in the EpCAM⁺ cells than in the EpCAM⁻

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cells (Figure 5B and Supplementary Figure 3E; see supplementary material online at www.gastrojournal.org). EpCAM⁺ cells produced tumors with a mixture of both EpCAM⁺ and EpCAM⁻ cells in xenografts, and these cells invaded in the capsule and muscles of the leg adjacent to the tumor (Figure 5C). EpCAM⁺ cells derived from tumors again maintained their tumor-initiating capacity, tumor morphology, and invasive ability in an *in vivo* serial transplantation experiment (Figure 5D). Occasionally, EpCAM⁻ cell fractions produced a few small tumors that always contained a mixture of EpCAM⁺ and EpCAM⁻ cells (data not shown), indicating that the contaminated EpCAM⁺ cells from FACS sorting contribute to the tumor-initiating ability.

To further validate whether EpCAM⁺ HCC cells were tumor-initiating cells, we isolated EpCAM⁺ HCC cells from 2 cases of AFP⁺ (>600 ng/mL serum AFP) HCC clinical specimens using MACS. Consistently, 1×10^4 EpCAM⁺ cells could induce tumors in NOD/SCID mice, but up to 1×10^6 EpCAM⁻ cells failed to do so (Table 1). In addition, similar to HCC cell lines, fresh EpCAM⁺ tumor cells from 2 clinical HCC specimens were more efficient in forming spheroids *in vitro* than EpCAM⁻ cells (Supplementary Figure 4; see supplementary material online at www.gastrojournal.org).

FACS analysis results indicate that a majority of EpCAM⁺ cells express CD133 in HuH7 cells but not in HuH1 cells (Figure 2B), which prompted us to compare the tumorigenic capacity of EpCAM⁺ and CD133⁺ cells in these cell lines. Noticeably, EpCAM⁺ HuH1 cells showed marked tumor-initiating capacity compared with CD133⁺ HuH1 cells (Figure 5E and F), whereas EpCAM⁺ and CD133⁺ cells had similar tumorigenic ability in HuH7 cells (data not shown).

GSK-3 β Inhibition Augments EpCAM⁺ HCC Cells

To determine the role of Wnt/ β -catenin signaling²⁸ in EpCAM⁺ HCC cells (Figure 1B), we first treated

HuH1, HuH7, and HLF cells with a GSK-3 β inhibitor BIO (Figure 6A), which activates Wnt/ β -catenin signaling (Figure 6B) and maintains undifferentiation of embryonic stem cells.³⁸ 6-bromindirubin-3'-oxime (BIO) increased the EpCAM⁺ cell population in HuH1 and HuH7 cells when compared with the control methylated BIO (MeBIO) (Figure 6A). In contrast, BIO had no effect on the CD90⁺ cell population, which is more tumorigenic than the CD90⁻ cell population in HLF (Figure 6A and data not shown). Enrichment of EpCAM⁺ cells was provoked further by the treatment of Wnt10B-conditioned media in HuH7 cells (Figure 6C).³⁴ BIO induced morphologic alteration of HuH7 cells because most cells became small and round when compared with MeBIO and suppressed EpCAM⁻ AFP⁻ cell populations (Figure 6D). Moreover, BIO induced *TACSTD1*, *MYC*, and *hTERT* expression and spheroid formation (Figure 6E and F).

EpCAM Blockage by RNA Interference

One of the hallmarks of CSCs is its resistance to conventional chemotherapeutic agents resulting in tumor relapse and thus targeting CSCs is critical to achieve successful tumor remission. Consistently, 5-FU could increase the EpCAM⁺ population and spheroid formation of HuH1 and HuH7 cells (Figure 7A and B) (data not shown), suggesting a differential sensitivity of EpCAM⁺ and EpCAM⁻ HCC cells to 5-FU. In contrast, EpCAM blockage via RNA interference dramatically decreased the population of EpCAM⁺ cells (Figure 7C), and significantly inhibited cellular invasion, spheroid formation, and tumorigenicity of HuH1 cells (Figure 7D–F). Thus, EpCAM may serve as a molecular target to eliminate HCC cells with stem/progenitor cell features.

Discussion

The cellular origin of HCC is currently in debate. In this study, we found that EpCAM can serve as a marker to enrich HCC cells with tumor-initiating ability and with some stem/progenitor cell traits. EpCAM is expressed in many human cancers with an epithelial origin.³⁹ During embryogenesis, EpCAM is expressed in fertilized oocytes, embryonic stem cells, and embryoid bodies, suggesting its role in early stage embryogenesis.⁴⁰ Furthermore, a recent article indicated that EpCAM is expressed in colonic and breast CSCs.⁴¹ Taken together, these data suggest a critical role of EpCAM in CSCs as well as embryonic and somatic stem cells. Consistently, we found that EpCAM expression is regulated by Wnt/ β -catenin signaling²⁹ and tumorigenic and highly invasive HpSC-HCC is orchestrated by a subset of cells expressing EpCAM and AFP with stem cell-like features and self-renewal and differentiation capabilities regulated by Wnt/ β -catenin signaling (this study). Thus, EpCAM may be a common gene expressed in undifferentiated normal cells and HCCs with activated Wnt/ β -catenin signaling. It may act as a downstream molecule

Table 1. The Tumor-Initiating Capacity of EpCAM⁺ Cells From Clinical HCC Specimens

HCC patients			Tumor incidence (mice with tumors/ total no. of mice injected)		
No.	% of EpCAM ⁺ HCC cells	Groups	No. of cells injected	2 months	3 months
1	5.2	EpCAM ⁺	1×10^3	0/3	0/3
			1×10^4	2/3	2/3
		EpCAM ⁻	1×10^5	2/2	2/2
			1×10^6	0/3	0/3
			1×10^6	0/2	0/2
2	1.4	EpCAM ⁺	1×10^3	0/2	0/2
			1×10^4	0/1	1/1
		EpCAM ⁻	1×10^4	0/3	0/3
			1×10^5	0/2	0/2

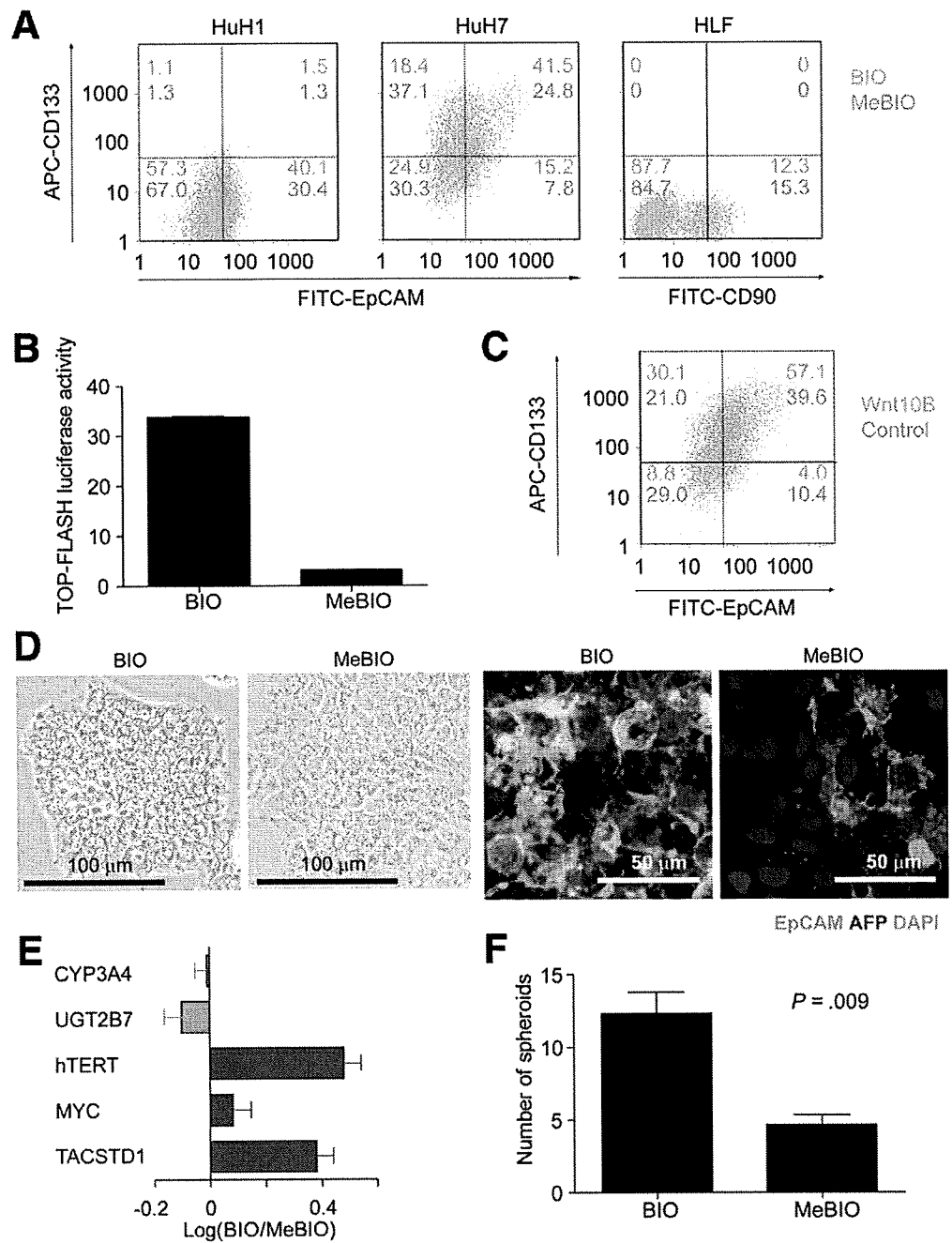


Figure 6. Wnt/ β -catenin signaling augments EpCAM⁺ HCC cells. (A) Flow cytometer analysis of HuH1, HuH7, and HLF cells treated with 2 μ mol/L of BIO (orange) or MeBIO (green) for 10 days and stained with anti-EpCAM, anti-CD133 and anti-CD90 antibodies. (B) TOP-FLASH luciferase assays of HuH7 cells treated with 2 μ mol/L of BIO or MeBIO. (C) Flow cytometer analysis of HuH7 cells cultured in normal media (Dulbecco's modified Eagle medium supplemented with 10% FBS) or Wnt10B conditioned media (details are described in the Materials and Methods section). Cells were cultured in each medium for 2 weeks. (D) Representative phase-contrast images (left panel: scale bar, 100 μ m) or IF images (right panel: scale bar, 50 μ m) of HuH7 cells treated with 2 μ mol/L of BIO or MeBIO for 14 days. (E) Quantitative reverse transcription-polymerase chain reaction analysis of representative HCC-related genes in HuH7 cells treated with 2 μ mol/L of BIO or MeBIO for 14 days. (F) Spheroid formation assay of HuH7 cells treated with 2 μ mol/L of BIO or MeBIO for 14 days (mean \pm SD). FITC, fluorescein isothiocyanate.

to maintain HCC stemness and serve as a good marker for HCC initiating cells.

CD133 or CD90 have been used to identify potential hepatic CSCs.^{35,42} CD133 is expressed in normal and malignant stem cells of the neural, hematopoietic, epithelial, hepatic, and endothelial lineages,^{23,43,44} suggesting that CD133 is also a common marker to detect normal cells and CSCs. Captivatingly, EpCAM expression overlaps with CD133 expression in normal human colon tissues and colorectal cancer tissues, yet CD133⁺ and CD133⁻ cells are equally tumorigenic.⁴⁵ Similarly, we found that EpCAM⁺ and EpCAM⁻ HuH1 cells equally expressed CD133, but only EpCAM⁺ cells de-

veloped large hypervascular tumors. Our data suggest that EpCAM may be a better marker than CD133 to enrich HCC tumor-initiating cells from AFP⁺ tumors. We also found that CD90 expression was limited to HCC cell lines that are EpCAM⁻ AFP⁻, and Wnt/ β -catenin signaling had little effect on CD90⁺ cell enrichment. These results suggest that the expression patterns of various stem cell markers in tumor-initiating cells with stem/progenitor cell features may be different in each HCC subtype, possibly owing to the heterogeneity of activated signaling pathways in normal stem/progenitor cells where these tumor-initiating cells may originate. Therefore, it would be useful to

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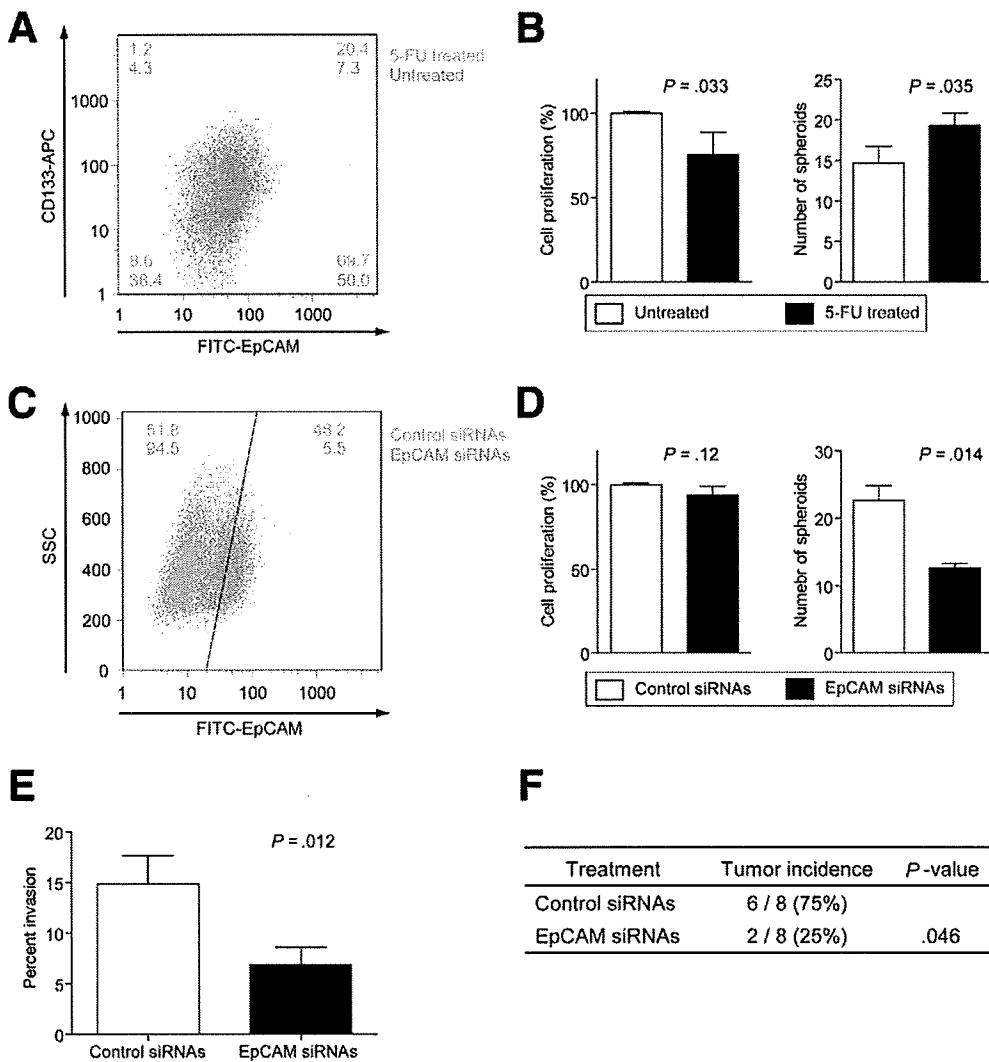


Figure 7. EpCAM blockage inhibits the tumorigenic and invasive capacity of EpCAM⁺ HCC cells. (A) Enrichment of EpCAM⁺ cells after 5-FU treatment. HuH1 cells refer as control or without treatment (green) or treated with 2 μ g/mL of 5-FU (orange) for 3 days and analyzed by FACS using anti-EpCAM and anti-CD133 antibodies. (B) Spheroid formation of HuH1 cells treated with 2 μ g/mL of 5-FU for 3 days. (C) FACS analysis of HuH1 cells treated with a control siRNA (orange) or EpCAM-specific siRNA (green) at day 3 after transfection. (D) Spheroid formation or (E) invasive capacity of EpCAM⁺ HuH1 cells transfected with a control siRNA or EpCAM-specific siRNA. Experiments were performed in triplicate and the data are shown as mean \pm SD. (D) siRNAs. (F) Inhibition of tumor formation in vivo by EpCAM gene silencing. EpCAM⁺ HuH1 cells were transfected with siRNA oligos and 1000 cells were injected 24 hours after transfection.

comprehensively investigate the expression patterns of stem cell markers to characterize the population of CSCs that may correlate with the activation of their distinct molecular pathways.

CSCs may be more resistant to chemotherapeutic agents than differentiated tumor cells possibly owing to an increased expression of adenosine triphosphate-binding cassette transporters and anti-apoptotic proteins.⁴ Thus, the development of an effective strategy to target CSC pools together with conventional chemotherapies is essential to eradicate a tumor mass.¹⁴ By blocking the programs that activate self-renewal and/or inhibit asymmetric division, CSC features could be destemmed.^{46,47} Consistently, EpCAM blockage could inhibit cellular invasion and tumorigenicity of EpCAM⁺ HCC cells, revealing the feasibility of targeting a CSC marker to destem CSC features. EpCAM may induce c-Myc,⁴⁸ a common molecular node activated in HpSC-HCC.²⁷ c-Myc, together with Oct3/4, Sox2, and Klf4, can induce pluripotent stem cells from adult fibroblasts.⁴⁹ It is possible that EpCAM blockage to inhibit hepatic CSCs may

result in a suppression of c-Myc signaling. Encouragingly, EpCAM-specific antibodies are currently in phase II clinical trials.⁵⁰ Furthermore, a recent study indicated that EpCAM⁺ circulating tumor cells identified by a unique microfluidic platform can be used to monitor outcomes of patients undergoing systemic treatment.⁵¹ Therefore, it may be useful to combine EpCAM antibodies with conventional chemotherapy to target both CSCs and non-CSCs for the treatment of HCC.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.12.004.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

FACS and MACS Analyses

Cultured cells were trypsinized, washed, and re-suspended in Hank's balanced salt solutions (Lonza, Basel, Switzerland) supplemented with 1% HEPES and 2% fetal bovine serum. Cells then were incubated with FITC-conjugated anti-EpCAM monoclonal antibody Clone Ber-EP4 (DAKO, Carpinteria, CA) on ice for 30 minutes, and EpCAM⁺ and EpCAM⁻ cells were isolated by a BD FACSAria cell sorting system (BD Biosciences). For magnetic separation, cells were labeled 24 hours after enzymatic dissociation with primary EpCAM antibody (mouse IgG1; Dako), subsequently magnetically labeled with rat anti-mouse IgG1 Microbeads, and separated on a MACS LS column (Miltenyi Biotec, Inc, Auburn, CA). All the procedures were performed according to the manufacturer's instructions. The purity of sorted cells was evaluated by FACS. Fixed cells also were analyzed by FACS using a FACSCalibur (BD Biosciences). Anti-EpCAM antibody VU-1D9, anti-CD133/2 clone 293C3 (Miltenyi Biotec Inc), and anti-CD90 clone 5E10 (Stem-Cell Technologies Inc, Vancouver, British Columbia, Canada) were used to detect EpCAM⁺, CD133⁺, or CD90⁺ cells. Intracellular AFP levels were examined by a BD Cytotfix/Cytoperm Fixation/Permeabilization Kit (San

Jose, CA) and anti-AFP rabbit polyclonal antibody (DAKO).

Quantitative Reverse Transcription-Polymerase Chain Reaction and IHC Analyses

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The expression of selected genes was determined in triplicate using the Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described.¹ Genes expressed in embryonic stem cells were determined in quadruplicate using TaqMan Human Stem Cell Pluripotency Array (Applied Biosystems). IHC analyses with specific antibodies were performed essentially as previously described.¹ Confocal fluorescence microscopic analysis was performed essentially as previously described.²

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Supplementary Table 1. Clinicopathologic Characteristics of HpSC-HCC and MH-HCC Cases Used for Oligonucleotide Microarray Analyses

Parameters	HpSC-HCC (n = 60)	MH-HCC (n = 96)	P value ^a
Mean age, y (SD)	46.0 ± 10.7	52.9 ± 10.5	.0004
Sex: male/female	50/10	87/9	.18
Cirrhosis: yes/no/no data	56/4	88/7/1	.72
Median AFP level, ng/mL (25%–75%)	1706 (865–5915)	11.8 (4.0–48.6)	<.0001
Histologic grade ^b			
I–II	14	41	
II–III	44	48	
III–IV	2	5	
No data	0	2	.031
Mean tumor size, cm (SD)	5.1 ± 3.0	4.4 ± 3.0	.088
Multinodular: yes/no	16/44	15/81	.09
Portal vein invasion, yes/no ^c	11/49	9/87	.10
TNM classification			
I	24	46	
II	22	42	
III	14	8	.03
Virus status: HBV/HBV + HCV/unknown	56/4/0	95/0/1	.43

^aMann–Whitney *U* test or χ^2 test.^bEdmondson–Steiner.^cMacroscopic portal vein invasion.**Supplementary Table 2.** Clinicopathologic Characteristics of HpSC-HCC and MH-HCC Cases Used for IHC

Parameters	HpSC-HCC (n = 24)	MH-HCC (n = 55)	P value ^a
Mean age, y (SD)	46.4 ± 9.4	58.4 ± 11.9	<.0001
Sex: male/female	20/4	48/7	.64
Cirrhosis: yes/no	23/1	46/9	.14
Median AFP level, ng/mL (25%–75%)	1620 (887–3166)	12 (9.3–21.9)	<.0001
Histologic grade ^b			
I–II	12	32	
II–III	8	21	
III–IV	4	2	.13
Mean tumor size, cm (SD)	7.1 ± 3.6	5.2 ± 3.6	.014
Multinodular: yes/no	4/20	16/39	.24
Portal vein invasion: yes/no ^c	12/12	12/43	.012
TNM classification			
I	4	19	
II	8	20	
III	12	16	.14
Virus status: HBV/HCV/unknown	21/2/1	32/21/2	.026

^aMann–Whitney *U* test or χ^2 test.^bEdmondson–Steiner.^cMacroscopic portal vein invasion.

Supplementary Table 3. Top 10 List of Canonical Pathways Activated in HpSC-HCC From Ingenuity Pathway Analysis

Pathways	Genes included in cluster A
Axonal guidance signaling	
Up	ROBO2, ARPC5L (includes EG:81873), SEMA4G, PDGFRB, PLCB1, PRKCD, FGFR3, FZD5, MERTK, DDR1, LINGO1, SEMA3C
Down	PIK3C3, IGF1, PIK3C2G, MAP2K2, ARHGEF15
Transforming growth factor- β signaling	
Up	PDGFRB, FGFR3, MERTK, UBD, DDR1, SMAD5
Down	MAP2K2, HNF4A
Integrin signaling	
Up	ARPC5L (includes EG:81873), PDGFRB, FGFR3, GRB7, MERTK, ITGB5, DDR1, DDEF1
Down	PIK3C3, MYLK, PIK3C2G, MAP2K2
Apoptosis signaling	
Up	PDGFRB, BAK1, CYCS, FGFR3, MERTK, DDR1
Down	MAP3K5, MAP2K2
G2/M DNA damage checkpoint regulation	
Up	YWHAZ, CCNB2, UBD, WEE1
Down	CDKN2A, GADD45A
ERK/MAPK signaling	
Up	ELF3, PDGFRB, YWHAZ, PRKCD, FGFR3, MERTK, DDR1
Down	PIK3C3, DUSP1, PIK3C2G, ESR1, MAP2K2
Wnt/ β -catenin signaling	
Up	DKK1, SOX9, FZD5, UBD, TCF7L2, CSNK1E
Down	CDKN2A, RARG
PI3K/AKT signaling	
Up	PDGFRB, YWHAZ, FGFR3, MERTK, DDR1
Down	MAP3K5, MAP2K2, GYS2
Amyloid processing	
Up	BACE2, CSNK1E, MAPK13
Down	
Leukocyte extravasation signaling	
Up	PRKCD, CLDN4, CLDN1, MMP11, MAPK13
Down	PIK3C3, CLDN2, PIK3C2G, MAP2K2

NOTE. The top 10 pathways were selected based on the significance for the enrichment of the genes with a particular canonical signaling pathway determined by the one-sided Fisher exact test ($P < .01$).

Supplementary Table 4. Top 10 List of Canonical Pathways Activated in MH-HCC From Ingenuity Pathway Analysis

Pathways	Genes included in cluster B
Lipopolysaccharide/interleukin-1-mediated inhibition of RXR function	
Up	SULT1C2, ACSL4, ACSL3, FABP5, GSTP1
Down	NR1I2, NR1I3, CYP7A1, ALDH1L1, ABCB1, SLC10A1, SLC27A2, CD14, GSTM1, ALDH6A1, GSTM4, ACSL5, CES2 (includes EG:8824), FMO3, SULT2A1 (includes EG:6822), GSTA1, CYP2C8, LC27A5, CYP3A7, ABCG5, ALDH8A1, APOC4 (includes EG:346), CYP3A4, ACSL1, ABCB11, FMO4, MAOA
Xenobiotic metabolism signaling	
Up	SULT1C2, PRKCD, GSTP1, MAPK13
Down	NR1I2, NR1I3, ALDH1L1, ABCB1, UGT2B15, MAP2K2, UGT2B7, PPARGC1A, GSTM1, PIK3C3, ALDH6A1, GSTM4, CES2 (includes EG:8824), MAP3K5, FMO3, PIK3C2G, SULT2A1 (includes EG:6822), CYP1A2, GSTA1, CYP2C8, CYP3A7, NQO2, ALDH8A1, CYP3A4, CES1 (includes EG:1066), FMO4, MAOA
Hepatic cholestasis	
Up	ADCY3, PRKCD
Down	CD14, ABCG5, NR1I2, CYP7A1, CYP7B, CYP8B1, ABCB1, ESR1, SLC10A1, ABCB11, ABCB4, HNF4A
Aryl hydrocarbon receptor signaling	
Up	GSTP1
Down	CDKN2A, NQO2, GSTM1, ALDH8A1, ALDH6A1, ALDH1L1, GSTM4, ESR1, CYP1A2, GSTA1, RARG
NRF2-mediated oxidative stress response	
Up	DNAJA4, PRKCD, GSTP1
Down	NQO2, GSTM1, AOX1, PIK3C3, GSTM4, MAP3K5, SOD1, PIK3C2G, MAP2K2, FKBP5, GSTA1
Complement system	
Up	
Down	C8A, C1R, MASP1, C6, C8B, MASP2
Coagulation system	
Up	
Down	SERPINC1, KLKB1, F9, KNG1 (includes EG:3827), F11
Acute-phase response signaling	
Up	MAPK13
Down	APCS, RBP5, C1R, MAP3K5, HRG, MAP2K2, KLKB1, SAA4
p53 signaling	
Up	THBS1
Down	CDKN2A, PIK3C3, SNAI2, GADD45A, PIK3C2G, GADD45B
LXR/RXR activation	
Up	HMGCR
Down	CD14, ABCG5, APOA5, CYP7A1, APOC4 (includes EG:346)

LXR/RXR, liver X receptor/retinoid X receptor; NRF2, NF-E2-related factor 2.

NOTE. The top 10 pathways were selected based on the significance for the enrichment of the genes with a particular canonical signaling pathway determined by the one-sided Fisher exact test ($P < .01$).

Activation of lipogenic pathway correlates with cell proliferation and poor prognosis in hepatocellular carcinoma[☆]

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Background/Aims: Metabolic dysregulation is one of the risk factors for the development of hepatocellular carcinoma (HCC). We investigated the activated metabolic pathway in HCC to identify its role in HCC growth and mortality.

Methods: Gene expression profiles of HCC tissues and non-cancerous liver tissues were obtained by serial analysis of gene expression. Pathway analysis was performed to characterize the metabolic pathway activated in HCC. Suppression of the activated pathway by RNA interference was used to evaluate its role in HCC *in vitro*. Relation of the pathway activation and prognosis was statistically examined.

Results: A total of 289 transcripts were up- or down-regulated in HCC compared with non-cancerous liver ($P < 0.005$). Pathway analysis revealed that the lipogenic pathway regulated by sterol regulatory element binding factor 1 (*SREBF1*) was activated in HCC, which was validated by real-time RT-PCR. Suppression of *SREBF1* induced growth arrest and apoptosis whereas overexpression of *SREBF1* enhanced cell proliferation in human HCC cell lines. *SREBF1* protein expression was evaluated in 54 HCC samples by immunohistochemistry, and Kaplan–Meier survival analysis indicated that *SREBF1*-high HCC correlated with high mortality.

Conclusions: The lipogenic pathway is activated in a subset of HCC and contributes to cell proliferation and prognosis.

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Keywords: Hepatocellular carcinoma; Serial analysis of gene expression; Lipogenesis; Gene expression profiling; Sterol regulatory element binding factor 1

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Abbreviations: HCC, hepatocellular carcinoma; *SREBF1*, sterol regulatory element binding factor 1; HBV, hepatitis B virus; HCV, hepatitis C virus; SAGE, serial analysis of gene expression; RT-PCR, reverse transcription-polymerase chain reaction; IHC, immunohistochemistry; FADS1, fatty acid desaturase 1; SCD, stearoyl CoA desaturase; FASN, fatty acid synthase; si-RNA, short interfering-RNA; CLD, chronic liver disease; PCNA, proliferating cell nuclear antigen; IGF, insulin-like growth factor.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently occurring malignancies in the world [1]. The major risk factors associated with HCC include chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol abuse, and exposure to aflatoxin B1 [2]. HCC usually develops from liver cirrhosis, which involves continuous inflammation and hepatocyte regeneration, suggesting that reactive oxygen species and DNA damage are involved in the process of hepatocarcinogenesis [3].

The development of gene expression profiling technologies including DNA microarrays and serial analysis

of gene expression (SAGE) have enhanced our ability to identify inventory transcripts and global genetic alterations in HCC [4–10]. In general, these methods have demonstrated that transcripts associated with cell growth are up-regulated, whereas those related to inhibition of cell growth are down-regulated, in HCC [11]. It is difficult, however, to decipher molecular pathways activated during hepatocarcinogenesis.

Epidemiological studies suggest that metabolic dysregulation in the liver increases the risk of HCC development. For example, diabetes is associated with a 2-fold increase in the risk of HCC [12]. Obesity and hepatic steatosis also increase the risk of HCC [13–15]. Furthermore, recent studies indicate that HCV infection provokes hepatic steatosis, which may be a vulnerable factor for liver inflammation and HCC development [16,17]. Thus, dysregulation of a metabolic pathway may play a crucial role to promote HCC growth, but the molecular mechanism is still obscure. In this study, we have utilized SAGE [18,19], which enables us to monitor the differential expression of all genes, to determine the global changes in gene expression that occur during hepatocarcinogenesis.

2. Materials and methods

2.1. Tissue samples

All HCC tissues, adjacent non-cancerous liver tissues, and normal liver tissues were obtained from 69 patients who underwent hepatectomy from 1997 to 2005 in Kanazawa University Hospital. Normal liver tissue samples were obtained from patients undergoing surgical resection of the liver for treatment of metastatic colon cancer. HCC and surrounding non-cancerous liver samples were obtained from patients undergoing surgical resection of the liver for the treatment of HCC. The samples used for SAGE, real-time reverse-transcription (RT)-PCR analysis, and immunohistochemistry (IHC) are listed in Supplemental Table 1. All samples used for SAGE and real-time RT-PCR analysis were snap-frozen in liquid nitrogen. Four normal liver tissues and 20 HCCs and their corresponding non-cancerous liver tissues were used for real-time RT-PCR analysis; seven of these HCC samples, along with 47 additional HCC samples, were formalin-fixed paraffin-embedded and used for IHC. HCC and adjacent non-cancerous liver were histologically characterized as described [20].

All strategies used for gene expression analysis as well as tissue acquisition processes were approved by the Ethics Committee and the Institutional Review Board of Kanazawa University Hospital. All procedures and risks were explained verbally, and each patient provided written informed consent.

2.2. SAGE

Total RNA was purified from each homogenized tissue sample using a ToTally RNA extraction kit (Ambion, Inc., Austin, TX), and polyadenylated RNA was isolated using a MicroPoly (A) Pure kit (Ambion). A total of 2.5 µg mRNA per sample was analyzed by SAGE [18]. SAGE libraries were randomly sequenced at the Genomic Research Center (Shimadzu-Biotechnology, Kyoto, Japan), and the sequence files were analyzed with SAGE 2000 software. The size of each SAGE library was normalized to 300,000 transcripts per library, and the abundance of transcripts was compared by SAGE 2000 soft-

ware. Monte Carlo simulation was used to select genes with significant differences in expression between two libraries without multiple hypothesis testing correction ($P < 0.005$) [21]. Each SAGE tag was annotated using a gene-mapping web site (<http://www.ncbi.nlm.nih.gov/SAGE/index.cgi>).

2.3. Analysis of signaling networks

Ingenuity Pathways Analysis software (Ingenuity® Systems, www.ingenuity.com) was used to investigate the molecular pathways activated in an HCC SAGE library compared with an adjacent non-cancerous liver SAGE library. All reliable transcripts statistically up-regulated in HCC were investigated and annotated with biological processes, protein-protein interactions, and gene regulatory networks, using a reference-based data file with statistical significance. All identified pathways were screened individually. MetaCore™ software (GeneGo Inc., St. Joseph, MI) was used to evaluate candidate transcription factors responsible for up-regulation of transcripts in HCC.

2.4. RT-PCR

A 1-µg aliquot of each total RNA was reverse-transcribed using SuperScript II reverse-transcriptase (Invitrogen, Carlsbad, CA). Real-time RT-PCR analysis was performed using ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Using the standard curve method, quantitative PCR was performed in triplicate for each sample-primer set. Each sample was normalized relative to β-actin. The assay IDs used were Hs00231674_m1 for sterol regulatory element binding factor 1 (*SREBF1*); Hs00203685_m1 for fatty acid desaturase 1 (*FADS1*); Hs00748952_s1 for stearoyl CoA desaturase (*SCD*); Hs00188012_m1 for fatty acid synthase (*FASN*); and Hs99999_m1 for β-actin. *SREBF1a* and *SREBF1c* mRNA levels were assayed by semi-quantitative RT-PCR [22].

2.5. RNA Interference targeting *SREBF1*

Si-RNAs targeting *SREBF1* were constructed using a *Silencer*™ siRNA Construction kit (Ambion) according to the manufacturer's protocol. We constructed two different si-RNAs, targeting different sites of *SREBF1* (*SREBF1-1*; CAGTGGCACTGACTCTTCC, *SREBF1-2*; TCTACGACCAGTGGGACTG). Control si-RNA duplexes targeting scramble sequences were also synthesized (Dharmacon Research, Inc., Lafayette, CO). Lipofectamine 2000™ reagent (Invitrogen) was used for transfection according to the manufacturer's instructions.

2.6. Cell proliferation assay

Cell proliferation assays were performed using a Cell Titer96 Aqueous kit (Promega, Madison, WI). Results are expressed as the mean optical density (OD) of each five-well set. All experiments were repeated at least twice.

2.7. Soft agar assay

To each well of a six-well plate, containing a base layer of 0.72% agar in growth medium, was added 1×10^4 cells, suspended in 2 ml of 0.36% agar with growth medium (DMEM supplemented with 10% FBS), and the plates were incubated at 37 °C in a 5% CO₂ incubator for 2 weeks. The numbers of colonies in each well were counted as previously described [23].

2.8. TUNEL assay

A DeadEnd™ Colorimetric TUNEL System (Promega) was used to measure nuclear DNA fragmentation as described previously [24].

2.9. Annexin V staining

To evaluate apoptotic cell death, Annexin V binding to cell membranes was evaluated using Annexin V-FITC antibodies and FAC-SCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ), as described by the manufacturer.

2.10. Focus assay

HuH7 cells and Hep3B cells were transiently transfected with pCMV7 or pCMV7-*SREBF1c* vectors (kindly provided by Dr. Hitoshi Shimano) using Lipofectamine 2000™ reagent (Invitrogen), as described by the manufacturer. A total of 2×10^3 cells were seeded on six-well plates 48 h after transfection, and cultured in usual media with 400 ng/ml of Geneticin for 9 days. The foci were fixed with ice-cold 100% methanol and stained with 0.5% crystal violet solution. All experiments were performed in triplicates.

2.11. Western blotting

Whole cell lysates were prepared using RIPA lysis buffer. Antibodies used were rabbit polyclonal antibodies to phospho-GSK-3 β (ser9) (Cell Signaling Technology Inc., Danvers, MA), rabbit anti-sterol regulatory element binding protein-1 (encoded by *SREBF1*) polyclonal antibody H-160 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and β -actin (Sigma-Aldrich Japan K.K., Tokyo, Japan). Immune complexes were visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ) as described in the manufacturer's protocol.

2.12. Immunohistochemistry

Rabbit anti-*SREBF1* polyclonal antibody H-160 (Santa Cruz Biotechnology, Inc.) and mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody PC10 (Calbiochem, San Diego, CA) were used to evaluate the immunoreactivity of HCC samples, using a DAKO EnVision+™ Kit, as described by the manufacturer. The signal intensity of *SREBF1* was scored as negative, low, or high determined by the representative staining of the normal liver tissue and cirrhotic liver tissue (Supplemental Fig. 1). HCC was referred as *SREBF1*-high if *SREBF1* expression in the tumor was higher than that in the cirrhotic liver tissue. PCNA index was evaluated as previously described [25].

2.13. Statistical analysis

Kruskal–Wallis test was used to compare the differentially expressed genes, as shown by real-time PCR, among normal liver, CLD, and HCC tissues. Mann–Whitney U test was also used to evaluate the statistical significance of differences of gene expression between CLD and HCC tissues. Spearman's correlation coefficient was used to assess correlations between the expression levels of *SREBF1*, *FADS1*, *SCD*, and *FASN*. Univariate Cox proportional hazards regression analysis was used to evaluate the association of gene expression and clinicopathologic parameters with patient outcomes. All statistical analyses were performed using SPSS software (SPSS software package; SPSS Inc., Chicago, IL) and GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Gene expression profiling of HCC

We constructed two SAGE libraries from a HCC–HBV tissue and a corresponding non-cancerous tissue (chronic liver disease (CLD)–HBV). We also used two

previously described SAGE libraries, from an HCC–HCV sample and a corresponding non-cancerous tissue sample (CLD–HCV) [4]. After excluding tags detected only once in each library, to avoid the contamination of tags derived from sequence errors, we selected 105,288 tags corresponding to the 9731 genes in all libraries. Using Monte Carlo simulation, we compared the differentially expressed transcripts in HCC and corresponding CLD libraries. Compared with their corresponding CLD libraries, there were statistically significant increases or decreases in 140 transcripts in the HCC–HBV library and in 197 transcripts in the HCC–HCV library ($P < 0.005$).

The HCC–HBV library contained one SAGE tag encoding the HBV–X region, which was increased more than 35-fold compared with its expression in the corresponding CLD–HBV library (Supplemental Table 2). We identified two additional SAGE tags, encoding unknown genes (GTTCTAAAGG, GCATTATGAT), which were expressed more than 10-fold in the HCC–HBV library than in the corresponding CLD–HBV library. The HCC–HBV library also contained tags associated with lipogenesis, at greater than 10-fold abundance, in the HCC–HBV library; these including tags for steroyl-CoA desaturase, fatty acid synthase, and fatty acid desaturase 1.

In contrast, SAGE tags associated with the immune response were up-regulated in the HCC–HCV library. These included tags for Th1-type chemokines, including chemokine ligand 10 (C–X–C motif), chemokine ligand 9 (C–X–C motif), and major histocompatibility complex classes IA and IB (Supplemental Table 3). In addition, tags associated with lipogenesis were increased in the HCC–HCV library, including tags for 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 and cytochrome P450, family 51, subfamily A, polypeptide 1. Taken together, the differential gene expression patterns may exist in HCC–HBV and HCC–HCV. HBV–X and lipogenesis-related genes are activated in HCC–HBV, whereas genes associated with inflammation as well as lipogenesis are activated in HCC–HCV.

3.2. Analysis of molecular pathways activated in HCC

To further characterize the gene expression patterns of HCC–HBV and HCC–HCV, we performed pathway analysis on SAGE data. Using MetaCore™ software, we found that the candidate transcription factors activated were distinct in each HCC library (Table 1). Several of these transcription factors, including NF- κ B, c-Myc, c-Jun, and HNF4- α , have been reported to be activated in HCC [26–29]. In addition, our findings indicated that the transcription factor *SREBF1* may be activated in both HCC–HBV and HCC–HCV (to avoid a confusion, we use HUGO symbol *SREBF1* to indicate both gene/protein name).

Table 1
Candidate transcription factors that regulate molecular pathways activated in HCC.

SAGE library	Transcription factor	Molecular processes	P-value
HCC-HCV	NF- κ B	Antigen presentation	0.004
		Antigen processing	
		Defense response	
		Immune response	
	SREBF1	Cholesterol biosynthesis	0.05
		Lipid biosynthesis	
		β -Glucoside transport	
	SP1	Negative regulation of lipoprotein metabolism	0.05
		Electron transport; drug metabolism	
	IRF1	Oxygen and reactive oxygen species metabolism	0.05
Cell-substrate junction assembly; wound healing			
Immune response			
HCC-HBV	HNF4- α	Antigen presentation; antigen processing	0.002
		Defense response; positive regulation of cell	
	HNF1	Lipid transport	0.01
		Fatty acid metabolism	
		Smooth muscle cell proliferation	
	SP1	Acute-phase response; lipid transport	0.01
		Negative regulation of lipid catabolism	
	c-Jun	β -Glucoside transport	0.03
		Negative regulation of lipoprotein metabolism	
		Zinc ion homeostasis; response to biotic stimulus	
	C/EBP- α	Nitric oxide mediated signal transduction	0.03
		Copper ion homeostasis; fatty acid biosynthesis	
	SREBF1	Progesterone catabolism; progesterone metabolism	0.03
		Regulation of lipid metabolism;	
		Prostaglandin metabolism	
	c-Myc	Lipid transport; negative regulation of lipid catabolism	0.03
		Negative regulation of lipoprotein metabolism	
	USF1	β -Glucoside transport	0.03
		Positive regulation of interleukin-8 biosynthesis	
		Lipid biosynthesis; fatty acid biosynthesis	
PPAR- α	Fatty acid metabolism	0.03	
	Negative regulation of lipid catabolism		
COUP-TFI	Negative regulation of lipoprotein metabolism	0.03	
	Fatty acid biosynthesis; fatty acid metabolism		
C/EBP- β	Fatty acid desaturation;	0.03	
	Activation of pro-apoptotic gene products		
	Release of cytochrome c from mitochondria		
C/EBP- β	Fatty acid metabolism	0.03	
	Smooth muscle cell proliferation		
C/EBP- β	Smooth muscle cell proliferation	0.03	
	Lipid transport		
C/EBP- β	Smooth muscle cell proliferation	0.03	
	Acute-phase response		
	Regulation of interleukin-6 biosynthesis		
C/EBP- β	Fat cell differentiation	0.03	
	Inflammatory response		

These findings were evaluated by other pathway analysis software, Ingenuity Pathways Analysis (IPA). We applied the signaling network analysis to the transcripts up-regulated in the HCC libraries ($P < 0.005$). We found that the top signaling network activated in HCC-HBV contained several pathways involved in ERK/MAPK signaling, PPAR signaling, linoleic acid metabolism, and fatty acid metabolism (Supplemental Fig. 2A). Similarly, pathways involved in interferon signaling, NF- κ B signaling, antigen presentation, PPAR signaling, linoleic

acid metabolism, and fatty acid metabolism were included in the top signaling network activated in HCC-HCV (Supplemental Fig. 2B). Consistent with the results of transcription factor analysis by MetaCoreTM, pathway analysis indicated that SREBF1 participates in the lipogenesis pathway in both HCC-HBV and HCC-HCV (blue nodes in Supplemental Fig. 2A and B). SREBF1, a major regulator of the lipogenesis pathway, binds to sterol regulatory elements on the genome [30], but less is known about its role in