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# EpCAM-Positive Hepatocellular Carcinoma Cells Are Tumor-Initiating Cells With Stem/Progenitor Cell Features

TARO YAMASHITA,* JUNFANG JI,* ANURADHA BUDHU,* MARSHONNA FORGUES,* WEN YANG,‡ HONG-YANG WANG,‡ HULIANG JIA,§ QINGHAI YE,§ LUN-XIU QIN,§ ELAINE WAUTHIER,‖ LOLA M. REID,‖ HIROSHI MINATO,¶ MASAO HONDA,¶ SHUICHI KANEKO,¶ ZHAO-YOU TANG,§ and XIN WEI WANG*

*Liver Carcinogenesis Section, Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland; ‡International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Institute, Shanghai, China; §Liver Cancer Institute and Zhongshan Hospital, Fudan University, Shanghai, China; ‖Department of Cell and Molecular Physiology, University of North Carolina School of Medicine, Chapel Hill, North Carolina; and the ¶Liver Disease Center and Kanazawa University Hospital, Kanazawa University, Kanazawa, Japan

***Background & Aims:*** **Cancer progression/metastases and embryonic development share many properties including cellular plasticity, dynamic cell motility, and integral interaction with the microenvironment. We hypothesized that the heterogeneous nature of hepatocellular carcinoma (HCC), in part, may be owing to the presence of hepatic cancer cells with stem/progenitor features.** ***Methods:*** **Gene expression profiling and immunohistochemistry analyses were used to analyze 235 tumor specimens derived from 2 recently identified HCC subtypes (EpCAM$^+$ α-fetoprotein [AFP$^+$] HCC and EpCAM$^-$ AFP$^-$ HCC). These subtypes differed in their expression of AFP, a molecule produced in the developing embryo, and EpCAM, a cell surface hepatic stem cell marker. Fluorescence-activated cell sorting was used to isolate EpCAM$^+$ HCC cells, which were tested for hepatic stem/progenitor cell properties.** ***Results:*** **Gene expression and pathway analyses revealed that the EpCAM$^+$ AFP$^+$ HCC subtype had features of hepatic stem/progenitor cells. Indeed, the fluorescence-activated cell sorting–isolated EpCAM$^+$ HCC cells displayed hepatic cancer stem cell–like traits including the abilities to self-renew and differentiate. Moreover, these cells were capable of initiating highly invasive HCC in nonobese diabetic, severe combined immunodeficient mice. Activation of Wnt/β-catenin signaling enriched the EpCAM$^+$ cell population, whereas RNA interference-based blockage of EpCAM, a Wnt/β-catenin signaling target, attenuated the activities of these cells.** ***Conclusions:*** **Taken together, our results suggest that HCC growth and invasiveness is dictated by a subset of EpCAM$^+$ cells, opening a new avenue for HCC cancer cell eradication by targeting Wnt/β-catenin signaling components such as EpCAM.**



Tumors originate from normal cells as a result of accumulated genetic/epigenetic changes. Although considered monoclonal in origin, tumor cells are heterogeneous in their morphology, clinical behavior, and molecular profiles.[1,2] Tumor cell heterogeneity has been explained previously by the clonal evolution model[3]; however, recent evidence has suggested that heterogeneity may be owing to derivation from endogenous stem/progenitor cells[4] or de-differentiation of a transformed cell.[5] This hypothesis supports an early proposal that cancers represent "blocked ontogeny"[6] and a derivative that cancers are transformed stem cells.[7] This renaissance of stem cells as targets of malignant transformation has led to realizations about the similarities between cancer cells and normal stem cells in their capacity to self-renew, produce heterogeneous progenies, and limitlessly divide.[8] The cancer stem cell (CSC) (or tumor-initiating cell) concept is that a subset of cancer cells bear stem cell features that are indispensable for a tumor. Accumulating evidence suggests the involvement of CSCs in the perpetuation of various cancers including leukemia, breast cancer, brain cancer, prostate cancer, and colon cancer.[9–13] Experimentally, putative CSCs have been isolated using cell surface markers specific for normal stem cells. Stem cell–like features of CSC have been confirmed by functional in vitro clonogenicity and in vivo tumorigenicity assays. For example, leukemia-initiating cells in nonobese diabetic, severe combined immunodeficient (NOD/SCID) mice are CD34$^{++}$CD38$^-$.[11] Breast cancer CSCs are CD44$^+$CD24$^{-/low}$ cells, whereas tumor-initiating cells of the brain, colon, and prostate are CD133$^+$ [10,12,13] CSCs are considered more metastatic and drug-/radiation-resistant than non-CSCs in the tumor, and are responsible for cancer relapse. These findings warrant the development of treatment strategies that can specifically eradicate CSCs.[14,15]

*Abbreviations used in this paper:* AFP, α-fetoprotein; BIO, 6-bromoindirubin-3′-oxime; CSC, cancer stem cell; FACS, fluorescence-activated cell sorting; 5-FU, 5-fluorouracil; HpSC, hepatic stem cell; IF, immunofluorescence; IHC, immunohistochemistry; MACS, magnetic-activated cell sorting; MeBIO, 1-methyl-BIO; MH, mature hepatocyte; PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA.


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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's[33] 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 BioCoat 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 $\mu$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.

BASIC-LIVER, PANCREAS, AND BILIARY TRACT

## 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×) yielded 793 genes that were expressed differentially between HpSC-HCC and MH-HCC ($P < .001$). Hierarchical cluster analyses revealed 2 main gene clusters that were up-regulated (cluster A; 455 genes) or down-regulated (cluster B; 338 genes) in HpSC-HCC (Figure 1*A*). Pathway analysis indicated that the enriched genes in cluster A were associated significantly with known stem cell signaling pathways such as transforming growth factor-$\beta$, Wnt/$\beta$-catenin, PI3K/Akt, and integrin ($P < .01$) (Figure 1*B*). 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 1*C* 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 1*D*). Consistently, HpSC-HCC patients had a high frequency of macroscopic and microscopic portal vein invasion (Figure 1*E*).

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 1*F*, *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 1*F*, *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 $\beta$-catenin) in 6 HCC cell lines (Figure 2*A*). All 3 AFP$^+$ cell lines (HuH1, HuH7, and Hep3B) expressed EpCAM, CD133, and cytoplasmic/nuclear $\beta$-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

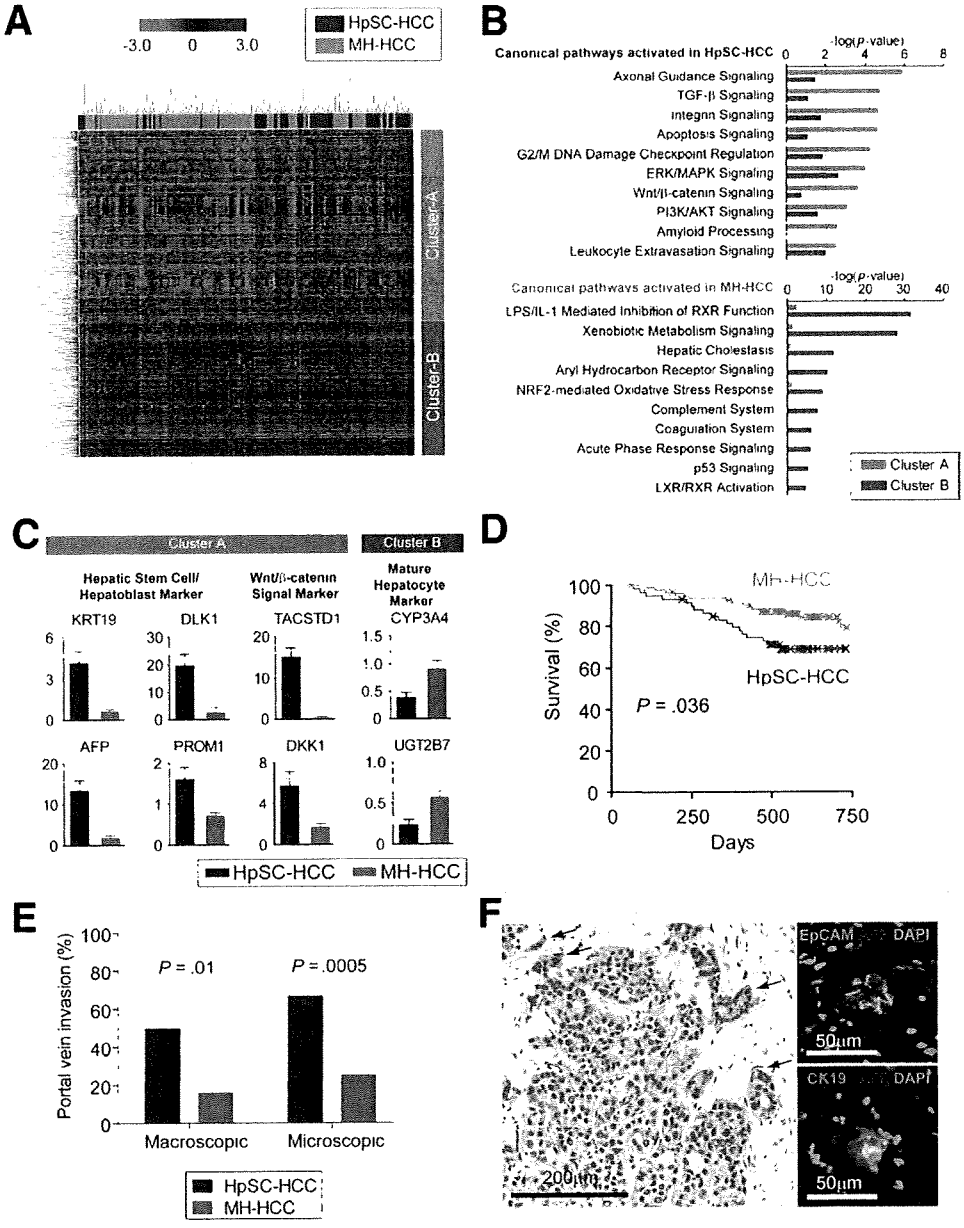



**Figure 1.** HpSC–HCC represents a subset of invasive HCCs with CSC features. (*A*) Hierarchical cluster analysis based on 793 HpSC–HCC–coregulated genes in 156 HCC cases. Each cell in the matrix represents the expression level of a gene in an individual sample. Red and green cells depict high and low expression levels, respectively, as indicated by the scale bar. (*B*) Pathway analysis of HpSC–HCC–coregulated genes. The top 10 canonical signaling pathways activated in cluster A (*upper panel*) or cluster B (*lower panel*) with statistical significance ($P < .01$) are shown. (*C*) Expression patterns of well-known HpSC and MH markers in each HCC subtype as analyzed by microarray. (*D*) Kaplan–Meier survival analysis of the cases used for array analysis. (*E*) Frequency of macroscopic and microscopic portal vein invasion in HpSC-HCC and MH-HCC used for IHC. (*F*) Representative images of EpCAM, AFP, and CK19 staining in HpSC–HCC samples analyzed by IHC and IF. EpCAM staining illustrates heterogeneous expression of EpCAM in HpSC-HCC (*left panel*). EpCAM$^+$ cells were disseminated in the invasive border (*left panel black arrows*) with expression of AFP (*right top panel*) and CK19 (*right bottom panel*).

EpCAM$^+$ and CD133$^+$, but no CD90$^+$ cells, whereas AFP$^-$ cell lines had a subpopulation of CD90$^+$ cells but no EpCAM$^+$ or CD133$^+$ cells (Figure 2*B*). 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 2*A* and *B* and Supplementary Figure 1*A*; 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 3*A*). 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 3*B* and Supplementary Figure 1*B*; 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 3*B*). 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*

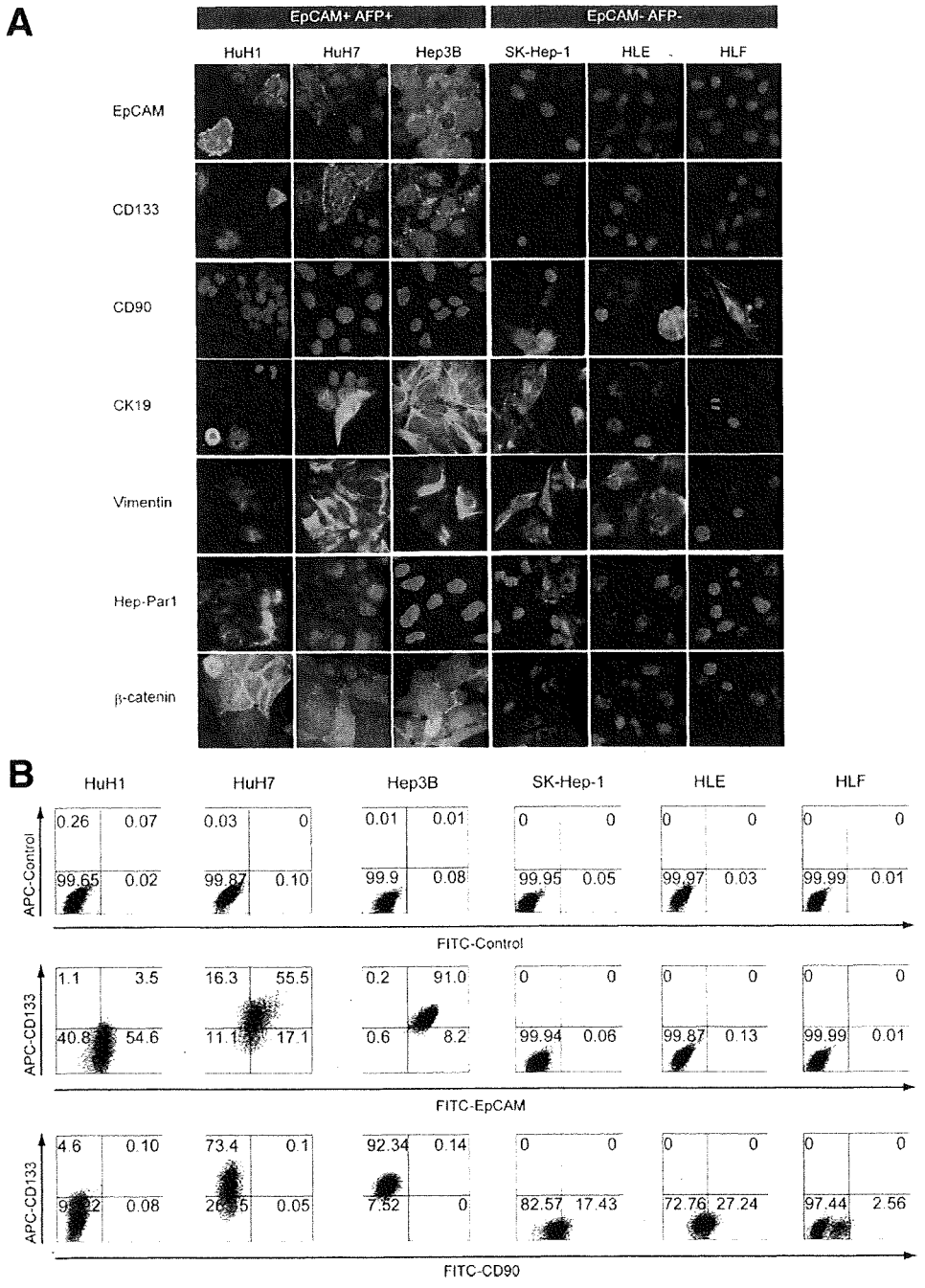



**Figure 2.** Characterization of hepatic stem cell marker expression in HCC cell lines. (*A*) IF analysis of 6 HCC cell lines (EpCAM$^+$ AFP$^+$ cell lines: HuH1, HuH7, and Hep3B; EpCAM$^-$ AFP$^-$ cell lines: SK-Hep-1, HLE, and HLF) stained with anti-EpCAM, anti-CD133, anti-CD90, anti-CK19, anti-Vimentin,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

(known mature hepatocyte markers) were significantly higher in EpCAM$^-$ HuH7 cells (Figure 3*C*, *left upper panel*). This expression pattern was reminiscent of human HpSC cells (Figure 3*C*, *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 expression 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 3*C*, *right panel*; and Supplementary Figure 1*C*; 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.

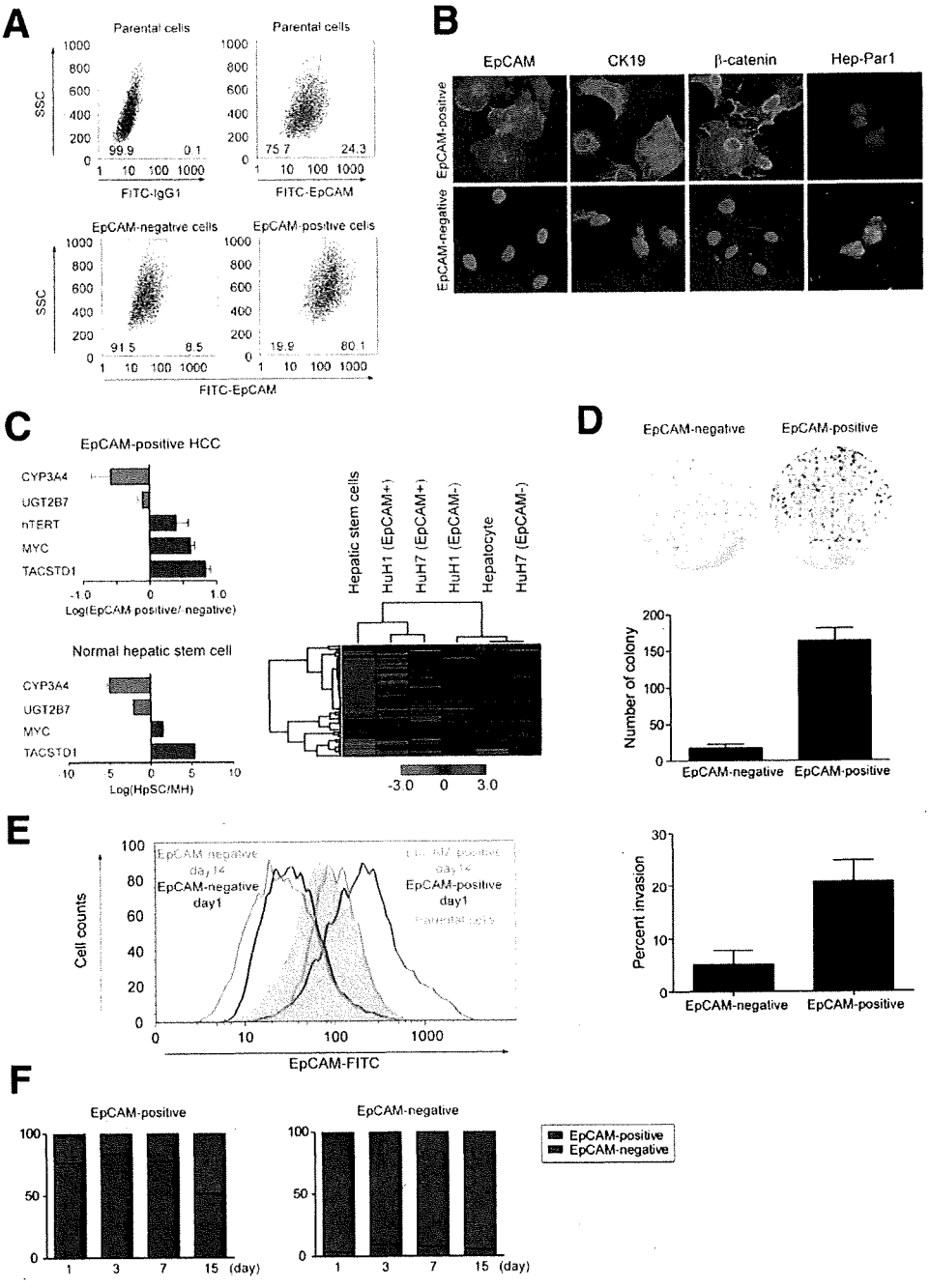



**Figure 3.** Characterization of EpCAM$^+$ and EpCAM$^-$ cells in HuH7 cells. (*A*) FACS analysis of EpCAM$^+$ and EpCAM$^-$ cells on day 1 after cell sorting. (*B*) IF analysis of cells stained with anti-EpCAM, anti-AFP, anti-CK19, or anti-β-catenin antibodies. (*C*) Quantitative reverse-transcription polymerase chain reaction analysis of EpCAM$^+$ and EpCAM$^-$ HuH7 cells (*left upper panel*) or HpSCs and MHs (*left lower panel*). Experiments were performed in triplicate. Hierarchical cluster analysis of HpSC, MH, and EpCAM$^+$ and EpCAM$^-$ HCC cells using a panel of genes expressed in human embryonic stem cells (*right panel*). Gene expression was measured in quadruplicate. (*D*) Representative photographs of the plates containing colonies derived from 2000 EpCAM$^+$ or EpCAM$^-$ HuH7 cells (*upper panel*). Colony formation experiments were performed in triplicate (mean ± SD) (*middle panel*). Cell invasiveness of EpCAM$^+$ and EpCAM$^-$ cells using the Matrigel invasion assay (*lower panel*). (*E*) Flow cytometer analysis of EpCAM$^+$ and EpCAM$^-$ HuH7 cells stained with anti-EpCAM at days 1 and 14 after cell sorting. (*F*) Percentage of sorted EpCAM$^+$ and EpCAM$^-$ cells after culturing for various times as analyzed by IF. Numbers of EpCAM$^+$ and EpCAM$^-$ cells were counted in 3 independent areas of chamber slides at days 1, 3, 7, and 15 after cell sorting. The average percentages of EpCAM$^+$ or EpCAM$^-$ cells are depicted as *red* or *blue*, respectively.

The isolated EpCAM$^+$ HuH7 cells formed colonies efficiently whereas EpCAM$^-$ cells failed to do so (Figure 3*D*, *upper* and *middle panels*; and Supplementary Figure 2*A* 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 3*D*, *lower panel*; and Supplementary Figure 2*B* 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 3*E*). However, a small percentage of EpCAM$^+$ cells remained constant in sorted EpCAM$^-$ HuH7 cells. FACS analysis confirmed the results of IF analysis (Figure 3*F* and Supplementary Figure 2*C* 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.

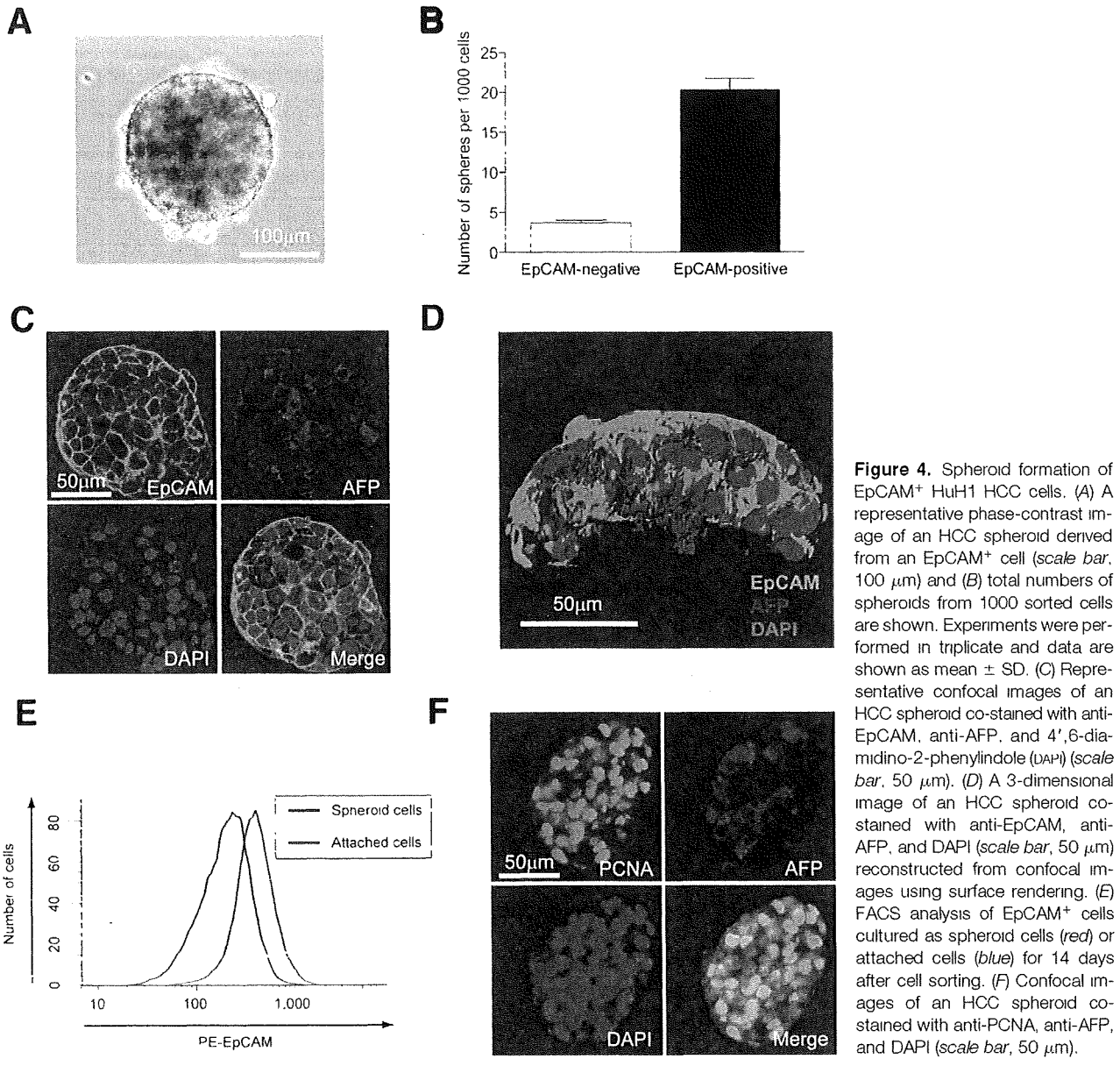



**Figure 4.** Spheroid formation of EpCAM$^+$ HuH1 HCC cells. (*A*) A representative phase-contrast image of an HCC spheroid derived from an EpCAM$^+$ cell (*scale bar*, 100 μm) and (*B*) total numbers of spheroids from 1000 sorted cells are shown. Experiments were performed in triplicate and data are shown as mean ± 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 μm). (*D*) A 3-dimensional image of an HCC spheroid co-stained with anti-EpCAM, anti-AFP, and DAPI (*scale bar*, 50 μm) 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 μm).



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 μm in diameter after 14 days of culture (Figure 4*A* and *B*). Interestingly, all cells in a spheroid were EpCAM$^+$, whereas AFP expression was relatively heterogeneous (Figure 4*C* 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 4*E*). Most spheroid cells also abundantly expressed proliferating cell nuclear antigen (PCNA), implying active cell proliferation (Figure 4*F* 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.

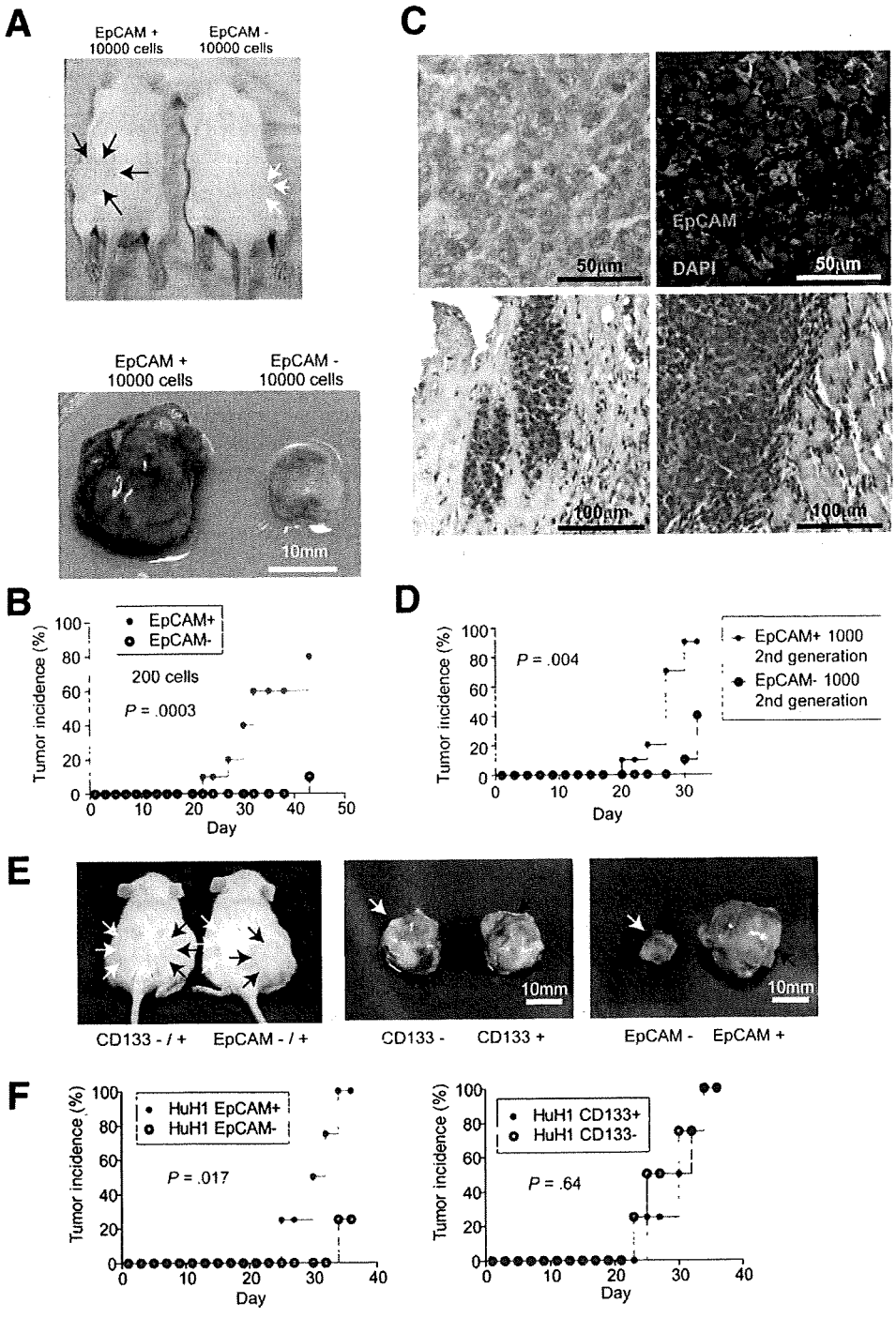



**Figure 5.** Tumorigenic and invasive potential of EpCAM$^+$ HCC cells. (*A*) Representative NOD/SCID mice (*upper panel*) with subcutaneous tumors (*lower panel*) from EpCAM$^+$ (*black arrows*) or EpCAM$^-$ (*white arrows*) HuH1 cells. (*B*) Tumorigenicity of 200 sorted HuH1 cells. (*C*) Histologic 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 5*A* and Supplementary Figure 3*A*; see supplementary material online at www.gastrojournal.org). Similar results were obtained with HuH7 cells (Supplementary Figure 3*B–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$^-$

cells (Figure 5*B* and Supplementary Figure 3*E*; 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 5*C*). EpCAM$^+$ cells derived from tumors again maintained their tumor-initiating capacity, tumor morphology, and invasive ability in an in vivo serial transplantation experiment (Figure 5*D*). 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, $1 \times 10^4$ EpCAM$^+$ cells could induce tumors in NOD/SCID mice, but up to $1 \times 10^6$ 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 2*B*), 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 5*E* and *F*), whereas EpCAM$^+$ and CD133$^+$ cells had similar tumorigenic ability in HuH7 cells (data not shown).

### *GSK-3β Inhibition Augments EpCAM$^+$ HCC Cells*

To determine the role of Wnt/β-catenin signaling[28] in EpCAM$^+$ HCC cells (Figure 1*B*), we first treated HuH1, HuH7, and HLF cells with a GSK-3β inhibitor BIO (Figure 6*A*), which activates Wnt/β-catenin signaling (Figure 6*B*) and maintains undifferentiation of embryonic stem cells.[38] 6-bromoindirubin-3′-oxime (BIO) increased the EpCAM$^+$ cell population in HuH1 and HuH7 cells when compared with the control methylated BIO (MeBIO) (Figure 6*A*). In contrast, BIO had no effect on the CD90$^+$ cell population, which is more tumorigenic than the CD90$^-$ cell population in HLF (Figure 6*A* and data not shown). Enrichment of EpCAM$^+$ cells was provoked further by the treatment of Wnt10B-conditioned media in HuH7 cells (Figure 6*C*).[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 6*D*). Moreover, BIO induced *TACSTD1*, *MYC*, and *hTERT* expression and spheroid formation (Figure 6*E* and *F*).

**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$^+$ | $1 \times 10^3$ | 0/3 | 0/3 |
| | | | $1 \times 10^4$ | 2/3 | 2/3 |
| | | | $1 \times 10^5$ | 2/2 | 2/2 |
| | | EpCAM$^-$ | $1 \times 10^5$ | 0/3 | 0/3 |
| | | | $1 \times 10^6$ | 0/2 | 0/2 |
| 2 | 1.4 | EpCAM$^+$ | $1 \times 10^3$ | 0/2 | 0/2 |
| | | | $1 \times 10^4$ | 0/1 | 1/1 |
| | | EpCAM$^-$ | $1 \times 10^4$ | 0/3 | 0/3 |
| | | | $1 \times 10^5$ | 0/2 | 0/2 |

### *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 7*A* 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 7*C*), and significantly inhibited cellular invasion, spheroid formation, and tumorigenicity of HuH1 cells (Figure 7*D–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 signaling[29] 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

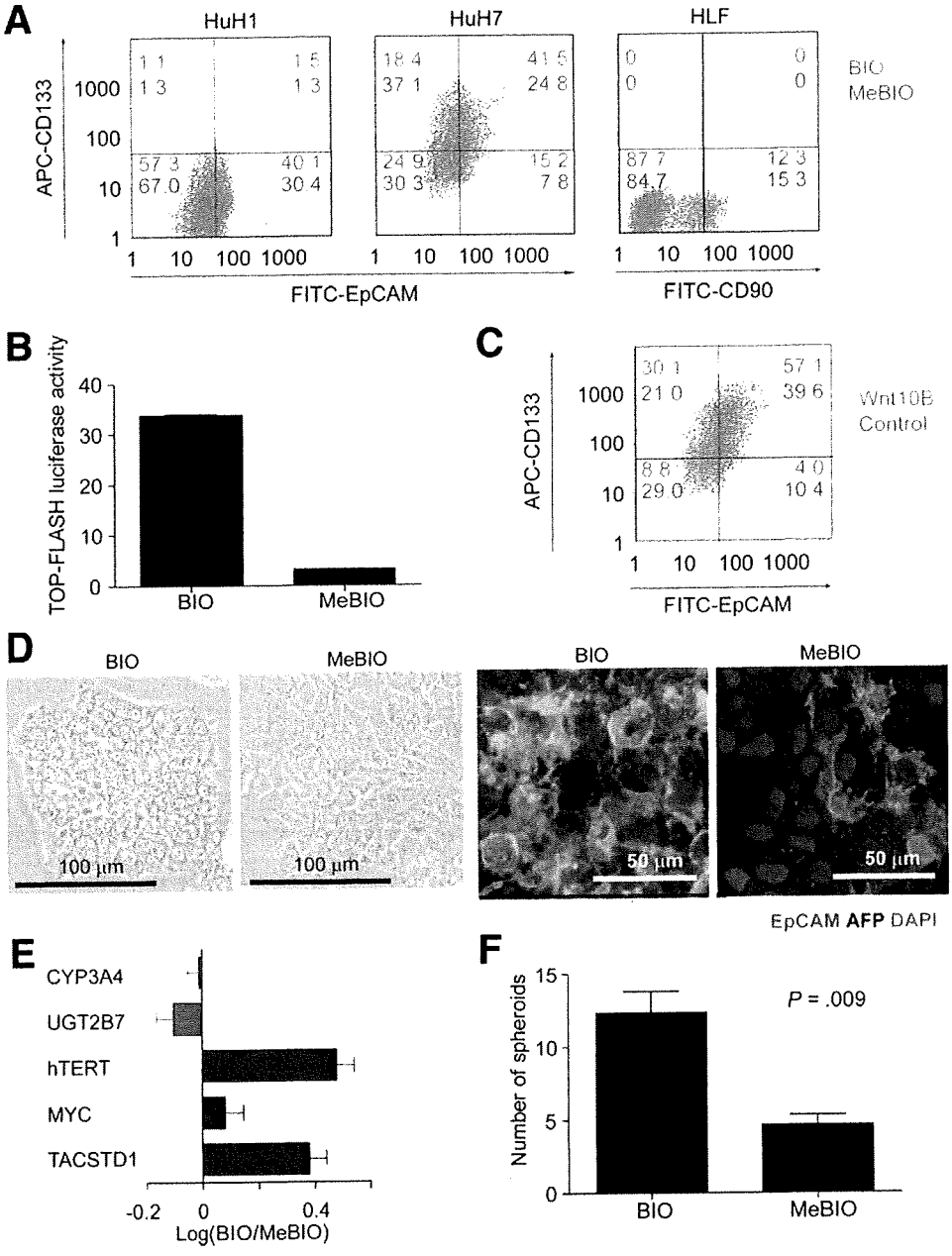
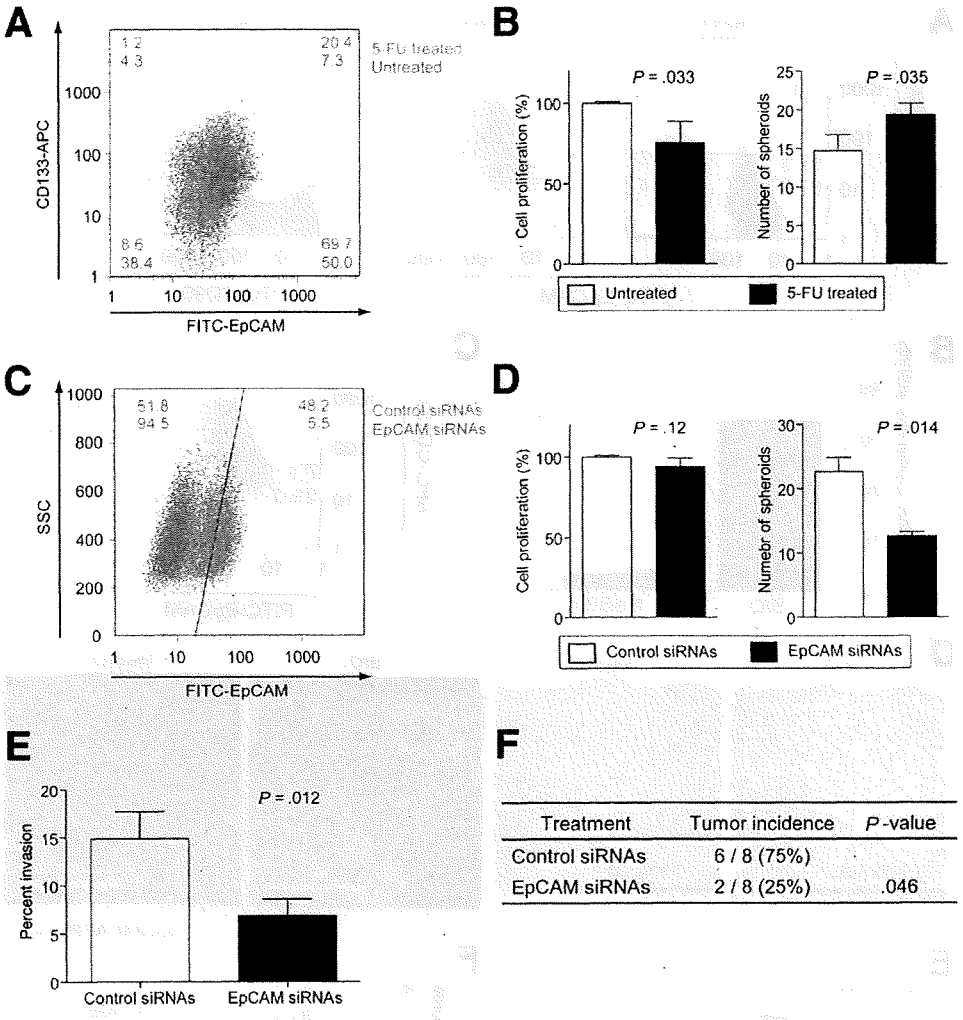



**Figure 6.** Wnt/β-catenin signaling augments EpCAM$^+$ HCC cells. (*A*) Flow cytometer analysis of HuH1, HuH7, and HLF cells treated with 2 μmol/L of BIO (*orange*) or MeBIO (*green*) for 10 days and stained with anti-EpCAM, anti-CD133 and anti-CD90 antibodies. (*B*) TOP-FLASH luciferase assays of HuH7 cells treated with 2 μmol/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 μm) or IF images (*right panel*: *scale bar*, 50 μm) of HuH7 cells treated with 2 μmol/L of BIO or MeBIO for 14 days. (*E*) Quantitative reverse transcription-polymerase chain reaction analysis of representative HpSC-HCC–related genes in HuH7 cells treated with 2 μmol/L of BIO or MeBIO for 14 days. (*F*) Spheroid formation assay of HuH7 cells treated with 2 μmol/L of BIO or MeBIO for 14 days (mean ± 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 developed 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




| Treatment | Tumor incidence | P-value |
|---|---|---|
| Control siRNAs | 6 / 8 (75%) | |
| EpCAM siRNAs | 2 / 8 (25%) | .046 |

**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 $\mu$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 $\mu$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 ofEpCAM$^+$ HuH1 cells transfected with a control siRNA or EpCAM-specific siRNA. Experiments were performed in triplicate and the data are shown as mean ± 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.

### References

1. Fialkow PJ. Clonal origin of human tumors. Biochim Biophys Acta 1976;458:283–321.

2. Heppner GH. Tumor heterogeneity. Cancer Res 1984;44:2259–2265.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100:57–70.
4. Jordan CT, Guzman ML, Noble M. Cancer stem cells. N Engl J Med 2006;355:1253–1261.
5. Clarke MF, Dick JE, Dirks PB, et al. Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res 2006;66:9339–9344.
6. Potter VR. Phenotypic diversity in experimental hepatomas: the concept of partially blocked ontogeny. The 10th Walter Hubert Lecture. Br J Cancer 1978;38:1–23.
7. Sell S. Cellular origin of cancer: dedifferentiation or stem cell maturation arrest? Environ Health Perspect 1993;101(Suppl 5): 15–26.
8. Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea—a paradigm shift. Cancer Res 2006;66:1883–1890.
9. Al Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100:3983–3988.
10. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. Nature 2004;432:396–401.
11. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730–737.
12. Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007; 445:111–115.
13. O'Brien CA, Pollett A, Gallinger S, et al. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445:106–110.
14. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005;5:275–284.
15. Rich JN. Cancer stem cells in radiation resistance. Cancer Res 2007;67:8980–8984.
16. Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
17. Sell S, Pierce GB. Maturation arrest of stem cell differentiation is a common pathway for the cellular origin of teratocarcinomas and epithelial cancers. Lab Invest 1994;70:6–22.
18. Thorgeirsson SS, Grisham JW. Hepatic stem cells. Semin Liver Dis 2003;23:301.
19. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31:339–346.
20. Lee JS, Heo J, Libbrecht L, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med 2006;12:410–416.
21. Sigal SH, Brill S, Fiorino AS, et al. The liver as a stem cell and lineage system. Am J Physiol 1992;263:G139–G148.
22. Schmelzer E, Wauthier E, Reid LM. The phenotypes of pluripotent human hepatic progenitors. Stem Cells 2006;24:1852–1858.
23. Schmelzer E, Zhang L, Bruce A, et al. Human hepatic stem cells from fetal and postnatal donors. J Exp Med 2007;204:1973–1987.
24. Dan YY, Riehle KJ, Lazaro C, et al. Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages. Proc Natl Acad Sci U S A 2006;103:9912–9917.
25. Zaret KS. Regulatory phases of early liver development: paradigms of organogenesis. Nat Rev Genet 2002;3:499–512.
26. Shafritz DA, Oertel M, Menthena A, et al. Liver stem cells and prospects for liver reconstitution by transplanted cells. Hepatology 2006;43:S89–S98.
27. Yamashita T, Forgues M, Wang W, et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res 2008;68:1451–1461.
28. Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature 2005;434:843–850.
29. Yamashita T, Budhu A, Forgues M, et al. Activation of hepatic stem cell marker EpCAM by Wnt-β-catenin signaling in hepatocellular carcinoma. Cancer Res 2007;67:10831–10839.
30. Budhu A, Forgues M, Ye QH, et al. Prediction of venous metastases, recurrence and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. Cancer Cell 2006;10:99–111.
31. Ye QH, Qin LX, Forgues M, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. Nat Med 2003;9:416–423.
32. Wu CG, Forgues M, Siddique S, et al. SAGE transcript profiles of normal primary human hepatocytes expressing oncogenic hepatitis B virus X protein. FASEB J 2002;16:1665–1667.
33. Kubota H, Reid LM. Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. Proc Natl Acad Sci U S A 2000;97:12132–12137.
34. Yoshikawa H, Matsubara K, Zhou X, et al. WNT10B functional dualism: beta-catenin/Tcf-dependent growth promotion or independent suppression with deregulated expression in cancer. Mol Biol Cell 2007;18:4292–4303.
35. Yang ZF, Ho DW, Ng MN, et al. Significance of CD90(+) cancer stem cells in human liver cancer. Cancer Cell 2008;13:153–166.
36. Dontu G, Abdallah WM, Foley JM, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003;17:1253–1270.
37. Fang D, Nguyen TK, Leishear K, et al. A tumorigenic subpopulation with stem cell properties in melanomas. Cancer Res 2005; 65:9328–9337.
38. Sato N, Meijer L, Skaltsounis L, et al. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med 2004;10:55–63.
39. Balzar M, Winter MJ, de Boer CJ, et al. The biology of the 17-1A antigen (Ep-CAM). J Mol Med 1999;77:699–712.
40. Trzpis M, McLaughlin PM, de Leij LM, et al. Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. Am J Pathol 2007;171:386–395.
41. Dalerba P, Dylla SJ, Park IK, et al. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A 2007;104:10158–10163.
42. Ma S, Chan KW, Hu L, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007;132:2542–2556.
43. Yin AH, Miraglia S, Zanjani ED, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997;90: 5002–5012.
44. Fargeas CA, Corbeil D, Huttner WB. AC133 antigen, CD133, prominin-1, prominin-2, etc.: prominin family gene products in need of a rational nomenclature. Stem Cells 2003;21:506–508.
45. Shmelkov SV, Butler JM, Hooper AT, et al. CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. J Clin Invest 2008; 118:2111–2120.
46. Hill RP, Perris R. "Destemming" cancer stem cells. J Natl Cancer Inst 2007;99:1435–1440.
47. Piccirillo SG, Reynolds BA, Zanetti N, et al. Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. Nature 2006;444:761–765.
48. Munz M, Kieu C, Mack B, et al. The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. Oncogene 2004;23:5748–5758.

49. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–872.
50. Chaudry MA, Sales K, Ruf P, et al. EpCAM an immunotherapeutic target for gastrointestinal malignancy: current experience and future challenges. Br J Cancer 2007;96:1013–1019.
51. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 2007;450:1235–1241.

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*Reprint requests*

Address requests for reprints to: Xin Wei Wang, PhD, Liver Carcinogenesis Section, Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, 37 Convent Drive, Building 37, Room 3044A, MSC 4258, Bethesda, Maryland 20892-4258. e-mail: xw3u@nih.gov; fax: (301) 496-0497.

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Microarray data are available publicly at http://www.ncbi.nlm.nih.gov/geo/ (accession number: GSE5975).

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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 resuspended 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 (StemCell Technologies Inc, Vancouver, British Columbia, Canada) were used to detect $EpCAM^+$, $CD133^+$, or $CD90^+$ cells. Intracellular AFP levels were examined by a BD Cytofix/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]

### References

1. Yamashita T, Forgues M, Wang W, et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res 2008;68:1451–1461.
2. Wang W, Budhu A, Forgues M, et al. Temporal and spatial control of nucleophosmin by the Ran-Crm1 complex in centrosome duplication. Nat Cell Biol 2005;7:823–830.

**Supplemetary 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 |

[a]Mann–Whitney *U* test or $\chi^2$ test.
[b]Edmondson–Steiner.
[c]Macroscopic 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 (9.3–219) | < .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 |

[a]Mann–Whitney *U* test or $\chi^2$ test.
[b]Edmondson–Steiner.
[c]Macroscopic 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 (includesEG: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 ($P < .01$).

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

Taro Yamashita[1], Masao Honda[1,2], Hajime Takatori[1], Ryuhei Nishino[1], Hiroshi Minato[3], Hiroyuki Takamura[4], Tetsuo Ohta[4], Shuichi Kaneko[1,*]

[1]*Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, 13-1 Takara-Machi, Kanazawa 920-8641, Japan*
[2]*Department of Advanced Medical Technology, Kanazawa University School of Health Sciences, 13-1 Takara-Machi, Kanazawa 920-8641, Japan*
[3]*Pathology Section, Kanazawa University Hospital, 13-1 Takara-Machi, Kanazawa 920-8641, Japan*
[4]*Department of Gastroenterologic Surgery, Kanazawa University Graduate School of Medical Science, 13-1 Takara-Machi, Kanazawa 920-8641, Japan*

***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.**


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

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* Corresponding author. Tel.. +81 76 265 2231, fax: +81 76 234 4250.
*E-mail address:* skaneko@m-kanazawa.jp (S. Kaneko).

*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

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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 software. 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*); Hs001 88012_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 $1 \times 10^4$ 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% $CO_2$ 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 FACSCalibur 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 $2 \times 10^3$ 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-3β (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-κB, 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).