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## EpCAM-positive hepatocellular carcinoma cells are tumor initiating cells with stem/progenitor cell features

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### Abstract

**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) may be, in part, due 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 two recently identified HCC subtypes (EpCAM<sup>+</sup> AFP<sup>+</sup> HCC and EpCAM<sup>−</sup> AFP<sup>−</sup> HCC). These subtypes differed in their expression of alpha-fetoprotein (AFP), a molecule produced in the developing embryo, and EpCAM, a cell surface hepatic stem cell marker. Fluorescence-activated cell sorting (FACS) was used to isolate EpCAM<sup>+</sup> HCC cells, which were tested for hepatic stem/progenitor cell properties.

**Results**—Gene expression and pathway analyses revealed that the EpCAM<sup>+</sup> AFP<sup>+</sup> HCC subtype had features of hepatic stem/progenitor cells. Indeed, the FACS-isolated EpCAM<sup>+</sup> 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 NOD/SCID mice. Activation of Wnt/ $\beta$ -catenin signaling enriched the EpCAM<sup>+</sup> cell population, while RNA interference-based blockage of EpCAM, a Wnt/ $\beta$ -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<sup>+</sup> cells, opening a new avenue for HCC cancer cell eradication by targeting Wnt/ $\beta$ -catenin signaling components such as EpCAM.

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## INTRODUCTION

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<sup>1, 2</sup>. Tumor cell heterogeneity has previously been explained by the clonal evolution model<sup>3</sup>, however, recent evidence has suggested that heterogeneity may be due to derivation from endogenous stem/progenitor cells<sup>4</sup> or de-differentiation of a transformed cell<sup>5</sup>. This hypothesis supports an early proposal that cancers represent “blocked ontogeny”<sup>6</sup> and a derivative that cancers are transformed stem cells<sup>7</sup>. 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<sup>8</sup>. The cancer stem cell (CSC) (or Tumor Initiating Cell) concept is that a subset of cancer cells bears 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<sup>9-13</sup>. 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 NOD/SCID mice are CD34<sup>++</sup>CD38<sup>-</sup><sup>11</sup>. Breast cancer CSCs are CD44<sup>+</sup>CD24<sup>-/low</sup> cells while tumor initiating cells of the brain, colon and prostate are CD133<sup>+</sup><sup>10, 12, 13</sup>. 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<sup>14, 15</sup>.

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide<sup>16</sup>. Although the cellular origin of HCC is unclear<sup>17, 18</sup>, HCC has heterogeneous pathologies and genetic/genomic profiles<sup>19</sup>, suggesting that HCC can initiate in different cell lineages<sup>20</sup>. The liver is considered as a maturational lineage system similar to that in the bone marrow<sup>21</sup>. Experimental evidence indicates that certain forms of hepatic stem cells (HpSC), present in human livers of all donor ages, are multipotent and can give rise to hepatoblasts (HB)<sup>22-23</sup>, which are, in turn, bipotent progenitor cells that can progress either into the hepatocytic or biliary lineages<sup>22, 24</sup>. Alpha-fetoprotein (AFP) is one of the earliest markers detected in the liver bud specified from the ventral foregut<sup>25, 26</sup>, but its expression has only been found in HB and to a lesser extent in committed hepatocytic progenitors, not in later lineages nor in normal human HpSC<sup>22</sup>. Recent studies also indicate that EpCAM is a biomarker for HpSC as it is expressed in HpSCs and HBs<sup>22-24</sup>.

We recently identified a novel HCC classification system based on EpCAM and AFP status<sup>27</sup>. Gene expression profiles revealed that EpCAM<sup>+</sup> AFP<sup>+</sup> HCC (referred to as Hepatic Stem Cell-like HCC; HpSC-HCC) has progenitor features with poor prognosis, whereas EpCAM<sup>-</sup> AFP<sup>-</sup> HCC (referred to as Mature Hepatocyte-like HCC; MH-HCC) have adult hepatocyte features with good prognosis. Wnt/ $\beta$ -catenin signaling, a critical player for maintaining embryonic stem cells<sup>28</sup>, is activated in EpCAM<sup>+</sup> AFP<sup>+</sup> HCC, and EpCAM is a direct transcriptional target of Wnt/ $\beta$ -catenin signaling<sup>29</sup>. Moreover, EpCAM<sup>+</sup> AFP<sup>+</sup> HCC cells are more sensitive to  $\beta$ -catenin inhibitors than EpCAM<sup>-</sup> HCC cells *in vitro*<sup>29</sup>. 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<sup>+</sup> HCC cells are highly invasive and tumorigenic, and have activated Wnt/ $\beta$ -catenin signaling. We also demonstrate 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 Board of the respective Institutes. The microarray data from clinical specimens are publicly available (GEO accession number, GSE5975)<sup>27</sup>. Array data from a total of 156 HCC cases (155 hepatitis B virus-positive) corresponding to two subtypes of HCC, i.e., HpSC-HCC and MH-HCC, were used to search for HpSC-HCC-associated genes (Suppl Table 3). A total of 79 formalin fixed and paraffin-embedded HCC samples were used for IHC analyses (Suppl Table 4), 56 of which were also used in a recent study<sup>30</sup>. The classification of HpSC-HCC and MH-HCC were based on previously described criteria<sup>27</sup>.

### 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<sup>31</sup>. Normal human MHs, provided by the University of Pittsburgh through LTPADS, were cultured as previously described<sup>32</sup>. Human HpSCs were isolated from fetal livers and cultured in Kubota's Medium as previously described<sup>33</sup>. Wnt10B conditioned medium was prepared as described<sup>34</sup>. Embryonic stem cell culture medium was prepared using Knockout<sup>TM</sup> DMEM supplemented with 18% of Serum Replacement (Invitrogen). The pTOP-FLASH and pFOP-FLASH luciferase constructs were previously described<sup>29</sup>. 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<sup>+</sup> 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 successfully plated. The clones which grew well were selected two weeks after seeding and used for IF analysis. The 5FU 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<sup>+</sup> HCC cells (supplemental materials).

### Clonogenicity, spheroid formation, invasion, quantitative RT-PCR and immunohistochemistry (IHC) assays

For colony formation assays, 2,000 EpCAM<sup>+</sup> or EpCAM<sup>-</sup> 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 1,000 EpCAM<sup>+</sup> or EpCAM<sup>-</sup> 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<sup>TM</sup> Matrigel Matrix Cell Culture Inserts and Control Inserts (BD Biosciences) essentially as previously described<sup>31</sup>. RT-PCR and IHC assays are described in details in the supplemental materials.

### Tumorigenicity in NOD/SCID Mice

Six-week-old NOD/SCID mice (NOD/NCrCRI-*Prkdc<sup>scid</sup>*) were purchased from Charles River (Charles River Laboratories, Inc., Wilmington, MA). The protocol was approved by the NCI-Bethesda Animal Care and Use Committee. Cells were suspended in 200  $\mu$ l of DMEM and Matrigel (1:1), and subcutaneous injection was performed. The size and incidence of subcutaneous tumors were recorded. For histological evaluation, tumors were formalin-fixed

paraffin-embedded or directly embedded in O.C.T. compound (Sakura Finetek, Torrance, CA) and stored in  $-80^{\circ}\text{C}$ .

### RNA interference

A 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 nM of siRNA duplex was used for each transfection.

### Statistical Analyses

The class comparison and gene clustering analyses were performed as previously described<sup>30</sup>. The canonical pathway analysis was performed using Ingenuity Pathways Analysis (v5.5, Ingenuity® Systems). The association of HCC subtypes and clinicopathological characteristics was examined using either Mann-Whitney U-tests or  $\chi^2$  tests. 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 two recently identified prognostic subtypes of HCC, i.e., 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 while 96 cases were defined as MH-HCC with a good prognosis, based on EpCAM and AFP status<sup>27</sup>. A class comparison analysis with univariate t-tests and a global permutation test (x1000) yielded 793 genes that were differentially expressed between HpSC-HCC and MH-HCC ( $P < 0.001$ ). Hierarchical cluster analyses revealed two main gene clusters that were upregulated (cluster-A; 455 genes) or downregulated (cluster-B; 338 genes) in HpSC-HCC (Fig 1A). Pathway analysis indicated that the enriched genes in cluster-A were significantly associated with known stem cell signaling pathways such as TGF- $\beta$ , Wnt/ $\beta$ -catenin, PI3K/Akt and integrin ( $P < 0.01$ ) (Fig 1B). In contrast, genes in cluster-B were significantly associated with mature hepatocyte functions such as xenobiotic metabolism, complement system, and coagulation system ( $P < 0.01$ ). Noticeably, known HpSC markers such as *KRT19* (CK19), *TACSTD1* (EpCAM), *AFP*, *DKK1*, *DLK1* and *PROM1* (CD133) were significantly upregulated in HpSC-HCC while known liver maturation markers such as *UGT2B7* and *CYP3A4* were more abundantly expressed in MH-HCC (Fig 1C) (Suppl Tables 1-2). Kaplan-Meier survival analysis revealed that HpSC-HCC patients had a significantly shorter survival than MH-HCC patients ( $P = 0.036$ ) (Fig 1D). Consistently, HpSC-HCC patients had a high frequency of macroscopic and microscopic portal vein invasion (Fig 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<sup>+</sup> and EpCAM<sup>-</sup> tumor cells in each tumor (Fig 1F, **left panel**). Noticeably, many of the EpCAM<sup>+</sup> tumor cells were located at the invasion border zones and were often disseminated at the invasive front (black arrows). Immunofluorescence (IF) analysis revealed that HCC cells located at the invasive front co-expressed EpCAM, CK19, and AFP (Fig 1F, **right panels**). Noticeably, HpSC-HCC patients were significantly younger than MH-HCC patients (Suppl Tables 3-4). Enrichment of EpCAM<sup>+</sup> AFP<sup>+</sup> tumor cells at the tumor invasive front suggested their involvement in HCC invasion and metastasis.

## Isolation and characterization of EpCAM<sup>+</sup> cells in HCC

The results above suggest that HpSC-HCC may be organized in a hierarchical fashion in which EpCAM<sup>+</sup> tumor cells act as stem-like cells with an ability to differentiate into EpCAM<sup>-</sup> tumor cells. To test this hypothesis, we first evaluated the expression pattern of seven hepatic stem/maturation markers (EpCAM, CD133, CD90, CK19, Vimentin, Hep-Par1, and  $\beta$ -catenin) in six HCC cell lines (Fig 2A). All three AFP<sup>+</sup> cell lines (HuH1, HuH7, and Hep3B) expressed EpCAM, CD133, and cytoplasmic/nuclear  $\beta$ -catenin, whereas the other three AFP<sup>-</sup> cell lines (SK-Hep-1, HLE, and HLF) did not, consistent with the microarray data. Interestingly, AFP<sup>+</sup> cell lines had no CD90<sup>+</sup> cell population which was recently identified as hepatic CSCs<sup>35</sup>, whereas AFP<sup>-</sup> cell lines had such a population. Consistently with the IF data, FACS analysis showed that AFP<sup>+</sup> cell lines had a subpopulation of EpCAM<sup>+</sup> and CD133<sup>+</sup> but no CD90<sup>+</sup> cells, whereas AFP<sup>-</sup> cell lines had a subpopulation of CD90<sup>+</sup> cells but no EpCAM<sup>+</sup> nor CD133<sup>+</sup> cells (Fig 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 two human HCC cell lines (HuH1 and HuH7) to isolate EpCAM<sup>+</sup> cells since both lines were heterogeneous in EpCAM, AFP, CK19 and  $\beta$ -catenin expression (Fig 2A-B and Suppl Fig 1A)<sup>29</sup>. We successfully enriched EpCAM<sup>+</sup> and EpCAM<sup>-</sup> populations from HuH7 cells by fluorescence activated cell sorting (FACS), with >80% purity in EpCAM<sup>+</sup> cells and >90% in EpCAM<sup>-</sup> cells one day after sorting (Fig 3A). Similar results were obtained when the purity check was done immediately post-sorting (data not shown). EpCAM<sup>+</sup> cells were also positive for CK19 and  $\beta$ -catenin (Fig 3B and Suppl Fig 1B) and most were AFP<sup>+</sup> (data not shown). In contrast, EpCAM<sup>-</sup> cells were negative for these markers but positive for HepPar1, a monoclonal antibody specific to hepatocytes (Fig 3B). Consistent with the microarray data described above, the levels of *TACSTD1*, *MYC* and *hTERT* (known HpSC markers) were significantly increased in EpCAM<sup>+</sup> HuH7 cells, while the levels of *UGT2B7* and *CYP3A4* (known mature hepatocyte markers) were significantly higher in EpCAM<sup>-</sup> HuH7 cells (Fig 3C, **left upper panel**). This expression pattern was reminiscent of human HpSC cells (Fig 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 containing 96 selected human stem cell-related genes. Although a differential expression pattern of stem cell related genes was evident among HpSC, EpCAM<sup>+</sup> HuH1 and EpCAM<sup>+</sup> HuH7 cells, EpCAM<sup>+</sup> HCC cells were more closely related to HpSC cells while EpCAM<sup>-</sup> HCC cells were more closely related to diploid adult mature hepatocytes (Fig 3C **right panel**, Suppl Fig 1C). Thus, it appeared that EpCAM<sup>+</sup> HCC cells had a gene expression pattern that is more closely related to HpSC than EpCAM<sup>-</sup> HCC cells.

The isolated EpCAM<sup>+</sup> HuH7 cells formed colonies efficiently while EpCAM<sup>-</sup> cells failed to do so (Fig 3D **upper and middle panel**, and Suppl Fig 2A **for HuH1 cells**). In addition, EpCAM<sup>+</sup> HuH7 cells were much more invasive than EpCAM<sup>-</sup> cells ( $P < 0.03$ ) (Fig 3D **lower panel** and Suppl Fig 2B **for HuH1 cells**). The EpCAM<sup>+</sup> fraction decreased with time in sorted EpCAM<sup>+</sup> HuH7 cells from >80% to 50% (Fig 3E). However, a small percent of EpCAM<sup>+</sup> cells remained constant in sorted EpCAM<sup>-</sup> HuH7 cells. FACS analysis confirmed the results of IF analysis (Fig 3F and Suppl Fig 2C **for HuH7 and HuH1 cells, respectively**), suggesting that EpCAM<sup>+</sup> cells could differentiate into EpCAM<sup>-</sup> cells, eventually allowing an enriched EpCAM<sup>+</sup> fraction to revert back to parental cells after 14 days of culture. In contrast, EpCAM<sup>-</sup> cells maintained their EpCAM<sup>-</sup> status. In addition, we successfully isolated 12 HuH1 and 2 HuH7 colonies from 192 single cell-plated culture wells. However, all colonies were heterogeneous in EpCAM and AFP expression and no colony was completely EpCAM<sup>-</sup> (data not shown). Taken together, these results indicate that EpCAM<sup>+</sup> HCC cells resemble HpSC

features. It appears that EpCAM<sup>+</sup> cells, but not EpCAM<sup>-</sup> cells, have self-renewal and differentiation capabilities with the ability to form colonies from a single cell, and produce both EpCAM<sup>+</sup> and EpCAM<sup>-</sup> cells.

It has been previously demonstrated that stem/progenitor cells and cancer stem/progenitor cells can form spheroids *in vitro* in a non-attached condition<sup>36, 37</sup>. Consistently, EpCAM<sup>+</sup> cells could form spheroids efficiently, reaching to about 150~200  $\mu\text{m}$  in diameter after 14 days culture (Fig 4A-B). Interestingly, all cells in a spheroid were EpCAM<sup>+</sup> while AFP expression was relatively heterogeneous (Fig 4C-D, Suppl Movie 1). Rarely, a few spheroids derived from an EpCAM<sup>-</sup> cell fraction were positive for EpCAM (data not shown), suggesting that these spheroids were derived from contaminated residual EpCAM<sup>+</sup> cells by FACS sorting. All spheroid cells maintained EpCAM expression while half of the attached cells lost EpCAM expression when the EpCAM<sup>+</sup> fraction was cultured for 14 days (Fig 4E). Most spheroid cells also abundantly expressed PCNA, implying active cell proliferation (Fig 4F and Suppl Movie 2). Thus, a subset of EpCAM<sup>+</sup> cells, but not EpCAM<sup>-</sup> cells, can form spheroids.

### EpCAM<sup>+</sup> HCC cells as tumor initiating cells

EpCAM<sup>+</sup> HCC cells, but not EpCAM<sup>-</sup> HCC cells, could efficiently initiate invasive tumors in NOD/SCID mice (Fig 5). For example, 10,000 EpCAM<sup>+</sup> HuH1 cells produced large hypervascular tumors in 100% of mice whereas EpCAM<sup>-</sup> cell fractions produced only small and pale looking tumors in 30% of mice 4 weeks after injection (Fig 5A, Suppl Fig 3A). Similar results were obtained with HuH7 cells (Suppl Fig 3B-D). As little as 200 EpCAM<sup>+</sup> cells could initiate tumors in 8 of 10 injected mice, whereas 200 EpCAM<sup>-</sup> cells produced only one tumor among 10 injected mice at 6 weeks after transplantation, and the tumor sizes were much larger in the EpCAM<sup>+</sup> cells than the EpCAM<sup>-</sup> cells (Fig 5B and Suppl Fig 3E). EpCAM<sup>+</sup> cells produced tumors with a mixture of both EpCAM<sup>+</sup> and EpCAM<sup>-</sup> cells in xenografts, and these cells invaded in the capsule and muscles of the leg adjacent to the tumor (Fig 5C). EpCAM<sup>+</sup> cells derived from tumors again maintained their tumor initiating capacity, tumor morphology and invasive ability in an *in vivo* serial transplantation experiment (Fig 5D). Occasionally, EpCAM<sup>-</sup> cell fraction produced a few small tumors that always contain a mixture of EpCAM<sup>+</sup> and EpCAM<sup>-</sup> cells (data not shown), indicating that the tumor-initiating ability is contributed by the contaminated EpCAM<sup>+</sup> cells from FACS sorting.

To further validate whether EpCAM<sup>+</sup> HCC cells were tumor initiating cells, we isolated EpCAM<sup>+</sup> HCC cells from two cases of AFP<sup>+</sup> (>600 ng/ml serum AFP) HCC clinical specimens using MACS. Consistently,  $1 \times 10^4$  EpCAM<sup>+</sup> cells could induce tumors in NOD/SCID mice, but up to  $1 \times 10^6$  EpCAM<sup>-</sup> cells failed to do so (Table 1). In addition, similar to HCC cell lines, fresh EpCAM<sup>+</sup> tumor cells from two clinical HCC specimens were more efficient in forming spheroids *in vitro* than EpCAM<sup>-</sup> cells (Suppl Fig 4).

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

### GSK-3 $\beta$ inhibition augments EpCAM<sup>+</sup> HCC cells

To determine the role of Wnt/ $\beta$ -catenin signaling<sup>28</sup> in EpCAM<sup>+</sup> HCC cells (Fig 1B), we first treated HuH1, HuH7, and HLF cells with a GSK-3 $\beta$  inhibitor BIO (Fig 6A), which activates Wnt/ $\beta$ -catenin signaling (Fig 6B) and maintains undifferentiation of embryonic stem cells<sup>38</sup>. BIO increased the EpCAM<sup>+</sup> cell population in HuH1 and HuH7 cells when compared to the control methylated BIO (MeBIO) (Fig 6A). In contrast, BIO had no effect on the CD90<sup>+</sup> cell

population that is more tumorigenic than the CD90<sup>-</sup> cell population in HLF (Fig 6A and data not shown). Enrichment of EpCAM<sup>+</sup> cells was further provoked by the treatment of Wnt10B-conditioned media in HuH7 cells (Fig 6C)<sup>34</sup>. BIO induced morphological alteration of HuH7 cells as most cells became small and round when compared to MeBIO and suppressed EpCAM<sup>-</sup> AFP<sup>-</sup> cell population (Fig 6D). Moreover, BIO induced *TACSTD1*, *MYC*, and *hTERT* expression and spheroid formation (Fig 6E-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<sup>+</sup> population and spheroid formation of HuH1 and HuH7 cells (Fig 7A-B) (data not shown), suggesting a differential sensitivity of EpCAM<sup>+</sup> and EpCAM<sup>-</sup> HCC cells to 5-FU. In contrast, EpCAM blockage via RNA interference dramatically decreased the population of EpCAM<sup>+</sup> cells (Fig 7C), and significantly inhibited cellular invasion, spheroid formation and tumorigenicity of HuH1 cells (Fig 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 epithelium origin<sup>39</sup>. During embryogenesis, EpCAM is expressed in fertilized oocytes, embryonic stem cells and embryoid bodies, suggesting its role in early stage embryogenesis<sup>40</sup>. Furthermore, a recent paper indicates that EpCAM is expressed in colonic and breast CSCs<sup>41</sup>. 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/ $\beta$ -catenin signaling<sup>29</sup> 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/ $\beta$ -catenin signaling (this study). Thus, EpCAM may be a common gene expressed in undifferentiated normal and HCC with activated Wnt/ $\beta$ -catenin signaling. It may act as a downstream molecule 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<sup>35, 42</sup>. CD133 is expressed in normal and malignant stem cells of the neural, hematopoietic, epithelial, hepatic and endothelial lineages<sup>23, 43, 44</sup>, suggesting that CD133 is also a common marker to detect normal and CSCs. Captivatingly, EpCAM expression overlaps with CD133 expression in normal human colon tissues and colorectal cancer tissues, yet CD133<sup>+</sup> and CD133<sup>-</sup> cells are equally tumorigenic<sup>45</sup>. Similarly, we found that EpCAM<sup>+</sup> and EpCAM<sup>-</sup> HuH1 cells equally expressed CD133 but only EpCAM<sup>+</sup> 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<sup>+</sup> tumors. We also found that CD90 expression was limited to HCC cell lines that are EpCAM<sup>-</sup> AFP<sup>-</sup>, and Wnt/ $\beta$ -catenin signaling had little effect on CD90<sup>+</sup> 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 due 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 comprehensively investigate the expression patterns of stem cell markers to characterize the population of CSC that may correlate with the activation of their distinct molecular pathways.

CSCs may be more resistant to chemotherapeutic agents than differentiated tumor cells possibly due to an increased expression of ATP-binding cassette transporters and anti-apoptotic proteins<sup>4</sup>. Thus, development of an effective strategy to target CSC pools together with conventional chemotherapies is essential to eradicate a tumor mass<sup>14</sup>. By blocking the programs that activate self-renewal and/or inhibit asymmetric division, CSC features could be “destemmed”<sup>46, 47</sup>. Consistently, EpCAM blockage could inhibit cellular invasion and tumorigenicity of EpCAM<sup>+</sup> HCC cells, revealing the feasibility of targeting a CSC marker to “destem” CSC features. EpCAM may induce c-Myc<sup>48</sup>, a common molecular node activated in HpSC-HCC<sup>27</sup>. c-Myc, together with Oct3/4, Sox2, and Klf4, can induce pluripotent stem cells from adult fibroblasts<sup>49</sup>. 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<sup>50</sup>.

Furthermore, a recent study indicates that EpCAM<sup>+</sup> circulating tumor cells identified by a unique microfluidic platform can be used to monitor patient outcomes undergoing systemic treatment<sup>51</sup>. 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 Material

Refer to Web version on PubMed Central for supplementary material.

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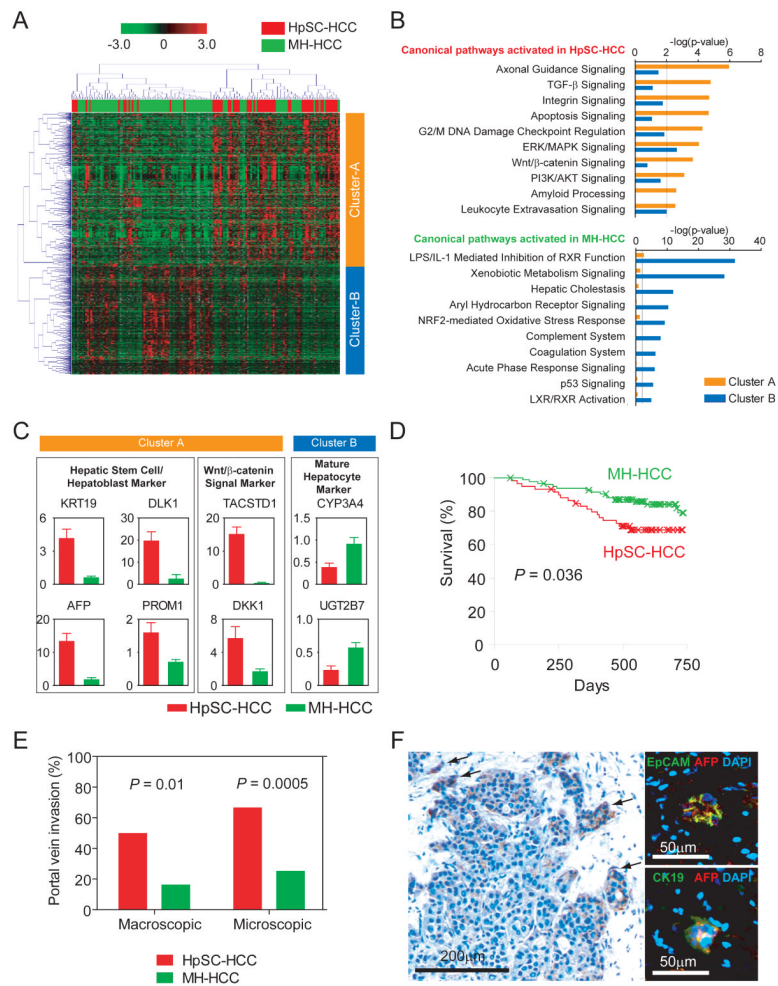
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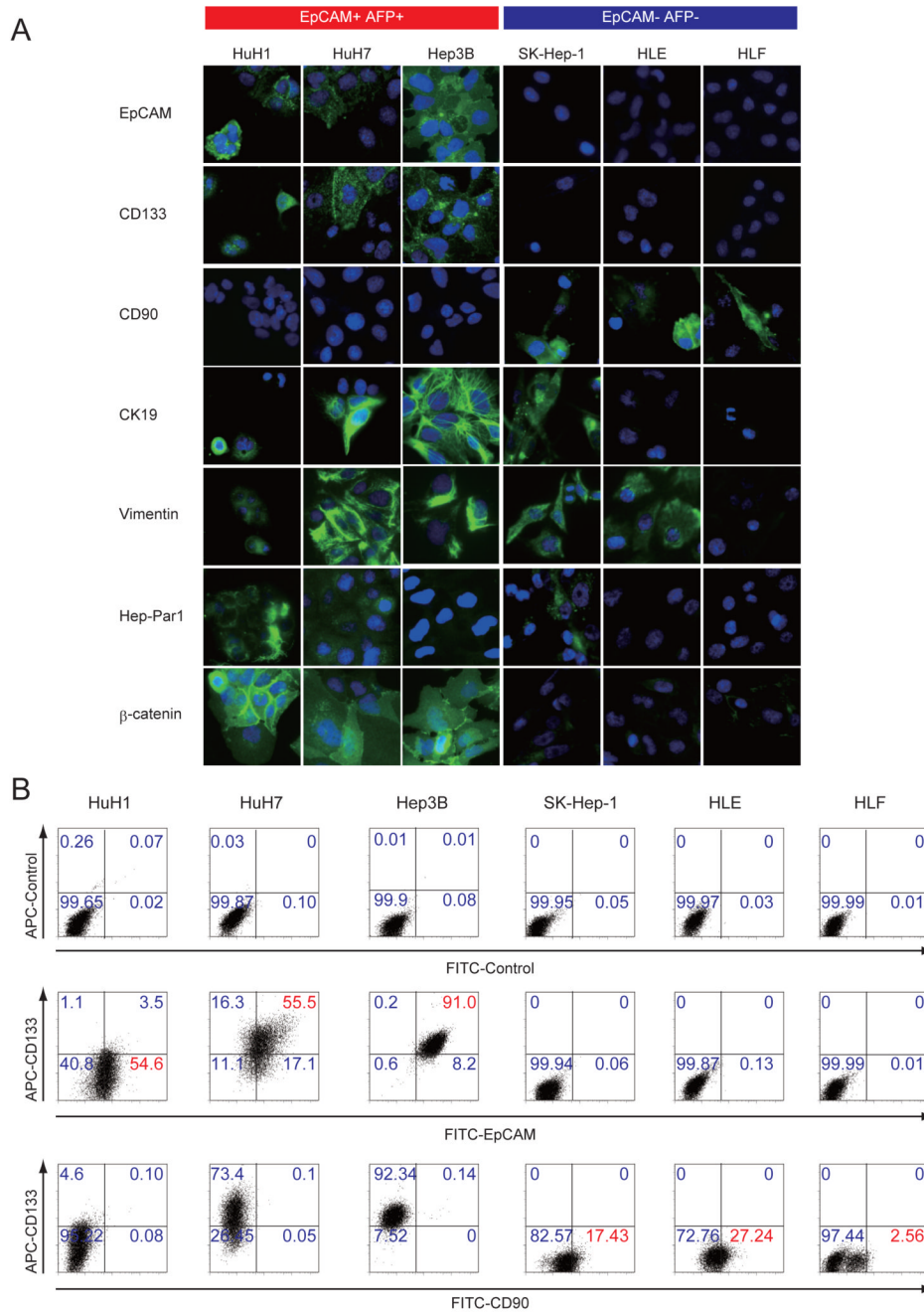


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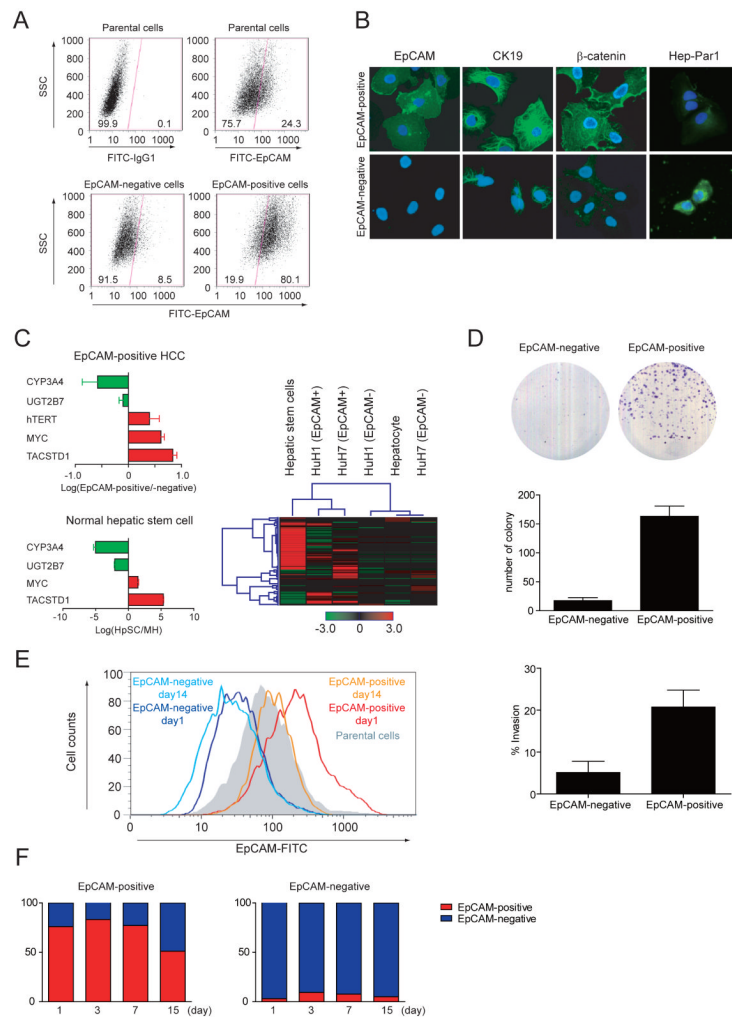
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**Figure 1.**

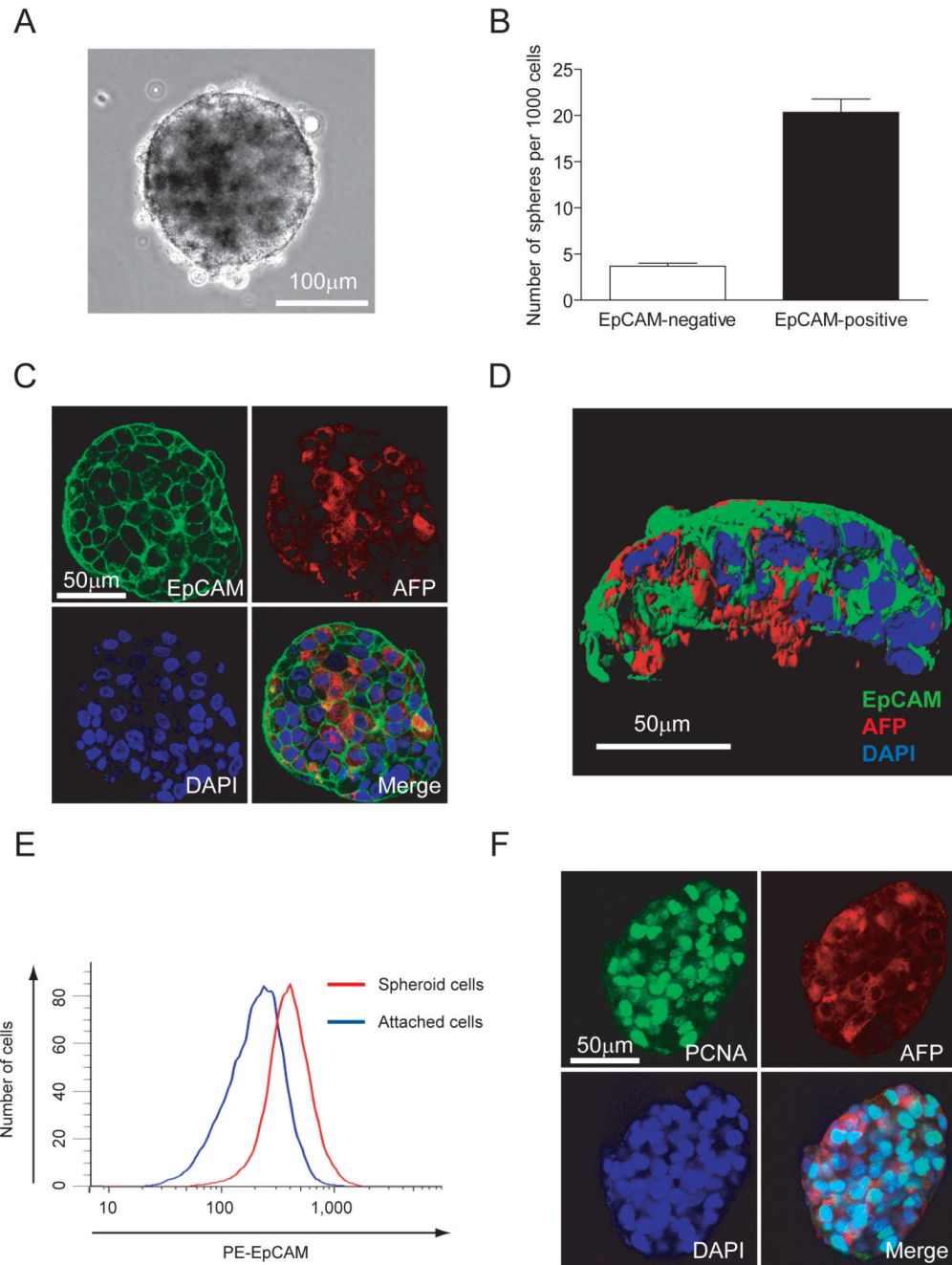
HpSC-HCC represents a subset of invasive HCCs with cancer stem cells (CSC) Features. (A) Hierarchical cluster analysis based on 793 HpSC-HCC-coreregulated genes in 156 HCC cases. Each cell in the matrix represents the expression level of a gene in an individual sample. Red and green cells depict high and low expression levels, respectively, as indicated by the scale bar. (B) Pathway analysis of HpSC-HCC-coreregulated genes. The top ten canonical signaling pathways activated in cluster-A (upper panel) or cluster-B (lower panel) with statistical significance ( $P < 0.01$ ) 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<sup>+</sup> cells were disseminated in the invasive border (left panel black arrows) with expression of AFP (right top panel) and CK19 (right bottom panel).



**Figure 2.** Characterization of hepatic stem cell marker expression in HCC cell lines. (A) IF analysis of six HCC cell lines (EpCAM<sup>+</sup> AFP<sup>+</sup> cell lines; HuH1, HuH7, and Hep3B, EpCAM<sup>-</sup> AFP<sup>-</sup> 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 six HCC cell lines stained with anti-EpCAM, anti-CD133, and anti-CD90 antibodies.

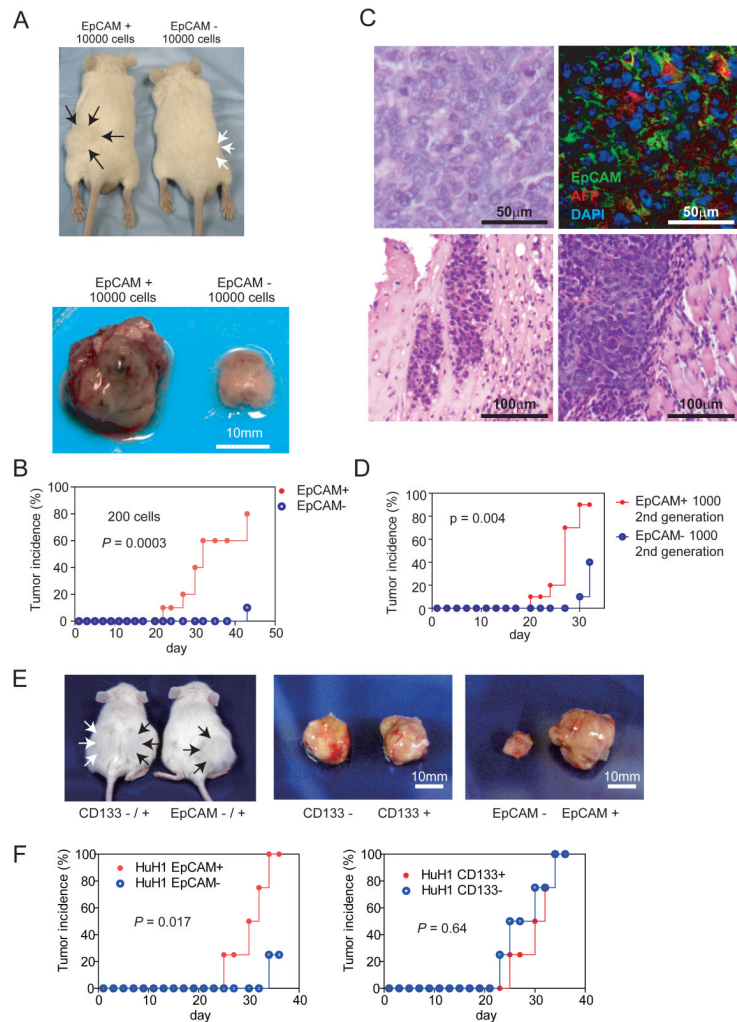
**Figure 3.**

Characterization of EpCAM<sup>+</sup> and EpCAM<sup>-</sup> cells in HuH7 cells. (A) FACS analysis of EpCAM<sup>+</sup> and EpCAM<sup>-</sup> cells day 1 after cell sorting. (B) IF analysis of cells stained with anti-EpCAM, anti-AFP, anti-CK19, or anti- $\beta$ -catenin antibodies. (C) qRT-PCR analysis of EpCAM<sup>+</sup> and EpCAM<sup>-</sup> HuH7 cells (left upper panel) or hepatic stem cells (HpSC) and mature hepatocytes (MH) (left lower panel). Experiments were performed in triplicate. Hierarchical cluster analysis of HpSC, MH, EpCAM<sup>+</sup> and EpCAM<sup>-</sup> 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 2,000 EpCAM<sup>+</sup> or EpCAM<sup>-</sup> HuH7 cells (upper panel). Colony formation experiments were performed in triplicate (mean  $\pm$  SD) (middle panel). Cell invasiveness of EpCAM<sup>+</sup> and EpCAM<sup>-</sup> cells using the Matrigel invasion assay (lower panel). (E) Flow cytometer analysis of EpCAM<sup>+</sup> and EpCAM<sup>-</sup> HuH7 cells stained with anti-EpCAM at day 1 and 14 after cell sorting. (F) Percent of sorted EpCAM<sup>+</sup> and EpCAM<sup>-</sup> cells after culturing with various times as analyzed by IF. Numbers of EpCAM<sup>+</sup> and EpCAM<sup>-</sup> cells were counted in three independent areas of Chamber Slides at day 1, 3, 7, and 15 after cell sorting. The average percentages of EpCAM<sup>+</sup> or <sup>-</sup> cells are depicted as red or blue, respectively.



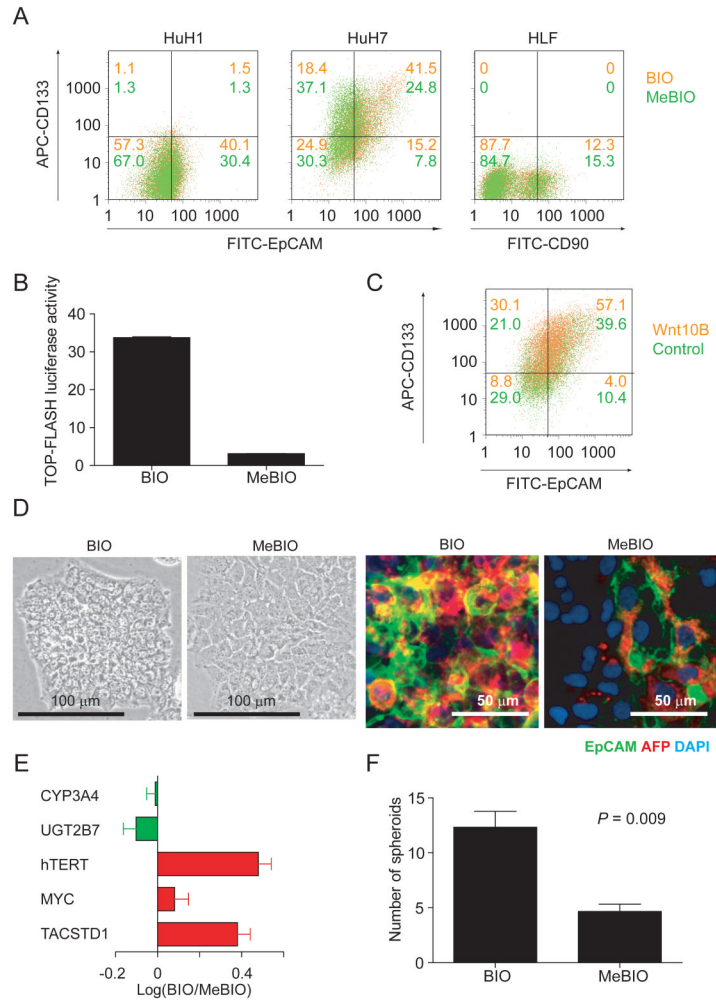
**Figure 4.** Spheroid formation of EpCAM<sup>+</sup> HuH1 HCC cells. A representative phase contrast image of an HCC spheroid derived from an EpCAM<sup>+</sup> cell (scale bar = 100  $\mu$ m) (A) and total numbers of spheroids from 1,000 sorted cells (B) are shown. Experiments were performed in triplicate and data are shown as mean  $\pm$  SD. (C) Representative confocal images of an HCC spheroid co-stained with anti-EpCAM, anti-AFP and DAPI (scale bar = 50  $\mu$ m). (D) A 3-D image of an HCC spheroid co-stained with anti-EpCAM, anti-AFP and DAPI (scale bar = 50  $\mu$ m) reconstructed from confocal images using surface rendering. (E) FACS analysis of EpCAM<sup>+</sup> cells cultured as spheroids 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  $\mu\text{m}$ ).

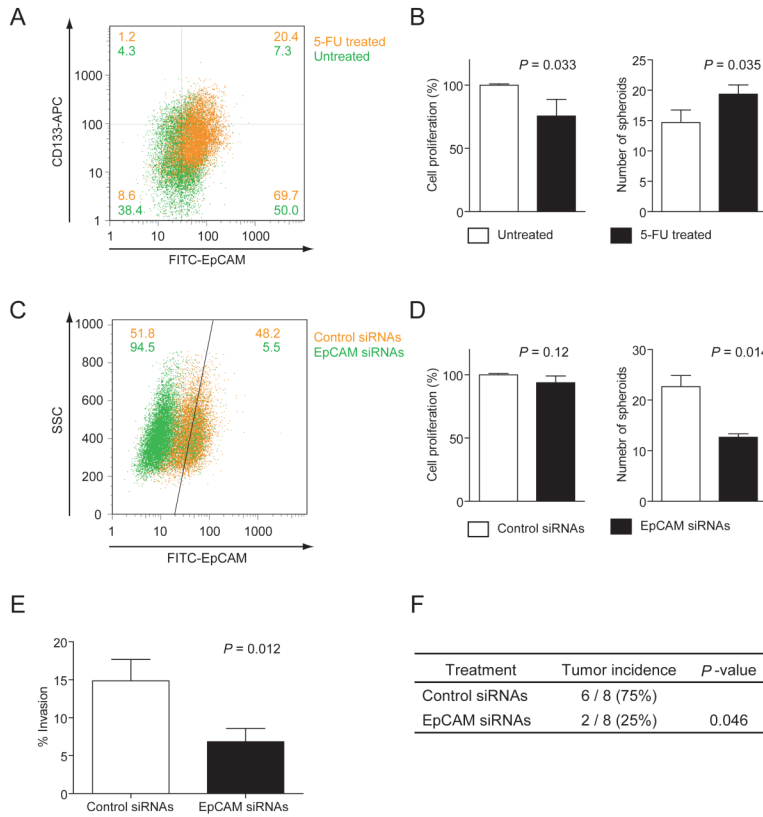
**Figure 5.**

Tumorigenic and invasive potential of EpCAM<sup>+</sup> HCC cells. (A) Representative NOD/SCID mice (upper panel) with subcutaneous tumors (lower panel) from EpCAM<sup>+</sup> (black arrows) or EpCAM<sup>-</sup> (white arrows) HuH1 cells. (B) Tumorigenicity of 200 sorted HuH1 cells. (C) Histological analysis of EpCAM<sup>+</sup> 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 DAPI (right upper panel) (scale bar = 50  $\mu$ m). (D) Tumorigenicity of 1,000 sorted cells derived from an EpCAM<sup>+</sup> HuH1 xenograft. Data are generated from 10 mice in each group. (E) Representative NOD/SCID mice (left panel) with subcutaneous tumors from CD133<sup>+</sup> (black arrows) or CD133<sup>-</sup> (white arrows) (mid panel) and EpCAM<sup>+</sup> (black arrows) or EpCAM<sup>-</sup> (white arrows) (right panel) HuH1 cells. (F) Tumorigenicity of 1,000 HuH1 cells sorted by anti-EpCAM (left panel) or anti-CD133 (right panel) antibodies.





**Figure 6.** Wnt/ $\beta$ -catenin signaling augments EpCAM<sup>+</sup> HCC cells. (A) Flow cytometer analysis of HuH1, HuH7, and HLF cells treated with 2 $\mu$ M 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 $\mu$ M of BIO (red) or MeBIO (green). (C) Flow cytometer analysis of HuH7 cells cultured in the normal media (DMEM supplemented with 10% FBS) or Wnt10B conditioned media (details are described in the Materials and Methods). Cells were cultured in each medium for 2 weeks. (D) Representative phase contrast images (left panel, scale bar = 100  $\mu$ m) or IF images (right panel, scale bar = 50  $\mu$ m) of HuH7 cells treated with 2 $\mu$ M of BIO or MeBIO for 14 days. (E) qRT-PCR analysis of representative HpSC-HCC related genes in HuH7 cells treated with 2 $\mu$ M of BIO or MeBIO for 14 days. (F) Spheroid formation assay of HuH7 cells treated with 2 $\mu$ M of BIO or MeBIO for 14 days (mean  $\pm$  SD).



**Figure 7.** EpCAM blockage inhibits the tumorigenic and invasive capacity of EpCAM<sup>+</sup> HCC cells. (A) Enrichment of EpCAM<sup>+</sup> cells after 5-FU treatment. HuH1 cells were without (green) or treated with 2 μg/ml of 5FU for 3 days and analyzed by FACS using anti-EpCAM and anti-CD133 antibodies. (B) Spheroid formation of HuH1 cells treated with 2 μg/ml of 5FU (red) 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. Spheroid formation (D) or invasive capacity (E) of EpCAM<sup>+</sup> HuH1 cells transfected with a control siRNA or EpCAM-specific siRNA. Experiments were performed in triplicate and the data are shown as mean ± SD. (F) Inhibition of tumor formation *in vivo* by EpCAM gene silencing. EpCAM<sup>+</sup> HuH1 cells were transfected with siRNA oligos and 1,000 cells were injected 24 hours after transfection.

Table 1

The tumor-initiating capacity of EpCAM<sup>+</sup> cells from clinical HCC specimens.

HCC Patients		Groups	No. of cells injected	Tumor incidence (mice with tumors/total # of mice injected)	
No.	% of EpCAM <sup>+</sup> HCC cells			2 months	3 months
1	5.2	EpCAM <sup>±</sup>	1 × 10 <sup>3</sup>	0/3	0/3
			1 × 10 <sup>4</sup>	2/3	2/3
			1 × 10 <sup>5</sup>	2/2	2/2
2	1.4	EpCAM <sup>±</sup>	1 × 10 <sup>5</sup>	0/3	0/3
			1 × 10 <sup>6</sup>	0/2	0/2
			1 × 10 <sup>3</sup>	0/2	0/2
		EpCAM <sup>±</sup>	1 × 10 <sup>4</sup>	0/1	1/1
			1 × 10 <sup>5</sup>	0/3	0/3
		EpCAM <sup>-</sup>	1 × 10 <sup>5</sup>	0/2	0/2