

# Epithelial Cell Adhesion Molecule Regulates Tumor Initiation and Tumorigenesis via Activating Reprogramming Factors and Epithelial-Mesenchymal Transition Gene Expression in Colon Cancer<sup>\*[5]</sup>

Received for publication, June 1, 2012, and in revised form, September 16, 2012. Published, JBC Papers in Press, September 18, 2012, DOI 10.1074/jbc.M112.386235

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**Background:** EpCAM is highly expressed on tumor and tumor-initiating cells.

**Results:** EpCAM induces reprogramming factor and EMT gene expression, which regulates tumor self-renewal and tumorigenesis.

**Conclusion:** EpCAM-mediated self-renewal and initiation of tumor cells are regulated by inducing reprogramming factors expressions.

**Significance:** Our data reveal the mechanism underlying EpCAM-mediated tumor initiation and tumorigenesis of tumor-initiating cells in colon cancer.

Epithelial cell adhesion molecule (EpCAM) is highly expressed in epithelial-transformed neoplasia and tumor-initiated cells (TICs), but the role that EpCAM plays in the stemness properties of TICs is still unclear. Here we show that EpCAM and reprogramming factors (*c-Myc*, *Oct4*, *Nanog*, and *Sox2*) were concomitantly elevated in TICs, which were shown to have superior self-renewal, invasiveness, and tumor-initiating abilities. Elevation of EpCAM enhanced tumorsphere formation and tumor initiation. Knockdown of EpCAM inhibited the expressions of reprogramming factors and epithelial-mesenchymal transition genes, thereby suppressing tumor initiation, self-renewal, and invasiveness. In addition, EpCAM, especially intracellular domain of EpCAM (EpICD), bound to and activated the promoter of reprogramming factors. Treatment with the inhibitor of  $\gamma$ -secretase (DAPT) led to the blockage of the expressions of reprogramming factors and epithelial-mesenchymal transition genes, which was accompanied by the reduction of tumor self-renewal and invasion. Furthermore, the increased release of EpEX enhanced production of EpICD and regulated the expression of reprogramming factors. Together, these findings suggest that EpCAM plays an important role in regulating cancer-initiating abilities in TICs of colon cancer. This discovery can be used in the development of new strategies for cancer therapy.

Tumor-initiating cells (TICs),<sup>2</sup> a subpopulation of cancer cells, are indispensable to tumor. They share many similar bio-

logical properties with embryonic stem cells (ESCs). Specifically, TICs, like ESCs, undergo molecular regulations such as perpetuating proliferation, persistent self-renewal, and unlimited differentiation (1). In addition, TICs are considered to be more drug-resistant and metastatic than non-TICs (2, 3). Several genes and transcriptional factors in maintaining stemness properties of ESCs have also been shown to be associated with tumor and TICs. Among these, the reprogramming factors (*OCT4*, *SOX2*, *NANOG*, and *c-MYC*), which possess the ability to reprogram adult somatic cells into pluripotent stem cells (4, 5), are reported to be up-regulated in initiated tumor cells (6–8). *c-MYC* is the enhancer of this process, and it has long been considered to play an oncogenic role in the formation of tumors. Additionally, elevated expression of *OCT4*, *SOX2*, and *NANOG* not only helps to maintain the stemness properties of TIC but also plays an essential role in the tumorigenic ability of TIC (7–9). However, little is known about the role of these four reprogramming factors in self-renewal and initiating potentials of tumor cells. The signaling mechanism underlying the regulation of these four factors is also unclear.

Epithelial cell adhesion molecule (EpCAM) is expressed in several types of carcinoma and has been used as a target to enrich TICs (10) and to isolate circulating tumor cells (11). The extracellular domain of EpCAM (EpEX) is composed of two epidermal growth factor-like domains and a cysteine-poor region, whereas the intracellular domain (EpICD) is composed of a short 26-amino acid fragment. EpCAM was previously thought to be a cellular adhesion molecule only, but recent studies have discovered that nuclear translocation of EpICD not only functions as a signaling transducer (12) but also correlates with tumor malignancy.

We have found previously that overexpression of EpCAM and/or the accumulation of EpICD is associated with undifferentiated status of ESCs (13). Additionally, the expression of EpCAM is involved in the reprogramming process of induced pluripotent stem cells (14). Therefore, it is necessary to unveil the mechanism and functional roles of EpCAM and EpICD in

<sup>\*</sup> This work was supported by Academia Sinica and Taiwan National Science Council Grants NSC-99-3111-B-001-007 and NSC-100-2325-B-001-011 (to H.-C. W.).

<sup>§</sup> This article contains supplemental Tables S1 and S2 and Figs. S1–S6.

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<sup>2</sup> The abbreviations used are: TIC, tumor-initiated cell; EpCAM, epithelial cell adhesion molecule; EMT, epithelial-mesenchymal transition; EpICD, intracellular domain of EpCAM; ESC, embryonic stem cell; EpEX, extracellular domain of EpCAM; qPCR, quantitative PCR; TACE, TNF- $\alpha$ -converting enzyme; TAPI, TACE inhibitor; DAPT, gamma-secretase inhibitor.

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TICs. In this study, we found that EpCAM induces expressions of reprogramming factors (*OCT4*, *SOX2*, *NANOG*, and *c-MYC*) and EMT genes. These factors collectively are found to have tumorigenic potential and play an instrumental role in the promotion of tumor initiation. Additionally, the increased release of EpEX enhances EpICD cleavage and regulates the expression of reprogramming genes.

### EXPERIMENTAL PROCEDURES

**Cell Culture**—The following human cell lines were used: oral cancer (FaDu and SAS), colorectal cancer (HCT116), and hepatocellular carcinoma (Hep3B) were purchased from ATCC (Manassas, VA) and were grown in DMEM supplemented with 5% or 10% FBS. Primary culture of normal nasal mucosal epithelia was adopted from surgery of patients with nasal polyposis.

**Spheroid Assay**—Cells ( $5\text{--}10 \times 10^3$ ) were seeded on an ultra-low 6-well plate (Corning, New York, NY) and maintained in DMEM/F-12 supplemented twice a week with B27, EGF (10 ng/ml), and FGF (25 ng/ml) (Invitrogen). For serum-induced differentiation assay, tumorspheres were cultured in Matrigel-coated plate supplemented with DMEM containing 10% FBS for 7 days to induce the differentiation process. To evaluate tumorigenic potential of tumorsphere, tumorspheres were trypsinized into single cells, passed through a cell strainer (BD Falcon, Franklin Lake, NJ) to prevent cell clumps, and then subjected to further analysis.

**Colony Formation and Invasion Assay**—The cells ( $5\text{--}10 \times 10^3$ ) were seeded in 6-well plate, incubated for 10 days, and then fixed and stained with crystal violet. For invasion assay, cells ( $1 \times 10^5$ ) were seeded in a Transwell insert (8- $\mu\text{m}$  filters; Corning) coated with Matrigel (BD Biosciences, La Jolla, CA). After a 24-h incubation, the cells were fixed with methanol for 10 min. The uninvaded cells were removed by cotton swap; the invaded cells were stained with DAPI, imaged under inverted fluorescent microscopy (Zeiss) and quantified using ImageJ software.

**Plasmid Constructions**—Full-length human EpCAM was cloned into pcDNA3.1-v5-His. The pEpEX<sub>291</sub> (composed of the extracellular and transmembrane domain of EpCAM) and pEpICD plasmids were constructed from pcDNA3.1-EpCAM. Luciferase reporter activities were constructed by inserting the PCR fragments of *c-MYC* (−1224/+47 related to transcriptional start site), *OCT4* (−2616/+1), and *NANOG* (−1590/+250) into pGL4.1 plasmid (Promega, Madison, WI). Lentivirus the encoding small hairpin RNA of EpCAM (pLKO-shEpCAM) and the control plasmid pLKO-AS1 were obtained from RNAi Core Facility (Academia Sinica, Taipei, Taiwan).

**Lentivirus Infection**—HEK293T packaging cells were co-transfected with packaging plasmid (pCMV- $\Delta\text{R8.91}$ ), envelope (pMDG), and hairpin pLKO-RNAi vectors using a PolyJET transfection kit (SignaGen Laboratories, Ijamsville, MD). At 48 h post-transfection, virus-containing supernatants were collected, mixed with fresh medium containing polybrene (8  $\mu\text{g}/\text{ml}$ ), and incubated with target cells for another 48 h. The transduced cells were selected with puromycin (4  $\mu\text{g}/\text{ml}$ ) for 4 days.

**Luciferase Reporter Assay**—The Cells were seeded in a 24-well plate and co-transfected with pcDNA3.1-expressing vectors (EpCAM, EpICD, or EpEX; 400 ng) and reprogramming gene-relative promoters (pGL4-Oct4-Luc, pGL4-Nanog-Luc,

pGL4-Sox2-Luc, or pGL4-c-Myc-Luc; 100 ng) by PolyJET for 24 h. Promoter activities were measured using a Dul-Glo luciferase kit (Promega, Madison, WI). The transfected efficiency was normalized by co-transfection with pRL-TK (20 ng) as an internal control.

**Chromatin Immunoprecipitation**—The protein-DNA complexes were cross-linked using 1% formaldehyde and quenched by adding glycine to a final concentration of 200 mM. The chromatin complexes were sonicated to an average size of 250 bp by MISONIX Sonicator 3000. For immunoprecipitation, 4  $\mu\text{g}$  of anti-EpICD (A20, Santa Cruz Biotechnology) was incubated with protein A beads (Invitrogen) for 4 h. The immunocomplexes were further incubated with chromatin for another 4 h. The bound fraction was isolated by protein A beads according to the manufacturer's instructions, and the immunocomplexes were subjected to reverse cross-linking. The immunoprecipitated DNA was recovered by PCR purification kit (Qiagen), and the purified DNA were subjected to real time quantitative PCR for further analysis. All of the ChIP data were calculated by immunoprecipitation/input for each gene and were further normalized to the level of GAPDH promoter, which was defined as 1.0.

**Real Time Quantitative PCR**—Total RNAs were extracted by TRIzol reagent (Invitrogen) and reverse transcribed by SuperScript III reverse transcriptase (Invitrogen). cDNA were amplified with KAPA SYBR fast PCR mix (Kapa Biosystems, Woburn, MA), and quantitative PCR was performed using a LightCycler480 System (Roche Applied Science). The human colon cancer array V (Origene, Rockville, MD) was analyzed in an ABI9600 thermocycler (Applied Biosystems). The results were expressed as fold change relative to control sample by equation  $\Delta\Delta C_T$ . *GAPDH* or *18S rRNA* were used as an internal normalization control. All of the primer sequences are provided in supplemental Table S2.

**Immunoprecipitation and Western Blotting**—Cell-free supernatants were passed through a Vivaspin concentrator (Sartorius Stedim Biotech) to eliminate molecules with a molecular weight under 10,000. An equal amount of proteins (1000  $\mu\text{g}$ ) was incubated with Dynabeads protein G (Invitrogen Dynal AS, Oslo, Norway) in conjunction with anti-EpCAM (N-ter) antibody (3  $\mu\text{g}$ ) at 4 °C for 4 h. The mixtures were washed twice with ice-cold radioimmune precipitation assay buffer and boiled in SDS sample buffer. For Western blotting, the cells were extracted with radioimmune precipitation assay buffer containing protease inhibitor mixture (Roche Applied Science). An equal amount of proteins were separated on SDS-PAGE and then transferred to PVDF membranes (Millipore, Bedford, MA). The membrane was blocked with 1% BSA and incubated with specific primary antibodies overnight. Appropriated HRP-conjugated secondary antibodies (1:5000; Jackson ImmunoResearch) were incubated for 1 h at room temperature, and expression of protein was detected using an ECL kit (Millipore, Temecula, CA). All the information of antibodies used was listed in supplemental Table S1.

**Immunofluorescence**—The cells grown on coverslips were fixed with 4% paraformaldehyde at room temperature for 10 min and switched to methanol at −20 °C for 6 min and 25 °C for 10 min, followed by blocking with 1% BSA in PBS. The cover-

slips were then incubated with an indicated primary antibody for 3 h at room temperature and washed twice with PBS. Secondary antibodies, goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 568 (1:250; Invitrogen), were incubated for 1 h. DAPI (1:500) was used for nuclear counterstaining. The slides were observed under confocal microscopy (TCS SP5; Leica, Wetzlar, Germany).

**Flow Cytometric Analysis and Cell Sorting**—Trypsinized cells were washed twice with FACS buffer (1% FBS and 20 mM HEPES in PBS) and then incubated on ice with FITC-conjugated EpCAM or PE-CD133 antibodies for 30 min. The cells were washed twice with FACS buffer and analyzed using FACSCanto II flow cytometry equipment (BD Bioscience) and FACSiva software (BD Bioscience).

**Immunohistochemistry**—Human colon cancer tissue microarray (TMA BC05011) was purchased from Biomax (Rockville, MD). The use of tissue microarray was approved by Human Subject Research Ethics Committee, IRB, Academia Sinica (AS-IRB02-100067). Immunostaining was performed using HRP-polymer IHC kit (BioGenex, Fremont, CA) according to the manufacturer's directions. Tissue microarray was scanned by TissueFAX, and the expression of EpICD was quantified by HistoQuest software (TissueGnostics GmbH, Vienna, Austria). The data were represented as diaminobenzidine (DAB)-positive percentage  $\times$  DAB-mean intensity.

**Animal Study**— $2 \times 10^6$  of HCT/pLKO and HCT/EpCAM shRNA cells were injected subcutaneously into dorsal flank of 6-week-old NOD/SCID mice. Tumor volume was measured by electric caliper every 3 days during experiments and was calculated using the following equation: length  $\times$  (width)<sup>2</sup>  $\times$  0.52. For *in vivo* limiting dilution assay, spheroid or adherent cells were dissociated into single cells and mixed with Matrigel (1:1). The cell mixtures were implanted into both sides of the dorsal flank of NOD/SCID mice. Tumor incidence was defined when a tumorous mass reached 100 mm<sup>3</sup>, and growth was monitored until it reached 1000 mm<sup>3</sup>. Tumor latency was defined by average day for tumor to grow to 100 mm<sup>3</sup>. All animals were kept in SPF room and treated according to the animal care protocol established by Academia Sinica Animal Committee.

**Statistical Analysis**—All of the data were derived from at least three-independent experiments. The values are expressed as the means  $\pm$  S.D. The significant difference from the respective control for each experimental test condition was calculated using Student's *t* test, unless specified otherwise. *p* values of <0.05 (\*) or <0.01 (\*\*) were considered significant. Survival analysis was performed by log-rank test. Correlation co-efficient was assayed by Spearman's analysis.

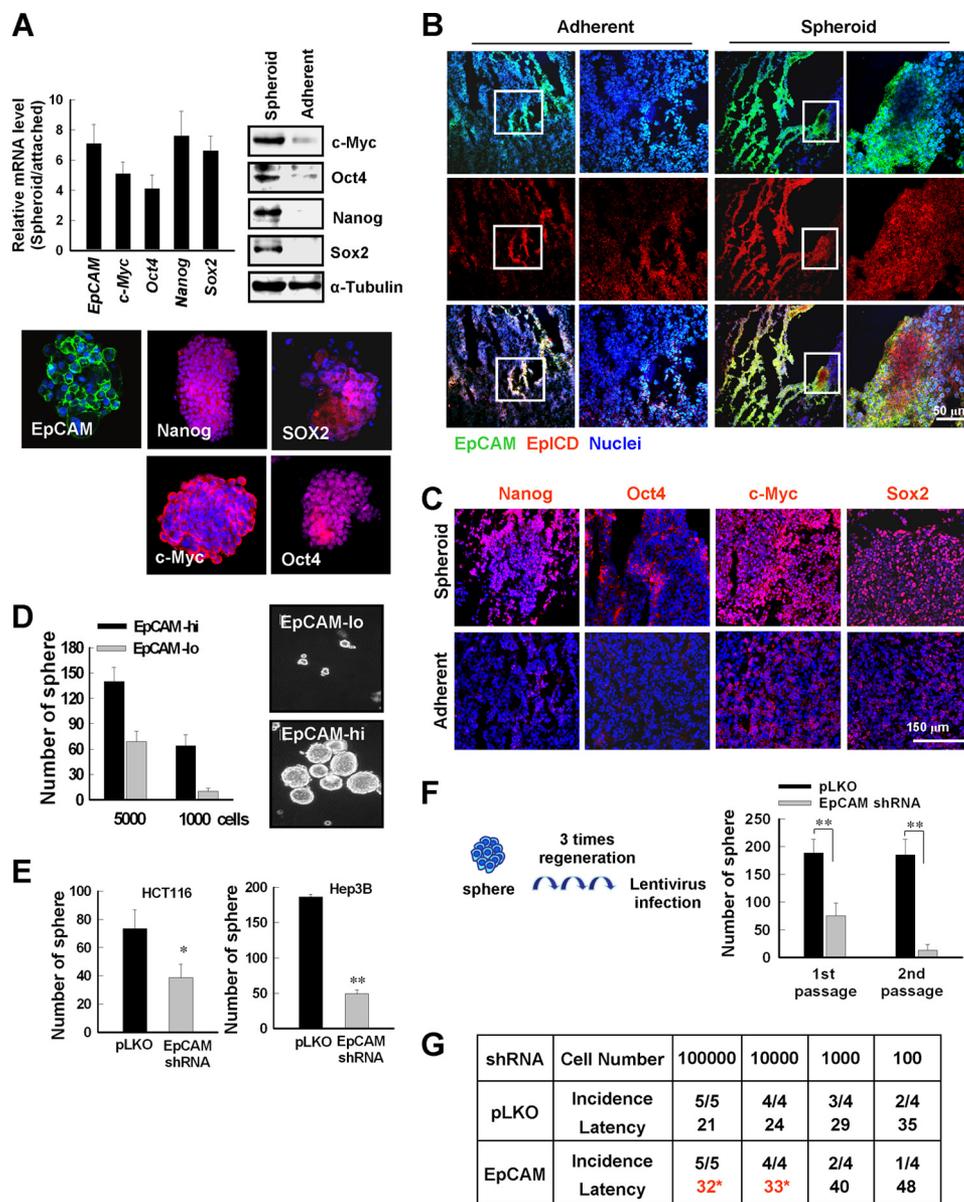
## RESULTS

**Elevation of EpCAM Is Associated with Reprogramming Factors Expression, Self-renewal, and Tumor-initiating Properties**—We cultivated the attached cancer cells into the tumorsphere and found that EpCAM expression was elevated in the tumorspheres of HCT116 (colon cancer), SAS (oral carcinoma), FaDu (pharynx carcinoma), and Hep3B (hepatoma) (Fig. 1A and supplemental Fig. S1, A–C). Double staining of EpCAM and CD133 showed that CD133-positive cells were localized in population with high EpCAM expressions (supple-

mental Fig. S1D). Further evaluation of the tumorigenic potential of tumorsphere showed that tumorsphere-derived cells possessed both superior growth and invasive properties *in vitro* and tumor-initiating ability *in vivo* (supplemental Fig. S2, A–C). In addition, tumorsphere-derived xenograft had a more aggressive phenotype (supplemental Figs. S2D), with persistent self-renewal and EpCAM expression (supplemental Fig. S2, E and F). However, the EpCAM expression level declined after sequential passages in the differentiated condition (data not shown). Additionally, the elevations of EpCAM and CD133 in the Hep3B tumorsphere were both decreased after cultured in the differentiated condition (supplemental Fig. S1D), suggesting that up-regulation of EpCAM may be associated with the undifferentiated status of TICs. Because the expression of reprogramming factors, c-Myc, Oct4, Nanog, and Sox2, is associated with undifferentiated status of ESCs and the self-renewal property of TICs, we next examined the correlation between these factors and EpCAM. The results showed that the expressions of EpCAM and reprogramming factors were both up-regulated in tumorsphere and tumorsphere-derived xenografts when compared with that in adherent tumor cell-derived xenografts (Fig. 1, A–C). Moreover, tumorsphere formation was greater in EpCAM-enriched cells (Fig. 1D), but it was reduced in EpCAM knockdown tumor cells (Fig. 1E). To further evaluate the importance of EpCAM in the self-renewal of TICs, HCT116 tumorspheres were transduced with EpCAM shRNA. The results showed that knockdown of EpCAM in tumorsphere impaired self-renewal and tumor-initiating abilities (Fig. 1, F and G, and supplemental Fig. S3). Suppression of EpCAM in tumorsphere increased the formation time of tumor burden, suggesting that up-regulation of EpCAM may be important in regulating reprogramming gene expression and tumor initiation.

**EpCAM and EpICD Regulate Reprogramming Gene Expression**—We further examined whether EpCAM can regulate reprogramming factors expression. Real time PCR data showed that the expression of c-Myc, Oct4, Nanog, and Sox2 mRNA were up-regulated in EpCAM-enriched or -overexpressed cells (Fig. 2, A and B). Conversely, these expressions were down-regulated in EpCAM knockdown or low expressed cells (Fig. 2, A and C). Luciferase assay indicated that overexpression of EpCAM induced promoter activities of c-Myc, Oct4, Nanog, and Sox2 (Fig. 2D). Because nuclear translocation of EpICD has been reported to play a role in mediating EpCAM signaling, we tested whether EpICD can control reprogramming factors. Immunofluorescent staining data showed that a higher expression of soluble EpICD was detected in spheroid-derived tumor sections than that in adherent cells-derived tumors (Fig. 1B). Additionally, soluble EpICD were found in both cytoplasm and nucleus of HCT116 cells but not in DAPT (a  $\gamma$ -secretase inhibitor)-treated cells (supplemental Fig. S4A). In the latter, most of EpICDs were found to be co-localized with membrane-bound EpEX instead (supplemental Fig. S4A). Western blot analysis confirmed the presence of EpICD in 293T/EpCAM-v5 cells, but the expression of EpICD was reduced after the treatment with DAPT (supplemental Fig. S4B). Treatment with DAPT not only inhibited EpCAM-induced promoter activities of c-Myc, Oct4, Nanog, and Sox2 (Fig. 2E), but it also decreased tumorsphere

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**FIGURE 1. Elevation of EpCAM is associated with reprogramming factors expression, self-renewal, and tumor initiation abilities.** *A*, increasing EpCAM and reprogramming gene expressions in tumorsphere. Real time qPCR (*top left*), Western blotting (*top right*), and immunofluorescence staining (*bottom*) analyses of the expressions of EpCAM, c-Myc, Oct4, Nanog, and Sox2 in spheroid and adherent HCT116 cells. *B*, tumor cryosections from adherent or spheroid xenografts were stained with EpCAM (green), EpICD (red), and DAPI (blue). Each of the *right panels* shows high magnification ( $\times 400$ ) from the boxed region of the *left panel* ( $\times 200$ ). *C*, immunofluorescent staining analysis of c-Myc, Oct4, Nanog, and Sox2 expression in spheroid or adherent cells-derived xenograft. *D*, spheroid formation from sorted EpCAM high and low expressed cells were counted in triplicate. Representative images of tumorspheres from EpCAM high and low expressed cells are shown. *E*, suppression of EpCAM reduces tumorsphere formation in Hep3B and HCT116 cells. Bar, 50  $\mu\text{m}$ . *F*, knockdown of EpCAM in tumorsphere impairs self-renewal ability *in vitro*. The experimental procedures are shown above. \*\*,  $p < 0.01$  denotes a significant difference. *G*, *in vivo* limiting dilution assay demonstrates that EpCAM knockdown in tumorsphere inhibits tumor-initiating capability. \*,  $p < 0.01$  was analyzed by log-rank test.

formation (Fig. 2*G*). Further analysis of EpICD in regulating reprogramming factors showed that EpICD induced the promoter activation of c-Myc, Oct4, Nanog, and Sox2 (Fig. 2*F*). Chromatin immunoprecipitation assay also indicated that EpICD occupied c-MYC (proximal upstream region instead of exon 1), OCT4 (distal upstream region), NANOG (upstream region), and SOX2 (downstream region) in HCT116 cells (Fig. 2*H*), but not in normal nasal mucosa cells, which expressed a low level of EpCAM (data not shown). These data indicate that EpICD may regulate the activation of reprogramming genes and thereby modulate TICs.

*EpCAM Regulates EMT Progression and Tumorigenesis*—In addition to reprogramming factors, we also assessed the effect of EpCAM on tumorigenic potential. The results showed that knockdown of EpCAM reduced colony formation and invasion (Fig. 3, *A* and *B*), and treatment of DAPT decreased tumor invasion (Fig. 3*C*). Moreover, suppression of EpCAM inhibited xenograft tumor growth *in vivo* (Fig. 3*D*). Analysis of RNA samples obtained from xenograft tumors showed that reprogramming genes (c-Myc, Oct4, and Nanog) and EpCAM were significantly down-regulated in EpCAM knockdown groups (Fig. 3*E*). In addition, compared with the control groups, EpCAM

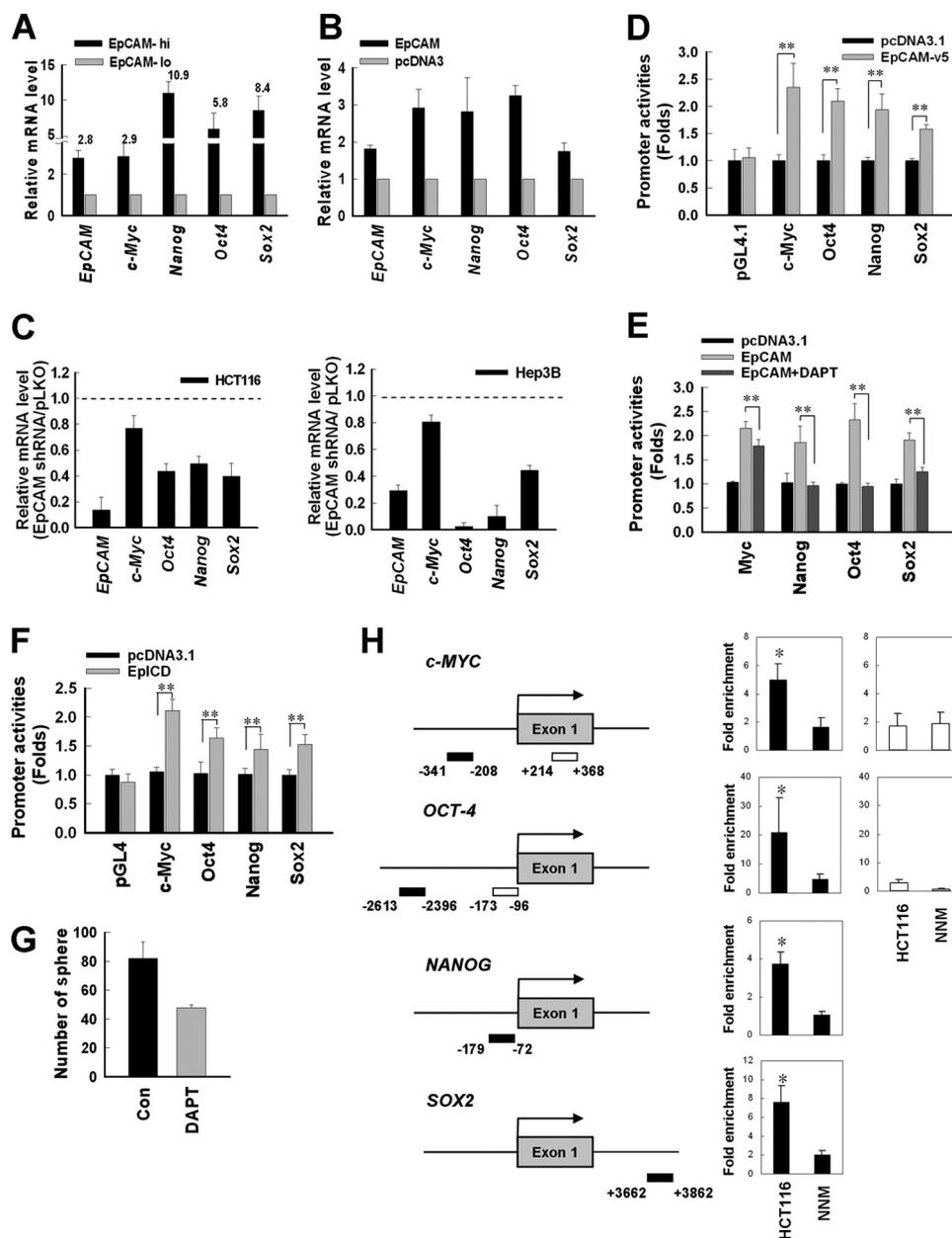


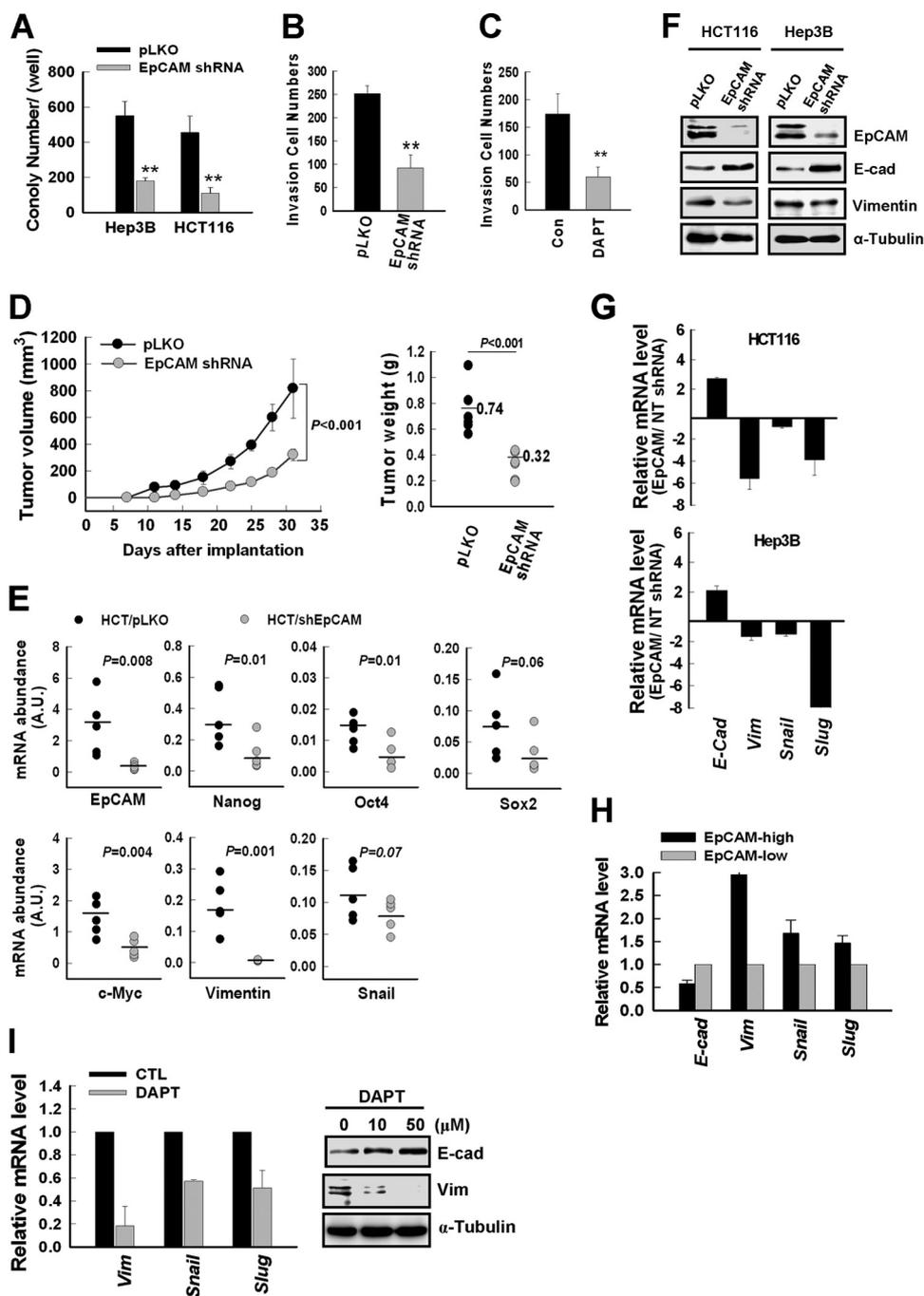
FIGURE 2. **EpCAM regulates expression of reprogramming genes (c-MYC, OCT4, NANOG, and SOX2).** A, real time qPCR analysis of gene expression in EpCAM high and low expressed HCT116 cells. B, ectopic expression of EpCAM in HEK293 cells, and reprogramming gene expression were analyzed by real time qPCR. C, real time qPCR analysis of EpCAM and reprogramming genes mRNA level in EpCAM knockdown Hep3B and HCT116 cells. D, overexpression of EpCAM up-regulates transcriptional activities of c-Myc, Oct4, Nanog, and Sox2. Promoter activities were assessed by luciferase assay. E, DAPT inhibits EpCAM-induced c-Myc, Oct4, Nanog, and Sox2 transcriptional activities. F, EpiCD induces transcriptional activation of reprogramming genes. G, DAPT inhibits tumorsphere formation. H, quantitative ChIP analysis of DNA occupancy by EpiCD on c-MYC, OCT4, NANOG, and SOX2 genes in HCT116 and normal nasal mucosa cells (NNM) cells. \*,  $p < 0.05$  represents significance. Con, control.

knockdown groups had a reduction in EMT markers, such as vimentin and Snail (Fig. 3E). Sox2 and Snail had a modest but insignificant decrease in EpCAM knockdown xenograft. We also found an increase in E-cadherin with a concomitant decrease in vimentin both in protein and mRNA levels in EpCAM knockdown cells (Fig. 3, F and G). Similar results from immunofluorescent analyses also depicted the changes that occurred in epithelial markers (the up-regulation of E-cadherin and cytokeratin 18) and in mesenchymal marker (the down-regulation of vimentin) after EpCAM was knocked down (supplemental Fig. S5). Other EMT regulatory transcriptional factors such as Snail and Slug were simultaneously reduced in both

EpCAM knockdown cells and EpCAM low expressed cells (Fig. 3, G and H). However, the expression of EMT genes was attenuated in DAPT-treated cells (Fig. 3I). These findings suggest that EpCAM may involve in regulating EMT progression and promoting tumorigenesis.

*Extracellular Domain of EpCAM Triggers EpCAM Signaling and Regulates Reprogramming Gene Expression*—To further address the importance of EpCAM in controlling reprogramming gene expression, we constructed an EpiCD deleted vector (EpEX<sub>291</sub>-v5), which contained the extracellular and transmembrane domains of EpCAM (Fig. 4A). Intriguingly, the promoter activities of c-MYC, OCT4, NANOG, and SOX2 were

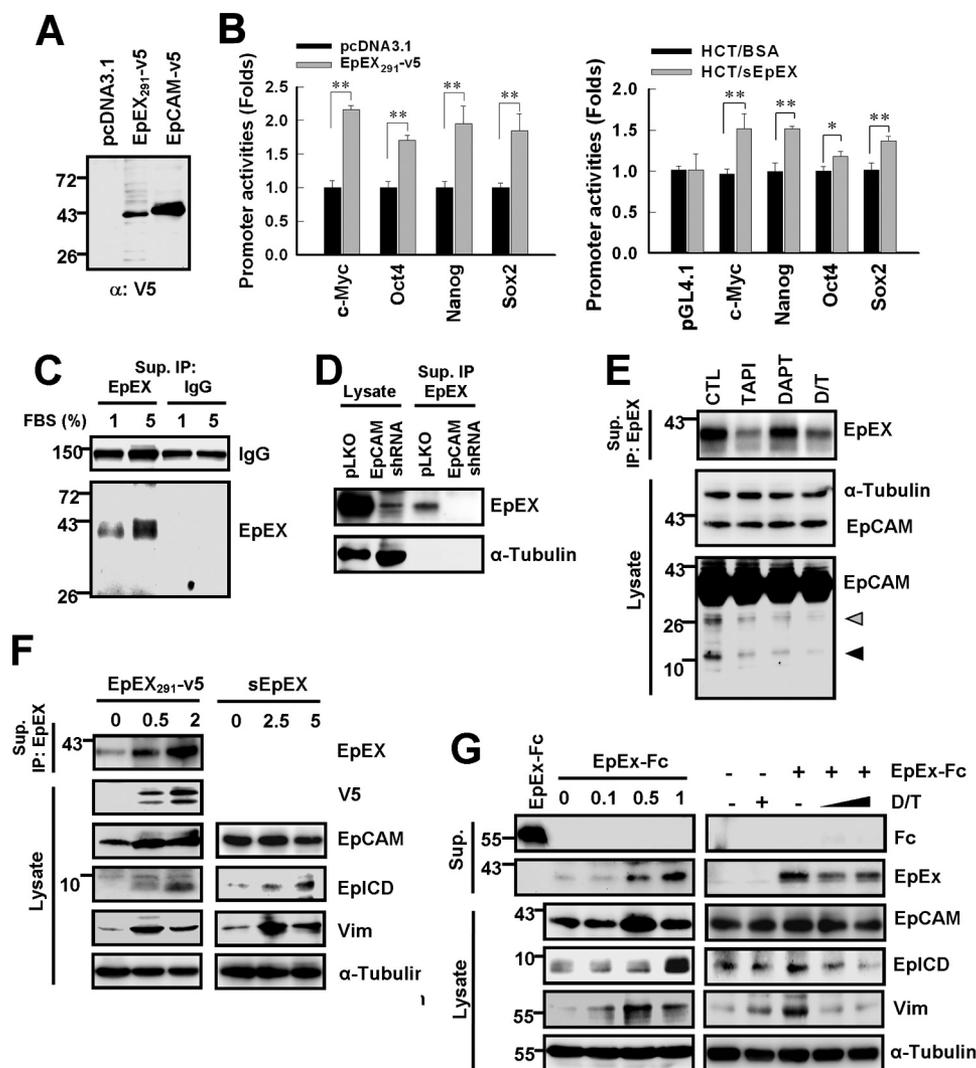
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**FIGURE 3. Suppression of EpCAM attenuates EMT progression and tumorigenesis.** A and B, suppression of EpCAM inhibits colony formation (A) and invasion (B) of tumor cells *in vitro*. C, DAPT inhibits tumor invasion. D, suppression of EpCAM reduces tumor growth *in vivo*. Tumor volumes were measured every 3 days (left panel), and tumor weights were measured at the end of the experiments (right panel) ( $n = 6$ ). E, RNA from tumor extractions was analyzed by real time qPCR. The data are presented as the means  $\pm$  S.D., and the  $p$  value was analyzed by  $t$  test. F and G, Western blot (F) and real time qPCR (G) analyses of EMT gene expression in EpCAM knockdown Hep3B and HCT116 cells. H, real time qPCR analysis of EMT gene expression in EpCAM high and low sorted cells. I, DAPT attenuates EMT gene expression. CTL or Con, control.

enhanced in EpEX<sub>291</sub>-v5-transfected HCT116 cells (Fig. 4B). Similar results were observed when cells were treated with soluble EpEX (Fig. 4B), suggesting that shedding or releasing of extracellular domain of EpCAM might also play a part in coordinating EpCAM regulation, and the increasing production of EpEX might enhance EpICD signaling. To test this hypothesis, cultured supernatant from HCT116 cells were immunoprecipitated with antibody against extracellular domain of EpCAM. The release of EpEX increased in the presence of the serum (Fig.

4C) but decreased in the EpCAM knockdown cells (Fig. 4D). Moreover, treatment with DAPT blocked EpICD but not EpEX cleavage (12), whereas treatment with a TNF- $\alpha$ -converting enzyme inhibitor (TAPI) or TAPI/DAPT inhibited both EpICD and EpEX cleavage (Fig. 4E). Forced expression of EpEX<sub>291</sub>-v5 in HCT116 cells resulted in an increase release of total EpEX in culture supernatants. Similarly, ectopic expression of EpEX resulted in an increased release of EpEX from 293T/EpCAM cells (data not shown). The inductions of EpICD cleavage and



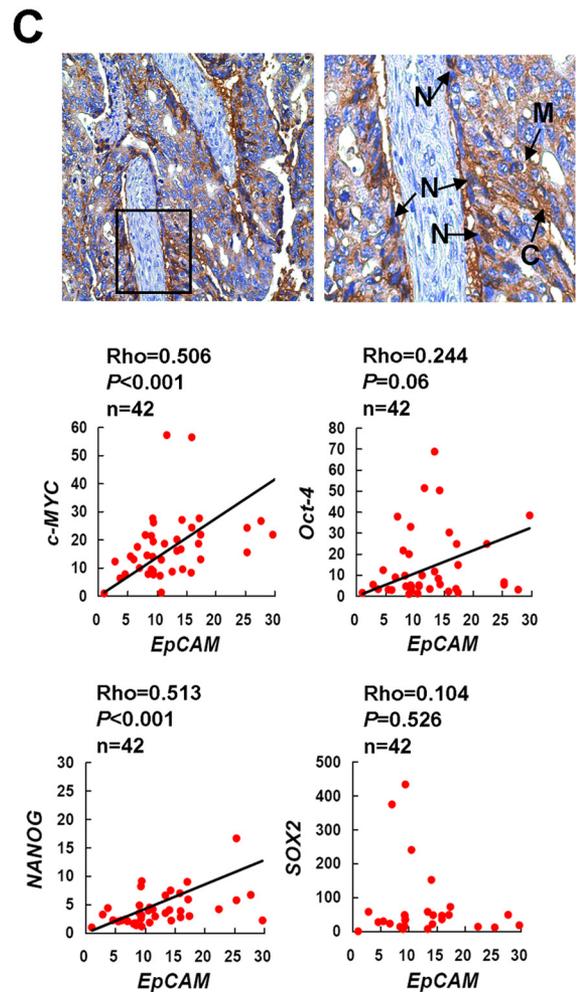
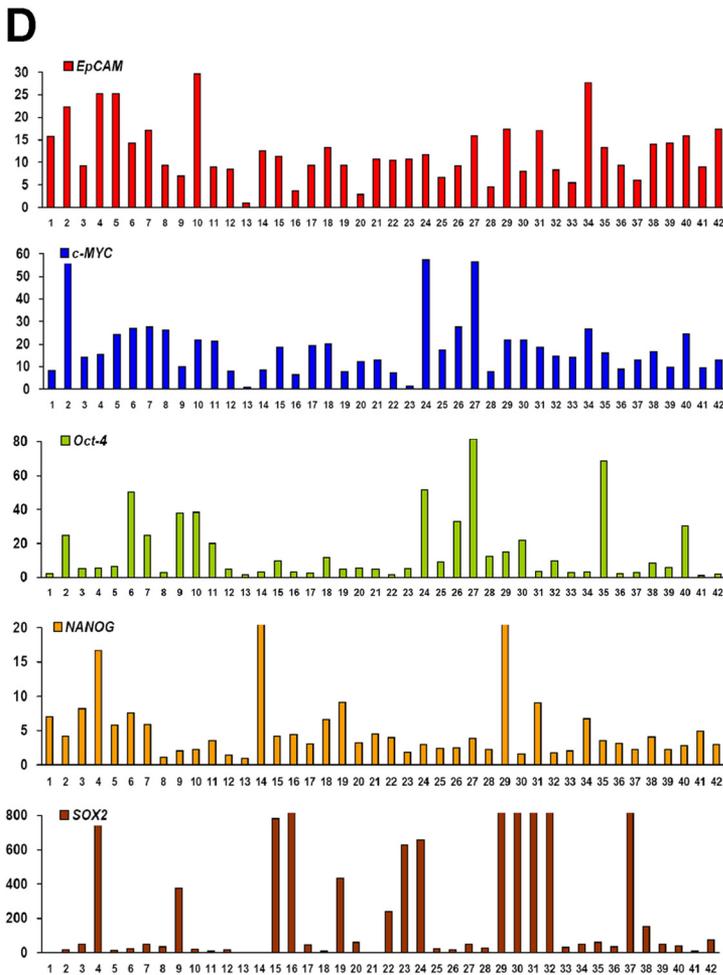
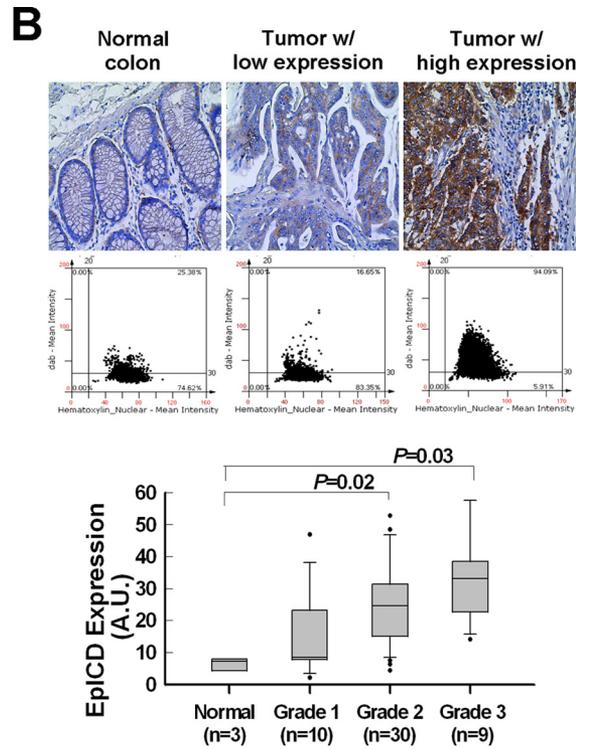
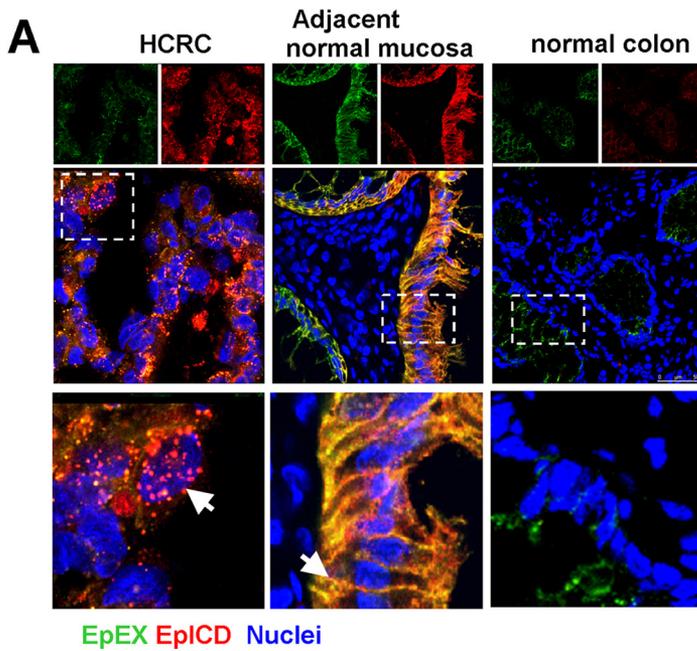
**FIGURE 4. EpEX modulates reprogramming gene expression.** *A*, protein expressions of EpEX<sub>291</sub> and full-length EpCAM in 293T transfectants. *B*, EpEX induces promoter activities of c-Myc, Nanog, Oct4, and Sox2. The cells were either transfected with pEpEX<sub>291</sub> (left panel) or added soluble EpEX (sEpEX; 2 μg/ml) (right panel); promoter activities were assessed by luciferase assay. BSA was used for control treatment. *C*, extracellular release of EpEX in EpCAM-expressed cells. Immunoprecipitation (IP) and Western blotting analyses of the releasing of EpEX level in culture supernatants (sup.) of HCT116 cells. *D*, identification of EpEX in culture supernatant from HCT/LKO and from HCT/EpCAM shRNA cells. *E*, HCT116 cells were treated with DAPT (50 μM), TAPI (40 μM), or both (D/T) for 24 h, the culture supernatants were immunoprecipitated with EpEX antibody, and whole cell lysates were subjected to Western blotting with anti-EpEX (middle panel) or anti-EpICD (bottom panel) antibodies. Black arrowhead, soluble EpICD; gray arrowhead, intermediate EpICD. *F*, HCT116 cells were transfected with pEpEX<sub>291</sub>-v5 (0.5, 2 μg) (left panel) or treated with sEpEX (2.5, 5 μg/ml) (right panel), and the culture supernatants and cell lysates were analyzed as described above. *G*, cells were treated with EpEX-Fc (0.1, 0.5, 1 μg/ml) in the absence (left panel) or presence (right panel) of DAPT/TAPI (20, 40 μM) for 24 h, and the culture supernatants were pulled down by protein G followed by Western blot analysis using anti-EpEX antibody. Antibody against human Fc was used for detecting the contamination of EpEX-Fc. The cell lysates were analyzed as described above. Lane 1, EpEX-Fc was used as a positive control. CTL, control.

vimentin expression were detected in both EpEX<sub>291</sub>-v5 transfectants and sEpEX-treated cells (Fig. 4F). Similar results were also obtained in EpEX-Fc-treated cells. EpEX-Fc treatment induced the release of endogenous EpEX from EpCAM and promoted the cleavage of EpICD, but these phenomena were not observed in the presence of DAPT/TAPI, suggesting that the release of endogenous EpEX and the cleavage of EpICD were inhibited by DAPT/TAPI (Fig. 4G). Moreover, immunofluorescent analysis of human colon cancer specimens further revealed that some tumor cells expressing soluble EpICD in the nucleus lost their membrane-EpEX detection signal, whereas other tumor cells or their adjacent mucosa cells showed intact co-localization of EpICD and EpEX in the cell membrane. There was a low expression of either EpEX or EpICD in normal

colon tissue with no detectable expression of EpICD in the nucleus of normal colon tissue (Fig. 5A).

**EpCAM/EpICD Expression in Human Colon Cancer Correlates with Reprogramming Factors**—We further analyzed the expressions of EpCAM and EpICD and their associations with reprogramming factors and tumor malignancy in human colon cancer specimens. Expression of EpICD was increased in cancerous colon tissues, compared with those in normal ones. The expression level of EpICD in cancer appeared to be associated with tumor grade (Fig. 5B). Twenty-four of 49 tumor sections were positive with nuclear EpICD signals. However, the nuclear EpICD signals were observed only in certain numbers of tumor cells within the positive sections (Fig. 5, A and C), suggesting that nuclear translocation of EpICD may be a dynamically tran-

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sient regulation. Further evaluation of the correlation between EpCAM and the four reprogramming factors in the 42 colon cancer patients through analysis of the mRNA level showed that the expression of EpCAM was positively correlated with *c-MYC* (co-efficiency = 0.501; moderate correlation), *NANOG* (co-efficiency = 0.513; moderate correlation), and *OCT4* (co-efficiency = 0.244; minor correlation) (Fig. 5D).

## DISCUSSION

Tumor initiation and EMT progression play an important role in cancer formation, with a myriad of genes participating and cooperating with each other throughout these processes. Although the relevance of EpCAM with tumor malignancy has been well documented, most studies focus the effect of EpCAM on the proliferation (15–18) or the invasiveness (19, 20) of tumor cells. Other studies used EpCAM as a surface marker for identifying tumor-initiating cells (10, 11, 21, 22). The genetic profile regulated by EpCAM in the self-renewal and initiation of TICs, however, has yet to be elucidated. Up-regulations of EpCAM and  $\alpha$ -fetoprotein (*AFP*) have been observed in the hepatic stem cell of hepatocellular carcinoma (10). Elevated co-expression of EpCAM and other TICs genes, such as *CD133*, *CD44*, and *LGR5*, have been detected in tumorspheres (23). In addition, several reprogramming genes, such as *NANOG*, *SOX2*, *NOTCH*, and *LIF*, have been found to intensely promote tumorsphere formation and tumor initiation (9, 24, 25). Alternatively, up-regulation of EpCAM has been shown both to increase the reprogramming process of induced pluripotent stem cells by suppressing p53 and p21 expression and to maintain undifferentiated status of ESCs through the controlling of the pluripotent gene expression (13, 14, 26). However, there has been no direct evidence linking the regulation of EpCAM in these reprogramming genes in TICs. Our data show that EpCAM regulated *c-Myc*, *Oct4*, *Nanog*, and *Sox2* in cancer cells (Figs. 1 and 2). Suppression of EpCAM repressed these gene expressions, which resulted in the inhibition of self-renewal, growth, and tumor-initiating abilities. Analysis of the cancer tissue further confirmed that the expression of EpCAM was positively correlated with *c-Myc*, *Oct4*, and *Nanog*. Because the expression of self-renewal genes in tumor cells has been identified to be closely linked to the promotion of tumor initiation and tumorigenesis (7–9), we proposed that these factors are involved in EpCAM-mediated tumor initiation in TICs.

The intracellular domain of EpCAM, EpICD, has been reported to play a dominant role in EpCAM signaling (12). Nuclear translocation of EpICD participates in gene modulation in both ESCs and tumor cells. In ESCs, nuclear EpICD binds to stemness genes (13). In tumor cells, the accumulation of nuclear EpICD is correlated with tumor malignancy and

poor prognosis of thyroid, prostate, head and neck, and breast cancers (27, 28). EpICD is regulated by proteolysis through TNF- $\alpha$ -converting enzyme (TACE) and  $\gamma$ -secretase (presenilin 2) in collaboration with FHL2 and Tcf/Lef1. Treatment with either the inhibitor of TACE or presenilin 2 suppresses EpCAM-dependent genes (12, 16). However, these two enzymes are required for the shedding of several transmembrane proteins including amyloid peptide, cadherin, Notch, as well as EpCAM. Some of these proteins, such as Notch and EpCAM, are involved in tumorigenesis. Our data were consistent with previous findings that accumulation of nuclear EpICD was detected in tumor cells (27, 28), tumorsphere-derived xenografts, and tumor tissues (Fig. 1). Treatment with the inhibitors of TACE (TAPI) or presenilin 2 (DAPT) blocked the cleavage of EpEX and EpICD, which was accompanied by the suppressions of self-renewal gene expression, promoter activation, and tumorsphere formation elicited by EpCAM (Figs. 2 and 4). Additionally, overexpression of EpICD induced promoter activities of *Oct4*, *Nanog*, *Sox2*, and *c-Myc* (Fig. 2F). Recent studies also delineated that EpICD promoted cell cycle progression and that the addition of DAPT inhibited EpCAM-induced cyclin D1 transactivation (15). These findings suggest that EpICD indeed participates in tumor self-renewal and tumorigenesis and that  $\gamma$ -secretase inhibitor may possess the potential of being developed into cancer therapeutics. However, we cannot rule out the possibility of other potential effects from the intracellular signaling events by  $\gamma$ -secretase inhibitor. Moreover, the presence of soluble EpICD in either cytoplasm or nucleus was not expressed homogeneously in any tumor cells, suggesting that the cleavage of EpICD might be a dynamic process (Fig. 5). Notably, parts of tumor cells with high expression of EpICD in the nucleus lost their membrane-EpEX detection signal. Therefore, pathologic correlations with surface EpCAM expression have to include nuclear EpICD staining to correctly elucidate the role of EpCAM in tumorigenesis.

We also found that the increased release of EpEX may enhance EpICD cleavage and trigger EpCAM signaling (Fig. 4). Recent studies demonstrated that the release of EpEX and EpICD was determined by cellular density (29), and contact growth of rat marrow stromal cells promotes TACE enzyme activity (30), suggesting that cell-cell interaction may be a factor in initiating EpEX activation. In our experiments, the addition of EpEX in HCT116, which are EpCAM-positive cells, induced transcriptional activity of self-renewal genes, possibly through the activation of endogenous EpCAM/EpICD triggered by EpEX (Fig. 4, C–G). However, treatment with EpEX in 293T, which is lacking of EpCAM, did not induce self-renewal gene activation (supplemental Fig. S6). These data suggest that

**FIGURE 5. Correlation between EpCAM (EpICD) and reprogramming gene expression and cancer malignancy.** *A*, cellular localization of EpEX and EpICD in human colorectal carcinoma (HCR), in adjacent normal mucosa, and in normal colon tissue. Representative images are shown in a separate image by EpEX (green) and EpICD (red) (top panel) and merged image (middle panel), and the bottom panel shows enlargements of regions in dashed boxes in respective images above. Arrows indicate cellular localization of EpICD in the nucleus (human colorectal carcinoma) and in the membrane where co-localization with EpEX (adjacent normal mucosa) occurred. *B*, immunohistochemistry analysis of EpICD protein expression by human colon cancer tissue microarray. Representative images are shown in randomly enlarged local view (top panel); scatter plot of EpICD quantification in the whole tissue section (middle panel); and box plot of EpICD expression in normal and tumor with different grade (bottom panel). The *p* value was analyzed by *t* test. *C*, cellular localization of EpICD expresses in membrane (M), cytosol (C), and nucleus (N). The right image is shown in enlarged local view from the boxed region of the left image. *D*, expressions of EpCAM, *c-Myc*, *Nanog*, *Oct4*, and *Sox2* mRNA level in 42 human colon cancer patients were determined by qPCR analysis (upper graphs). Correlation between EpCAM and reprogramming genes was evaluated by Spearman's analysis (lower graphs).

## EpCAM Regulates Self-renewal of Tumor-initiating Cells

EpICD acts as a signaling transducer co-activator, whereas the release of EpEX further triggers EpCAM/EpICD activation. Moreover, treatment with EpEX-Fc increased the release of endogenous EpEX, which was accompanied by the induction of EpICD cleavage and vimentin expression. However, these events were inhibited in the presence of TAPI/DAPT (Fig. 4G), suggesting that enhancing of regulated intramembrane proteolysis by EpEX might participate in the cleavage of EpCAM and accompanied signaling activation. In analogy to Notch pathway, interaction of Notch with its associated protein causes Notch extracellular domain disassociation and triggers Notch-ICD activation (31). A recent study reported that shedding of extracellular domain of E-cadherin may further support ErbB2 signaling (32); however, the mechanism by which the soluble EpEX enhances the disassociation of EpEX and EpICD still needs to be investigated.

The induction of EMT has been shown not only to promote tumor metastasis but also to generate cells with stem-like signatures (33, 34). However, the mechanisms of EpCAM-regulated EMT gene expression and EMT-mediated stemness have yet to be clarified. Previous studies indicated that EpCAM induced the invasion of breast cancer through its interruption of E-cadherin (19). In addition, a mutation of EpCAM that lacks EpICD has been reported to abrogate cadherin-mediated junction (35), suggesting that EpICD not only negatively regulates cell-cell interaction, but it may also be associated with increased tumor metaplastic properties and mobility. Our study found that EpCAM regulates Snail, Slug, and vimentin. Knocking down EpCAM or blocking the release of EpICD suppressed the expression of Snail, Slug, and vimentin, thus reducing tumor invasiveness (Fig. 3). A previous study showed that the overexpression of c-Myc abrogates p21<sup>CIP1</sup>-mediated EMT gene repression (36). Overexpressions of Nanog and Oct4 in lung cancer induce Snail and N-cadherin production, which result in provoking mesenchymal-like morphological changes and increasing tumor self-renewal ability (37). In addition, association of EpICD with  $\beta$ -catenin/Lef1 bound to Tcf4 promoter, and the activation of Tcf4 has been reported to regulate Slug expression. Moreover, up-regulation of Snail inherits cells with stem cell properties and tumor malignancy in colonospheres (38). We propose that EpCAM regulates EMT progression, possibly through direct activation of Tcf4-dependent Slug expression or through the secondary effect via induction of c-Myc, Oct4, and Nanog expression. Additionally, interruption of cadherin by EpICD might also participate in regulating cell mobility.

Because EpCAM is overexpressed in tumor and TICs, it is often explored as a potential target for antibody-based therapies, such as adecatumumab, catumaxomab, bispecific EpCAM/CD3 antibody (39–41), and EpCAM antibody-carried nanovesicular drugs (42). Our study confirmed that EpCAM is an attractive therapeutic target for both tumor and TICs. We provide evidence that elevation of EpCAM, especially EpICD, promotes tumorigenesis in TICs through the up-regulation of reprogramming genes and EMT process. The release of EpEX may further enhance EpEX cleavage and trigger EpICD signaling in an autocrine or paracrine manner, which consequently promotes tumor initiation and progression. Therefore, the

development of therapeutic antibodies targeting EpCAM or the inhibition of EpICD activation may hold great potential in eradicating both tumor and TICs in colon cancer.

*Acknowledgments*—We thank the Core Facility of the Institute of Cellular and Organismic Biology and the RNAi core, Academia Sinica for assistance.

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