Transplant depletion of p53 followed by transduction of c-Myc and K-Ras converts ovarian stem-like cells into tumor-initiating cells

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Although the existence of tumor-initiating cells (T-ICs) in several types of human cancer has been documented, the contribution of somatic stem cells to the development of T-ICs has remained unclear. Here, we show that normal mouse ovary contains epithelial cell adhesion molecule (EpCAM)-expressing stem-like cells that possess the ability to differentiate into cytokeratin 8 (CK8)-expressing epithelial progeny cells. Furthermore, RNA interference-mediated transient depletion of the tumor suppressor p53 followed by retrovirus-mediated transfer of c-Myc and K-Ras oncoproteins in EpCAM-expressing ovarian stem-like cells resulted in the generation of ovarian T-ICs. The established ovarian T-ICs gave rise to hierarchically organized lethal tumors in vivo and were able to undergo peritoneal metastasis. Finally, subsequent RNA interference-mediated knockdown of p53 in tumor cells triggered the expansion of EpCAM-expressing stem-like tumor cells and induced further tumor growth. These data reveal a role for p53 in the development and expansion of ovarian stem-like tumor cells and subsequent malignant progression.

Introduction

Ovarian cancer is the leading cause of death from gynecological malignancies. The clinical outcome of women with advanced ovarian cancer is poor even after treatment with aggressive surgery and intensive chemotherapy. Even though the cancer may respond to primary therapy, most tumors undergo relapse in the peritoneal cavity that is associated with chemoresistant residual cells (1). Although human ovarian tumor-initiating cells (T-ICs) have not been fully characterized, such stem-like cancer cells are thought to play a key role in chemoresistance and relapse of cancer. Improved targeted gene therapies and chemosensitization strategies are therefore needed to eradicate T-ICs in ovarian cancer. Ovarian cancer research has been hampered, however, by a lack of appropriate model systems with which to investigate the molecular mechanisms of tumor initiation and progression in relation to the biology of somatic stem cells and T-ICs.

Abbreviations: CK8, cytokeratin 8; CSC, cancer stem cell; DMEM, Dulbecco’s modified Eagle’s medium; EpCAM, epithelial cell adhesion molecule; GFP, green fluorescent protein; K06, Kusabira Orange; mRNA, messenger RNA; OSE, ovarian surface epithelium; PCR, polymerase chain reaction; RT, reverse transcription; siRNA, small interfering RNA; TBS, Tris-buffered saline; T-IC, tumor-initiating cell.

Evidence suggests that human cancers are derived from somatic stem cells and are composed of hierarchies of cells sustained by T-ICs (2,3). Whereas most ovarian epithelial cancers are thought to arise from the ovarian surface epithelium (OSE) (4,5), the cells of origin for such tumors have remained obscure (6,7). The identification of cell surface markers common to ovarian epithelial stem cells and T-ICs may provide insight into the origin of T-ICs.

Epithelial cell adhesion molecule (EpCAM) (8,9), also known as epithelial-specific antigen, is a type I transmembrane glycoprotein that is expressed specifically in epithelial tissues and is overexpressed in some epithelial cancers (10,11). In normal tissues, EpCAM is expressed in several types of epithelial stem/progenitor cells and contributes to tissue development (12–14). On the other hand, a subpopulation of EpCAM-positive (EpCAMPOS) cells has been identified as T-ICs in human colon (15), breast (16), liver (17) and pancreatic (18) cancers. In addition, increased EpCAM expression in human ovarian cancer has been associated with poor clinical outcome (19). Those data suggest the possibility that EpCAM is a common marker for epithelial cells and T-ICs in ovarian tissue.

Genetic alterations of oncogenes and tumor suppressor genes have been implicated in ovarian cancer formation and progression (1). Proto-oncogenes such as those for c-Myc and K-Ras are often amplified, overexpressed or mutated in ovarian epithelial cancer (1,2,20), whereas dysfunction of signaling by the tumor suppressor p53 pathway has been identified as the most common defect in high-grade serous or undifferentiated adenocarcinoma of the ovary (22,23). Whether p53 dysfunction plays a role in the generation or affects the behavior of ovarian T-ICs has remained unknown, however. Investigation of the roles of p53 signaling in T-ICs and in their precursor cells will require the establishment of appropriate ovarian cancer models.

In the present study, we identify a subpopulation of EpCAMPOS cells as candidate ovarian epithelial stem-like cells and we were able to generate ovarian T-ICs from these cells. We also found that EpCAMPOS cells isolated from hierarchically organized ovarian tumors possess cancer stem cell (CSC) traits and are regulated by p53.

Materials and methods

Mice

C57BL/6 mice were obtained from CLEA Japan and were bred and maintained according to institutional guidelines. All animal experiments were performed in accordance with protocols approved by the animal ethics committee of Keio University.

Cell preparation

Ovarian tissues were surgically isolated from 7-week-old C57BL/6 mice and dissociated by incubation for 3–4 h at 37°C with collagenase (300 U/ml) and hyaluronidase (100 U/ml). Red blood cells in the tissue digest were lysed by exposure to NH4Cl. A single-cell suspension was obtained by sequential dissociation of the remaining tissue fragments by gentle pipetting in the presence of dispase (5 mg/ml) and DNase (0.1 mg/ml) followed by filtration through a 40 μm nylon mesh. All reagents were from STEMCELL Technologies (Vancouver, Canada).

Flow cytometry

Cell sorting and flow cytometric analysis were performed with the use of a FACSAria Cell Sorter (BD Biosciences, Tokyo, Japan) or MoFlo flow cytometer (DakoCytomation, Glostrup, Denmark). Cells were incubated with antibodies for 15 min. APC-conjugated antibody to EpCAM (G8.8) and Pacific blue-conjugated antibodies to CD44 and to CD117 were obtained from R&D Systems (Minneapolis, MN) and incubated with anti-human IgG (Southern Biotech, Birmingham, AL) for 30 min. Cells were then analyzed with a FACSAria (BD Biosciences) or a MoFlo cell sorter (DakoCytomation).
Small interfering RNA transfection
The sequences of small interfering RNA (siRNA, chimeric RNA–DNA) duplexes (Japan Bioservice, Saitama, Japan) were 5'-GUAUCUCCCCUCCCCUCAUU TT-3' and 5'-AUGAGGGGGGAGAGUGAUCC-3' for p53 siRNA and 5'-CG AGCAGGGAUUACUUCGAT-3' and 5'-UCAGAAUUUCCGCGAGCC-3' for luciferase siRNA (GL-2, control). Cells were transfected with the annealed siRNAs for 72 h in the presence of Lipofectamine RNAi Max reagent (Invitrogen, Tokyo, Japan).

Mouse ovarian tumor model
EpCAMPOS cells in the ovaries of 7-week-old mice were isolated by FACS and were transfected with p53 siRNA in adherent cultures. Retroviruses encoding human c-Myc or K-RasG12V were generated by transfection of Plat-E packaging cells with the retroviral vectors pMXs-c-Myc-IRES-enhanced green fluorescence protein (EGFP) (24) or pGCDN-K-RasG12V-IRES-Kusabira Orange (KoD) (25) with the use of the FuGENE HD transfection reagent (Roche, Mannheim, Germany). The isolated retroviruses were then used to infect sorted EpCAMPOS cells in floating culture. The cells were then planted in 50 μl of DMEM-F12 into the left ovarian bursa of 7-week-old syngeneic recipient mice.

Transplantation assays
Sorted tumor-derived cells were suspended in DMEM-F12 and injected orthotopically, intraperitoneally or subcutaneously into 7-week-old syngeneic female C57BL/6J mice. TIC frequencies were estimated with the use of ELDA software for limiting dilution analysis (26).

Histology and immunofluorescence analysis
Tissue was fixed in 10% buffered formalin, embedded in paraffin and sectioned at a thickness of 4 μm. Sections were stained with hematoxylin and eosin for histological analysis. For immunofluorescence staining, tissue sections were incubated at room temperature for 30 min with Tris-buffered saline (TBS) containing 0.2% Triton X-100 and 60 min with TBS containing 3% bovine serum albumin before exposure overnight at 4°C to primary antibodies diluted in TBS containing 1.5% bovine serum albumin. The sections were then incubated with Alexa Fluor 488- or Texas red-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) diluted in TBS containing 0.2% bovine serum albumin and were mounted in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Cultured cells were fixed with 4% paraformaldehyde and incubated at room temperature for 60 min with primary antibodies, for 60 min with appropriate secondary antibodies and for 5 min with Hoechst 33342 (Invitrogen) for detection of nuclei. Sections and cells were viewed with a Biorevo BZ-9000 fluorescence microscope (Keyence, Tokyo, Japan). EpCAM was detected with the rat monoclonal antibody 9D8 (Santa Cruz Biotechnology, Santa Cruz, CA), cytokeratin 8 (CK8) was detected with rabbit polyclonal antibodies (Abcam, Cambridge, UK), and CA125 was detected with rabbit polyclonal antibodies (Abbiotec, San Diego, CA).

In vivo fluorescence imaging
Twenty-four hours before tumor imaging, mice were injected intravenously with 2 nmol of Integrilense 750 (VisEn Medical, Bedford, MA). Imaging was performed with the use of an in vivo fluorescence imager ClairviewOPT (Shimadzu Corporation, Kyoto, Japan) with a 785 nm laser diode for excitation and an 845/855 nm band-pass filter for detection of Integrilense 750 fluorescence. For quantitative comparisons, the total fluorescence signal intensities in the region of interest corresponding to each tumor were determined.

Sphere-forming assay
Sorted EpCAMPOS cells or tumor-derived cells were seeded in low-attachment plates and grown in serum-free DMEM-F12 supplemented with 20 ng/ml epidermal growth factor, 10 ng/ml basic fibroblast growth factor (Peprotech, Rocky Hill, NJ) and B27 (1:50; Gibco, Karlsruhe, Germany). The number of spheres generated by sorted normal EpCAMPOS cells or tumor-derived cells were counted after 7 or 5 days, respectively.

reverse transcription-polymerase chain reaction analysis
Total RNA was extracted from sorted EpCAMPOS cells or primary tumor-derived cells with the use of Isogen (Nippon Gene, Tokyo, Japan) and was subjected to reverse transcription (RT)–polymerase chain reaction (PCR) analysis with specific primers; GAPDH messenger RNA (mRNA) was examined as an internal control. Quantitative RT–PCR analysis was performed with the use of the Thermal Cycler Dice Real Time System (Takara Bio, Tokyo, Japan). The amplification protocol comprised an initial incubation at 95°C for 2 min, 40 cycles of 95°C for 30 s and 60°C for 30 s, followed by dissociation curve analysis to confirm specificity. Primer sets for RT–PCR analysis (forward and reverse, respectively) were as follows: p53, 5'-GCCAAGTCTCTGTTATGTG-3' and 5'-GCTGTGAACCTTCTGGATAG-3'; MUC16, 5'-TGACCC-ACTTAGTTGGAAGA-3' and 5'-GTACCCGCAAGACGAT-3'; EpCAM, 5'-AGACTGTGGTCTGTGGAGAATCAACAG-3' and 5'-GTTACCTTGTCTTGTTCTCAGCGA-3'; CD44, 5'-AACTTGCAAGATTTCCACGCA-3' and 5'-GGCTGCACTGTCATC-3'; CD117, 5'-GACCCAATCTCTTGGATTGTC-3' and 5'-GTGGTTGAGCATTCAGG-3'; Snail, 5'-GCCGAAGGGCCCAAATGATTCGA-3' and 5'-AGATGAGGCAGCCGC-3'; Slug, 5'-ATGCTGCGCAGCTCCTACT-3' and 5'-GTCATCTAATGCGGATTCTGACA-3' (quantitative RT–PCR), 5'-GGTGAAGGTCTGGTGGAAAGC-3' and 5'-CTACCCCATTTTGATGTCG-3' (semiquantitative RT–PCR).

Establishment of mouse ovarian tumor cell lines expressing human EpCAM protein
The full-length human EpCAM (hEpCAM) complementary DNA was amplified by PCR from the complementary DNA of HCT116 cells by the following primers: EpCAM-sense (5'-CGCCTCTGAGATGGCCCGCCCCGGGAGTCGC-3') containing an Xhol site (underlined) and EpCAM-antisense (5'-ATAAGAATGGCGCCGCTTTGCAHCGTGTC-3') containing a NotI site (underlined). The PCR fragments were digested with Xhol and NotI and then ligated into pMXs-IG expression plasmids (27). Retroviral gene transfer into EpCAMPOS tumor cells was performed as described previously (24). hEpCAM-expressing cells were sorted with APC-conjugated antibodies to human EpCAM (Miltenyi Biotec, Auburn, CA) using Mollo flow cytometer.

Immunoblot analysis
Immunoblot analysis was performed as described previously (28). In brief, equal amounts of cell lysate protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and exposed to anti-EpCAM antibody (R&D systems, Minneapolis, MN). Immune complexes were detected with Chemiluminescence Reagent Plus (PerkinElmer Japan, Yokohama, Japan).

Matrigel invasion assay
BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA) were used in accordance with the manufacturer's instructions. Established mouse ovarian tumor cells (5×104) in serum-free DMEM-F12 were seeded onto Matrigel-coated filters in the upper chambers. In the lower chambers, DMEM-F12 containing 10% FBS was added as a chemoattractant. After 24 h of incubation, cells on the upper surface of the filters were removed with a cotton swab, and the filters were fixed with 100% methanol and stained with Diff-Quik. The invasive ability of the tumor cells was expressed as the mean number of cells in three fields.

Statistical analysis
Data are presented as means ± SDs and were analyzed with the unpaired Student's t-test. A P value of <0.05 was considered statistically significant.

Results
EpCAM as a potential marker for epithelial stem-like cells in adult mouse ovary and expansion of ovarian stem-like cells induced by p53 depletion in vitro
The identity of epithelial stem cells responsible for the maintenance and regenerative repair of the adult ovarian epithelium has remained unclear. Given that EpCAM is a marker of several types of stem cell (13, 14, 29), we examined the expression of EpCAM in the normal mouse ovary. Immunohistofluorescence analysis revealed that most EpCAMPOS cells were located near the OSE, a single layer of cells that covers the surface of the ovary, as well as in the ovarian medulla near blood vessels (Figure 1A). EpCAM expression was largely undetectable in OSE cells, which express CK8 at a high level (Figure 1A), suggesting that EpCAMPOS ovarian cells are distinct from differentiated epithelial cells of the OSE. Flow cytometric analysis with antibodies to EpCAM revealed that the frequency of EpCAM-POS cells in the adult ovary ranged from 2.9 to 3.9% of total cells (mean of 3.6%) (Figure 1B). For examination of the differentiation capacity of EpCAMPOS cells, sorted EpCAMPOS cells were cultured on Matrigel, a reconstituted basement membrane, for 10 days. Individual EpCAMPOS cells formed multicellular spherical structures consisting of EpCAMPOSCK8POS and OSE-like EpCAMNEGCK8POS...
cells (Figure 1C), whereas EpCAMNEG cells failed to form CK8POS spherical cysts. These results thus suggested that EpCAMPOS ovarian cells are able to form an OSE-like structure composed by EpCAMNEGCK8POS cells on reconstituted basement membrane. Furthermore, culture of sorted EpCAMPOS or EpCAMNEG cells in culture dishes in the presence of FBS revealed that EpCAM expression was downregulated in adherent cells and that more CK8POS cells were derived from EpCAMPOS cells than from EpCAMNEG cells (Figure 1D), suggesting that the EpCAMPOS cell population might contain epithelial stem or progenitor cells that give rise to the OSE. To test this hypothesis, we examined whether the EpCAMPOS cell population contains stem-like cells with sphere-forming ability. Sorted EpCAMPOS cells seeded on low-attachment plates in serum-free medium supplemented with epidermal growth factor, basic fibroblast growth factor and B27 formed non-adherent spherical clusters of cells within several days after plating (Figure 1E), suggesting that EpCAMPOS cells indeed include stem-like cells that possess sphere-forming ability.

Given that loss of p53 expression increases the size of stem cell populations (30), we examined the effect of RNA interference-mediated depletion of p53 (supplementary Figure 1 is available at Carcinogenesis Online) on sphere formation by sorted EpCAMPOS cells.
EpCAM\textsuperscript{POS} cells transfected with a siRNA specific for p53 mRNA yielded more and larger spheres under non-adherent conditions compared with those transfected with a control siRNA (Figure 1E). This result thus suggested that p53 controls the expansion of EpCAM\textsuperscript{POS} epithelial stem-like cells.

Transient suppression of p53 followed by retroviral transduction of oncogenes in EpCAM\textsuperscript{POS} cells generates stem-like tumor cells that give rise to lethal ovarian tumors

Normal stem cells and progenitor cells are candidates for cells of origin of T-ICs (2,31). We therefore attempted to generate ovarian T-ICs by the introduction of oncogenes into sorted EpCAM\textsuperscript{POS} cells with a retroviral gene delivery system. Given that knockdown of p53 induced expansion of stem-like cells in the EpCAM\textsuperscript{POS} population, we transfected the sorted EpCAM\textsuperscript{POS} cells with p53 siRNA before their infection with a retrovirus encoding c-Myc plus GFP as a marker (24) or with a retrovirus encoding the K-Ras\textsuperscript{G12V} mutant plus humanized KuO as a marker (25) (Figure 2A). Immunofluorescence analysis revealed that the efficiency of retroviral infection was higher in cells transfected with p53 siRNA than in those transfected with control siRNA (Figure 2B), suggesting that p53 ablation increased the rate of retroviral infection in EpCAM\textsuperscript{POS} cells. We next examined the ability of the retrovirus-infected EpCAM\textsuperscript{POS} cells (5 \times 10^5) to form ovarian tumors in vivo by transplanting the cells into the ovarian bursa of syngeneic immunocompetent mice. Only cells depleted of p53 and infected with both the c-Myc and K-Ras\textsuperscript{G12V} retroviruses formed lethal ovarian tumors in vivo (Figure 2C and D), suggesting that suppression of p53 expression is essential for oncogene-induced transformation of sorted EpCAM\textsuperscript{POS} cells. The established ovarian tumors spread from the ovary to the intraperitoneal and retroperitoneal organs, resulting in massive abdominal distension by hemorrhagic ascites, within 4–5 weeks after transplantation (Figure 2E). These results thus suggested that transient knockdown of p53 together with forced expression of both c-Myc and K-Ras\textsuperscript{G12V} was able to transform EpCAM\textsuperscript{POS} cells isolated from adult mouse ovary.

To examine the characteristics of cells derived from the primary ovarian tumors, we sorted tumor cells positive for both GFP and KuO (supplementary Figure 2A is available at Carcinogenesis Online) and assayed these cells for sphere-forming ability. The tumor-derived cells indeed formed spheres (supplementary Figure 2B is available at Carcinogenesis Online), with cells of these spheres also forming new spheres on serial passage in vitro, and they gave rise to histologically similar tumors on serial allograft passage in vivo (Figure 2F, supplementary Figure 2C, available at Carcinogenesis Online). Furthermore, Matrigel invasion analysis revealed that tumor-derived cells show highly invasive character when cells were incubated in FBS-containing medium (supplementary Figure 2D is available at Carcinogenesis Online). Thus, the established ovarian tumors contain stem-like tumor cells and invasive tumor cells.

The established ovarian tumors expressed CA125 (supplementary Figure 2E is available at Carcinogenesis Online), which is a marker of human ovarian epithelial cancer (1) and CK8 (supplementary Figure 2F is available at Carcinogenesis Online). RT–PCR analysis also revealed that the abundance of MUC16 (CA125) mRNA was increased in primary tumor-derived cells compared with normal EpCAM\textsuperscript{POS} cells (Figure 2G), indicating that an increase in MUC16 gene expression is associated with ovarian epithelial tumorigenesis in this model. We confirmed that the p53 gene was not mutated in the established ovarian tumor cells by DNA sequence analysis.

Established mouse ovarian tumors harbor EpCAM\textsuperscript{POS} stem-like tumor cells

To investigate whether the established mouse ovarian tumors are organized as hierarchies of cells sustained by T-ICs, we subjected tumor-derived cells to flow cytometric analysis with antibodies to EpCAM. EpCAM\textsuperscript{POS} tumor cells constituted 5.3–13.8% (mean of 10.4%) of the GFP and KuO double-positive cell population of primary ovarian tumors (Figure 3A). To compare the tumorigenic potential of EpCAM\textsuperscript{POS} and EpCAM\textsuperscript{NEG} tumor cells, we sorted EpCAM\textsuperscript{POS} and EpCAM\textsuperscript{NEG} primary tumor cells and serially transplanted them orthotopically into syngeneic mice. EpCAM\textsuperscript{POS} tumor cells developed secondary ovarian tumors at a frequency of 83.3% (five of six) with as few as 10 cells. In contrast, \geq 500 EpCAM\textsuperscript{NEG} tumor cells were necessary to initiate lethal ovarian tumor formation (Table I, supplementary Figure 3A is available at Carcinogenesis Online). Furthermore, secondary ovarian tumors generated by EpCAM\textsuperscript{POS} primary tumor cells contained a percentage of EpCAM\textsuperscript{POS} cells similar to that apparent for primary tumors (Figure 3A). These results suggested that highly tumorigenic EpCAM\textsuperscript{POS} tumor cells give rise to less tumorigenic EpCAM\textsuperscript{NEG} cells in vivo, possibly through differentiation.

To examine further the change in the EpCAM\textsuperscript{POS} population in primary tumor-derived cells, we cultured sorted EpCAM\textsuperscript{NEG} or EpCAM\textsuperscript{POS} tumor cells in vitro for 2 weeks. Although only a few EpCAM\textsuperscript{POS} tumor cells arose from the EpCAM\textsuperscript{NEG} population, EpCAM\textsuperscript{NEG} tumor cells frequently emerged from the EpCAM\textsuperscript{POS} population (Figure 3A), suggesting that the established ovarian tumors are hierarchically organized and that EpCAM\textsuperscript{POS} tumor cells give rise to EpCAM\textsuperscript{NEG} tumor cells. We next investigated the sphere-forming ability of EpCAM\textsuperscript{POS} and EpCAM\textsuperscript{NEG} tumor cells sorted from primary ovarian tumors. The sphere-forming activity of EpCAM\textsuperscript{POS} tumor cells was markedly greater than that of EpCAM\textsuperscript{NEG} tumor cells (Figure 3B), suggesting that EpCAM\textsuperscript{POS} tumor cells are maintained in an immature state compared with EpCAM\textsuperscript{NEG} tumor cells. Together, these data showed that EpCAM\textsuperscript{POS} tumor cells are highly efficient at tumor initiation and possess the ability to differentiate into EpCAM\textsuperscript{NEG} tumor cells.

Given that EpCAM\textsuperscript{POS} cells in primary ovarian tumors showed the characteristics of T-ICs, we next investigated the expression of CD44 and CD117, which were reported to be markers of human ovarian T-ICs (32), by flow cytometric analysis and quantitative RT–PCR. We found that EpCAM\textsuperscript{POS} tumor cells manifested high expression of CD44 and CD117 compared with EpCAM\textsuperscript{NEG} cells (Figure 3C, supplementary Figure 3B is available at Carcinogenesis Online), suggesting that these are common markers for human and mouse ovarian T-ICs.

EpCAM expression enhances the tumor-initiating ability and is regulated by c-Myc and K-Ras oncogenes in ovarian stem-like cells

To examine whether EpCAM expression impacts tumor-initiating ability of ovarian tumor cells, we expressed human EpCAM in EpCAM\textsuperscript{NEG} tumor cells (supplementary Figure 3C is available at Carcinogenesis Online). Forced expression of EpCAM enhanced tumor initiation ability in less tumorigenic EpCAM\textsuperscript{NEG} cells (Table I), suggesting that EpCAM expression plays a role in tumor formation.

To elucidate the mechanism underlying both c-Myc and K-Ras\textsuperscript{G12V}-induced conversion of ovarian stem-like cells, we next investigated the expression of EpCAM mRNA in ovarian stem-like cells transduced with c-Myc and K-Ras\textsuperscript{G12V}. Transduction of both c-Myc and K-Ras\textsuperscript{G12V} maintained high expression of EpCAM in ovarian stem-like cells compared with transduction of c-Myc or K-Ras\textsuperscript{G12V} alone (supplementary Figure 3D is available at Carcinogenesis Online). These results suggested that c-Myc and K-Ras\textsuperscript{G12V} oncogenes co-operatively induce EpCAM expression leading to the promotion of tumor-forming ability in ovarian tumor cells.

EpCAM\textsuperscript{POS} stem-like tumor cells have a high potential for peritoneal metastasis

Given that a key clinical characteristic of human ovarian cancer is its capacity for peritoneal metastasis, we next attempted to generate a mouse model of such metastasis by intraperitoneal injection of established ovarian T-ICs. IntegriSense in vivo imaging, which is able to detect integrin \( \alpha v \beta 3 \) expressing newly sprouting vasculature in tumor and thereby quantify tumor size, revealed that primary tumor-derived EpCAM\textsuperscript{POS} cells generated extensive disseminated tumors within
2 weeks of injection (Figure 4A). In contrast, primary tumor-derived EpCAMNEG cells showed little ability to form disseminated tumors in the peritoneal cavity (Figure 4A). Consistent with the results obtained with the IntegriSense in vivo imaging system, the total weight of disseminated tumors generated by EpCAMPOS tumor cells was significantly higher than that of those generated by EpCAMNEG tumor cells (Figure 4B). Furthermore, these disseminated tumors invaded intraperitoneal and retroperitoneal organs including the omentum, intestine and kidneys (Figure 4C). Together, these observations suggested that EpCAMPOS tumor cells, but not EpCAMNEG tumor cells, play a key role in peritoneal dissemination. Transplantation of EpCAMPOS T-ICs combined with the use of a non-invasive and quantitative fluorescence imaging system thus proves a useful model for the study of peritoneal metastasis.

**Fig. 2.** Generation of ovarian T-ICs by transient knockdown of p53 and retroviral transduction of oncogenes in sorted EpCAMPOS cells. (A) Generation of an ovarian tumor model. EpCAMPOS ovarian cells were isolated by FACS, maintained in adherent culture for transfection with p53 siRNA and transferred to floating culture for retroviral transduction of c-Myc and K-RasG12V genes individually or in combination. The cells were then transplanted into the left ovarian bursa of syngeneic recipient mice. (B) The efficiency of sorted EpCAMPOS cell infection with retroviruses encoding c-Myc (plus GFP) and K-RasG12V (plus KuO) was examined by immunofluorescence analysis. The cells were transfected with control or p53 siRNAs before infection. Scale bars, 200 μm. (C) Summary of the tumorigenic potential of sorted EpCAMPOS cells transfected with control or p53 siRNAs and infected with retroviruses encoding c-Myc or K-RasG12V individually or in combination. The incidence of tumor formation within 8 weeks of cell transplantation (5 x 10⁴ cells) was scored. Data represent the number of tumors per number of injections. (D) Survival curves for recipient mice transplanted with 5 x 10⁴ c-Myc/K-RasG12V-expressing EpCAMPOS cells transfected with p53 siRNA (blue, n = 12) and that with control siRNA (gray, n = 5). (E) Macropscopic appearance of an ovarian tumor at 36 days after cell transplantation. The tumor spread from the ovary to the intraperitoneal and retroperitoneal organs, resulting in massive abdominal distension by hemorrhagic ascites. Tumors were macroscopically solid and cystic with the cysts containing bloody fluid. Scale bar, 2 cm. (F) Histopathology of primary and secondary ovarian tumors. Hematoxylin and eosin (H&E) staining revealed that the tumors consisted mostly of undifferentiated epithelial cells, resembling human poorly differentiated adenocarcinoma of the ovary. Nuclear pleomorphism and mitotic activity were prominent. Scale bars, 100 μm. (G) RT–PCR analysis of MUC16 (CA125) mRNA in equal amounts of total RNA isolated from sorted EpCAMPOS cells (non-transformed) and primary tumor-derived cells. The mRNA for GAPDH was examined as an internal control.

Generation of mouse ovarian stem-like tumor cells
Given that EpCAM<sup>POS</sup> tumor cells showed metastatic ability, we next checked the expression of epithelial-mechenchymal transition regulatory genes, Snail, Slug and Twist. The expression level of Snail was slightly higher in EpCAM<sup>POS</sup> cells than EpCAM<sup>NEG</sup> cells, whereas the expressions of Slug and Twist were not associated with expression status of EpCAM (supplementary Figure 4 is available at Carcinogenesis Online). Thus, metastatic behavior of EpCAM<sup>POS</sup> tumor cells was not simply due to the expression levels of these epithelial-mechenchymal transition regulatory genes.

**Fig. 3.** Established mouse ovarian tumors contain EpCAM<sup>POS</sup> stem-like tumor cells. (A) Flow cytometric analysis of EpCAM expression in GFP and KuO double-positive cells derived from a primary ovarian tumor (i) and from a secondary ovarian tumor formed by transplantation of EpCAM<sup>POS</sup> cells from the primary tumor (ii). EpCAM<sup>NEG</sup> (iii) and EpCAM<sup>POS</sup> (iv) cells derived from a primary ovarian tumor were maintained under adherent culture conditions for 2 weeks and then analyzed for EpCAM expression by flow cytometry. Shaded profiles were obtained with an isotype-matched control antibody. (B) Sphere-forming ability of sorted EpCAM<sup>POS</sup> and EpCAM<sup>NEG</sup> cells derived from primary tumors. Spheres were imaged by GFP and KuO fluorescence microscopy (scale bars, 500 μm), and the numbers of spheres formed from the indicated numbers of cells per well are presented as means ± SDs from three independent experiments. The number of spheres formed with a diameter of >100 μm was counted. **P < 0.01.** (C) Flow cytometric analysis of EpCAM, CD44 and CD117 expression in primary tumor-derived cells. Cells were stained with antibody to EpCAM as well as with antibodies to CD44 or to CD117.

p53 regulates expansion of ovarian stem-like tumor cells and tumor growth

In epithelial ovarian cancer, mutation or loss of p53 function is one of the most frequent genetic abnormalities (60–80%) (1) and high expression of EpCAM is correlated with poor prognosis (19). Furthermore, dysfunction of p53 pathway is frequently associated with undifferentiated histology in several types of epithelial ovarian cancer (23). Thus, we hypothesized that dysfunction of p53 pathway contributes to the expansion of EpCAM<sup>POS</sup> stem-like cells and thereby
impacts malignant behavior of epithelial ovarian cancer. To test this hypothesis, we next investigated whether p53 ablation might result in expansion of the EpCAMPOS population among tumor cells and thereby promote tumor growth (Figure 5A). Quantitative RT–PCR analysis revealed that transfection of primary tumor-derived cells with p53 siRNA resulted in depletion of \( p53 \) mRNA (Figure 5B). Flow cytometric analysis showed that the size of the EpCAMPOS population among these tumor-derived cells was increased by transfection with p53 siRNA \( \text{in vitro} \) (Figure 5C), suggesting that p53 restricts the expansion of the EpCAMPOS cell population. To examine the effect of this expansion of the EpCAMPOS population on ovarian tumor development \( \text{in vivo} \), we subcutaneously or orthotopically injected tumor cells transfected with control or p53 siRNAs into recipient mice. Tumor cells transfected with p53 siRNA showed enhanced tumor growth compared with those transfected with control siRNA (Figure 5D and E). Furthermore, Kaplan–Meier analysis revealed that the survival of recipients orthotopically injected with tumor cells transfected with p53 siRNA was reduced compared with that of those transplanted with tumor cells transfected with control siRNA (Figure 5F). These results suggested that the increase of numbers of EpCAMPOS stem-like tumor cells by p53 ablation is...
Fig. 5. p53 regulates the expansion of EpCAMPOS tumor cells and consequent ovarian tumor growth. (A) Experimental protocol. (B) Cells positive for both GFP and KuO were isolated from primary ovarian tumors, transfected with p53 or control siRNAs and subjected to real-time RT–PCR analysis of p53 mRNA. Data were normalized by the amount of GAPDH mRNA and are means ± SDs from three independent experiments. **P < 0.01. (C) Flow cytometric analysis of EpCAM expression in primary tumor-derived cells transfected with p53 or control siRNAs. (D) In vivo growth of primary tumor-derived cells transfected with p53 or control siRNAs. Cells (5 × 10⁴) were implanted subcutaneously into the flank of immunocompetent mice. Data are means ± SDs for three mice. *P < 0.05. (E) In vivo imaging of Integrifluorescence in tumor-bearing mice. Primary tumor-derived cells (1 × 10⁵) that had been transfected with p53 or control siRNAs were transplanted orthotopically into recipients at 14 days after transplantation. Quantitative data are means ± SDs for three mice. *P < 0.05. (F) Survival curves for recipient mice transplanted orthotopically with primary tumor-derived cells (5 × 10⁴) that had been transfected with p53 (blue, n = 10) or control siRNAs (gray, n = 12). **P < 0.01. (G) Sphere-forming ability of GFP and KuO double-positive cells isolated from secondary tumors initiated by primary tumor cells transfected with p53 or control siRNAs. The cells were seeded in low-attachment plates at a density of 3 × 10³ cells per well. Data are means ± SDs from three independent experiments. **P < 0.01.
associated with tumor growth and poor prognosis in mouse ovarian cancer model.

To confirm that the enhancement of tumor growth by p53 knockdown was indeed due to the expansion of stem-like tumor cells, we investigated the frequency of stem-like tumor cells that possess sphere-forming ability. Cells isolated from the secondary tumors formed by the p53 siRNA-transfected cells yielded a significantly larger number of spheres compared with those isolated from the secondary tumors formed by control siRNA-transfected cells (Figure 5G). This result provided further evidence that the enhanced growth of tumors formed by p53 siRNA-transfected cells was associated with expansion of stem-like tumor cells. Together, these results suggested that the expansion of EpCAMPOS stem-like tumor cells by p53 ablation enhances the malignant potential of epithelial ovarian cancer.

Discussion

We have identified a subpopulation of EpCAMPOS cells as candidates for ovarian stem or progenitor cells that have the ability to yield CK8-expressing epithelial progeny. Furthermore, we established a mouse model of ovarian cancer by RNA interference-mediated transient knockdown of p53 followed by retroviral transduction of c-Myc and K-RasG12V oncogenes in primary mouse ovarian EpCAMPOS cells. Established mouse ovarian tumors manifested a hierarchical organization and contained stem-like tumor cells whose expansion was promoted by additional p53 ablation.

In normal mouse ovary, EpCAM-expressing cells were found to be localized predominantly in the proximity of the OSE and in the ovarian medulla near blood vessels. The expression of EpCAM in primary ovarian cells was downregulated after in vitro cultivation under normal conditions. Furthermore, EpCAMPOS ovarian cells, but not EpCAMNEG cells, gave rise to CK8-expressing epithelial progeny in vitro. These findings are consistent with the results of previous studies showing that EpCAM expression is upregulated in somatic stem cells and is immediately downregulated during differentiation (29,33). EpCAMPOS ovarian stem-like cells might therefore play a role in the maintenance and regenerative repair of the OSE as a result of their ability to yield CK8-expressing epithelial cells.

We also found that p53 knockdown induced the expansion of sphere-forming stem-like cells and promoted efficient retroviral transduction. Only EpCAMPOS cells transfected with p53 siRNA formed tumors, whereas transduced with the combination of c-Myc and K-RasG12V oncogenes. EpCAMPOS cells with normal p53 gene expression thus failed to form tumors even when transduced with both of these oncogenes. In addition, regardless of the level of p53 expression, EpCAMPOS cells transfected with only a single oncogene (c-Myc or K-RasG12V) failed to form tumors. Together, these results indicated that transient suppression of the p53 pathway predisposes EpCAMPOS ovarian cells to transformation by c-Myc and K-RasG12V. Furthermore, we found that EpCAM expression enhanced the tumor-forming ability in EpCAMNEG cells, indicating that EpCAM is a functional marker for mouse ovarian T-ICs. Consistent with our results, it has been recently reported that the expression of EpCAM confers the tumor-forming ability to non-tumorigenic human embryonic kidney 293T cells (9). Taken together, c-Myc and K-RasG12V oncogenes enhance EpCAM expression and thereby promote the conversion of p53-silenced stem-like cells into T-ICs.

Poorly differentiated human breast tumors were recently shown to contain a higher number of CSCs than well-differentiated tumors, suggesting that CSC content affects biological and molecular heterogeneity of cancers (34). The tumor suppressor p53 was found to negatively regulate the self-renewal ability of CSCs as well as to reduce CSC content in an ErbB2 transgenic model of breast cancer (30). Furthermore, the absence of functional p53 has been shown to enhance the yield of induced pluripotent stem (iPS) cells, suggesting that p53 serves as a gatekeeper of self-renewal (35). In the present study, we found that RNA interference-mediated depletion of p53 triggers the expansion of EpCAMPOS stem-like tumor cells that play a key role in ovarian tumor development. In human ovarian cancer, p53 mutation and EpCAM expression are frequently observed (36), and loss of p53 function has been associated with the pathogenesis of high-grade serous or undifferentiated adenocarcinoma of the ovary (37,38). Consistent with these clinical observations, ovarian carcinogenesis models based on oncogene-induced transformation of cells in which p53 is deficient or inactivated have been found to give rise to the development of undifferentiated tumors (39–43). Together, these findings suggest that dysfunction of the p53 pathway potentiates the self-renewal ability of ovarian stem-like tumor cells and thereby might affect the biological and molecular heterogeneity of ovarian cancers.

In conclusion, our experimental mouse model should facilitate further studies toward a comprehensive understanding of somatic stem cells and CSCs in ovarian tissue. Ultimately, such studies will be imperative to determine whether eradication of CSCs is critical for effective therapy.

Supplementary material

Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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References
