Evidence for Cancer Stem Cells in Human Endometrial Carcinoma

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Abstract

Emerging evidence indicates that the highly regenerative human endometrium harbors rare populations of epithelial progenitor cells. In tumors of other regenerative epithelial tissues, rare cancer stem cells (CSC) have been identified that may have originated from normal epithelial stem/progenitor cells. We hypothesized that CSC are responsible for epithelial neoplasia associated with endometrial carcinoma, the most common gynecologic malignancy in women. Stem cell characteristics of single cells isolated from endometrial carcinoma tissues from women ages 62 ± 11.8 years (n = 34) were assessed. Twenty-five of 28 endometrial carcinoma samples contained a small population of clonogenic cells [0.24% (0-1.84%)], with no significant difference in cloning efficiency between the three grades of endometrial carcinoma or between endometrial carcinoma and normal endometrial epithelial samples. Isolated endometrial carcinoma cells transplanted under the kidney capsule of immunocompromised mice in serial dilution (2 × 106-1 × 104 cells) generated tumors in 8 of 9 samples with morphologies similar to the parent tumors. These tumors recapitulated cytokeratin, vimentin, estrogen receptor-α, and progesterone receptor expression of the parent tumor, indicating that tumor-initiating cells likely differentiated into cells comprising the endometrial carcinoma tissue. Individual clones underwent serial clonal subculture 2.5 to 4 times, with a trend of increasing number of subclonings with increasing tumor grade, indicating increasing self-renewal with greater malignancy. Clonally derived endometrial carcinoma cells also expressed the self-renewal genes BMI-1, NANOG, and SOX-2. Isolated cells from primary tumors were serially transplanted 3 to 5 times in nonobese diabetic/severe combined immunodeficient mice, showing self-renewal in vivo. This evidence of cells with clonogenic, self-renewing, differentiating, and tumorigenic properties suggests that a CSC population may be responsible for production of endometrial carcinoma tumor cells. [Cancer Res 2009;69(21):8241–8]

Introduction

Endometrial carcinoma is the fourth most common cancer in the United States and the most common gynecologic malignancy (1). The lifetime risk for developing endometrial carcinoma is 1 in 41 (1), with an estimated 40,100 new cases and 7,470 deaths from the disease in the United States in 2008 (1). Of the two types of endometrial carcinoma type I is the most common (85% of cases4; ref. 2), generally affecting premenopausal/perimenopausal women. It arises in a setting of unopposed estrogen, often associated with complex atypical endometrial hyperplasia (2, 3), mutations in PTEN and KRAS genes, and microsatellite instability (2, 4). It is often of low tumor grade and has a good prognosis, as it is usually diagnosed early due to abnormal uterine bleeding (2, 3). Type II endometrial carcinoma, however, typically affects postmenopausal women, arising in atrophic endometrium independent of estrogen, and may be preceded by endometrial intraepithelial carcinoma (2). It is associated with mutations in TP53 and HER-2/neu genes (2, 4) and has a poor prognosis (2). The survival rate of endometrial carcinoma is 84% at 5 years (1).

As carcinogenic events can be acquired over many years, it is believed that only adult stem/progenitor cells have a lifespan sufficient long enough to accumulate the genetic damage necessary to give rise to cancer stem cells (CSC) hypothesized to initiate carcinomas (2, 5). Recently, a small population of endometrial epithelial stem/progenitor cells were identified in normal human endometrium (6, 7) as clonogenic cells (6) and within the side population (8, 9). Dispersed human endometrial cells transplanted under the kidney capsule of immunocompromised mice reconstituted endometrial tissue, suggesting endometrial stem/progenitor cell activity (10). Endometrial carcinoma is characterized by abnormal endometrial epithelial cell proliferation resulting in an elevated epithelial-to-stromal ratio (2). It is possible that human endometrial stem/progenitor cells are targets of carcinogenesis in this tissue (2, 5, 11), acquiring genetic mutations enabling their transformation into CSC likely responsible for initiation, maintenance, and progression of endometrial carcinoma.

CSC were first identified and characterized in acute myeloid leukemia (12) as rare leukemia colony-forming cells that recapitulated the tumor when grafted into mice (5, 12, 13). Recently, rare, phenotypically distinct CSC were identified in human breast, pancreas, brain, colon, and prostate tumors (14–19) that were more efficient at initiating tumors when transplanted into immunocompromised mice, or forming clones, compared with other tumor cells (15–19). Evidence for CSC in an ovarian serous adenocarcinoma, another gynecologic malignancy, was shown when a small number of cells formed spheroids in vitro and produced tumors when transplanted into nude mice (20, 21). A further 17 human ovarian cancer tissues transplanted under the kidney capsule of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice produced tumors phenotypically similar to the parent tumor (21). These studies

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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suggest that many cancers develop from a small subset of cells with self-renewal and tumor-initiating properties, which are responsible for maintaining the heterogeneous tumor cell population (22).

The incidence and mortality of endometrial carcinoma is expected to increase in the foreseeable future, as mortality has been largely unaffected by early detection and treatment modalities, and risk factors, such as obesity and ageing, increase in western women (23). It is important to determine if endometrial carcinoma is established and maintained by a CSC population to develop new drug treatment options. Preliminary evidence for the presence of endometrial CSC was established when some cells from the EMTOKA cell line formed clones in vitro (24). In our study, we focus on primary human endometrial carcinoma and show that some tumor cells possess the CSC properties of clonogenicity, self-renewal, differentiation, and tumorigenicity, which may be responsible for the initiation, maintenance, and progression of endometrial carcinoma.

Materials and Methods

Patient samples. Endometrial carcinoma or hyperplasia samples were collected from 34 women (62 ± 11.8 years) undergoing hysterectomy. Tumors were graded by histopathologists and comprised 2 hyperplasias, 14 grade 1, 9 grade 2, 1 grade 2 to 3, 4 grade 3 tumors (all type I), and 4 type II tumors (Supplementary Table S1). Samples were obtained from the Victorian Cancer Biobank and collected with patients' written informed consent in accordance with ethics approval obtained from Southern Health Institutional Review Board. Some samples were obtained from patients at Massachusetts General Hospital following informed written consent or as discarded tissue as outlined in the Institutional Review Board approval.

Tissue samples were collected in HEPES-buffered DMEM and Ham’s F-12 (Invitrogen) containing 5% newborn calf serum (CSL) and 1% antibiotic-antimycotic solution (final concentrations: 100 µg/mL penicillin G, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B; Invitrogen), stored at 4°C, and dissociated within 24 h.

Tissue dissociation into single-cell suspensions. Single-cell suspensions were prepared as described previously (6) with minor modifications. Briefly, tissue was manually dissociated into <0.5 mm fragments and then digested with 20 µg/mL collagenease type II (Worthington Biochemical) and 0.64 µg/mL trypsin (Invitrogen) was used for 15 to 30 min to ensure complete dissociation into single cells. Cells were filtered through consecutive 40 µm and 35 µm cell strainers. Leukocytes were removed with anti-CD45– (+) antibodies (BD Biosciences). Epithelial cells were not separated from endometrial carcinoma cell suspensions with EpCAM Dynabeads because immunohistochemistry revealed that many epithelial endometrial carcinoma cells did not express EpCAM.

Tissue culture and colony-forming assays. Single endometrial carcinoma cells were cultured in DMEM/F-12 supplemented with 10% FCS (HyClone, Thermo Fisher Scientific), 2 mmol/L glutamine (Invitrogen), and 1% antibiotic-antimycotic (Invitrogen). Cells were cultured at clonal densities (100-300 per cm²) on 2% gelatin (Sigma-Aldrich)-coated tissue culture ware (BD Biosciences) at 37°C in 5% CO₂ in air. Cloning plates were monitored daily until cell adhesion, to ensure clones were derived from single cells, and examined every 2 to 3 days and medium was changed weekly. After 5 to 7 weeks in culture, plates were fixed in 10% formaldehyde/PBS for 10 min and stained with Harris hematoxylin (6). Clones (>50 cells) were counted on ≥3 plates per sample and averaged. Colony-forming efficiency was determined as a percentage [(number of colonies) / (number of cells seeded) × 100; ref. 6].

RNA isolation and PCR. Self-renewal and pluripotency gene expression was assessed in 10 samples of freshly isolated and 6 clonally derived endometrial carcinoma cells. RNA was isolated using Trizol (Invitrogen) for samples >100,000 cells or otherwise with RNAqueous microcolumns (Ambion) according to the manufacturer’s instructions. Embryonic carcinoma cell RNA was used as positive controls (gift from Dr. P. Verma). RNA (0.1-0.5 µg) was reverse transcribed to cDNA (Superscript III, random primers, and glyceraldehyde 3-phosphate dehydrogenase) and 1 µL of the reverse transcriptase reaction products was amplified in 25 µL with primers (10 pmol) for β-Catenin, BMI-1, Cytokeratin-8, Sox-2, and GAPDH (internal control; Supplementary Table S2) in an Applied Biosystems GeneAMP PCR system 2700. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. Embryonic carcinoma products were excised from the gel using QIAquick gel extraction kits (Qiagen) and sequenced.

Tumorigenicity of dissociated single endometrial cancer cells. Procedures performed on animals were approved by the Monash Medical Centre Animal Ethics Committee. Freshly isolated cells from human endometrial carcinoma tissue were transplanted in serial dilution (2 × 10⁶-1 × 10⁷ cells) under the kidney capsule of 6- to 8-week-old female NOD/SCID mice anesthetized with ketamine (10 mg/mL; Ellar Laboratories) and xylazine (5 mg/mL; Ellar Laboratories). A 2 cm dorsal incision was made and kidneys were externalized. Isolated endometrial carcinoma cells encapsulated in fibrin gel (50 µg fibrinogen; Calbiochem, EMD Biosciences; 2 µL thrombin, 1,000 US/mL; Sigma-Aldrich) were placed under the kidney capsule. Mice were s.c. injected with 100 ng estradiol valerate (Sigma-Aldrich) in 100 µL peanut oil immediately following surgery and fortnightly thereafter. Mice were sacrificed 12 to 16 weeks after implantation and necropsy. Kidneys were fixed in 10% buffered formalin overnight, embedded in paraffin, sectioned to 4 to 5 µm, and stained with H&E. The sections were examined in a blinded manner by a pathologist (B.K.) unaware of the source of transplanted cells. The presence or absence of tumor in the kidneys, morphologic classification and differentiation based on features such as mucinous/papillary differentiation, and pattern of the tumor based on glandular architectural complexity/solid components were analyzed. Mice were graded [International Federation of Gynecology and Obstetrics (FIGO) system] based on the percentage of tumor showing a solid architecture and nuclear grade of tumor cells (25, 26).

Self-renewal in vitro. Two to 6 weeks after seeding, three individual primary epithelial clones (>400 cells; Fig. 1, middle and right) generated from single cells were recloned to examine for self-renewal. Well-separated individual clones were trypsinized (0.25%) in cloning rings (Sigma-Aldrich) and replated at <10 cells/cm² to generate secondary clones (27). After 2 to 4 weeks in culture, three secondary clones were recloned to generate tertiary clones. Serial recloning continued in this manner until cloning activity was exhausted.

Serial transplantation of primary human endometrial carcinoma explants. Procedures performed on animals were approved by Massachusetts General Hospital Institutional Review Board. Primary endometrial carcinoma or tumor explants were processed as described previously (28) with minor modifications. Endometrial carcinoma tissue was minced to 2 mm³ pieces and dissociated in HBBS (Cambrex)-2% FCS-1 mL EDTA (Sigma-Aldrich) containing 1 mg/mL collagenase type II (Sigma) and 0.025% Dnase I (Sigma) at 37°C for 1 h. Cells were filtered through a 100 µm mesh filter (BD Biosciences) and washed in PBS, resuspended in ACK lysis buffer (Cambrex) for 30 s at room temperature to lyse RBC, washed in PBS, and centrifuged over Ficoll (Pharmacia) (GE Healthcare Life Sciences). The remaining tumor-derived cells were washed with PBS, resuspended in DMEM (Mediatech) containing 2% FCS, 1-glyceraldehyde (100 units/mL), penicillin (1%), streptomycin (1%), and 2.5 µg/mL amphotericin B (Sigma) and incubated at 37°C, 5% CO₂ in a humidified chamber for 1 h to collect nonadherent endometrial carcinoma epithelial cells. Nonviable cells were eliminated using the Dead Cell Removal Kit (Miltenyi Biotec). Tumor cells were resuspended in 1:1 PBS/Matrigel and injected s.c. into the right dorsal side of NOD/SCID mice. Mice were assessed regularly for tumor formation. Once tumors reached <10 × 10 mm, mice were euthanized, tumors were removed, and single-cell suspensions were prepared as above, except that HBBS-2% FCS-1 mL EDTA was the wash solution. Mouse cells were depleted using H-2Kd+ antibodies (BD Biosciences) and MACS separation columns (Miltenyi Biotec) as per manufacturers’ recommendations. Cells were suspended in 1:1 PBS/Matrigel and reinjected s.c. into NOD/SCID mice.

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**Immunohistochemistry.** Paraffin-embedded tumor sections (parent tissue) from blocks used in pathologic diagnosis and paired normal endometrial tissue and tumors from transplanted endometrial carcinoma cells were dewaxed before citrate buffer (0.01 mol/L in PBS) antigen retrieval. Sections were blocked with 3% H2O2 (Biotech Pharmaceuticals) followed by DakoCytonation Protein Block (DakoCytonation). Primary antibodies cytokeratin (1:100; 180 mg/L; DakoCytonation), vimentin (1:50; 59 mg/L; Zymed), and proliferating cell nuclear antigen (1:100; 90 mg/L; Novocastra Laboratories) were diluted in 0.1% bovine serum albumin/PBS and incubated for 1 h at 37°C or overnight at 4°C for estrogen receptor-α (Erbα; 1:200; 250 mg/L; DakoCytonation), progesterone receptor (PR; 1:500; 200 mg/L; Neomarkers), and PTEN (1:500; 1 mg/mL; Cascade Biosciences). Negative controls were matching mouse IgG isotypes at the same concentration. Sections were washed with PBS and incubated with secondary antibody (Envision+ System, anti-mouse; DakoCytonation) for 30 min at room temperature. Staining was visualized using 3,3′-diaminobenzidine chromogen (Sigma), counterstained with Mayer’s hematoxylin (Amber Scientific), and analyzed using a Zeiss AxioSkop microscope and image acquisition with Zeiss AxioCam ICc3 and Zeiss AxioVision Release 4.5.3.

**Results**

**Cloning efficiency of human endometrial cancer cells.** The clonogenic activity of single endometrial carcinoma cells was examined to determine if individual tumor cells initiated clones in vitro. In this prospective study, most samples (28 of 31) contained colony-forming units/cells (CFU); cells (Fig. 1A) that adhered within 7 days of isolation and produced clones. One sample per grade of type I samples failed to adhere and initiate CFU. Clones varied in their appearance. The majority were similar to normal human epithelial clones (Fig. 1A, middle and right), some to stromal clones, whereas others comprised epithelial cells with stromal features. The proportions of these clonal morphologies varied between samples but were predominantly epithelial. The cloning efficiency of all endometrial carcinoma samples was 0.24% (median; range, 0.18%; n = 24), which was not significantly different (P = 0.14) from normal human endometrial epithelial samples (median, 0.03%; range, 0–0.63%; n = 19; ref. 6). Similarly, there was no difference in cloning efficiency between type I endometrial carcinoma (median, 0.25%; range, 0–1.84%; n = 22) and normal human endometrial epithelial samples (P = 0.11). Comparison between type I and II or hyperplasia samples was not possible because of the small sample sizes of the latter two. No significant difference was observed between the grades of type I endometrial carcinoma [0.13% (0–0.94%), grade 1 (n = 11); 0.68% (0–1.84%), grade 2 (n = 8); and 0.24% (0–0.41%), grade 3 (n = 3); P = 0.12], indicating that rare cells have the potential to initiate clones of daughter cells in all grades and both types of endometrial carcinoma, suggesting the presence of CSC.

Clones from individual samples were divided into three groups based on diameter (Supplementary Fig. S1). The majority of clones were small (diameter <5 mm), which was unaffected by type I endometrial carcinoma tumor grade. Cloning efficiency was unaffected by concurrent adenomyosis (P = 0.1; n = 20), lymphovascular invasion (P = 0.13; n = 23), or myometrial invasion (P = 0.73; n = 23) of the parent tissue but was significantly higher when associated with leiomyoma (P = 0.01; n = 24; data not shown).

**Expression of self-renewal genes.** BMI-1 and β-Catenin are self-renewal genes commonly found in endometrial carcinoma (29–31). Freshly isolated Cytokeratin-8–expressing endometrial carcinoma and clonally derived epithelial endometrial carcinoma cells expressed BMI-1 and β-Catenin, indicating that the clonally derived cells express self-renewal genes and maintain a cancer phenotype in culture (Fig. 1D). Sox-2 and Nanog are associated with pluripotency and self-renewal in human embryonic stem cells (32) and were expressed in clonally derived cells and in freshly isolated samples (Fig. 1C; Supplementary Table S2), indicating that cells capable of forming clones have greater self-renewal potential than other tumor cells while maintaining their epithelial cancer phenotype.

**Tumorigenicity of human endometrial carcinoma cells.** The tumor-initiating capacity of human endometrial carcinoma cells was determined by transplanting freshly isolated single-cell suspensions in serial dilution under the kidney capsule of...
The transplanted tumors expressed differentiation markers typical of endometrial carcinoma (2) recapitulating cytokeratin, vimentin, ERα, and PR expression of parent tumors (Fig. 3; Supplementary Table S3), indicating that surviving tumor-initiating cells (TIC) produced differentiated tumor cells comprising the bulk of the xenograft. Heterogeneous immunostaining was occasionally observed between and within individual transplant and parent tumors; 7 of 8 and 6 of 8 epithelial-like cells were cytokeratin positive and PR positive, respectively. Heterogeneous vimentin staining of epithelial cells was noted in most samples (Fig. 3) as observed previously in endometrial carcinoma (33); however, consistency between transplants and parent tumors was maintained (Fig. 3). ERα staining was heterogeneous and mainly located in the epithelial cells, but some staining was observed in surrounding stroma (Fig. 3A). The large number of proliferating cell nuclear antigen-stained nuclei indicates that the tumor cells in the transplants and parent tumors were highly proliferative (Fig. 3B). PTEN inactivation is frequently involved in type I endometrial carcinoma and is preceded by monoclonal loss of PTEN expression in individual glands (34). We compared PTEN immunolocalization in the transplants with paired samples of the original parent endometrial carcinoma tumors and normal adjacent endometrium. Normal endometrial tissue showed typical heterogeneity between individual glands and more than half were PTEN null (Supplementary Fig. S2). The parent tumors showed less heterogeneity (Supplementary Fig. S2, right), whereas the epithelial elements in the transplants were PTEN null in all but one case. This homologous lack of PTEN staining is suggestive of a clonal origin of the tumors. Collectively, these data suggest that the transplanted endometrial carcinoma cells contained a small number of TIC capable of establishing new tumors in a foreign host with similar morphology and marker expression as the original tumor, indicating the capacity of the TIC to recapitulate the original tumor and differentiate in vivo.

**Self-renewal of endometrial cancer TIC.** Self-renewal was assessed in vivo using a serial transplantation model (28). Primary endometrial carcinoma single-cell suspensions s.c. injected into NOD/SCID mice established tumors in this site. Serial transplantation of human cells harvested from these tumors developed further tumors for up to five passages, regardless of grade, and final serial transplanted tumors expressed the differentiation markers ERα, PR, cytokeratin, and vimentin in a similar pattern to the original parent tumor (Fig. 4; Supplementary Table S4), supporting the hypothesis that a subpopulation of endometrial carcinoma cells has self-replicative capacity.

### Discussion

This study shows that a small population of tumor cells residing within human endometrial carcinoma tissues are clonogenic and initiate and differentiate into tumors resembling the original tumor when transplanted into a foreign host and self-renew in vitro and in vivo. The population of cells possessing these properties are possible endometrial CSC.

In this comprehensive study of 32 primary endometrial carcinoma tumors and 2 hyperplasias, we provide the only evidence to date for potential CSC across all grades and both types of endometrial carcinoma and its precursor lesion. Although many cancers have shown self-renewal in culture (18, 37–40), this is the only work showing a trend of increasing self-renewal with increasing endometrial carcinoma tumor grade in vitro. Recently, it was shown that cells with in vivo self-renewal properties existed in four higher-grade endometrial carcinoma samples (28). These studies used ≥2.6 million primary human endometrial carcinoma cells to initiate primary tumors. Fewer cells were required in serial transplants, and two quaternary tumors were established with 10,000 cells (28). The present comprehensive study complements and extends this work (28), because as few as 10,000 primary human endometrial carcinoma cells established xenograft tumors in host mice in 50% of cases, indicating that the frequency of TIC present in primary endometrial carcinoma tissues was similar to that of serially transplanted endometrial carcinoma cells. Further, we showed self-renewal capability of endometrial carcinoma cells in vitro by serial cloning. We also showed that clonally derived CYTOKERATIN-8–expressing endometrial carcinoma cells

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</tr>
<tr>
<td>0</td>
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<td>1 × 10⁴</td>
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<td>5 × 10⁴</td>
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<tr>
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<tr>
<td>7.5 × 10⁵</td>
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<tr>
<td>1 × 10⁶</td>
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<td>2 × 10⁶</td>
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NOTE: Freshly isolated cells were transplanted under kidney capsules of NOD/SCID mice and tumors were harvested 12 to 16 wk later. The numbers of tumors/transplants are shown. ND, not done.
expressed several self-renewal genes, *BMI-1*, *β*-CATENIN, *SOX-2*, and *NANOG*. Furthermore, endometrial carcinoma cells could be serially transplanted and continued to develop tumors. Together, these data suggest that a subpopulation of endometrial carcinoma cells are TIC that undergo self-renewing divisions *in vivo*. Tumors initiated by transplanted endometrial carcinoma cells had similar histoarchitecture to parent tumors, a feature maintained on serial transplantation, confirming previous data (28) and extending this finding to all grades of type I endometrial carcinoma. Transplanted cells from grade 2/3 and type II parent tumors resulted in more aggressive tumor phenotypes, also evident by strong proliferating cell nuclear antigen staining, whereas grade I tumors were mainly confined to the subcapsular location. Our evidence also suggests that the progeny of the few TIC capable of surviving the transplantation procedures may have differentiated into ERα- and PR-expressing cells in a similar pattern to the primary tumor. Vimentin stained both epithelial and stromal cells in this study, similar to previous observations in endometrial carcinoma (33). It is possible that vimentin-positive epithelial cells are either undifferentiated or are undergoing epithelial-to-mesenchymal transition (41). The cell clones with morphology intermediate between epithelial and stromal cells also suggest epithelial-to-mesenchymal transition in the progeny of clonogenic cells.

Figure 2. Histology of transplant and parent tumors. H&E staining of tumors expressed several self-renewal genes, *BMI-1*, *β*-CATENIN, *SOX-2*, and *NANOG*. Furthermore, endometrial carcinoma cells could be serially transplanted and continued to develop tumors. Together, these data suggest that a subpopulation of endometrial carcinoma cells are TIC that undergo self-renewing divisions *in vivo*. Tumors initiated by transplanted endometrial carcinoma cells had similar histoarchitecture to parent tumors, a feature maintained on serial transplantation, confirming previous data (28) and extending this finding to all grades of type I endometrial carcinoma. Transplanted cells from grade 2/3 and type II parent tumors resulted in more aggressive tumor phenotypes, also evident by strong proliferating cell nuclear antigen staining, whereas grade I tumors were mainly confined to the subcapsular location. Our evidence also suggests that the progeny of the few TIC capable of surviving the transplantation procedures may have differentiated into ERα- and PR-expressing cells in a similar pattern to the primary tumor. Vimentin stained both epithelial and stromal cells in this study, similar to previous observations in endometrial carcinoma (33). It is possible that vimentin-positive epithelial cells are either undifferentiated or are undergoing epithelial-to-mesenchymal transition (41). The cell clones with morphology intermediate between epithelial and stromal cells also suggest epithelial-to-mesenchymal transition in the progeny of clonogenic cells.

CFU activity, self-renewal, and differentiation are properties of CSC, allowing them to generate additional CSC and phenotypically diverse tumor cells (42). Single endometrial carcinoma or endometrial hyperplasia cells initiated clones with a similar frequency and variability as observed in breast cancer, retinoblastoma, myeloma, and some brain tumors (18, 37, 38, 43) and was not different to normal human endometrial epithelial cells (6), suggesting that a small number of endometrial carcinoma cells may be CSC. More differentiated progenitor cells may have contributed to the observed cloning efficiency, as a range of clone sizes were observed. It is possible that CSC only initiated the larger clones in line with the lower frequency of TIC. TIC from the more aggressive type II endometrial carcinoma appeared more efficient at initiating tumors *in vivo* than type I cells, but paradoxically type II tumor cloning efficiency was lower. This may be due to the culture conditions, optimized for normal human endometrial epithelial cells, which resemble type I more than type II tumor cells. TIC have been identified in xenografts from a range of human cancers, including endometrial carcinoma in this study (18, 20, 29, 37, 39, 43). Most required a minimum of 10,000 to 25,000 unselected cells to recapitulate tumor immunophenotype (15, 17, 19, 38, 44), and in some, 1 million cells were required (45, 46). Not all dilutions of transplanted endometrial carcinoma cells gave rise to tumors, and not all cells formed clones or underwent self-renewal, suggesting heterogeneity of endometrial carcinoma cell function and CSC potential, conforming to the CSC hypothesis. Our study shows that the potential CSC identified in endometrial carcinoma have similar properties to those characterized in mammary, colon, pancreatic, and prostate cancers, producing further evidence that CSC play a role in the initiation and progression of malignant human tumors.

One limitation of this study was that endometrial carcinoma cells were xenografted into two ectopic rather than orthotopic sites, which provides a more appropriate niche, but unfortunately cells are expelled from the uterus before they attach (47). The larger number of endometrial carcinoma cells required to establish tumors following s.c. compared with subrenal capsular transplantation is not unexpected given the high frequency of cell death anticipated with s.c. transplantation and suggests that the level of vascularization is an important factor. This ability of endometrial carcinoma cells to adapt to new environments may be considered as another CSC property. Stromal cell or cancer-associated fibroblast contamination may be a second limitation of our studies, as variable proportions of stromal cells were observed in endometrial carcinoma samples. It is possible that nonepithelial cells act as niche cells promoting tumor growth, and for this reason, we did not remove them. Although cancer-associated fibroblasts/stromal cells may have made a small contribution to the cloning efficiency and promoted tumor growth *in vivo*, this issue can only be addressed once endometrial carcinoma stem cell markers are identified. A final limitation is the variability of tumor grades within single endometrial carcinoma samples. Pathology reports often noted that endometrial carcinoma tissues were not of uniform grade, which may account for minor variations in marker expression observed between transplants and parent tumors as well as within samples. Further evidence of the heterogeneity of endometrial carcinoma cellular composition is indicated by focal staining for ERα, PR, and vimentin within endometrial carcinoma samples as noted previously (33). Future discovery of markers for endometrial carcinoma CSC will enable their prospective isolation for genomic and molecular characterization. Nevertheless, our study shows evidence for potential CSC in endometrial carcinoma.
Figure 3. Differentiation marker analysis of transplanted and parent endometrial carcinoma tumors. Immunohistochemistry of representative tumors derived from transplanted cells (A-D, top) and corresponding parent tumors (A-D, bottom). A to C, type I: grade 1 (A), grade 2 (B), and grade 3 (C). D, type II transplants and parent tumors. CK, cytokeratin; Vim, vimentin (brown with blue hematoxylin counterstain); K, kidney. Transplants were highly proliferative as indicated by proliferating cell nuclear antigen (PCNA) staining. Arrows, representative ERα or PR nuclear staining (brown). Bar, 100 μm.
Type I endometrial carcinoma commonly arises in a setting of unopposed estrogen, which stimulates proliferation of normal endometrial epithelial cells, increasing the opportunity for them to acquire mutations or epigenetic changes conferring self-renewal ability. Mutations and loss of heterozygosity of the PTEN gene result in PTEN-null glands and commonly occur in type I endometrial carcinoma (34). Rare PTEN-null glands have been observed in normal endometrium and these increase in number under conditions of unopposed estrogen, generating precursor hyperplastic lesions, some of which progress to endometrial carcinoma (34). We observed a lack of PTEN immunoreactivity in most tumors derived from transplanted type I endometrial carcinoma cells, even when the parent tumor comprised both PTEN-expressing and PTEN-null epithelial elements. Together, these observations suggest that the tumors initiated from transplanted cells could be monoclonal in origin and may have been initiated from a TIC.

An increased cloning efficiency was observed in samples from women with concurrent leiomyoma, another estrogen-dependent disease. Associated leiomyoma may represent cases where estrogen concentrations are higher, and the accompanying increased cellular turnover of self-renewing endometrial carcinoma CSC may result in increased numbers of CFU in the tumor. Alternatively, leiomyomas are associated with many connective tissue elements that are hypothesized to contain a reservoir of growth factors implicated in cellular proliferation (48).

In this study, we have shown that some cells within a large number of endometrial carcinoma samples of all grades of type I tumors are capable of forming colonies, have TIC and differentiation capacity, and can self-renew in vitro and in vivo. In the few type II endometrial carcinoma tumors examined, we showed that some cells were clonogenic, self-renewed in vitro, and had TIC and differentiation activity. This study lays the groundwork for future studies to identify markers enabling the prospective isolation of endometrial carcinoma CSC required to confirm their existence and role in the development of human endometrial carcinoma. Together with the identification of normal human endometrial epithelial stem cell markers, the development and progression of endometrial carcinoma can be investigated, allowing the identification of potential drug targets selective for CSC but sparing normal endometrial stem cells. Such treatments will be particularly useful for early-stage

Table 2. Serial cloning analysis of human endometrial cancer CFU

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Median subclonings (range)</th>
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<tr>
<td>Hyperplasia</td>
<td>2.5 (2-3)</td>
<td>2</td>
</tr>
<tr>
<td>Grade 1</td>
<td>3 (1-5)</td>
<td>11</td>
</tr>
<tr>
<td>Grade 2</td>
<td>3 (3-4)</td>
<td>5</td>
</tr>
<tr>
<td>Grade 3</td>
<td>3.5 (3-4)</td>
<td>2</td>
</tr>
<tr>
<td>Type II</td>
<td>4 (1-4)</td>
<td>3</td>
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NOTE: Median number of replatings averaged from three clones per patient sample.

Figure 4. Differentiation marker analysis of serially transplanted tumors by immunohistochemistry. Representative sample of a grade 1 endometrial carcinoma. Parent tumor (A-D, left), primary xenograft tumor (A-D, middle), and fifth serially transplanted tumor (P5; A-D, right). A, ERα; B, PR; C, vimentin; D, cytokeratin (brown; blue, hematoxylin counterstain). Bar, 20 μm.
endometrial carcinoma candidates where the uterus may be conserved and for late-stage cases where hysterectomy is not curative and current treatments target the bulk tumor cells rather than CSC.

Disclosure of Potential Conflicts of Interest

C.E. Gargett gave a lecture and provided expert advice at an internal workshop for Merck-Serono. The other authors disclosed no potential conflicts of interest.

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