Clear Cell and Endometrioid Carcinomas: are their differences attributable to distinct cells of origin?

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Running Title: Distinct cellular origins for endometrioid and clear cell cancers

Abstract

Endometrial epithelium is the presumed tissue of origin for both eutopic and endometriosis-derived clear cell and endometrioid carcinomas. We had previously hypothesized that the morphological, biological and clinical differences between these cancers are due to subtype-restricted underlying mutations. Although some mutations and genomic landscape features are more likely to be found in one of these histotypes, we were not able to identify a single class of mutations that was exclusively present in one histotype and not the other. This lack of genomic differences led us to an alternative hypothesis that these cancers arise from distinct cells of origin within endometrial tissue, and it is the cellular context that accounts for their differences. In a proteomics screen, we have identified CTH as a marker for clear cell carcinoma differentiation, as it is expressed at high levels in clear cell carcinomas of the ovary and endometrium. We analyzed normal Müllerian tissues and found CTH was expressed in ciliated cells of endometrium (both eutopic endometrium and endometriosis) and fallopian tube. We have since determined that other ciliated cell markers are expressed in clear cell carcinomas whereas endometrial secretory cell markers are expressed in endometrioid carcinomas. To determine whether the ciliated endometrial cells are uterine derived we developed a 3D organoid culture system, which reliably produced both ciliated and secretory cells. Clear cell carcinoma is an IL-6 driven tumour and lineage experiments on bronchial epithelium have shown that IL-6 is an essential pathway in maintaining the population of ciliated cells. Taken together we hypothesize that endometrioid carcinomas are derived from cells of secretory cell lineage whereas clear cell carcinomas are derived from cells of endometrial origin that share features with a ciliated cell lineage.

Keywords (3-10)

Clear cell cancer; endometrioid cancer; endometriosis; ciliated cells

Introduction

Ovarian cancers are subdivided into histotypes that display distinct histological and clinical features [1-3]. The different properties of the histotypes can be partially explained by their histogenesis.

The majority of ovarian cancers are proposed to have extra-ovarian origins with the ovary providing a rich “soil”, permissive for the growth of cancerous cells (as reviewed by [4]). For example it is likely that the majority of high-grade serous (HGS) ovarian cancers originate in the fallopian tube [5-7]. The model of HGS genesis begins with the appearance of p53 signature in fallopian tube secretory cells, progressing to a serous tubal intraepithelial carcinoma (STIC) lesion prior to becoming invasive cancer [8-10]. The origins of the other subtypes of ovarian cancer are not as well characterized. Clear cell ovarian cancer (CCOC) and endometrioid ovarian cancer (ENOC) are both associated with ovarian endometriotic cysts, or endometriomas, which are thought to be the precursor lesion of these cancers [11]. The presence of endometrioma confers up to a 3 times increased risk of CCOC and ENOC [12]. Furthermore, endometriosis, both adjacent and distal to cancer, has been found to contain mutations common to the associated cancer, indicating a clonal relationship [13, 14]. However, as these mutations can be found in endometriotic lesions without associated cancer and at sites with minimal transformational potential, the role they play in oncogenesis is uncertain [13, 15]. This finding also suggests that clonal relationships between adjacent CCOC and ENOC may reflect the mutations in a shared endometriotic cyst and not a common process of transformation or cell of origin.

It remains unclear, however, how endometriosis gives rise to two distinct subtypes of ovarian cancer. ARID1A mutations, while slightly more common in CCOC, are frequent in both subtypes [16]. Mutations in CTTNB1 are more frequent in ENOC, but can also be found in CCOC [17]. There is not a single genetic mutation that is unique to either subtype and genomic landscape features are not mutually exclusive [18]. In an effort to further understand the differences between the three most common subtypes of ovarian cancer, we undertook a global proteomic analysis. From these studies, we identified cystathionine gamma lyase (CTH) to be highly expressed in CCOC compared to ENOC and HGS [19].

In the current studies, we build on the proteomic results to provide correlative evidence that proteins expressed in CCOC tend to be expressed in ciliated cells of the endometrium. Conversely, ENOC and secretory epithelial cells tend to express some of the same proteins. We propose a cell context-based model of endometriosis-associated cancers that explains how endometriosis gives rise to two different ovarian cancer histotypes.

Methods and Materials

Immunohistochemistry

Tissue microarrays (TMAs) were constructed using duplicate 0.6mm cores of formalin fixed paraffin embedded (FFPE) materials of 485 primary ovarian epithelial, sex cord stromal and germ cell tumors. 4 µm sections of the TMAs or FFPE embedded normal fallopian tube (5 cases), endometrium (5 cases), typical endometriosis (4 cases), atypical endometriosis adjacent to CCOC (4 cases) or atypical endometriosis adjacent to ENOC (3 cases) were sectioned onto Superfrost+ glass slides. The slides were processed using the automated Ventana Benchmark and Discovery systems (Ventana Medical Systems). Description of antibodies and optimization are found in Supplemental Methods. This include a modified protocol for estrogen receptor alpha (ER) staining designed to provide a dynamic range of expression in

Müllerian tissue, as opposed to the protocol used for staining breast carcinomas; this was done by decreasing the primary antibody concentration so as to be able to distinguish between weak and intermediate or strong staining. Slides were stained with antibodies for CTH (1:1000, LSBio); Ezrin (1:1000, Sigma); ER (1:100, ThermoFisher); MTHFD1 (1:50, Sigma) and Napsin A (1:100, Cell Marque). Scoring of the TMAs was assessed by an anatomical pathologist (BT-C). Positivity was evaluated by H-Score, a combination of staining intensity and percentage of tumor cell staining. Staining intensity was scored as 0 (none or staining in less than 10% tumor cells), 1 (weak), 2 (moderate), or 3 (strong) and each score multiplied by the percentage of cells (0-100%) staining. Therefore, H scores ranged from 0 to 300.

Immunofluorescence

Heat immobilized sections (4µm) were deparaffinised and subjected to steam heat for antigen retrieval. Slides were blocked with normal goat serum and probed with primary antibodies as follows: CTH (1:100; mouse monoclonal clone 1E12, LSBio); CTH (1:50; rabbit polyclonal, LS-C312801, Lifespan Bioscience); Ezrin (1:100; rabbit polyclonal, HPA021616, Sigma); ER (1:200; rabbit monoclonal SP1, ThermoFisher); FOXJ1 (1:100; mouse clone 3-19, Abcam) and MTHFD1 (1:100; rabbit polyclonal, HPA000704, Sigma). Secondary antibodies used were goat anti-Mouse IgG conjugated Alexa 594 (1:500; A11005, ThermoFisher) and goat anti-Rabbit IgG conjugated Alexa 488 (1:500; A11008, ThermoFisher). Slides were mounted in Fluorshield mounting media containing DAPI (Sigma).

Organoid culture

Organoid culture of normal endometrium was performed using a modification of a previously described protocol for fallopian tube organoid culture [20]. Detailed protocol can be found in Supplemental Methods.

Results

Clear cell ovarian cancer markers are also expressed in normal Müllerian tissue

To better understand the biology underlying the different histotypes of ovarian cancer, we have previously performed whole proteome profiling of HGS, ENOC and CCOC tumors [19]. Of particular interest were the differences in ENOC and CCOC, as these tumors usually arise from the same precursor lesion, endometriosis. We found cystathionine gamma lyase (CTH) was higher in CCOC than in ENOC. These findings were confirmed by immunohistochemistry (IHC) on a tissue microarray (TMA) containing different subtypes of ovarian cancer (Supplemental Figure 1). We also find that CTH stains highly in endometrial clear cell carcinoma compared to endometrial endometrioid cancer (Supplemental Figure 1).

To determine the expression pattern of CTH in normal Müllerian tissues, we performed IHC on normal endometrium (secretory phase shown) and fallopian tube. In both tissues, CTH was very highly

expressed in ciliated cells, with much lower expression in secretory cells (Figure 1). Since endometriosis is thought to be the precursor lesion of CCOC, we examined expression of CTH and found that there was high CTH expression. These observations led us to examine the expression of a ciliated cell protein, Ezrin [21]. Similar to CTH, Ezrin is also highly expressed in ciliated cells of the endometrium, fallopian tube and in endometrioma, with little or no expression in secretory cells (Figure 1). In the endometrioma, the cells with strong Ezrin staining have visible cilia. We next examined expression of Napsin A, a known CCOC marker [22-24]. We found weakly positive expression of Napsin in typical endometriosis, and no Napsin expression observed in the normal endometrium and fallopian tube (Figure 1).

As we had observed previously, CTH was highly expressed in CCOC, and here we find CTH also highly expressed in atypical endometriosis adjacent to the tumor and clear cell borderline tumor (Figure 2). Ezrin and Napsin are also expressed in CCOC tumor, borderline tumor and adjacent endometriosis (Figure 2). Staining of the ovarian tumor TMA for Ezrin and Napsin revealed that they are both expressed at higher levels in CCOC than in ENOC (Supplemental Figure 1). The expression of ciliated markers in CCOC led us to postulate that ciliated cells are the cell of origin for this cancer. Since Napsin is not expressed in the normal Müllerian tissues, we hypothesize it's expression does not indicate cell of origin, but results from metabolic or other perturbations that occur later in the transformation process.

The observation that there are shared proteins in CCOC and ciliated cells led us to postulate the cells of origin of CCOC are similar to ciliated cells in ectopic endometrium. By extension, we questioned whether ENOC arises from a different cell population, i.e. the secretory cells.

Endometrioid ovarian cancer markers are expressed in secretory cells of normal Müllerian tissue

To determine whether there will be shared expression of some proteins in ENOC and secretory cells of Müllerian epithelium we studied methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) a protein expressed at a higher level in ENOC compared to CCOC in our proteomic data [19] and the estrogen receptor alpha (ER) which has been previously reported to be rarely expressed in CCOC [25-27]. We note that ER was not found to be differentially expressed in the proteomics screen likely because ER can also be expressed in the stroma. We examined the expression of MTHFD1 and ER by IHC staining of the ovarian tumor TMA and found that both are more highly expressed in ENOC compared to CCOC (Figure 3A). In the normal Müllerian tissue, we found that MTHFD1 displayed diffuse staining of the glandular epithelial cells, which are largely secretory cells (Figure 3B). In the fallopian tube, where ciliated cells are more numerous, it is more apparent that MTHFD1 expression is primarily in the secretory cells, with little or no expression in the ciliated cells. Furthermore, MTHFD1 is expressed in the secretory cells in endometriosis. Similarly, ER is expressed in the secretory cells of the endometrium, fallopian tube, and in endometriosis, but expressed at much lower levels in ciliated cells. Next, we examined ENOC tumors and adjacent endometriosis. We found that CTH, ER and MTHFD1 expression in the tumors phenocopied what is observed in the adjacent endometriosis (Figure 3C). Namely, that CTH has a weak diffuse pattern of staining (much weaker than what is observed in CCOC tumors), while ER and MTHFD1 positively stain both the tumor and adjacent endometriosis.

CCOC and ENOC markers in normal endometrial tissue

To verify that CTH was staining the ciliated cells and not the secretory cells, dual immunofluorescence in normal endometrium was performed. Dual staining of CTH and FOXJ1, a transcription factor specific for ciliated cells [28], reveals that CTH and FOXJ1 stain the same cells (Figure 4A). Similarly, Ezrin and CTH also both stain the same cells in the normal endometrium (Figure 4B). To verify that CTH was not staining the secretory cells, dual IF was performed for CTH/ER and CTH/MTHFD1. In both cases, the staining of CTH was mutually exclusive to the secretory cell markers (Figures 4C, 4D). Similar results were observed in normal fallopian tube (Supplemental Figure 2).

Ciliated cells in the endometrium are often thought to be metaplastic in nature [29]. To ensure that we are observing endometrium derived ciliated cells, we turned to an *in vitro* model system. We grew organoid cultures from normal endometrium samples (Figure 5A). The organoids have a pseudostratified columnar appearance, comparable to the pattern that is often observed in normal endometrial glands. The organoids are composed mostly of secretory cells, with occasional ciliated cells, similar to the composition of normal endometrial glands. The dual immunofluorescent staining pattern recapitulates the pattern observed in the normal endometrial glands (Figure 5B). The organoid culture demonstrates that the stem cell population of the normal endometrium is able to differentiate into both secretory and ciliated cells, a finding that has been recently observed by others using a similar, but not identical, protocol [30].

Discussion

The importance of cell context in shaping the oncogenic potential of mutations as well as the phenotype of resulting neoplasms is well known [31-33]. Clinical examples include the many unusual tumours that share the ETV6:NTRK3 gene fusion events [33, 34] or the presence of KRAS mutations as driver events in many cancers but also in endometriosis, a diseases generally thought to be non-neoplastic. This concept has been recapitulated in mouse models, including the demonstration that the same mutations lead to distinct cancer phenotypes depending on which follicular epithelial cells express canonical cancer mutations [35]. In a model of gynecologic cancer, the combined loss of PTEN and APC in ovarian surface epithelium lead to high grade endometrioid-like carcinomas, whereas the same events in secretory cells in the oviduct lead to conventional low grade endometrioid carcinomas that more accurately resemble the clinicopathological and immunohistochemical features of human endometrioid carcinoma, indicating the Müllerian cell of origin [36, 37]. For CCOC and ENOC, the finding of identical mutations; ARID1a, PIK3CA and CTNNB1, albeit at different frequencies in these histotypes, suggests that their clinical and phenotypic distinctions are not determined by mutation alone. Given these mutations can be found in non atypical deep infiltrating endometriosis, as well as cancer associated ovarian endometriomas, the usual precursor for CCOC and ENOC, it is uncertain where in the development of endometriosis the mutations occur and what role they play in that process [15]. We have recently shown that although genomic landscape features correlate with ovarian cancer histotype the relationship is not absolute [18]. The APOBEC signature is most commonly found in CCOC but can occur in ENOC, and whilst we found microsatellite instability only in ENOC in our cohort, MSI has

been described in CCOC in other studies [38, 39]. It is not known when in the transition from endometriosis to carcinoma these landscape features become apparent.

In our proposed model of CCOC and ENOC genesis, CCOC tumors arise from cells sharing features with a ciliated cell lineage, whereas ENOC tumors come from secretory cells or their precursors (Figure 5). Central to this model is an endometrial progenitor cell, which can differentiate towards a secretory or ciliated cell lineage, depending on cues provided from the microenvironment. In normal endometrium and in organoid culture, the secretory cell population is much more prevalent, however, a progenitor cell in an endometrioma will be exposed to different factors which may promote ciliated cell differentiation in addition to mutation, en route to CCOC.

One aspect of this model that needs to be addressed is the differences in prevalence of clear cell and endometrioid cancers of the ovary and the endometrium. There is roughly equal prevalence of clear cell and endometrioid subtypes in the ovary [40]. Conversely, there is a vast difference in the incidence of endometrioid and clear cell cancers of the endometrium, with endometrioid being common and clear cell tumors being very rare [41]. One explanation can be the relative numbers of ciliated and secretory cells in the endometrium, where ciliated cells are by far the minor population. Throughout the menstrual cycle, ciliated cells range from 5-20% of the epithelial population of the endometrium [42-44]. Tubal metaplasia of the endometrial glandular epithelium is more prevalent in post-menopausal endometrium, which is when the majority of endometrial clear cell cancers occur, although the two have not been linked before [29].

It is likely that the environment in the endometriotic cyst contributes to the transformation process. Inside the endometriotic cyst, trapped blood leads to high levels of reactive oxygen species, proteolytic enzymes and inflammation [45-47]. We believe that this environment may act on the progenitor cells to increase the number of cells on the ciliated cell lineage. Metaplasia is often linked to chronic inflammation and is thought to be a reprogramming of stem cells [48]. In the trachea, IL-6 has been demonstrated to promote ciliated cell differentiation [49]. The inflammatory cytokines such as IL-6 could be originating from the inflammatory cells within the endometriotic cyst, or from the ovarian stroma [50-52]. A large portion of CCOCs express IL-6 [53-55], and this autocrine IL-6 (or another bypass mechanism) may be an integral step in achieving stromal independence during oncogenesis. If inflammation drives cells towards a ciliated lineage and the potential for transformation to CCOC, then other microenvironmental factors, perhaps in combination with specific mutations or epigenomic changes could lead to oncogenesis along a secretory lineage towards ENOC. The organoid culture system described could be used to model the interplay between mutations, microenvironmental factors, and other modifiable risk factors, such as hormonal manipulation, to determine their role in differentiation and transformation.

It is interesting to note that in the CTH-high cells observed in the endometriosis adjacent to CCOC do not have any apparent cilia. These could either be cells earlier on the ciliated cell lineage that have not differentiated to the point of expressing cilia, or could be a de-differentiation of ciliated cells. Napsin is a marker for CCOC [22-24], however it is not expressed to any appreciable levels in normal fallopian tube, endometrium and has weak expression in endometriosis. It appears that some CCOC

markers give us insight into the cell of origins (CTH, Ezrin), while others, like Napsin, may be expressed as secondary events occurring later in the transformation process. Napsin and HNF1B, another well known marker for CCOC, as well as CTH are also expressed in hypersecretory glands within decidualized endometrium, the Arias –Stella reaction [56] (data not shown). Although Arias-Stella reaction can be a phenocopy of CCOC, it is not a precursor lesion, and may speak to the plasticity of Müllerian secretory cells in specific stromal microenvironments.

We note that a simple, yet intriguing question must be addressed before our proposed hypothesis that ciliated cells of the endometrium (or cells which share a lineage with terminally differentiated ciliated cells) are the cells of origin for clear cell carcinomas of the uterus and ovary is accepted. That is the question of why given the vast numbers of ciliated cells in the fallopian tube that we never see clear cell carcinomas of that organ. It is possible that the explanation goes beyond the importance of cell context and mutation underpinning the development of cancers, and demands consideration of the influence of particular stromal microenvironments. The fallopian tube is a highly evolved structure designed for two purposes: 1) The transit and nutrient support for egg and sperm, and 2) The prevention of implantation and resulting ectopic pregnancy, which would have been frequently lethal in the pre-surgical era. The stroma of the endometrium is absolutely distinct [57], in that it has a key role in the maintenance and replenishment of uterus as an ideal site for implantation and invasion, and ultimately the growth of an embryo. We therefore hypothesize that the paracrine interactions that underpin the tumour suppressive microenvironment of the fallopian tube and the invasion enhancing microenvironment of the uterus may ultimately explain the differences in the oncogenic pathways that are opened in these organ systems. This consideration may also explain why the most common subtype of ovarian cancer, high-grade serous, which are derived from secretory epithelial cells of the fallopian tube present as mass lesions outside of the proposed organ of origin.

Herein we have provided correlative evidence to formulate a new model of how endometriosis-associated ovarian cancers develop. We recognize that ciliated cells are assumed to be terminally differentiated [58] and the cell of origin for CCOC may share features with this lineage as opposed to being directly related to it. We also recognize that the rare cases of a true mixed CCOC and ENOC may reflect tumour cell plasticity. The model described is aligned with the burgeoning recognition of the importance of cell context in shaping oncogenic opportunity [31, 32, 35]. Although functional evidence from *in vitro* and likely *in vivo* modeling of the events described here will be required to test its validity; the model described offers a potential explanation for one of the most vexing questions in gynecologic pathology. Namely, how can two very different cancers emerge from the same precursor lesion, and contain the same mutations?

Acknowledgements

This work was funded by A Canadian Cancer Society Research Institute Impact grant to DGH. We wish to thank the Gray Family Ovarian Clear Cell Carcinoma Research Resource, which has provided funding for

this research. We also wish to acknowledge further support from the BC Cancer Foundation and the VGH and UBC Hospital Foundations. DGH holds a Canada Research Chair.

Statement of Author Contributions

DRC and DGH were responsible for conceptualization of the experimental design and development of the model of clear cell development from ciliated cells. DRC performed the organoid culture experiments and optimization of dual IF. BT-C scored all TMAs. BT-C, TN, ANK, LNH and CBG provided pathology support, review of cases and contributed to IHC antibody optimization. KML performed all IF and fluorescent microscopy. CS assisted with organoid culture experiments. ASC performed IHC experiments. JNM directs the tumor bank where the endometrial tissue for organoid culture was obtained. Manuscript was prepared by DRC and DGH, with input from CBG.

Conflicts of Interest

The authors have no conflicts of interest in relation to this manuscript.

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Figure Legends

Figure 1. Staining of clear cell ovarian cancer markers in normal tissues and typical endometriosis.

Images of CTH, Ezrin and Napsin A immunohistochemical staining in normal endometrium, fallopian tube and typical endometriosis, 40X magnification. Insets show high magnification (100X) of ciliated cells when visible.

Figure 2. Expression of clear cell ovarian cancer markers in clear cell ovarian cancer, clear cell borderline tumor and adjacent endometriosis. Top image shows clear cell ovarian tumor with adjacent clear cell borderline tumor and atypical endometriosis. Below are images of CTH, Ezrin and Napsin A immunohistochemical staining in representative areas, 40X magnification.

Figure 3. Endometrioid ovarian cancer markers are expressed in secretory cells of the endometrium and fallopian tube.

A. Immunohistochemical staining of ovarian cancer TMA showing expression MTHFD1 (left) and ER (right) in clear cell and endometrioid ovarian cancers. Error bars represent standard error of the mean. P-values were calculated using a Mann-Whitney non-parametric test. Representative images shown below. B. Images of MTHFD1 and ER immunohistochemical staining of normal endometrium, fallopian tube and typical endometriosis, 40X magnification. High magnification (100X) of ciliated cells when apparent. C. Immunohistochemical staining of CTH, ER and MTHFD1 in endometrioid ovarian tumor and endometriosis adjacent to the tumor.

Figure 4. CTH expression in normal endometrium is restricted to ciliated cells. Dual immunofluorescent staining of normal endometrial glands for CTH and FOXJ1 (A), CTH and Ezrin (B), CTH and ER (C) and, CTH and MTHFD1 (D). Nuclei are stained with DAPI (blue). 63X magnification.

Figure 5. Organoid cultures of normal endometrium express both secretory and ciliated cells. A. Phase contrast image of organoid culture were grown from normal endometrium. H&E of organoids at 20X and 50X magnification with ciliated cell indicated by arrow. B. Dual immunofluorescence staining of CTH and FOXJ1 (top), and CTH and ER (bottom), with nuclei stained with DAPI, 63X magnification.

Figure 6. Model of endometriosis associated cancers. Endometrial progenitor can differentiate into ciliated cells or secretory cells in normal endometrium or atypical endometriosis. Conditions in atypical endometriosis can lead to the development of clear cell ovarian cancer arising from a ciliated cell lineage or endometrioid ovarian cancer from a secretory cell lineage.

Supplemental Figure 1. CTH, Ezrin and Napsin expression is enriched in clear cell ovarian cancer. A. CTH staining of endometrial clear cell and endometrioid cancers. Staining of ovarian cancer TMA showing expression CTH (B), Ezrin (C) and Napsin (D) in clear cell and endometrioid ovarian cancers. Error bars represent standard error of the mean. P-values were calculated using a Mann-Whitney non-parametric test. Representative images shown below.

Supplemental Figure 2. CTH expression in normal fallopian tube in ciliated cells. Dual immunohistochemical staining of normal fallopian tube for CTH and FOXJ1 (A), CTH and Ezrin (B), CTH and ER (C) and, CTH and MTHFD1 (D). 63X magnification.

Figure 1

Endometriosis

Clear Cell Borderline

CCOC

CTH

Ezrin

Napsin

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

Endometrial
clear cell
carcinoma

Endometrial
endometrioid
carcinoma

B **C** **D**

Supplementary Figure S1

Supplementary Figure S2

Supplemental Methods

Optimization of Immunohistochemistry

For Napsin A, the clinical concentration (1:100) and conditions were used. All other antibodies were optimized to maximize sensitivity and minimize background staining. The ER antibody used clinically was originally optimized in breast tissue. The current conditions performed on the gynecological tissues were modified from the original protocol, at 1:100 using the HQ and ChromoMap Detection Kit instead.

For each antibody, optimizations were performed with normal fallopian tube sections on charged glass slides baked at 60°C for an hour. Initial dilutions as determined from previous publications or from manufacturer's suggestions, were trialed on two different chromogenic detection systems for initial comparisons. Additional antibody dilutions, antibody diluent, incubation time, antigen retrieval methods and/or chromogenic detection kits were assayed and altered as needed, until the optimal conditions were determined. All IHC were performed on the automated Ventana Benchmark and Discovery systems (Ventana Medical Systems).

Protein	CTH	Ezrin	ER	MTHFD1
Manufacturer	LSBio	Sigma	Thermo Fisher	Sigma
Antibody details	Mouse Clone: 1E12	Rabbit Polyclonal	Rabbit Clone: SP1	Rabbit Polyclonal
Catalogue Number	LS-C337259	HPA021616	RM-9101	HPA000704
Optimum Dilution	1:1000	1:1000	1:100	1:50
Antigen retrieval	Cell Conditioning 1 solution (Ventana) 64 minutes, 95°C	Cell Conditioning 1 solution (Ventana) 64 minutes, 95°C	Cell Conditioning 1 solution (Ventana) 32 minutes, 95°C	Cell Conditioning 1 solution (Ventana) 64 minutes, 95°C
Antibody incubation time	60 minutes, 37°C	60 minutes, 37°C	32 minutes, 37°C	60 minutes, 37°C
Chromogenic detection	DABMap kit (Ventana)	DABMap kit (Ventana)	Anti Rb HQ + Anti HQ-HRP ChromoMap DAB Detection Kit (Ventana)	DABMap kit (Ventana)

Organoid Culture

This study was approved by the Institutional Review Board (IRB) of the University of British Columbia and British Columbia Cancer Agency (H05-60119), and all patients provided written informed consent. Endometrial tissue samples, collected as part of the OVCARE gynecologic tissue bank, were obtained from women undergoing hysterectomy for benign conditions at the Vancouver General Hospital. Tissue was processed within 3h of surgery. From the small piece of uterus provided (approximately 4 cm X 4 cm), the endometrium was macrodissected with a scalpel from the rest of the tissue, washed in DPBS to remove excess blood and cut into small pieces using scissors. To dissociate the epithelial cells from connective tissue and extracellular

matrix, the minced tissue was incubated in 0.5 mg/ml collagenase (Sigma) for 1h, with stirring. After centrifugation (4 minutes, 300 x g), the cell pellet was resuspended in Advanced DMEM/F12 (ThermoFisher) containing 12 mM HEPES (ThermoFisher), 1% GlutaMAX (ThermoFisher), 5% fetal bovine serum (Hyclone), 10 ng/ml hEGF (ThermoFisher), 9 μ M ROCK inhibitor (Y-27632, Tocris) and 1% penicillin/streptomycin (ThermoFisher). The cells were plated and maintained in a 37°C, humidified incubator with 5% CO₂.

After 4-10 days in culture, when the cells had reached 80% confluence, the cells were trypsinized using TrypLE Express (ThermoFisher). Cells were centrifuged, counted and resuspended in growth factor-reduced Matrigel (ThermoFisher). Cells were plated into 24-well plates with 50 μ l of Matrigel containing 25,000 cells per well. Matrigel was allowed to solidify at 37°C for 30 minutes. The Matrigel was overlaid with Expansion Media: Advanced DMEM/F12 containing 12 mM HEPES, 1% GlutaMAX, 10 ng/ml hEGF, 9 μ M ROCK inhibitor, 2% B27 supplement (ThermoFisher), 1% N2 supplement (ThermoFisher), 100 ng/ml human noggin (Peprotech), 100 ng/ml human FGF10 (Peprotech), 1 mM nicotinamide (Sigma) and 0.5 μ M TGF β type I receptor inhibitor (SB431542, Tocris). Cultures were maintained in 37°C, humidified incubator with 5% CO₂. Media was changed every 3-4 days and organoids were passaged every 14-21 days.

To passage the organoids, cold DMEM/F12 was used to dissociate the Matrigel. Organoids were disrupted using mechanical force by vigorous pipetting with a glass capillary pipet. Cells were pelleted by centrifugation for 5 minutes at 300 x g, 4°C. The cell pellet was resuspended in Matrigel and plated in 24-well plates. The matrigel was overlaid with Expansion Media.

For immunohistochemical analysis, organoid cultures were formalin fixed and paraffin embedded. The Matrigel was dissolved with ice cold PBS, and the organoids fixed in 4% formaldehyde for 1 hour at room temperature. After dehydration in a series of alcohols, the organoids were embedded in paraffin. Sections (4 mm) were cut and put onto Superfrost+ glass slides and heat-immobilized prior to immunostaining.