Long-term, hormone-responsive organoid cultures of human endometrium in a chemically-defined medium

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Author Contributions

M.Y.T. and L.G. designed, carried out all experiments and data analyses; J.H. and T.C-D. assisted with experiments and data analyses; M.J.G. performed microarray analysis; M.H. performed EM analysis and assisted with confocal analysis; J.J.B. and H.C. provided endometrial specimens and input for the manuscript; L.F. and S.G.E.M. assisted with experiments; A.M and B.K.K. assisted with experimental design, analyses of results and preparation of manuscript; B.J.S and M.H. assisted with analyses of results and preparation of manuscript; M.Y.T., A.M. and G.J.B. wrote the manuscript.

In humans, the endometrium, the uterine mucosal lining, undergoes dynamic changes throughout the menstrual cycle and pregnancy. Despite the importance of the endometrium as the site of implantation and nutritional support for the conceptus, there are no long-term culture systems that recapitulate endometrial function *in vitro*. We adapted conditions used to establish human adult stem cell-derived organoid cultures to generate 3D cultures of normal and decidualised human endometrium. These organoids expand long-term, are genetically stable and differentiate following treatment with reproductive hormones. Single cells from both endometrium and decidua can generate a fully functional organoid. Transcript analysis confirmed great similarity between organoids and the primary tissue of origin. On exposure to pregnancy signals, endometrial organoids develop characteristics of early pregnancy. We also derived organoids from malignant endometrium, and so provide a foundation to study common diseases, such as endometriosis and endometrial cancer, as well as the physiology of early gestation.

Throughout adult reproductive life, the functional layer of the human endometrium undergoes a monthly cycle of regeneration, differentiation and shedding under the control of the hypothalamic-pituitary-ovarian (HPO) axis. The mucosa contains simple glands lined by secretory columnar epithelium, separated by intervening stroma. During the estrogen-dominated proliferative phase that follows menstruation, the mucosa regrows and then differentiates during the progesterone-dominated secretory phase. Implantation occurs ~7 days post-ovulation onto the ciliated luminal epithelium and stimulates transformation into the gestational endometrium, the true decidua of pregnancy, that provides a microenvironment essential for placentation. Up to ~10 weeks gestation, uterine glands provide histotrophic nutrition for the conceptus before the definitive hemochorial placenta is established[1, 2]. Animal models in mice and ruminants where glandular function is suppressed are unable to support implantation and pregnancy[3, 4]. Such models have revealed the molecular interactions involved between the trophectoderm and the uterine surface and the key cytokines secreted by the glands, such as leukemia inhibitory factor[5]. However, the composition of the secretions, and the gland/conceptus signalling dialogue during human placentation are unknown due to their inaccessibility *in vivo* and the absence of *in vitro* models. Suboptimal glandular development and/or functions may result in human pregnancy failure or predispose to complications of later pregnancy, such as growth restriction[6]. Thus, model systems to study these essential processes of human early pregnancy would have many biological and clinical applications.

Although stem/progenitor cells within the stromal compartment of the endometrium have been identified, suitable markers for glandular progenitors are unknown[7]. In mice, stem cells are probably present at the base of the glands[8]; similarly in primates, cells in the basal layer, that is not shed during menstruation, can generate both glandular and luminal epithelia[9, 10]. In humans, putative endometrial stem cells are the rare SSEA-1+, SOX9+ population with clonogenic ability[11, 12] but these are not fully characterised and it is unknown how they maintain

uterine glands. Previous culture systems of human endometrial glandular cells, including 3D cultures, do not fully recapitulate glandular features *in vivo*, and are not long-term or chemically defined[13, 14]. Establishing defined endometrial organoid cultures will offer possibilities for studying events during implantation and early pregnancy *in vitro* as human blastocysts can be cultured past the implantation phase of development[15, 16].

Organoids are self-organising, genetically stable, 3D culture systems containing both progenitor/stem and differentiated cells that resemble the tissue of origin. Human organoids have been derived from tissue-resident adult epithelial stem cells from gut, liver, pancreas, prostate and fallopian tube[17-21]. We have now generated long-term, chemically-defined 3D glandular organoid cultures from non-pregnant endometrium and decidua. The organoids recapitulate features of uterine glands *in vivo*; the ability to respond to hormonal signals, secrete components of uterine 'milk' and differentiate into ciliated luminal epithelial cells. Human endometrial organoids can be used to answer questions about uterine/placental cross-talk during placentation, and will provide a system for studying the pathogenesis and treatment of common conditions affecting women, such as endometriosis and endometrial cancer**.**

RESULTS

Long-term genetically-stable 3D organoid cultures can be established from human non-pregnant endometrium and decidua.

To generate endometrial organoids, we used tissue isolates enriched for epithelial cells, and allowed these to self-organise within Matrigel droplets with the basal medium that supports development of other human tissue organoids, containing EGF, Noggin and R-spondin-1 (ENR) (Fig. 1a). Because the signalling pathways maintaining endometrial gland stem/progenitor cells are unknown, we tested factors secreted by surrounding stromal cells, FGF10 and HGF[22-25]. Nicotinamide and the Alk3/4/5 inhibitor, A83-01, that blocks the TGFβ pathway were added as they are crucial in the establishment and/or long-term culture of other human organoid systems[18, 20, 26]. Decidual samples were initially used to optimise the culture conditions as they yield high cell numbers. Glandular cells were cultured for 7 days and passaged at 1:3. Organoid numbers were counted after another 7 days (Fig. 1b,c). A83-01, FGF10 and HGF with EGF, Noggin, R-spondin-1 and nicotinamide, expansion medium (ExM), gave the highest yield of cells (Fig. 1c, C8).

Organoid cultures were established in ExM within 1-2 passages (Fig. 1d). To assess the requirement for each culture component, 5000 cells were plated from established cultures (grown for >4 passages) in the absence of each factor, and the number of spheroids present after one week counted. Withdrawal of nicotinamide had the strongest effect, whilst the lack of Noggin, Rspondin-1, A83-01, EGF and HGF resulted in reduced numbers and/or smaller organoids (Fig. 1e, Supplementary Fig. 1a). FGF10 was maintained in the medium even though it had no effect on size or numbers of organoids (Fig. 1e), because it was important initially in establishing cultures and provides a physiological environment (Fig. 1b). ENR, A83-01 and

nicotinamide will maintain established cultures, but were not tested in differentiation experiments and long-term culture (Supplementary Fig. 1b). Organoid cultures were robustly established from decidual samples in ExM from 25/26 donors (derivation efficiency of 96%). Organoids were then successfully generated from non-pregnant secretory endometrium with 100% derivation efficiency (11/11) (Fig. 1f). Proliferative phase endometrium is infrequently sampled, but we did generate organoids from this phase (n=3) and from atrophic endometrium (n=1), demonstrating that our culture conditions can be used for tissue throughout the menstrual cycle, as well as pregnant and post-menopausal endometrium (Fig. 1f). The origin and characterization of established organoid cultures used for this study are summarized in Supplementary Table 1.

The established organoids can be expanded at passage ratios of 1:2 or 1:3 every 7-10 days for >6 months (reaching more than a 10^6-fold increase in the number of organoids). Markers of glandular epithelium (MUC1, E-CADHERIN, CK7 and EPCAM) are strongly expressed by the organoids (Fig. 1g,h,i). EPCAM and LAMININ are present at the baso-lateral membrane, showing epithelial polarity is intact (Fig. 1i). EdU pulse-labelling shows ~30% of cells are actively replicating (Fig. 1i). The organoids form cystic structures lined by columnar epithelium with secretions visible in the lumen. Electron microscopy reveals a microvillous, pseudostratified columnar epithelium supported by amorphous basement membrane material with basally-located nuclei (Fig. 1j). The cytoplasm contains plentiful rough endoplasmic reticulum and Golgi bodies, numerous secretory vesicles, with evidence of secretory activity from the apical surface (Fig. 1k, arrowheads). A major component of endometrial glandular secretions, glycogen, was visualized by vivid PAS staining (Fig. 1l). Thus, the appearances are highly similar to endometrial glands *in vivo*[27].

Next, the chromosomal stability of our endometrial organoids was checked by Comparative Genomic Hybridization (CGH) array. Genomic DNAs were compared between the patient and established organoid cultures at early passage (p) (2-4p) and between early and late cultures (8-15p) (Supplementary Fig. 1d-f). No significant DNA copy number abnormalities were identified during derivation or after continuous passaging for up to 5 months. These organoids can be frozen, thawed and regrown, allowing bio-banking of human endometrial cultures.

Established human endometrial gland organoids recapitulate molecular signature of glands *in vivo*.

To assess the similarity between organoids and the tissue of origin, we analysed the global gene expression profiles from established organoid lines (n=7), initial glandular digests, and cultured stromal cells from the same biopsy. Staining for MUC1 (glands) and VIMENTIN (stroma) confirmed enrichment of glands in our isolates and the purity of stromal cultures (Supplementary Fig. 2a-d). Hierarchical clustering analysis based on 15,475 probes (sd/mean >0.1) shows that the organoid cultures cluster more closely to glands than to stroma, confirming their glandular epithelial nature (Fig. 2a).

To define an endometrial glandular genetic signature, we compared glands and organoids to stroma. 287 genes were commonly upregulated in organoids and glands compared to stroma with a fold change of ≥1.5 (p≤0.01) (Fig. 2b). Gene ontology (GO) analysis shows enrichment for 'epithelial identity' and 'glandular function' (Fig. 2c,d). Markers of epithelial cells (*CDH1, CLDN10* and *EPCAM),* mucosal secretory cells (*PAX8 and MUC1*) and of uterine glandular products were all present (*PAEP*, *KLK11* and *MUC20*) (Fig. 2e). Murine genes involved in endometrial glandular development and function (*FoxA2, Sox17* and *Klf5)* also emerged[4, 28-31]. Using immunohistochemistry, we verified nuclear presence of FOXA2, SOX17 and PAX8 in all organoids and endometrial glandular cells throughout the cycle (Fig. 2f). Markers (*PROM1*, *AXIN2* and *LRIG1*) common to other epithelial progenitor cells[32, 33] were found (Fig. 2e), but in endometrium *LRIG1* transcripts are present in glands and luminal epithelium throughout the cycle and so their significance is uncertain (Fig. 2g, Supplementary Fig. 3a). Analysis of expression of other putative endometrial stem cell markers, *AXIN2* and SSEA1 was inconclusive[11]. Although *AXIN2* transcripts were found in glands *in vivo*, lack of a reliable antibody prevented further analysis (Supplementary Fig.3b). Only a few cells were SSEA-1+ in organoids, analysed by immunohistochemistry and flow cytometry (2-3%) and, after sorting SSEA-1+/- cells, organoids emerged from the SSEA-1-negative fraction (Supplementary Fig. 3c, d). Overall the gene signature of decidual organoids (n=6) is also very similar to non-pregnant endometrium (Supplementary Fig. 4a), with immunostaining of FOXA2, SOX17 and PAX8 and expression of *LRIG1* uniformly similar to *ex vivo* decidual glands (Supplementary Fig. 4b,c).

Apart from shared gene sets between glands and organoids, there are also genes only expressed in glands (421/652) or organoids (286/484) (Supplementary Fig. 5). GO terms for glands describe stromal interactions (integrin binding and extracellular matrix structural constituents), all absent *in vitro.* For organoids, *in vitro* proliferation, (cell division and mitotic nuclear division) dominated. Thus, differential gene expression between gland samples and organoids reflects their contrasting microenvironments.

A converse analysis to define a stromal cell signature (Supplementary Fig. 2e) revealed minimal contamination from endothelial cells (*CD31* or *CD34*) or leukocytes (*CD45*). GO analysis showed 'biological processes' typical of fibroblasts and 'molecular functions' (Supplementary Fig. 2f, g). Gene sets were enriched for stromal cell markers (*THY1*, *NT5E* and *IFITM1*)[34, 35], extracellular matrix proteins (*COL8A1, COL12A1*, *COL13A1* and *LAMA1*), and metalloproteinases (*MMP11, MMP2, MMP12, MMP27, MMP3*, *TIMP2* and *CTGF*) (Supplementary Fig. 2e). Genes encoding for components of WNT (*WNT2, WNT5A, RSPO3*), BMP (*BMP2, GREM1*) and MAPK (*FGF2*) signalling pathways also emerged, pathways already identified from our culture conditions.

Human endometrial gland organoids respond to sex hormones.

Unlike other mucosal epithelia, the endometrium responds dramatically to ovarian hormones, estrogen (E2) and progesterone (P4), which regulate cyclical proliferation and differentiation of endometrial glands with concomitant dynamic temporal and

spatial expression of their receptors, ERα and PR (Fig. 3a)[36-38]. Following menstruation, glands increase expression of ERα in response to rising E2 levels (proliferative phase). After ovulation, ERα expression declines in the early secretory phase whereas PR is maintained until mid-secretory (LH+7), after which both ERα and PR expression disappears[37].

To mimic the response of the organoid cultures to hormones, we exposed organoids to E2 followed by P4 (Fig. 3b). Under ExM conditions most cells show weak expression of ERα ($ERα^{low}$) with some $ERα^{high}$ (Fig. 3c, arrowheads) and $ERα^{negative}$ cells (Fig. 3c, arrows) present. Although most organoids are $PR^{negative}$, a few cells are PR^{high}; on serial sections these are also $ERα^{high}$. After exposure to E2 and P4, high expression of both ERα and PR is seen in most organoids similar to the situation *in vivo (*Fig. 3c*).* Organoid cultures derived from decidua showed similar responses (Supplementary Figure 6a).

We performed a microarray analysis of organoids in ExM, E2 alone or E2 and P4. Known genes upregulated by E2 and P4 in the mid-secretory phase *17βHSD2, PAEP, SPP1, LIF, IGFBP4, IGFBP5* and *CYCLIN A1* were all upregulated in hormonally-treated organoids (Fig. 3d)[39-42]. This was confirmed for several genes using qRT-PCR (Fig. 3e) and at the protein level for PAEP and SPP1 (Fig. 3 f,g). We also confirmed that the addition of cyclic adenosine monophosphate (cAMP) to the differentiation medium, a component used typically in decidualization protocols, enhances the expression of differentiation markers shown by increased expression of *PAEP* and *SPP1* (Supplementary Fig. 6b)[43].

Other hormonally-regulated endometrial genes emerged, including *OLFM4*, an intestinal stem cell marker[44]. In ExM, organoid cells were OLFM4-negative but a subset became OLFM4-positive after E2 treatment, similar to the proliferative phase *in vivo* (Fig. 3h, arrows). *Collagen 1A2 (COL1A2), chromogranin A (CHGA)* and *OVOL2* were also upregulated, whilst *HES1* and *SOX9* were downregulated. In summary, the phenotypic response of glandular endometrial organoids to ovarian sex hormones is characteristic of the early-mid secretory phase.

Signals from decidualised stroma and the placenta can further stimulate differentiation of human endometrial gland organoids.

If implantation occurs, the endometrium forms the true decidua of pregnancy in response to P4; decidualized stromal cells characteristically secrete Prolactin (PRL) [45] (Fig. 4a). Both PRL and signals from the conceptus are likely to stimulate uterine gland activity in early pregnancy (Fig. 4a)[46, 47]. To mimic pregnancy, we added placental hormones (Chorionic Gonadotropin, hCG and human Placental Lactogen, hPL) in combinations with PRL to ExM containing E2+P4+cAMP, referred to as Differentiation Medium (DM) (Fig. 4b).

The three hormones together stimulate maximal production of PAEP and a hypersecretory morphology characteristic of decidual glands *in vivo* (Fig. 4c). PRL has an additional effect by stimulating the formation of ciliated cells (identified by acetylated α-tubulin) (Fig. 4d). Similar findings were obtained using conditioned media from stromal cells decidualized *in vitro* for 10 days (Supplementary Figure 6c). As ciliated cells are only present *in vivo* in the uterine luminal epithelium and in

superficial glands, the organoids are undergoing both glandular and luminal differentiation.

SOX9, a marker of progenitor cells, is expressed in the base of endometrial glands *in vivo* and at high levels in the organoids[11, 48, 49] but is absent from decidual glands *in vivo.* Organoids cultured with both ovarian and pregnancy hormones undergo differentiation as SOX9 was downregulated (Fig. 4e). Thus, appropriate hormonal stimulation induces organoids to acquire a decidual-like phenotype characteristic of early pregnancy.

Human endometrial organoids have clonogenic ability and are bipotent

To assess for stem cell activity, we measured clonogenic ability by plating single cells from established organoid cultures by limiting dilution; drops containing single cells were marked and followed by time-lapse photography. Some cells formed an entire organoid over 7-14 days; the rest either did not divide or formed small dying spheroids (Fig. 5a). The organoid-forming efficiency of these cells, was 2-4% with 100 cells/drop and ~10-fold lower with 10 cells/drop (Supplementary Table 2). Single organoids can be expanded into clonal cultures and we now have grown 12 clonal lines from 5 independently-derived organoids (Fig. 5b). A single cell has bi-potent ability as it could generate the two main endometrial cell types: secretory (PAEP+) and ciliated (acetylated-α-tubulin+) cells (Fig. 5c). Formation of cilia was confirmed by EM (Fig. 5d).

Organoid cultures can be derived from endometrial cancer

Endometrial cancer is the commonest gynecological tumour. Organoids were derived from samples of tumours and the normal adjacent endometrium from post-menopausal women (Fig. 6). The morphology of the organoids resembles the primary tumour (FIGO Grade I Endometrioid Carcinoma) showing pleomorphic cells with hyperchromatic nuclei and disorganised epithelium. In places breaching of the basement membrane is obvious, and isolated cells are seen in the surrounding Matrigel. The organoids are positive for glandular markers such as MUC1 and SOX17, confirming their glandular origin.

DISCUSSION

Here, we describe a robust chemically-defined method for establishing genetically stable endometrial organoids from human non-pregnant endometrium and decidua that can be cultured long-term and recapitulate the molecular signature of endometrial glands *in vivo.* Several murine genes important for glandular development and function (*Foxa2*, *Klf5* and *Sox17*) are also expressed. The organoids functionally respond to sex hormones, E2 and P4, and when further stimulated with pregnancy (hCG, hPL) and stromal cell (PRL) signals, acquire characteristics of gestational endometrium, synthesising abundant PAEP (glycodelin) and SPP1 (osteopontin). PAEP and SPP1, components of glandular secretions,

'uterine milk', provide histotrophic nutrition to trophoblast before the hemochorial placenta is established.

Clonal organoid cultures generated from a single cell contain cells with extensive proliferative capacity, and both ciliated and secretory cells. Their gene signature includes markers of epithelial stem cells, *LRIG1, PROM1, AXIN2* and *SOX9*. Because we could generate SOX9-expressing organoids from non-proliferative, SOX9-, differentiated secretory phase endometrium and decidua, the few SOX9+ cells present mainly in the basal layer might expand[11]. Alternatively, plasticity of endometrial cells allows SOX9-negative differentiated cells to self-renew and reacquire SOX9 expression in our cultures. A similar reversion occurs in the liver, where non-Lgr5+ cells reacquire Lgr5 stem cell marker expression upon tissue injury[50].

Although organoids have been established from human fallopian tube with differentiation into both ciliated and secretory cells, neither the dramatic cyclical changes in response to E2 and P4, nor the process of decidualization induced by pregnancy occurs in the fallopian tube, a mucosal surface contiguous to endometrium[21]. Furthermore, the crucial site of embryo attachment is the luminal surface of the endometrium.

Endometrial organoids can be maintained and expanded in ExM, recapitulating pathways essential for culturing organoids from other organs - the FGF-MAPK, WNT-Rspondin, BMP-Noggin and TGFβ signalling pathways[51]. The contribution of endometrial stromal cells to these signalling pathways is revealed from our microarray analysis showing stromal transcripts encoding Rspondin-1 and FGF2. Further refinement of the method to replace Matrigel with a chemically-defined extracellular matrix would enhance the model in future[52]. The identity of the endometrial epithelial stem cells remains unknown although their presence is revealed by the long-term expansion and clonogenic activity of organoids, and we have defined the essential niche components for their maintenance.

We also recapitulate the glandular cyclical changes during the menstrual cycle triggered by sequential secretion of ovarian hormones, E2 and P4. Endometrial organoids acquire a differentiated phenotype characteristic of the mid-secretory phase, with upregulation of several genes (*17βHSD2*, *SPP1*, *LIF*) expressed at this time. Other genes, such as *OLFM4*, that may play key roles in regulating gland cell proliferation and function during the cycle were also identified.

Besides the direct effect E2 and P4 have on the glands, they also exert a paracrine effect via the stromal cells. Decidualized stroma secretes a wide range of proteins, including PRL whose function is unknown. Unlike the pituitary, decidual PRL is driven from an alternative promoter, derived from transposable elements (MER20)[53]. Our finding that addition of PRL induces ciliated cells suggests it may influence differentiation and function of the glands during early pregnancy.

The glands of gestational endometrium continue to differentiate and display a hypersecretory appearance with abundant PAEP production[54, 55]. In our organoid system, addition of trophoblast hormones (hCG and hPL) resulted in a similar appearance. This culture system will therefore allow further investigation of the essential (but understudied) period of histotrophic nutrition in the first trimester of

pregnancy before the hemochorial placenta is established. Additionally, we were able to derive organoids from endometrial adenocarcinomas. These common tumours in post-menopausal women are associated with increased exposure to estrogen that is a feature of obesity, nulliparity, treatment with tamoxifen and late menopause[56]. These can be used in the future to build a biobank to screen drugs and investigate the mutational changes, as has been done for colon cancers[57].

In summary, we describe a method for reliable chemically-defined, long-term culture of endometrial glands from non-pregnant endometrium and decidua that closely recapitulates the molecular and functional characteristics of their cells of origin. The organoid cultures can be frozen down without loss of their proliferative ability upon thawing, allowing the possibility to build up patient-specific bio-banks. This method will be an invaluable research tool to study new therapies for common pathologies of the endometrium, such as endometriosis and endometrial cancer, as well as investigating problems of implantation and the secretion of uterine histotroph during early pregnancy.

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Figure Legends

Figure 1. Long-term 3D organoid cultures can be established from human non-pregnant endometrium and decidua.

(a) Scheme for deriving organoids.
(b) Screening conditions for generating organoids. FGF10, A83-01, HGF and Nicotinamide added in combinations to generic organoid medium (ENR). Number of organoids derived under each condition (C2 to C9) shown relative to basal conditions (C1). Shown are decidual digests from 3 different patients. Source data in Supplementary Table 5.
(c) Representative images for conditions C1-C9 in Fig. 1b. Scale bar, 500 μm.
(d) Images of decidual gland isolates (passage 0) and organoids after one passage in Expansion Medium (ExM) (passage 1). Scale bar, 200 μm. Representative of all samples, summarized in Supplementary Table 1.
(e) Effect of withdrawal of growth factors from ExM. Organoids grown in ExM and each factor withdrawn: EGF, Noggin (NG), Rspondin-1 (RSPO1), FGF10, A8301, HGF and Nicotinamide (NIC). Organoids formed shown relative to ExM (%). Shown are decidual cultures derived from 3 different patients. Source data in Supplementary Table 5.
(f) Images of organoids established in ExM from proliferative (Prol.) endometrium (n=3), secretory (Sec.) endometrium (n=9), decidua (n=25) and post-menopausal (atrophic) endometrium (n=1). Scale bar, 100 μm.
(g) IHC of decidua (*in vivo*) and organoids for Mucin 1 (MUC-1). Scale bar, 50 μm. Representative of 6 decidual and endometrial samples, and organoids derived from 2 endometrial and 2 decidual samples from different patients.
(h) IF staining of organoid for E-CADHERIN (E-CAD) and CYTOKERATIN-7 (CK7). Scale bar, 50 μm. Experiment repeated twice (1 endometrial-derived and 1 decidua-derived organoids).
(i) IF staining of organoid for cell proliferation (uptake of EdU), epithelial marker EPCAM and basement membrane marker laminin (LAM). Scale bar, 50 μm. Experiment repeated twice (1 endometrial-derived and 1 decidua-derived organoids).
(j) Electron micrograph (EM) of organoid showing columnar epithelial cells with basally-located nuclei. Scale bar, 5 μm. Experiment repeated twice with different donors.
(k) EM showing secretory activity (black arrowheads). Scale bar, 1 μm. Experiment repeated twice with different donors.
(l) PAS staining for glycogen in endometrium and organoids. Scale bars, 50 μm (main image) and 10 μm (inset). Representative of 3 endometrial samples and 3 endometrial organoids.

Figure 2. Established human endometrial organoids recapitulate molecular signature of glands *in vivo*.

(a) Unsupervised hierarchical clustering analysis of global gene expression profiles by microarray of gland digests, stromal cells and corresponding established organoids from endometrium (n=7 independent donors). Analysis based on 15475 probes with sd/mean >0.1. Expression profiles of organoids cluster with glands while those of the stroma cluster in a separate tree.

(b) Venn diagram showing overlap of 287 genes significantly upregulated in glands and organoids with a fold change ≥1.5 ($p \leq 0.01$) relative to stroma.

(c) Gene ontology (GO) analysis of the 287 genes from (b) using HumanMine v2.2 database for GO Terms Biological processes and Benjamini Hochberg test correction with maximum p-value of 0.05. The top ten significantly enriched GO terms for each category are shown with the –log of their p-values and are enriched for terms describing epithelial tissue.

(d) Gene ontology (GO) analysis of the 287 genes from (b) using same method as in (c). The top ten significantly enriched GO terms describe epithelial cells with secretory function.

(e) Clustered heatmap of 287 genes commonly upregulated between organoids and glands compared to stroma from (b). Genes of interest are listed on the right. Epithelial markers (blue) (*EPCAM, CLD10, CDH1*), glandular products and markers of secretory cells (purple) (*MUC20, PAX8, PAEP, MUC1*), progenitor cell markers (cyan) (*LRIG1, PROM1, AXIN2*) and murine genes important for endometrial function (pink) (*SOX17, KLF5, FOXA2*).

(f) IHC for genes selected from microarray, FOXA2, SOX17 and PAX8, in proliferative and secretory endometrium and organoids. Scale bars, 50 μm (main image) and 10 μm (insets). Representative of 3 proliferative and 7 secretory endometrial samples and endometrial organoids derived from 8 different patients.

(g) ISH for *LRIG1* on proliferative and secretory endometrium and organoids. Negative control probe is for the bacterial gene *dapB*. Scale bars, 50 μm (main image) and 10 μm (insets). Representative of 3 proliferative and 3 secretory endometrial samples and endometrial organoids derived from 4 different patients.

Figure 3. Human endometrial organoids respond to sex hormones.

(a) Ovarian hormones, Estrogen (E2)(red) and Progesterone (P4)(blue), and the cycling endometrium. Expression of Estrogen Receptor (ERα)(dashed red) and Progesterone Receptor (PR)(dashed blue) are specific for glands of the functional layer. Adapted from Reference[37].

(b) Protocol for hormonal stimulation. Organoids grown in ExM, day 0 (d0), are primed with E2 for 48 h on day 4 (d4) followed by stimulation with P4 and cyclic AMP (cAMP) for 48 h.

(c) IHC for ERα and PR on organoids after hormonal stimulation. In ExM expression of ERα is weak, but some cells are either $ERα^{high}$ (arrowheads) or $ERα^{negative}$ (arrows). Few cells are positive for PR (arrowheads). After E2 and P4 treatment, levels of ERα and PR are higher. Scale bars, 50 μm (main image) and 10 μm (insets). Representative of endometrial organoids from 6 different patients and decidual organoids from 9 different patients.

(d) Clustered heatmap of selected genes from organoids grown in ExM, ExM+E2 or ExM+E2+P4+cAMP (n=3 donors). Shown are genes known to reflect differentiation in response to hormones (purple), uncharacterized genes (grey) and downregulated genes (cyan).
(e) QRT-PCR analysis for differentiation markers (*PAEP, SPP1, 17HSDβ2* and *LIF*) of organoids grown in ExM, ExM+E2 or ExM+E2+P4+cAMP. Shown is the mean±SEM levels of expression relative to housekeeping genes and ExM conditions (ΔΔCt). Data from endometrial organoids from n=6 different patients. Source data in Supplementary Table 5.
(f) Western blot for PAEP in organoids after hormonal stimulation. Levels of glycosylated and non-glycosylated PAEP increase upon exposure to E2 and E2+P4+cAMP. Ponceau S staining (Ponc S) for loading control. Experiment repeated twice using endometrial organoids from 2 patients. Unprocessed blots in Supplementary Figure 7.
(g) ELISA for SPP1 production by endometrial organoids upon exposure to hormones. Three independent experiments (Donors 1-3). SPP1 secretion increases following exposure to E2 and further after E2+P4+cAMP. Source data in Supplementary Table 5.
(h) IHC for OLFM4 on organoids under ExM, ExM+E2 and ExM+E2+P4+cAMP, and proliferative and secretory endometrium. Scale bars, 50 μm (main image) and 10 μm (insets). Representative of 2 proliferative and 2 secretory endometrial tissues and organoids derived from 3 different patients.

Figure 4. Signals from decidualised stroma and the placenta can further stimulate differentiation of human endometrial gland organoids.

(a) Hormonal environment of endometrium during the first trimester of pregnancy. Estrogen (E2) and Progesterone (P4) are ovarian products, human chorionic gonadotropin (hCG) and human placental lactogen (hPL) are secreted by trophoblast and prolactin (PRL) by decidualized stromal cells.
(b) Protocol for stimulation of endometrial organoids. Organoids are passaged and plated on day 0 (d0) in ExM. On d4, ExM is changed to Differentiation Medium (DM; ExM with E2+P4+cAMP). hCG, hPL and/or PRL were added for 8 d.
(c) IHC for PAEP on endometrial organoids under the following conditions: ExM, DM, DM with hCG/hPL or PRL or all three combined. Maximal production of PAEP and differentiated morphology of cells is seen upon exposure to DM with hCG, hPL and PRL. Scale bar, 50 μm. Representative of endometrial organoids derived from 3 different patients.
(d) IHC for acetylated α-tubulin to visualize cilia in secretory endometrium (Sec. Endom.) and endometrial organoids following stimulation with PRL. Ciliated cells (arrows) are present in the luminal epithelium (LE) and within organoids. GE, glandular epithelium. Scale bars, 50 μm (main image) and 10 μm (insets). Representative of 4 secretory endometrial samples and endometrial organoids derived from 4 different patients.

(e) IHC for SOX9 on endometrial glands *ex vivo* and *in vitro*. Organoids in ExM express high levels of SOX9 similar to proliferative endometrium (Prol. Endom.). After hormonal stimulation, SOX9 is downregulated in organoids (ExM+HCG+HPL+PRL) similar to glands in decidua. Scale bars, 50 μm (main image) and 10 μm (insets). Representative of 4 proliferative endometrial samples, 7 decidual samples and endometrial organoids derived from 4 different patients.

Figure 5. Human endometrial organoids have clonogenic ability and are bipotent.

(a) Phase-contrast images of (from top to bottom row): an organoid forming from a single cell; a single cell forming a spheroid with no further growth, and a single cell showing no growth. Images were taken every two days. Scale bar, 50 μm. Experiment was performed with 3 clonal lines derived from 2 endometrial and 1 decidual organoid cultures.
(b) Representative image showing expansion of a clonal culture at passage 1 (p1) from a single organoid (at passage 0, p0) in a 96-well. Scale bar, 500 μm. 12 clonal cultures were established from organoids from 5 different patient samples (4 endometrial-derived and 1 decidual-derived).
(c) IF on clonally-derived endometrial organoid cultures subjected to the full cocktail of hormonal stimuli to visualize two main endometrial epithelial cell types: ciliated cells (acetylated α-tubulin) (cyan) and secretory cells (PAEP) (red). Scale bars from left to right: 100 μm, 20 μm and 5 μm. Representative of 4 clonal lines derived from 2 different endometrial organoid cultures.
(d) EM on clonally-derived endometrial organoid cultures subjected to the full cocktail of hormonal stimuli showing basal bodies of fully formed cilia. Scale bars: 10 μm and 1 μm. Experiment performed twice using 1 clonal endometrial organoid culture.

Figure 6. Organoids can be derived from endometrial cancer.

Derivation of organoids from endometrial carcinomas. From left to right: H&E stained sections of normal atrophic endometrium showing gland surrounded by dense stroma and a FIGO Grade I endometrioid carcinoma with dense glandular structures from the same patient, scale bar, 100 μm; images of organoids derived from matched normal and malignant endometrium cultured in ExM (passage 1), scale bar, 100 μm; H&E stained sections showing marked differences in morphology between organoids derived from normal endometrium and those from tumours which show nuclear pleomorphism, a disorganized epithelium with irregular basement membrane and isolated cells present in surrounding Matrigel (arrows), scale bar, 20 μm; IHC for MUC-1 and SOX17 on tumour and normal organoids confirm their glandular origin, scale bar, 20 μm. Representative of organoids derived from 3 different endometrial carcinomas and 1 matching normal tissue.