

# 1 Long-term, hormone-responsive organoid cultures of human 2 endometrium in a chemically-defined medium

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## 40 Author Contributions

41 M.Y.T. and L.G. designed, carried out all experiments and data analyses; J.H. and T.C-D. assisted  
42 with experiments and data analyses; M.J.G. performed microarray analysis; M.H. performed EM  
43 analysis and assisted with confocal analysis; J.J.B. and H.C. provided endometrial specimens and  
44 input for the manuscript; L.F. and S.G.E.M. assisted with experiments; A.M and B.K.K. assisted with  
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1 **In humans, the endometrium, the uterine mucosal lining, undergoes dynamic**  
2 **changes throughout the menstrual cycle and pregnancy. Despite the**  
3 **importance of the endometrium as the site of implantation and nutritional**  
4 **support for the conceptus, there are no long-term culture systems that**  
5 **recapitulate endometrial function *in vitro*. We adapted conditions used to**  
6 **establish human adult stem cell-derived organoid cultures to generate 3D**  
7 **cultures of normal and decidualised human endometrium. These organoids**  
8 **expand long-term, are genetically stable and differentiate following treatment**  
9 **with reproductive hormones. Single cells from both endometrium and decidua**  
10 **can generate a fully functional organoid. Transcript analysis confirmed great**  
11 **similarity between organoids and the primary tissue of origin. On exposure to**  
12 **pregnancy signals, endometrial organoids develop characteristics of early**  
13 **pregnancy. We also derived organoids from malignant endometrium, and so**  
14 **provide a foundation to study common diseases, such as endometriosis and**  
15 **endometrial cancer, as well as the physiology of early gestation.**

16  
17 Throughout adult reproductive life, the functional layer of the human endometrium  
18 undergoes a monthly cycle of regeneration, differentiation and shedding under the  
19 control of the hypothalamic-pituitary-ovarian (HPO) axis. The mucosa contains  
20 simple glands lined by secretory columnar epithelium, separated by intervening  
21 stroma. During the estrogen-dominated proliferative phase that follows  
22 menstruation, the mucosa regrows and then differentiates during the progesterone-  
23 dominated secretory phase. Implantation occurs ~7 days post-ovulation onto the  
24 ciliated luminal epithelium and stimulates transformation into the gestational  
25 endometrium, the true decidua of pregnancy, that provides a microenvironment  
26 essential for placentation. Up to ~10 weeks gestation, uterine glands provide  
27 histotrophic nutrition for the conceptus before the definitive hemochorial placenta is  
28 established<sup>1, 2</sup>. Animal models in mice and ruminants where glandular function is  
29 suppressed are unable to support implantation and pregnancy<sup>3, 4</sup>. Such models  
30 have revealed the molecular interactions involved between the trophoblast and  
31 the uterine surface and the key cytokines secreted by the glands, such as leukemia  
32 inhibitory factor<sup>5</sup>. However, the composition of the secretions, and the  
33 gland/conceptus signalling dialogue during human placentation are unknown due to  
34 their inaccessibility *in vivo* and the absence of *in vitro* models. Suboptimal glandular  
35 development and/or functions may result in human pregnancy failure or predispose  
36 to complications of later pregnancy, such as growth restriction<sup>6</sup>. Thus, model  
37 systems to study these essential processes of human early pregnancy would have  
38 many biological and clinical applications.

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

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

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

## 19 20 **RESULTS**

### 21 22 **Long-term genetically-stable 3D organoid cultures can be established from** 23 **human non-pregnant endometrium and decidua.**

24 To generate endometrial organoids, we used tissue isolates enriched for epithelial  
25 cells, and allowed these to self-organise within Matrigel droplets with the basal  
26 medium that supports development of other human tissue organoids, containing  
27 EGF, Noggin and R-spondin-1 (ENR) (Fig. 1a). Because the signalling pathways  
28 maintaining endometrial gland stem/progenitor cells are unknown, we tested factors  
29 secreted by surrounding stromal cells, FGF10 and HGF<sup>22-25</sup>. Nicotinamide and the  
30 Alk3/4/5 inhibitor, A83-01, that blocks the TGF $\beta$  pathway were added as they are  
31 crucial in the establishment and/or long-term culture of other human organoid  
32 systems<sup>18, 20, 26</sup>. Decidual samples were initially used to optimise the culture  
33 conditions as they yield high cell numbers. Glandular cells were cultured for 7 days  
34 and passaged at 1:3. Organoid numbers were counted after another 7 days (Fig.  
35 1b,c). A83-01, FGF10 and HGF with EGF, Noggin, R-spondin-1 and nicotinamide,  
36 expansion medium (ExM), gave the highest yield of cells (Fig. 1c, C8).

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

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

12 The established organoids can be expanded at passage ratios of 1:2 or 1:3  
13 every 7-10 days for >6 months (reaching more than a 10<sup>6</sup>-fold increase in the  
14 number of organoids). Markers of glandular epithelium (MUC1, E-CADHERIN, CK7  
15 and EPCAM) are strongly expressed by the organoids (Fig. 1g,h,i). EPCAM and  
16 LAMININ are present at the baso-lateral membrane, showing epithelial polarity is  
17 intact (Fig. 1i). EdU pulse-labelling shows ~30% of cells are actively replicating (Fig.  
18 1i). The organoids form cystic structures lined by columnar epithelium with  
19 secretions visible in the lumen. Electron microscopy reveals a microvillous,  
20 pseudostratified columnar epithelium supported by amorphous basement membrane  
21 material with basally-located nuclei (Fig. 1j). The cytoplasm contains plentiful rough  
22 endoplasmic reticulum and Golgi bodies, numerous secretory vesicles, with evidence  
23 of secretory activity from the apical surface (Fig. 1k, arrowheads). A major  
24 component of endometrial glandular secretions, glycogen, was visualized by vivid  
25 PAS staining (Fig. 1l). Thus, the appearances are highly similar to endometrial  
26 glands *in vivo*<sup>27</sup>.

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

34

### 35 **Established human endometrial gland organoids recapitulate molecular** 36 **signature of glands *in vivo*.**

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

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

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

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

41

## 42 **Human endometrial gland organoids respond to sex hormones.**

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

1 spatial expression of their receptors, ER $\alpha$  and PR (Fig. 3a)<sup>36-38</sup>. Following  
2 menstruation, glands increase expression of ER $\alpha$  in response to rising E2 levels  
3 (proliferative phase). After ovulation, ER $\alpha$  expression declines in the early secretory  
4 phase whereas PR is maintained until mid-secretory (LH+7), after which both ER $\alpha$   
5 and PR expression disappears<sup>37</sup>.

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

14 We performed a microarray analysis of organoids in ExM, E2 alone or E2 and  
15 P4. Known genes upregulated by E2 and P4 in the mid-secretory phase *17 $\beta$ HSD2*,  
16 *PAEP*, *SPP1*, *LIF*, *IGFBP4*, *IGFBP5* and *CYCLIN A1* were all upregulated in  
17 hormonally-treated organoids (Fig. 3d)<sup>39-42</sup>. This was confirmed for several genes  
18 using qRT-PCR (Fig. 3e) and at the protein level for PAEP and SPP1 (Fig. 3 f,g).  
19 We also confirmed that the addition of cyclic adenosine monophosphate (cAMP) to  
20 the differentiation medium, a component used typically in decidualization protocols,  
21 enhances the expression of differentiation markers shown by increased expression  
22 of *PAEP* and *SPP1* (Supplementary Fig. 6b)<sup>43</sup>.

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

### 30 31 **Signals from decidualised stroma and the placenta can further stimulate** 32 **differentiation of human endometrial gland organoids.**

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

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

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

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

### 9 10 **Human endometrial organoids have clonogenic ability and are bipotent**

11 To assess for stem cell activity, we measured clonogenic ability by plating single  
12 cells from established organoid cultures by limiting dilution; drops containing single  
13 cells were marked and followed by time-lapse photography. Some cells formed an  
14 entire organoid over 7-14 days; the rest either did not divide or formed small dying  
15 spheroids (Fig. 5a). The organoid-forming efficiency of these cells, was 2-4% with  
16 100 cells/drop and ~10-fold lower with 10 cells/drop (Supplementary Table 2). Single  
17 organoids can be expanded into clonal cultures and we now have grown 12 clonal  
18 lines from 5 independently-derived organoids (Fig. 5b). A single cell has bi-potent  
19 ability as it could generate the two main endometrial cell types: secretory (PAEP+)  
20 and ciliated (acetylated- $\alpha$ -tubulin+) cells (Fig. 5c). Formation of cilia was confirmed  
21 by EM (Fig. 5d).

### 22 23 **Organoid cultures can be derived from endometrial cancer**

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

## 32 33 **DISCUSSION**

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

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

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

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

19 Endometrial organoids can be maintained and expanded in ExM,  
20 recapitulating pathways essential for culturing organoids from other organs - the  
21 FGF-MAPK, WNT-Rspondin, BMP-Noggin and TGF $\beta$  signalling pathways<sup>51</sup>. The  
22 contribution of endometrial stromal cells to these signalling pathways is revealed  
23 from our microarray analysis showing stromal transcripts encoding Rspodin-1 and  
24 FGF2. Further refinement of the method to replace Matrigel with a chemically-  
25 defined extracellular matrix would enhance the model in future<sup>52</sup>. The identity of the  
26 endometrial epithelial stem cells remains unknown although their presence is  
27 revealed by the long-term expansion and clonogenic activity of organoids, and we  
28 have defined the essential niche components for their maintenance.

29 We also recapitulate the glandular cyclical changes during the menstrual  
30 cycle triggered by sequential secretion of ovarian hormones, E2 and P4.  
31 Endometrial organoids acquire a differentiated phenotype characteristic of the mid-  
32 secretory phase, with upregulation of several genes (*17 $\beta$ HSD2*, *SPP1*, *LIF*)  
33 expressed at this time. Other genes, such as *OLFM4*, that may play key roles in  
34 regulating gland cell proliferation and function during the cycle were also identified.

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

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

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

7  
8 In summary, we describe a method for reliable chemically-defined, long-term  
9 culture of endometrial glands from non-pregnant endometrium and decidua that  
10 closely recapitulates the molecular and functional characteristics of their cells of  
11 origin. The organoid cultures can be frozen down without loss of their proliferative  
12 ability upon thawing, allowing the possibility to build up patient-specific bio-banks.  
13 This method will be an invaluable research tool to study new therapies for common  
14 pathologies of the endometrium, such as endometriosis and endometrial cancer, as  
15 well as investigating problems of implantation and the secretion of uterine histotroph  
16 during early pregnancy.

17

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20

21

## 1 **Figure Legends**

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

- 4
- 5 (a) Scheme for deriving organoids.
- 6 (b) Screening conditions for generating organoids. FGF10, A83-01, HGF and  
7 Nicotinamide added in combinations to generic organoid medium (ENR). Number  
8 of organoids derived under each condition (C2 to C9) shown relative to basal  
9 conditions (C1). Shown are decidual digests from 3 different patients. Source  
10 data in Supplementary Table 5.
- 11 (c) Representative images for conditions C1-C9 in Fig. 1b. Scale bar, 500  $\mu\text{m}$ .
- 12 (d) Images of decidual gland isolates (passage 0) and organoids after one passage  
13 in Expansion Medium (ExM) (passage 1). Scale bar, 200  $\mu\text{m}$ . Representative of  
14 all samples, summarized in Supplementary Table 1.
- 15 (e) Effect of withdrawal of growth factors from ExM. Organoids grown in ExM and  
16 each factor withdrawn: EGF, Noggin (NG), Rspondin-1 (RSPO1), FGF10, A8301,  
17 HGF and Nicotinamide (NIC). Organoids formed shown relative to ExM (%).  
18 Shown are decidual cultures derived from 3 different patients. Source data in  
19 Supplementary Table 5.
- 20 (f) Images of organoids established in ExM from proliferative (Prol.) endometrium  
21 (n=3), secretory (Sec.) endometrium (n=9), decidua (n=25) and post-menopausal  
22 (atrophic) endometrium (n=1). Scale bar, 100  $\mu\text{m}$ .
- 23 (g) IHC of decidua (*in vivo*) and organoids for Mucin 1 (MUC-1). Scale bar, 50  $\mu\text{m}$ .  
24 Representative of 6 decidual and endometrial samples, and organoids derived  
25 from 2 endometrial and 2 decidual samples from different patients.
- 26 (h) IF staining of organoid for E-CADHERIN (E-CAD) and CYTOKERATIN-7 (CK7).  
27 Scale bar, 50  $\mu\text{m}$ . Experiment repeated twice (1 endometrial-derived and 1  
28 decidua-derived organoids).
- 29 (i) IF staining of organoid for cell proliferation (uptake of EdU), epithelial marker  
30 EPCAM and basement membrane marker laminin (LAM). Scale bar, 50  $\mu\text{m}$ .  
31 Experiment repeated twice (1 endometrial-derived and 1 decidua-derived  
32 organoids).
- 33 (j) Electron micrograph (EM) of organoid showing columnar epithelial cells with  
34 basally-located nuclei. Scale bar, 5  $\mu\text{m}$ . Experiment repeated twice with different  
35 donors.
- 36 (k) EM showing secretory activity (black arrowheads). Scale bar, 1  $\mu\text{m}$ . Experiment  
37 repeated twice with different donors.
- 38 (l) PAS staining for glycogen in endometrium and organoids. Scale bars, 50  $\mu\text{m}$   
39 (main image) and 10  $\mu\text{m}$  (inset). Representative of 3 endometrial samples and 3  
40 endometrial organoids.

### 41 **Figure 2. Established human endometrial organoids recapitulate molecular** 42 **signature of glands *in vivo*.**

43

- 1 (a) Unsupervised hierarchical clustering analysis of global gene expression profiles  
2 by microarray of gland digests, stromal cells and corresponding established  
3 organoids from endometrium (n=7 independent donors). Analysis based on  
4 15475 probes with sd/mean >0.1. Expression profiles of organoids cluster with  
5 glands while those of the stroma cluster in a separate tree.
- 6 (b) Venn diagram showing overlap of 287 genes significantly upregulated in glands  
7 and organoids with a fold change  $\geq 1.5$  ( $p \leq 0.01$ ) relative to stroma.
- 8 (c) Gene ontology (GO) analysis of the 287 genes from (b) using HumanMine v2.2  
9 database for GO Terms Biological processes and Benjamini Hochberg test  
10 correction with maximum p-value of 0.05. The top ten significantly enriched GO  
11 terms for each category are shown with the  $-\log$  of their p-values and are  
12 enriched for terms describing epithelial tissue.
- 13 (d) Gene ontology (GO) analysis of the 287 genes from (b) using same method as in  
14 (c). The top ten significantly enriched GO terms describe epithelial cells with  
15 secretory function.
- 16 (e) Clustered heatmap of 287 genes commonly upregulated between organoids and  
17 glands compared to stroma from (b). Genes of interest are listed on the right.  
18 Epithelial markers (blue) (*EPCAM*, *CLD10*, *CDH1*), glandular products and  
19 markers of secretory cells (purple) (*MUC20*, *PAX8*, *PAEP*, *MUC1*), progenitor cell  
20 markers (cyan) (*LRIG1*, *PROM1*, *AXIN2*) and murine genes important for  
21 endometrial function (pink) (*SOX17*, *KLF5*, *FOXA2*).
- 22 (f) IHC for genes selected from microarray, *FOXA2*, *SOX17* and *PAX8*, in  
23 proliferative and secretory endometrium and organoids. Scale bars, 50  $\mu\text{m}$  (main  
24 image) and 10  $\mu\text{m}$  (insets). Representative of 3 proliferative and 7 secretory  
25 endometrial samples and endometrial organoids derived from 8 different patients.
- 26 (g) ISH for *LRIG1* on proliferative and secretory endometrium and organoids.  
27 Negative control probe is for the bacterial gene *dapB*. Scale bars, 50  $\mu\text{m}$  (main  
28 image) and 10  $\mu\text{m}$  (insets). Representative of 3 proliferative and 3 secretory  
29 endometrial samples and endometrial organoids derived from 4 different patients.

30 **Figure 3. Human endometrial organoids respond to sex hormones.**

- 31
- 32 (a) Ovarian hormones, Estrogen (E2)(red) and Progesterone (P4)(blue), and the  
33 cycling endometrium. Expression of Estrogen Receptor (ER $\alpha$ )(dashed red) and  
34 Progesterone Receptor (PR)(dashed blue) are specific for glands of the  
35 functional layer. Adapted from Reference<sup>37</sup>.
- 36 (b) Protocol for hormonal stimulation. Organoids grown in ExM, day 0 (d0), are  
37 primed with E2 for 48 h on day 4 (d4) followed by stimulation with P4 and cyclic  
38 AMP (cAMP) for 48 h.
- 39 (c) IHC for ER $\alpha$  and PR on organoids after hormonal stimulation. In ExM expression  
40 of ER $\alpha$  is weak, but some cells are either ER $\alpha^{\text{high}}$  (arrowheads) or ER $\alpha^{\text{negative}}$   
41 (arrows). Few cells are positive for PR (arrowheads). After E2 and P4 treatment,  
42 levels of ER $\alpha$  and PR are higher. Scale bars, 50  $\mu\text{m}$  (main image) and 10  $\mu\text{m}$   
43 (insets). Representative of endometrial organoids from 6 different patients and  
44 decidual organoids from 9 different patients.

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

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

- 25
- 26 (a) Hormonal environment of endometrium during the first trimester of pregnancy.  
27 Estrogen (E2) and Progesterone (P4) are ovarian products, human chorionic  
28 gonadotropin (hCG) and human placental lactogen (hPL) are secreted by  
29 trophoblast and prolactin (PRL) by decidualized stromal cells.
- 30 (b) Protocol for stimulation of endometrial organoids. Organoids are passaged and  
31 plated on day 0 (d0) in ExM. On d4, ExM is changed to Differentiation Medium  
32 (DM; ExM with E2+P4+cAMP). hCG, hPL and/or PRL were added for 8 d.
- 33 (c) IHC for PAEP on endometrial organoids under the following conditions: ExM, DM,  
34 DM with hCG/hPL or PRL or all three combined. Maximal production of PAEP  
35 and differentiated morphology of cells is seen upon exposure to DM with hCG,  
36 hPL and PRL. Scale bar, 50  $\mu$ m. Representative of endometrial organoids  
37 derived from 3 different patients.
- 38 (d) IHC for acetylated  $\alpha$ -tubulin to visualize cilia in secretory endometrium (Sec.  
39 Endom.) and endometrial organoids following stimulation with PRL. Ciliated cells  
40 (arrows) are present in the luminal epithelium (LE) and within organoids. GE,  
41 glandular epithelium. Scale bars, 50  $\mu$ m (main image) and 10  $\mu$ m (insets).  
42 Representative of 4 secretory endometrial samples and endometrial organoids  
43 derived from 4 different patients.

1 (e) IHC for SOX9 on endometrial glands *ex vivo* and *in vitro*. Organoids in ExM  
2 express high levels of SOX9 similar to proliferative endometrium (Prol. Endom.).  
3 After hormonal stimulation, SOX9 is downregulated in organoids  
4 (ExM+HCG+HPL+PRL) similar to glands in decidua. Scale bars, 50  $\mu\text{m}$  (main  
5 image) and 10  $\mu\text{m}$  (insets). Representative of 4 proliferative endometrial samples,  
6 7 decidual samples and endometrial organoids derived from 4 different patients.

7  
8 **Figure 5. Human endometrial organoids have clonogenic ability and are**  
9 **bipotent.**

- 10  
11 (a) Phase-contrast images of (from top to bottom row): an organoid forming from a  
12 single cell; a single cell forming a spheroid with no further growth, and a single  
13 cell showing no growth. Images were taken every two days. Scale bar, 50  $\mu\text{m}$ .  
14 Experiment was performed with 3 clonal lines derived from 2 endometrial and 1  
15 decidual organoid cultures.
- 16 (b) Representative image showing expansion of a clonal culture at passage 1 (p1)  
17 from a single organoid (at passage 0, p0) in a 96-well. Scale bar, 500  $\mu\text{m}$ . 12  
18 clonal cultures were established from organoids from 5 different patient samples  
19 (4 endometrial-derived and 1 decidual-derived).
- 20 (c) IF on clonally-derived endometrial organoid cultures subjected to the full cocktail  
21 of hormonal stimuli to visualize two main endometrial epithelial cell types: ciliated  
22 cells (acetylated  $\alpha$ -tubulin) (cyan) and secretory cells (PAEP) (red). Scale bars  
23 from left to right: 100  $\mu\text{m}$ , 20  $\mu\text{m}$  and 5  $\mu\text{m}$ . Representative of 4 clonal lines  
24 derived from 2 different endometrial organoid cultures.
- 25 (d) EM on clonally-derived endometrial organoid cultures subjected to the full cocktail  
26 of hormonal stimuli showing basal bodies of fully formed cilia. Scale bars: 10  $\mu\text{m}$   
27 and 1  $\mu\text{m}$ . Experiment performed twice using 1 clonal endometrial organoid  
28 culture.

29  
30 **Figure 6. Organoids can be derived from endometrial cancer.**

31  
32 Derivation of organoids from endometrial carcinomas. From left to right: H&E stained  
33 sections of normal atrophic endometrium showing gland surrounded by dense  
34 stroma and a FIGO Grade I endometrioid carcinoma with dense glandular structures  
35 from the same patient, scale bar, 100  $\mu\text{m}$ ; images of organoids derived from  
36 matched normal and malignant endometrium cultured in ExM (passage 1), scale bar,  
37 100  $\mu\text{m}$ ; H&E stained sections showing marked differences in morphology between  
38 organoids derived from normal endometrium and those from tumours which show  
39 nuclear pleomorphism, a disorganized epithelium with irregular basement membrane  
40 and isolated cells present in surrounding Matrigel (arrows), scale bar, 20  $\mu\text{m}$ ; IHC for  
41 MUC-1 and SOX17 on tumour and normal organoids confirm their glandular origin,  
42 scale bar, 20  $\mu\text{m}$ . Representative of organoids derived from 3 different endometrial  
43 carcinomas and 1 matching normal tissue.