

Review

Endometrial Organoids: A New Model for the Research of Endometrial-Related Diseases[†]

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Abstract

An ideal research model plays a vital role in studying the pathogenesis of a disease. At present, the most widely used endometrial disease models are cell lines and animal models. As a novel studying model, organoids have already been applied for the study of various diseases, such as disorders related to the liver, small intestine, colon, and pancreas, and have been extended to the endometrium. After a long period of exploration by predecessors, endometrial organoids (EOs) technology has gradually matured and maintained genetic and phenotypic stability after long-term expansion. Compared with cell lines and animal models, EOs have high stability and patient specificity. These not only effectively and veritably reflect the pathophysiology of a disease, but also can be used in preclinical drug screening, combined with patient derived xenografts (PDXs). Indeed, there are still many limitations for EOs. For example, the co-culture system of EOs with stromal cells, immune cell, or vascular cells is not mature, and endometrial cancer organoids have a lower success rate, which should be improved in the future. The investigators predict that EOs will play a significant role in the study of endometrium-related diseases.

Summary Sentence

This review describes the development and potential applications of endometrial organoids.

Key words: endometrial organoids, culture medium composition, hormone responsiveness, drug screening.

Introduction

As the inner lining of the uterus, the endometrium contains epithelial and stromal cells, and undergoes periodic changes in growth, differentiation, and degeneration under ovarian hormones [1–3]. The endometrium is associated with a number of gynecological problems, embracing infertility, endometrial hyperplasia, endometrial cancer, and endometriosis [3]. All of these endometrial diseases seriously affect a women's health, such as endometrial cancer (EC), which is the fourth most common type of female cancer, and the endometriosis influences approximately 6–10% of women of reproductive age

[4, 5]. The main obstacle of studying these diseases is the lack of ideal models. Existing research models have obvious shortcomings. Rodents do not have menstruation and cannot faithfully mimic the human situation. Primates, although have spontaneous menstruation, are high cost and ethically limited. Primary cells cannot maintain long-term culture in vitro, and there are transformed phenotypes in immortalized or carcinoma-derived endometrial cell lines [6–11]. Therefore, a reliable endometrial model is the key to study endometrium-related diseases, and endometrial organoids (EOs) are expected to conquer these problems. As a kind of 3D

culture system, organoids are self-organizing and genetically stable, which contain progenitor/stem cells and differentiated cells that resemble the original tissues [12]. Organoids derived from other tissues, such as the gastrointestinal tract, liver, pancreas, prostate, and fallopian tube, have already been established [13–17].

Boretto et al. and Turco et al. were the first scholars to report the study of EOs, including organoids derived from mouse and human endometrium [18, 19]. On these bases, Boretto et al. and Fitzgerald et al. further studied the characteristics of human EOs [20, 21], involving organoids from normal and abnormal endometrium. These researches described the specific composition of the EOs culture medium, the phenotypic (typical surface markers and structures) and genetic characteristics, and the responsiveness to hormones, and there were several similarities and differences in these studies. As an ideal study model, EOs have the potential to be used in pathophysiological research, drug screening for new therapy or drug testing for personalized medicine, regenerative medicine, and the EOs biobank of endometrium-related disease.

EOs culture came from mouse/human endometrium

The organoid is a novel model derived from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or adult stem/progenitor cells (ASCs), in order to recapitulate parental tissues. At present, EOs are mainly derived from ASCs, that is, endometrial tissue-derived stem cells. Boretto et al. successfully cultured mouse EOs and revealed that the endometrial tissues isolated at the estrous of mice were most efficient in forming EOs. In addition, the endometrium from 1-year-old mice that had already given birth could also develop into organoids [18]. As for human EOs, Boretto et al., Turco et al. and Fitzgerald et al. investigated the formation and characteristics of EOs, which ranged from normal endometrium to endometrium-related disease (endometriosis, endometrial hyperplasia, and endometrial cancer) [18–21]. Turco et al. established human EOs from the endometrium at different stages, which contained the proliferative, secretory, and postmenopausal endometrium, as well as the decidual endometrium during pregnancy. Among these, the success rate of organoids formation of secretory endometrium and decidua was the highest (100 and 96%, respectively) [19]. The culture process of EOs is shown in Figure 1.

Based on the researches on healthy EOs, Boretto et al. began to focus on the establishment of EOs related to gynecological diseases, such as endometriosis, endometrial hyperplasia, and endometrial cancer [20]. For endometriosis, Boretto et al. successfully developed EOs from various ectopic lesions at different clinical stages (American Society for Reproductive Medicine revised staging system (rASRM), stages I – IV). Simultaneously, they established patient-matched EOs from endometriosis patients, which are known as ectopic-endometrium organoids (EcEOs) and eutopic-endometrium organoids (EuEOs) together [20]. In addition, they also established organoids from the hyperplastic endometrium (HypEOs, divided into three categories: simple benign, complex atypical, and polyp) and endometrial cancer organoids (ECOs, derived from different grades and progression stages of endometrial cancer) [20]. Organoids from endometrial precancer and cancer pathologies recapitulated the disease phenotype and genetics. The efficiency of organoid formation varies among the endometrium of different nature. Healthy EOs and EuEOs (both 100%) were higher than HypEOs and EcEOs (70 and ~60%, respectively), and ECOs (40%) were the least efficient [20]. ECOs derived from different grade/stage endometrial cancers presented with morphological heterogeneity.

For example, ECOs from high-grade/stage cancer appeared to be dense without a visible lumen [20].

During the development of organoids culture, the introduction of single-cell genomic methods has put forward new analytic approaches to describe these organoid models with more resolution and less bias, when compared to the previous bulk RNA-sequencing experiment [22]. Fitzgerald et al. combined organoids with bulk RNA-sequencing and single-cell RNA-sequencing (scRNA-seq) to establish a high-resolution gene expression atlas of EOs [21]. They expounded how EOs respond to reproductive hormones and found that the organoid was heterogeneous, embracing specific subpopulations of cells that were hormone responsive, including stem cells [21]. Furthermore, they also explored the changes of relative receptors and ligands after exposure to hormones through the FANTOM5 database, which could predict some communication between different cell types [21]. It is worth noting that the successful establishment of EOs, long-term expansion, successful cryopreservation and thawing made it possible for EOs to be widely available.

The components of the EOs medium

Isolated epithelial cells were embedded in Matrigel, and cultured in a cocktail of growth and signaling factors. The formation and expansion of organoids was modulated by WNT3A/R-spondin 1 (RSPO1), and the culture system was usually regarded as the RSPO1-based culture method. The EOs culture medium usually contains ENRA, which means epidermal growth factor (EGF), Noggin, R-spondin-1, and A83-01. According to the discrepancies of experimental purposes and conditions, researchers would add different factors (Table 1), such as WNT3A (for mouse EOs), fibroblast growth factor 10 (FGF10), hepatocyte growth factor (HGF), nicotinamide, p38 inhibitor SB202190, and glycogen synthase kinase 3 β inhibitor CHIR99021 [18–20, 23]. For mouse EOs, Boretto et al. reported that WNT3A and RSPO1 are needed for an efficient culture and long-term expansion [18]. Compared to the low WNT proportion, mouse EOs possessed a more differentiated character under high WNT conditions, such as glandular-like lumen-containing shape, secretory activity, and decreased proliferative nature [18]. RSPO1 was the essential ingredient for the further expansion of mouse and human EOs, but WNT3A was only necessary for mouse EOs, and not for human EOs [18]. Turco et al. had named their EOs culture medium as Expansion Medium (ExM), and combined ExM with other six compounds (E2, P4, cAMP, hCG, hPL, and PRL) as the differentiation medium (DM) (Table 1) [19]. In addition, Turco et al. proved that omitting any of RSPO1, A83-01, Noggin, EGF and HGF would give rise to decreasing numbers and/or smaller organoids, while the withdrawal of nicotinamide had the strongest effect [19]. Boretto et al. reported that the omission of EGF significantly impaired both EcEOs and healthy EO expansion, and the removal of Noggin compromised EcEOs growth [18, 20]. A similar phenomenon was observed in organoid cultures derived from other organs. Huch et al. discovered that Noggin and nicotinamide were essential to maintain the pancreas organoids culture for more than 2 months [15]. This phenomenon might be correlated to the source of the tissues and the proportion of the ingredients. In order to improve the establishment efficiency of ECOs, Boretto et al. adjusted the types (adding insulin-like growth factor-1 (IGF1), HGF, and lipids) and concentration (lowering p38i concentration) of reagents, and further optimized the composition of the medium, and the success rate increased from 20 to 40% [20]. The detailed medium composition in different EOs studies and the function of the key factors are shown in Tables 1 and 2.

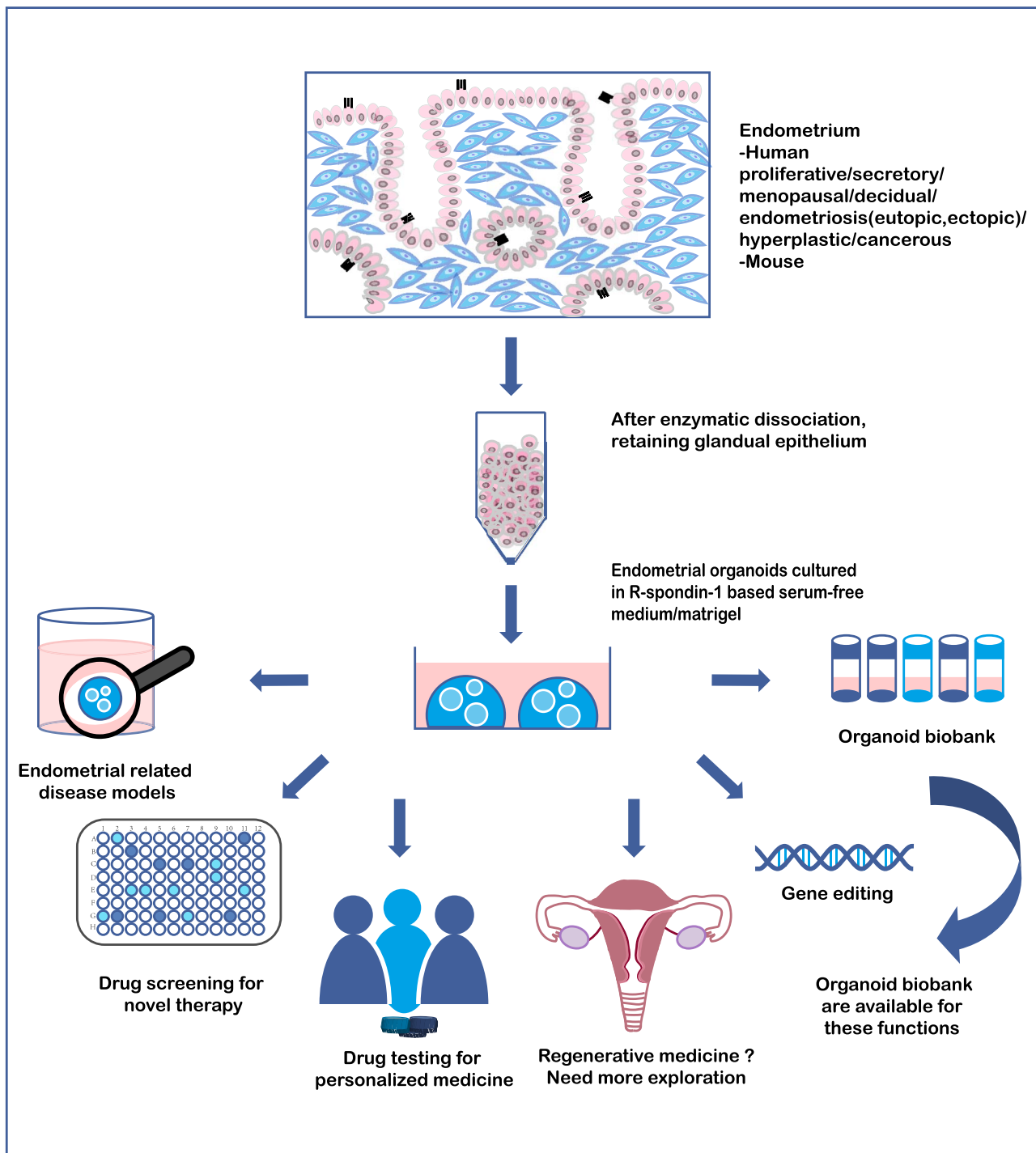


Figure 1. The culture of process of endometrial organoids (EOs) and their potential applications. EOs can be used for screening for novel therapy, drug testing for personalized medicine, as well as having the potential in regenerative medicine of endometrial-related diseases. Furthermore, gene editing could further assist these functions and all of these functions above can be realized based on the establishment of cryopreserved biobanks of healthy and diseased human EOs.

Some agents were not applied to EOs, but were present in organoids derived from other organs, such as the cAMP activator. Huch et al. discovered that in the liver organoids culture, adding Forskolin, which is a cAMP activator, could upregulate stem cell marker *LGR5* and downregulate differentiated-association genes, and increase the culturing longevity, but not the colony-forming efficiency [13].

Phenotypic (typical surface markers and structures) and genetics characteristics of EOs

Mouse EOs expressed epithelial markers pancytokeratin (PanCK) and E-cadherin, as well as estrogen receptor α (*ER* α), via immunohistochemical analysis [18]. Others such as mucus (analyzed by PAS staining) and apical microvilli (reviewed by transmission electron

Table 1. The components of EOs culture medium.

Author	Species	Medium components ^a									
		WNT3A ^b	ENRA ^c	NICO	FGF10	HGF	ITS	SB202190	E2 ^d	Y-27632 ^e	Others
Boretto et al. (2017)	Mouse	+	+	+	+	–	+	–	–	+	–
	Human	+	+	+	+	–	+	+	+	+	–
Turco et al. (2017)	Human	–	+	+	+	+	–	–	–	+	–
Boretto et al. (2019)	Human	–	+	+	+	–	+	+	+	+	bFGF
	(EMT & HYP)										
Haider et al. (2019)	Human (EC)	–	+	+	–	+	–	+	+	+	Lipid, IGF-1, IL-6
	Human	–	+	–	–	–	–	–	–	–	CHIR99021 PGE2

NICO Nicotinamide, FGF10 fibroblast growth factor 10, HGF hepatocyte growth factor, ITS insulin transferrin selenium, SB202190 p38 MAPK inhibitor, bFGF basic fibroblast growth factor, Lipid chemically defined lipid concentrate, IGF-1 insulin-like growth Factor-1, IL-6 interleukin-6, CHIR99021 glycogen synthase kinase 3 β inhibitor, PGE2 prostaglandin E2, EMT endometriosis, HYP hyperplasia, EC endometrial cancer.

^aThe basal medium components of EOs, such as DMEM/F12, N2, B27, N-acetyl L-cysteine, glutamine, HEPES and antibiotics, are not listed.

^bIn contrast to mouse EOs, WNT3A was not needed for further expansion and passaging of human EOs.

^cENRA consists EGF, Noggin, R-spondin-1 and A83–01 (TGF β receptor inhibitor).

^dE2 (β -estradiol) was only for organoid expansion.

^eY-27632 (ROCK signaling inhibitor) was only for organoid formation or dissociation at passaging.

Table 2. The common reagents and function of EOs culture medium.

Reagents	Function
WNT3A	An essential requirement for proliferation; inhibit the differentiation of epithelia
R-spondin-1	A WNT agonist to maintain stem cell populations; induce hyperplasia
EGF	Potent activator of tyrosine kinase receptor signaling to promote cell proliferation
Noggin	Induces expansion of crypt numbers; inhibition of BMP signals
Nicotinamide	Precursor of oxidized nicotinamide adenine dinucleotide; suppress sirtuin activity; inhibit differentiation, increase proliferation
A83–01	Inhibition of TGF- β receptors Alk4/5/7
SB202190	p38 inhibitor; inhibit differentiation, increase proliferation
CHIR99021	Glycogen synthase kinase 3 β inhibitor
Y-27632	A ROCK inhibitor; inhibit anoikis

microscopy, TEM) were also detected in mouse EOs, resembling the mouse endometrial epithelium in vivo [18]. A series of researchers had conducted in-depth research on human EOs [18, 19, 21]. Among them, Turco et al. gave the most detailed description of the specific cell surface markers and cellular typical structures. First, Turco et al. identified the EOs glandular epithelial nature by hierarchical clustering analysis. The EOs expressed E-cadherin, PanCK, Muc1 and EPCAM, which reflected the epithelial and glandular nature, and several nuclear markers (FOXA2, SOX17 and PAX8) [19]. The epithelial polarity was intact by presenting EPCAM, and showing Laminin at the basolateral membrane. Similar to mouse EOs, TEM revealed a microvillous, pseudostratified columnar epithelium supported by the basement membrane, and PAS staining had endometrial glandular secretions glycogen [19]. Analogously, epithelial nature (E-cadherin, PanCK), ER α and progesterone receptor (PR), mucus, cell polarization as well as secretory vacuoles, were also observed in EcEOs [20]. In addition to expressing EC-related markers in ECOs, Boretto et al. further found that they could also distinguish type I and type II EC, according to the expression of ER α and PR in ECOs [20]. As it is already known, type I EC is estrogen-dependent, while type II EC is estrogen-independent [24, 25].

Apart from phenotypic characteristics, EOs could also closely resemble the endometrium at the genetic level. The transcriptomic and genetic analyses of EOs reveal disease-associated traits and mutational landscape of the primary tumor. For instance, several important genes associated with signal pathway, hormonal response and adhesion/invasion factors differed among healthy EOs, EcEOs, and EuEOs. Compared with healthy EOs, the differential gene

of EcEOs derived from diverse stages were further analyzed by RT – qPCR. *LEF1*, *WNT11*, *LGR6*, *LIFR*, *SOX9*, *MMP2*, *IL-1 β* , and *IL-8*, which appeared to be upregulated, especially *LGR6* in the higher stages III – IV, *MMP2* in stages II and IV, *IL-1 β* , and *IL-8* in stages I – II EcEOs, while the progesterone-regulated gene *PAEP* was downregulated [20]. It is noteworthy that *LGR6* was one of the top regulated genes in EcEOs, when compared to EuEOs from individual endometriotic patients. As for EC, abundant somatic copy number alteration in primary tumors was reserved in the corresponding ECOs [20]. It was added that the gene signature of decidual EOs were fully similar to non-pregnant EOs [19]. All of these disease-derived EOs could recapitulate the disease phenotype (histological and molecular features) in vivo, and some differential gene expression levels between gland samples and organoids might be associated with their different microenvironments [19]. The genetic stable of organoids had also been observed in other organ-derived organoids, such as the pancreas, liver and prostate organoids [13, 15, 16].

EOs responsiveness to hormones

Boretto and colleagues found the number of proliferating cells (Ki67+) in mouse EOs increased after exposure to estrogen (E2), but not to progesterone (P4) [18]. Furthermore, several relevant genes appeared specific to regulation after treatment with E2 or P4, such as *Egf*, *Igf1*, *Lf* (increased by E2) and *Alox15*, *Lif*, and *Prss28* (upregulated by P4) [18]. Mouse EOs also responded to ovarian hormones in vivo. When treated by ovarian hormones, mouse EOs transplanted

under the kidney capsule of mouse could expand and assemble into an organized structure with glandular-type protuberances [18].

Same as in vivo, human EOs showed physiological, cycle-mimicking responses to hormones [18, 19, 21], which was usually not observed for endometrial epithelia to response to hormones in 2D culture system. Boretto et al. detected that the Ki67+ cell proportion in human EOs increased with the E2 treatment, but decreased with the addition of P4 (while reducing the E2 concentration to half) [18], which was similar to the situation in vivo [26–28]. In the level of protein and mRNA, the E2 treatment also upregulated the *OLFM4*, an intestinal stem cell marker [29], which was negative without E2 exposure [19, 21]. In addition to E2 and P4, Turco et al. further explored the effect of stimulation of cAMP, a second messenger, placental hormones (hCG and hPL) as well as PRL [19]. Among them, cAMP contributed decidualization of endometrial stromal cells in vivo [30]. After exposure to E2 followed by P4, the relevant genes *17βHSD2*, *PAEP*, *SPP1*, *LIF*, *IGFBP4*, *IGFBP5*, and *CYCLIN A1* all increased in hormonally treated organoids by microarray analysis, and *17βHSD2*, *PAEP*, *SPP1*, *LIF* were further confirmed by qRT-PCR [19]. In addition, after treatment of E2 followed by P4, the expression of both ERα and PR of treated organoids were higher than those untreated in most, but not all organoids [19]. However, Fitzgerald et al. confirmed that after E2 stimulation alone for 2 days, EOs exhibited an upregulation in ERα and PR, and after exposure to E2 + P4 for another 6 days, the decrease and even absence of ERα and PR appeared [21]. The difference might be correlated with concentration of hormones and the duration of P4 exposure in two studies. In vivo, the ERα and PR of the luminal epithelium (LE) and glandular epithelium (GE) would decline or disappear between the proliferative to the secretory phase of the menstrual cycle, especially after mid-secretory stage [31]. The addition of cAMP had upregulated the expression of differentiation markers PAEP (glycodelin) and SPP1 (osteopontin) at gene and protein level [19]. It is noteworthy that signals from decidualized stroma, PRL and signals from the placenta, hCG and hPL can further stimulated the differentiation of human EOs, synthesizing abundant PAEP and SPP1, producing more cilia, and downregulating the progenitor cells marker SOX9 [19].

Fitzgerald et al. further explored the influence of hormones on cell types of human EOs. In combining organoid and single-cell analysis, Fitzgerald et al. found 5 cell types, including ciliated, epithelial, proliferative, stem, and unciliated cells, in both control and E2-treated day six EOs [20]. In addition to these five cell types, there were also secretory cells in group of control, E2 + MPA (medroxyprogesterone), and E2 + MPA + cAMP treated day 12 EOs [20]. It noteworthy that after exposure to hormones, regardless of whether E2 or MPA, the proportion of stem cells decreased. Prolonged EOs culture and the addition of progesterone promoted the proportion of secretory cells [20]. In addition, the proportion of ciliated cells significantly increased following the E2 treatment, which indicated that cilia were one of the indicators of epithelial cell differentiation. This was consistent with the study conducted by Haider et al., in which E2 signaling induced ciliogenesis in the endometrium and organoids [23]. Combining E2 stimulation and NOTCH pathway inhibition greatly increased the ciliogenesis. However, inhibiting the NOTCH signaling alone was insufficient, that is, E2 plays a decisive role in cilia cell formation [23]. On the contrary, the proportion of proliferative type cells did not significantly change with the hormone intervention, and the cAMP had little effect on the cell variety in EOs [20]. The results of this research gave the investigators an idea of which types of epithelial cells were most

responsive to hormone stimulation. In addition, via scRNA-seq, they further identified in which cell *OLFM4* and *PAEP* (the expressions of these genes were increased mostly after exposure to E2 and P4, respectively) has a significantly increased expression. *OLFM4* predominantly upregulated in the stem and ciliated cells, and *PAEP* was predominantly upregulated in the ciliated, proliferative, secretory, and stem cell types [21]. Furthermore, the types and quantities of certain important receptors (such as COL1A2, IL1RN, SPP1, and TGM2) and ligands (such as CALM3, FN1, TIMP2) were also altered in the organoid with the hormone treatment, reflecting that the communication between different cell types changed under the stimulation of hormones [20]. Due to the hormone reactivity of EOs, researchers have considered that EOs plays a great auxiliary role in the study of abnormal pregnancy and hormone-related endometrial diseases. For example, if something goes wrong on either side of the secretory transformation of the glandular epithelium or the decidualization of the stromal cells, the pregnancy may fail or cause complications, including preeclampsia and fetal growth restriction [32, 33]. These endometrial changes are all correlated to hormone stimulation.

Con-culture of EOs and stromal cells

Organoids have advantages in pathogenesis research and drug screening, but classical EOs culture conditions (artificial extracellular matrix, Matrigel and EOs expansion medium) favor epithelial cells, and contaminated stromal cells are lost during subsequent passage. Several researches that studied organoids derived from other organs have already attempted to tackle the problem of co-culture, such as live organoids, co-culturing hPSC-derived hepatic endodermal cells with human endothelial, and human mesenchymal stem cells, thereby contributing to more closely resembling the liver cytoarchitecture [34]; Chakrabarti et al. successfully co-cultured the mouse-derived gastric cancer organoids with autologous immune cells used for the study of PD-L1/PD-1 interactions within the tumor microenvironment in vitro [35]. Mouse ASC-derived pancreatic islet organoids were co-cultured with endothelial cell, and this improved the ability of long-term expansion [36]. Murphy and Wiwatpanit et al. was the first to attempt to generate scaffold-free multicellular EOs, which were responsive to sex hormones, and contained epithelial and stromal cells [37]. They also applied this model to subsequent studies correlated to polycystic ovary syndrome [38]. Unlike the EOs summarized above, they combined the stromal and epithelial cells (1:3 ratio by volume) with 1.5% agarose 3D Petri Dishes using stromal cells as scaffolds, and cultured these in MammoCult growth medium [38]. It was noteworthy that in that culture system, similar to the native endometrial tissue, stromal cells provided the scaffold support for epithelial cells. In addition, 3D porous collagen scaffolds also made co-culture of EOs and stromal cells came true. Abbas et al. detected that stromal cells-only or EOs-only cultured on scaffolds could express the characteristics of corresponding cells and hormone reactivity, respectively, which was the basis of co-culture [39]. It should be noted that original EOs (established in Matrigel) and primary stromal cells were developed via classical culture methods, and that these were sequentially transferred to the scaffold [39]. Stromal cells were cultured in scaffolds with advanced DMEM/F-12 supplemented with 10% (v/v) FBS, glutamine and antibiotics, and co-cultured with EOs after 2 days. After washing several times, the medium was changed to EOs expansion medium [19, 39]. Lastly, after 10 days of co-culture, they verified the existence of stromal cells and epithelial

cells by immunofluorescence and flow cytometry [39]. The porous collagen scaffold-based model of the endometrium including both epithelial and stromal cells provided a new field for studies on endometrial related diseases. Therefore, the co-culture of epithelial cells and stromal cells will further mimic the endometrium *in vivo* via considering stromal-epithelial interactions. Indeed, these co-culture models also faced some challenges as to whether it could be cultured over a long period of time and maintain both phenotypic and genotypic stability simultaneously.

The potential function of EOs

Firstly, EOs have the basic function of *in vitro* models, and also possess certain extraordinary features. Compared to 2D cell cultures, EOs maximally retain the intrinsic properties of diseases. Compared to animal models, these are more convenient for conducting high-throughput detection. Organoids can maintain the genetic stability of original tissue, even for tumor significant genetic heterogeneity, which was better than tumor cell lines. Cell lines sometimes could not retain some rare mutations [40]. Furthermore, studies have shown that the barcode complexity of cell lines was progressively lost [41]. However, the barcode complexity of cell lines could be better preserved by injecting this into mouse [41]. These indicate that the maintenance of genetic stability was closely correlated to the extra cellular environment (ECM), and organoid-specific media may play a role in this regard. Consistent with organoids derived from other organs, EOs shed light into the pathophysiological exploration of endometrium-related disease, such as endometrial cancer, endometriosis, atypical hyperplasia of endometrium, as well as certain pregnant diseases. EOs retain the original characteristics of the disease, widely recapitulating the diversity of disease, and can be placed under expanded culture for a long time, relieving the pressure of insufficient tissues. The transcriptomic and genetic analyses of EOs could also reveal disease-associated traits. For instance, several important genes associated with signal pathway, hormonal response and adhesion/invasion factors differ between normal EOs, EcEOs and EuEOs [20]. Notably, Turco et al. successfully generated trophoblastic organoids [42]. Combining trophoblastic organoids with EOs would be more helpful for the study of pregnancy-related diseases in the future. Given that Abbas et al. measured the tissue stiffness at the human maternal-fetal interface, the investigators can try to optimize the culture method, such as modulating the Matrigel concentration when studying the maternal-fetal disorder [43]. Therefore, EOs can also be used as reliable disease models for the pathogenesis research of endometrium-related disease.

Secondly, organoids can be used for high-throughput drug screening in the development of novel drugs, which has already been applied in many cancers, such as colorectal cancer [44], breast cancer [45], lung cancer [46], and pancreatic cancer [47]. In addition to being superior to cell lines, Schutte et al. discovered that the response to various drugs between parallel organoids culture and patient-derived xenograft (PDX) was generally consistent [48], while PDX demanded more amount time and resources, and did not support high-throughput sequencing. Therefore, the combination of EOs and PDXs are beneficial for drug studies of endometrial-related diseases in the future, in which the selecting effective drugs via EOs can be validated in PDX models further before the clinical study. Furthermore, organoids can be tailored to individual patients, and applied for personalized medicine, especially for malignant tumors, which are highly heterogeneous, and prone to resistant drugs. ECO has been used to drug testing and appeared patient-specific drug responses [20].

Thirdly, organoids can also apply for regenerative medicine, which had already been proposed in liver organoids, kidney organoids, and islet cell organoids [49–51]. EOs might also possess potential in regenerative medicine. EOs, especially robust progesterone responsiveness and co-cultured with stromal cells, are amendable to cell replacement therapy for low-grade endometrial cancer, endometriosis and uterine related infertility, such as Asherman syndrome [52]. In the past, regenerative medicine related to the treatment of endometrial diseases has focused more on stem cells [53]. For example, endometrial epithelial cells generated from hiPSCs have been proposed to contribute to endometrial diseases treatment by cell replacement [52]. In contrast, if it works, considering the convenience of sampling, technology level and maturity of cell culture, the investigators consider that EOs are more advantageous in regenerative medicine. However, the application of EOs in regenerative medicine is still a hypothesis, as there is no specific research on it.

As discussed above, EOs can be used for studies of the pathophysiology of endometrial-related diseases, and researches of treatment, such as drug screening for novel therapy, drug testing for personalized medicine, as well as having the potential to play a role in the regenerative medicine of endometrial-related diseases. In addition, the combination of EOs and gene editing could further assist these functions, which has already been achieved in collaboration between CRISPR-based gene editing and organoids derived from cystic fibrosis disease [54]. Of note, all of these functions above can be realized based on the establishment of cryopreserved biobanks of healthy and diseased human EOs. The EOs development of all kinds of conditions is one of future endeavors of the investigators.

The limitations of EOs at present

Although EOs contain a lot of advantages, these also encounter some limitations at present. First, the improvement of ECM is also needed. At present, the main ECM in the EO culture is Matrigel purified from Engelbreth-Holm-Swarm mouse sarcoma [55]. Although it is effective in promoting organoid growth and self-organization, it may not completely resemble the human endometrial niche for animal-derived and ill-defined characteristics. In order to overcome these limitations, a number of synthetic and chemically defined hydrogels and extracellular matrix hydrogel derived from decellularized tissues and microstructured collagen scaffolds have been developed for 3D cell culture, as well as for organoids cultures [56–58]. In addition, collagen scaffolds and stromal cell induced-scaffolds via agarose 3D Petri Dishes have also been used in the co-culture of endometrial epithelial and stromal cells, indicating that the other types culture materials, rather than the Matrigel, are also feasible in the EO culture [37–39].

Second, the ECO efficiency was lower than other types of EOs, the same phenomenon happened in prostate, pancreas and colorectal cancers [16], and the purity of tumor organoids was also a challenge [16]. It might be possible to improve the efficiency of organoid culture by adjusting the composition of the medium, and selecting the metastatic lesions [16, 20]. Therefore, the efficiency of organoid formation and the underlying mechanism needs to be further explored.

Third, the present EO culture mainly pays attention to epithelial cells, but focuses less on other type of cells, such as stromal cells, vascular endothelial cells and immune cells, which are also critical for disease development. As described above, there have been studies that achieved the co-culture of endometrial epithelial cells and stromal cells. The EOs were cultured in new kinds of ECMs, rather than Matrigel. One of the ECMs was 3D porous collagen

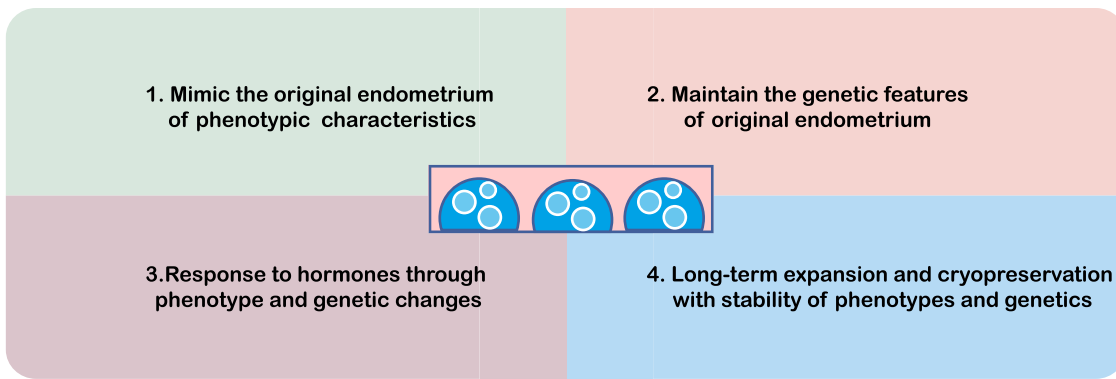


Figure 2. The advantages of EOs as an ideal model for the study of pregnant and gynecological diseases.

scaffolds, while the other ECMs were provided by stromal cells using agarose 3D Petri Dishes [38, 39]. Therefore, it can be observed that the exploration of new culture ECMs and co-culture methods promoted each other. However, these co-culture models are not mature and convincing enough, since these were not identified as to whether these could be both phenotypically and genotypically stably cultured in the long term. In addition, pregnant diseases and endometrial diseases are closely correlated to immune system abnormalities and vasculature dysfunction. For example, in terms of pregnancy, epithelium, decidualized stromal cells, resident immune cells, vasculature and placenta trophoblast communicate together to facilitate pregnancy establishment, and any kind of cell dysfunction would affect the final outcome. In order to explore the pathogenesis, the role of immune cells and vasculature must be considered. In studies on scaffold-free EOs via agarose 3D Petri Dishes, by detecting the typical surface markers, no immune cells were virtually present [38]. And there is currently no report on the role of the vasculature in EOs. Therefore, EOs should improve the protocol of co-culture of epithelial cells and stromal cells, striving to explore the technology of co-culture with immune cells, as well as endothelial cells.

Lastly, it has to be mentioned that there are still remain challenges for EO clinical applications in the future, such as how to establish and improve the EOs biobanking for drug screening efficiently, and how to develop an efficient evaluation system to judge the drug reactivity, in order to be truly effective in patients.

Discussion

The uterus is an important organ, and its main function is to conceive a fetus. In addition, the uterus is closely associated with many gynecological diseases, such as endometrial hyperplasia, endometrial cancer and endometriosis. On the base of the culture media of organoids derived from other organs, a great deal of researchers explored and summarized the culture medium suitable for EOs, which was an R-spondin1-based culture method, and slightly different in media composition between various types of endometrium. However, several key components were all contained in the culture medium (Table 2, the specific key ingredients and its concrete functions). Intriguingly, Forskolin, a cAMP activation, could slow the cell differentiation in liver organoids [13], but has a contradictory phenomenon in EOs. Turco et al. compared this to the exposure to E2 + P4, and the treatment with E2 + P4 + cAMP induced a more differentiated phenotype [19], which might be associated

with the ovarian sexual hormone responsiveness. The efficiency of EO formation varies depending on the nature of the endometrium, with the healthy endometrium in the secretory phase being highly efficient, and the successful rate of EOs derived from the EC being lower [19, 20]. For the colony forming ability, Turco et al. reported that the successful rate of EOs was inefficient (2–4%), which might be correlated to the stem cell nature of the organoid. A majority of studies have attempted to identify the markers of endometrial epithelial stem/progenitor cells, such as SSEA-1, SOX9, N-cadherin, LGR5 [59–62]. Epithelial cells with progenitor nature were considered to be located in the basalis layer of the premenopausal endometrium [63, 64], but these did not only existed in the basalis. Hapangama et al. discovered that the functionalis endometrium at the secretory phase from women with endometriosis comprised of basalis-like cells that expressed SSEA1 or SOX9, and the percentages of which were higher than that of the non-endometriosis group [62]. Therefore, in future studies, the investigators would figure out the proportion of stem cells in the functionalis and basalis layer, and determine how these vary in vivo at different stages in the menstrual cycle, which are beneficial for the original EO culture.

In fact, the influence of endometrial epithelial cells on the colony-forming of organoids is complicated, Turco et al. established the human EOs from SOX9 negative cells [19]. The same situation occurred in other organoids. Sato T et al. sorted Lgr5^{hi} cells, Lgr5^{low} cells and Lgr5^{ve} cells from mouse intestinal adenoma organoids, and found that although Lgr5^{hi} cells has the highest organoid-forming efficiency, Lgr5^{low} cells and Lgr5^{ve} cells also formed organoids with considerable efficiency [14]. Slightly different, Huch et al. reported that in mouse pancreas organoids culture, the clone forming ability of Lgr5⁺ cells was significantly higher than that of Lgr5⁻ cells, which were 16 and 1.6%, respectively [15]. Notably, the organoids derived from Lgr5⁻ cells could result in Lgr5⁺ mouse organoids, liver, or pancreas [14, 15]. Therefore, the ability of stem cells to establish organoids may be relatively stronger, but cells that do not temporally possess a stem cell nature can also form organoids with a relatively lower efficiency. More detailed mechanisms need further exploration.

As discussed above, as an ideal model for the study of pregnant and gynecological diseases, EOs have the following advantages (Figure 2): (1) mimic the endometrium of phenotypic characteristics, such as the expression of several classical epithelial markers (E-cadherin, PanCK, Muc1, and EPCAM) and ER α , PR, the formation of mucus, microvilli, cilia, and glandular polarization, which was supported by the presence of laminin at the basolateral side, and

of microvilli and cilia directed towards the lumen; (2) maintain the characteristics of the original endometrium in term of genetics, for instance, both healthy EOs and disease-related EOs reveal disease-associated traits at the transcriptomic and genetic level, and some mutations found in original tissues also presented with the corresponding organoids; (3) the response to hormones through phenotype and genetic changes, not only E2, P4 but also cAMP, hCG, hPL, and PRL, can influence the cell types, morphology, genetics, and cell product; (4) long-term expansion, cryopreservation, and thawing can maintain the stability of phenotypes and genetics, which solves the problem of insufficient original specimens and offers the possibility for long-term stable use (such as some prolonged drugs screening experiments). Taken together, EOs are expected to be an ideal pregnant and gynecological disease model in the future.

Supplementary data

Supplementary data is available at *BIOLRE* online.

Conflict of interest

The author reports no conflict of interest in this work.

Author contributions

Jin-Hua Leng, Shuang-Zheng Jia, Song Liu and Zhi-Yue Gu were responsible for studying conception and design. Zhi-Yue Gu, Shuang-Zheng Jia and Song Liu drafted the manuscript.

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