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Matrix scaffolds for endometrium-derived organoid models

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The uterus-lining endometrium is essential to mammalian reproduction, receiving and accommodating the embryo for proper development. Despite its key role, mechanisms underlying endometrial biology (menstrual cycling, embryo interaction) and disease are not well understood. Its hidden location in the womb, and thereby-associated lack of suitable research models, contribute to this knowledge gap. Recently, 3D organoid models have been developed from both healthy and diseased endometrium. These organoids closely recapitulate the tissue's epithelium phenotype and (patho)biology, including *in vitro* reproduction of the menstrual cycle. Typically, organoids are grown in a scaffold made of surrogate tissue extracellular matrix (ECM), with mouse tumor basement membrane extracts being the most commonly used. However, important limitations apply including their lack of standardization and xeno-derivation which strongly hinder clinical translation. Therefore, researchers are actively seeking better alternatives including fully defined matrices for faithful and efficient growth of organoids. Here, we summarize the state-of-the-art regarding matrix scaffolds to grow endometrium-derived organoids as well as more advanced organoid-based 3D models. We discuss remaining shortcomings and challenges to advance endometrial organoids toward defined and standardized tools for applications in basic research and translational/clinical fields.

KEYWORDS

endometrium, organoid, matrix scaffold, extracellular matrix, hydrogel

Introduction

The endometrium represents the dynamic tissue that lines the uterus which is essential for human reproduction, undergoing a menstrual cycle during which an embryo-receptive state is established. The underlying cellular and molecular mechanisms of the reiterative proliferative, secretory and menstrual phases are not well understood, with inaccessibility of these processes *in vivo* impeding in-depth investigation. Moreover, several disorders of

the endometrium (such as endometriosis and cancer) cause a significant health burden as well as highly impact fertility, and underlying pathogenic mechanisms remain unclear. Multiple study models have been developed to explore endometrium (patho) biology [extensively reviewed in (1–3)], but show important limitations such as failure to reliably maintain primary cells in long-term culture or their non-physiological nature (e.g. 2D, mouse models, immortalized cell lines). Thus, this research field has long been thwarted by lack of relevant (disease) models.

Recently, powerful new research models have been established in the form of organoids, shown to be highly instrumental and accurate to study endometrium biology and disease (4–6); and in detail reviewed in (2, 7, 8). Organoids are 3D cell constructions that *in vitro* self-develop from (diseased) tissue (stem) cells when embedded in a supporting ECM (hydrogel) scaffold, and cultured in an optimized, well-defined medium, specifically encompassing stem cell- and embryogenesis-regulatory factors (9) (Figure 1). In this review, we will delve into the (dis)advantages of different hydrogel scaffolds utilized to grow endometrial organoids. Hydrogels are polymer-based insoluble networks with a high H₂O composition and are divided by, among others, natural or synthetic origin, crosslinking method (physical or covalent bonds) and architecture (fibrous, macro- or nanoporous). Typically, a basement membrane (BM) extract derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma is used as a substitute for the tissue ECM to grow organoids (10). Commercially available variants include Matrigel, GelTrex and Cultrex BM extract. Matrigel has been the main ECM surrogate used for endometrium organoid culture (4, 5). Despite high level of success, Matrigel has important limitations (summarized in Table 1 and discussed in detail below), and better ECM-mimicking matrices are actively sought that support endometrial organoid culture as efficiently and reliably. Here, we first describe the characteristics of natural endometrium ECM as

ultimate but very challenging benchmark due to its very dynamic ECM composition (throughout the menstrual cycle). Then, we summarize the use of natural hydrogels for endometrium-derived organoid growth and discuss recent attempts to develop more defined matrix scaffolds. Finally, we present challenges and perspectives prevalent in the booming (endometrial) organoid field.

Native endometrium ECM as inspiration for defined organoid matrix development

ECM is the non-cellular network that connects the cellular components of a tissue, thereby establishing biochemical and biomechanical cues that determine key cellular processes such as differentiation, proliferation, homeostasis and migration (18, 19). The desired ECM surrogate for growing endometrium-derived organoids has to mimic the native endometrial ECM as closely as possible, both regarding composition and biomechanical properties.

The endometrial ECM mainly contains collagen IV, fibrillin, laminin and fibronectin (20) (Table 1). Importantly, ECM composition is dynamic throughout menstrual cycle and pregnancy (21, 22). For instance, fibronectin becomes more abundant in the proliferative as compared to the secretory phase 1, while laminin, collagen and fibrillin deposition increase in the secretory phase to promote embryo attachment and invasion (23) (Figure 1).

Changes in endometrial ECM composition have a direct impact on mechanical properties such as tissue stiffness (24). For example, the nonpregnant endometrium is less stiff than the endometrium of pregnant women (apparent elastic modulus $E = \sim 250$ Pa and ~ 1250 Pa, respectively) (24). Moreover, ECM composition and

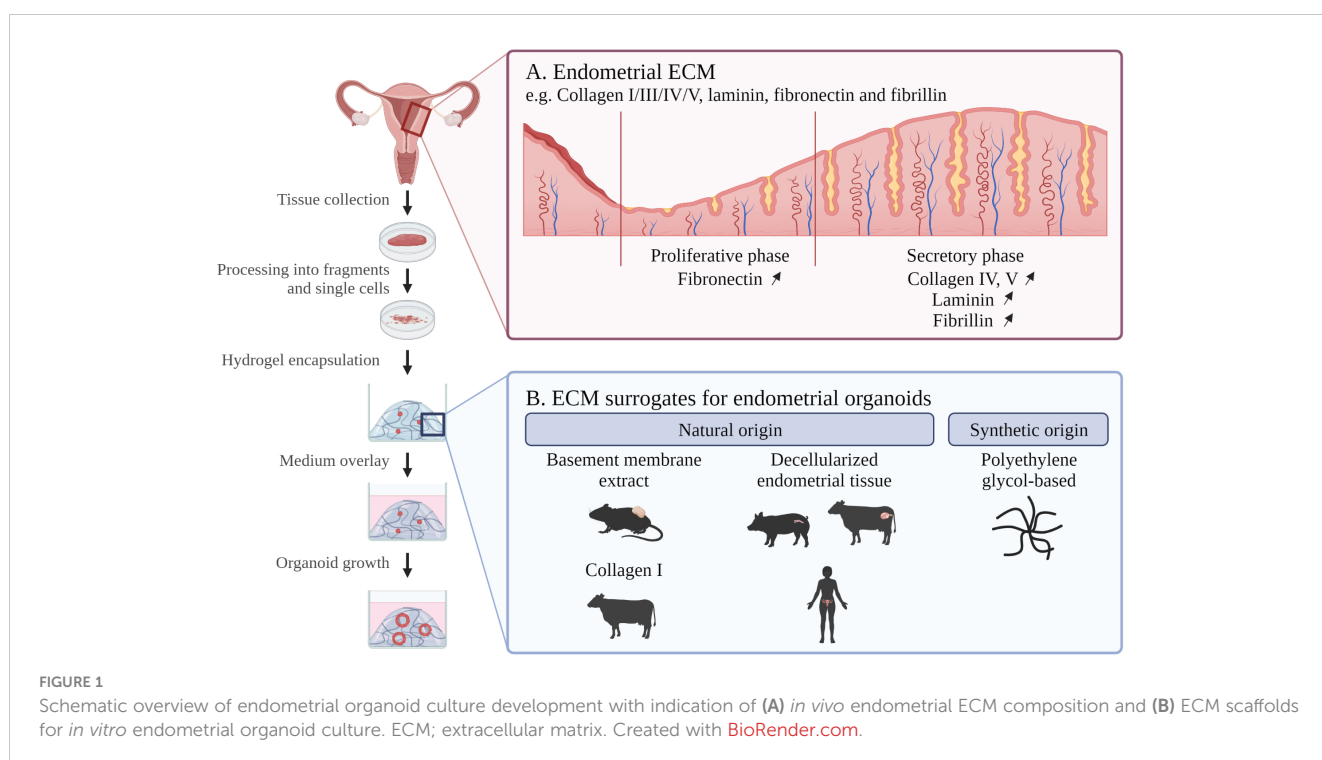


TABLE 1 Overview of the most successful ECM surrogates for endometrial organoid culture.

| | Hydrogel or scaffold material | Origin | Main components | Crosslinking method | Advantages | Disadvantages | Main references |
|-----------|---|-------------------------------------|---|--|--|--|-----------------|
| Natural | Basement membrane extract (e.g. Matrigel) | Engelbreth-Holm-Swarm mouse sarcoma | <ul style="list-style-type: none"> • Laminin (~60%) • Collagen IV (~30%) • Entactin (~8%) • Perlecan (~2%) • > 2000 other proteins | Physical and covalent crosslinking (temperature) | <ul style="list-style-type: none"> • Commercially available • Well-established protocols • Gentle organoid recovery method (temperature) | <ul style="list-style-type: none"> • Animal- and tumor-derived • Properties cannot be tailored • Batch-to-batch variability • Composition differs from <i>in vivo</i> ECM • Undefined ECM composition | (4, 5) |
| | Endometrial decellularized ECM | Porcine, bovine, human | <ul style="list-style-type: none"> • Collagen I and III • Elastin • Laminin • Fibronectin • > 1000 other proteins | Covalent crosslinking (ECM fiber self-assembly upon pH neutralization at 37°C) | <ul style="list-style-type: none"> • Retains biochemical structures of the native ECM • Patient-specific ECM for personalized research • FDA-approved | <ul style="list-style-type: none"> • Animal-derived (unless human patient-derived) • Batch-to-batch variability • Restricted availability • Undefined ECM composition | (11–13) |
| | Collagen | Bovine skin | <ul style="list-style-type: none"> • Collagen I (97%) and III (3%) | Physical and covalent crosslinking (pH and temperature) | <ul style="list-style-type: none"> • Commercially available • Composed of the most abundant ECM proteins seen in <i>in vivo</i> endometrium | <ul style="list-style-type: none"> • Animal-derived • Properties cannot be tailored • Batch-to-batch variability • Enzymatic (collagenase) recovery method | (14, 15) |
| Synthetic | Polyethylene glycol (PEG) | PEG | <ul style="list-style-type: none"> • PEG network • Protease-sensitive crosslinkers (sortase, MMP) • ECM-mimicking peptides (collagen and fibronectin) • Peptides binding cell-secreted basement membrane proteins and fibronectin | Covalent crosslinking (protease-degradable crosslinking peptides) | <ul style="list-style-type: none"> • Independent tuning of biochemical and biomechanical properties • Defined composition | <ul style="list-style-type: none"> • Enzymatic recovery method • Need for functionalization with cell-binding peptides • Purely elastic mechanical properties | (16, 17) |

structure give rise to complex nonlinear mechanics (19, 25). Among others, endometrial ECM is viscoelastic, meaning that it behaves both as viscous fluid and elastic solid. Viscoelasticity relies on bond remodeling (i.e. breaking and re-forming) between ECM proteins, mainly fibrin and collagen I (19, 26). This continuous remodeling is governed by secreted enzymes such as matrix metalloproteinases (MMPs) (27, 28), and, together with changes in biochemical composition, governs mechanical properties of the ECM. Resultant mechanosensing by the cells through their integrin receptors (e.g. for collagens and fibronectin) leads to activation of intracellular signaling pathways that ultimately influence biological processes such as proliferation and differentiation (19, 25, 29).

Finally, changes in ECM properties have been correlated with endometrial diseases. For example, the ECM remodeling MMPs have been identified as key factors in endometrial cancer [e.g. MMP11 is a

type I endometrial cancer marker (30)] as well as in formation and invasion of endometriotic lesions outside the uterus [e.g. high levels of MMP3 (31)]. To understand the relationship between ECM alterations and endometrial (patho-)biology, organoids may provide an excellent tool as the properties of the embedding ECM surrogate can be altered according to the research question.

Natural hydrogels as supporting scaffold for endometrium-derived organoids

As mentioned, the most used natural hydrogels for organoid culturing, like Matrigel, are BM extracts from EHS mouse sarcoma.

Matrigel consists of ~60% laminin (multiple isoforms), ~30% collagen IV, ~8% entactin (glycoprotein molecule bridging laminin and collagen IV), ~2% perlecan (heparin sulfate proteoglycan), and in addition up to 2000 other proteins (32) (Table 1). These hydrogels exhibit temperature-dependent gelation, remaining liquid when kept at low temperature (~5°C) and transitioning into a gel-like form above a defined temperature (e.g. 22–37°C for Matrigel). This property enables convenient encapsulation of the cells as well as their retrieval (Table 1). Typically, a purification (growth factor-reduced) format is used for organoid culturing to minimize any undesired effects of growth factors present in the BM extract, while factors specifically required for organoid growth are deliberately supplemented to the culture medium.

The first establishment of endometrium-derived organoids occurred in Matrigel (4, 5), further also used by the majority of subsequent endometrial organoid studies (Table 1). Indeed, Matrigel was found highly suitable for supporting the development and growth of endometrial organoids across species [i.e. human (4, 5); mouse (4); horse (33)], as well as throughout all menstrual phases in humans (4, 5, 34). Organoids could also recapitulate the different menstrual phases when exposed to the appropriate sex hormones (e.g. mucin production after progesterone treatment) (4, 5). Moreover, Matrigel has been used for both healthy (4, 5) and diseased endometrium, including endometriosis (6, 35, 36), cancer (5, 6) and Mayer-Rokitansky-Küster-Hauser syndrome (37). Importantly, organoid cultures derived from diseased endometrium reproduced key characteristics of the disorder such as mutational landscape (6), morphology (e.g. disorganized epithelium) (5) and gene expression (37). These organoid cultures have been instrumental to investigate endometrial hormonal responses (4, 5, 38), infections [*Chlamydia* (39)], drug screening (6), implantation (14, 40), pathological conditions (6, 37), and regenerative processes (41, 42).

Although highly successful, Matrigel, and generally BM extracts, have important limitations for organoid culturing (43) (Table 1). Firstly, their composition is not deeply defined and batch-to-batch variability may result in inconsistent experimental outcomes, although the reproducibility of the endometrial organoid protocol from the initial reports (4, 5) to follow-up studies (6, 14, 35, 38) does not point to major issues in this regard. Nevertheless, better standardization can be achieved by specifying protein concentrations in the BM extracts rather than just the proportion of Matrigel used in the culture (e.g. '8 mg/mL protein concentration in Matrigel' instead of '70%' or '1:20 in culture medium'), as protein concentrations can vary between batches and impact gel properties. Secondly, BM extracts do not highly mimic the original tissue's ECM composition, biochemistry and biomechanics. For instance, Matrigel contains a higher concentration of laminin compared to endometrial ECM, which is rich in collagen fibers (types I and III) and other glycoproteins such as fibronectin (10, 11, 44) (Table 1). It is worth noting that endometrial organoids can also produce their own ECM components, with laminin being observed at the outer edge of the organoid structures (6). Thirdly, Matrigel is a very soft viscoelastic hydrogel (E ranging between 70–330 Pa depending on batch and concentration) when

compared to endometrium ECM [250–1250 Pa (24)]. Importantly, it is not possible to tune the biochemical cues of Matrigel nor to increase its stiffness to match the *in vivo* situation of (diseased) endometrium. Lastly, the xeno- and tumor-origin of the BM extracts restricts clinical applicability of organoids cultured in these conditions, particularly for applications in regenerative medicine and potentially also drug screening and development (9, 43).

In addition to using these compound BM extracts, endometrial organoids have also been cultured in more pure ECM components (Table 1). For example, bovine skin collagen-based hydrogel was applied to co-culture epithelial endometrial cells (as organoids) with the stromal cell component of the tissue (14). These so-called 'assembloids' showed gland-like organoids surrounded by stromal cells, and allowed to study epithelial-stromal cell remodeling during embryo interaction (14). Collagen hydrogels, composed of fibrillar networks of collagen I (97%) and III (3%), contain the major collagen types present (and abundant) in natural endometrial ECM (11, 44, 45) (Table 1), and present a stiffness (shear modulus $G = \sim 700$ Pa) similar to that of native endometrium (14, 24, 46). Despite this fruitful application of collagen gels for endometrial assembloid culture, efficient development and expansion of pure (epithelial) organoids, as needed for extensive downstream analysis, was not reported. Another study developed collagen scaffolds through lyophilization, thereby optimizing pore size to allow efficient exchange of gas and nutrients while still providing sufficient structure (15). Primary endometrial stromal cells were seeded within this collagen scaffold and epithelial cells (derived from organoids but seeded as a 2D monolayer) were grown on top of it (15). The stromal cells were also able to produce collagen themselves (15). Both studies using collagen matrices confirmed the biological functionality of the co-cultures, as supported by hormonal responsiveness (14, 15). Although these collagen hydrogels do not have a tumor origin, they are still animal-derived and suffer from batch-to-batch variability (47). In addition, these hydrogels are not highly tunable regarding biochemical and biomechanical properties, which is needed to match the native tissue.

As the closest *in vivo*-mimicking natural hydrogel for growing endometrium-derived organoids, decellularized ECM (dECM) has been tested (Table 1). In one approach, soluble dECM, derived from porcine endometrium, was added to the organoid culture medium with the human endometrial cells (still) embedded in Matrigel, resulting in increased initial proliferation of the cells (12). The medium-dissolved dECM thus provided important biochemical cues. For instance, nicotinamide, previously shown to be essential for endometrial organoid growth (4, 5), was found dispensable in the presence of soluble dECM. In a second approach, cells were embedded in the endometrial dECM-derived hydrogel, obtained from bovine or human origin (11). Decellularization using sodium deoxycholate (SDC) resulted in better preservation of the native ECM structure and composition (such as abundant presence of collagens) than dECM obtained following sodium dodecyl sulfate (SDS) treatment. Moreover, stiffness of SDC-generated dECM hydrogel (storage modulus $G' = 380$ Pa) more closely resembled the stiffness of the intact (i.e. before

lyophilization and powder milling) decellularized tissue ($G' = 600$ Pa) than the hydrogel obtained using SDS treatment ($G' = 70$ Pa) or Matrigel ($G' = 75$ Pa). The optimized dECM hydrogel, from both bovine and human origin, supported human and mouse endometrium-derived organoid growth comparably to Matrigel. Proteomic analysis put forward that (human) endometrial organoids cultured in SDC-treated dECM hydrogel resembled the patient endometrial tissue better than organoids grown in Matrigel. Moreover, laminin was found to be important for endometrium organoid culturing (as shown for mouse), because addition of bovine-derived laminin to laminin-low (bovine) dECM hydrogel (as obtained using SDS) rescued organoid formation efficiency. Laminin addition also led to a change in morphology from crenelated to rounder structures, more similar to those found in SDC-obtained dECM hydrogel and Matrigel. Taken together, dECM-based matrices, mimicking the *in vivo* ECM better than other natural hydrogels, present a promising tool to enable organoid clinical application. In an alternative approach, organoids were dissociated to single cells which were seeded on top of a human dECM scaffold (13). The organoid-derived cells recellularized both luminal and glandular scaffold surfaces which may provide interesting perspectives for regenerative medicine (13).

Interestingly, dECM-based products have been approved by the US Food and Drug Administration (FDA) (48) which facilitates their clinical translation. However, use of human endometrium-derived dECM will not be straightforward given the limited availability (e.g. sufficient sample material is required if both dECM and organoids need to be derived from the same patient) and the uncertain reproducibility with inter-individual differences as well as intra-patient differences (e.g. along the menstrual cycle). Moreover, obtaining dECM at sufficient purification level may be expensive. Altogether, dECM is not a viable alternative yet for large-scale and long-term growth and expansion of endometrial organoids which is needed for the extensive downstream applications.

Other natural hydrogels have already been applied to develop organoids from several tissues but their application in endometrial organoid culturing remains unexplored. Examples include purified or recombinant ECM components such as fibrin and laminin (49) or hydrogels based on alginate, an FDA-approved polysaccharide derived from brown seaweed, with interesting tunable (visco-) elastic properties (50, 51).

Synthetic hydrogels as promising scaffolds for endometrium-derived organoid culture

To overcome the above-mentioned shortcomings of natural hydrogels to grow organoids in an efficient, reproducible, xeno-free as well as standardized manner, fully defined synthetic matrix scaffolds are needed and currently actively searched for (52, 53). Precise control of hydrogel composition and independent tuning of mechanical and biochemical properties would be an important

advantage over natural matrices. While several synthetic hydrogels have been developed for various tissue organoid models, only one has so far been reported to successfully support the culture of endometrial organoids (16¹; 17) (Table 1). The researchers designed a polyethylene glycol (PEG)-based hydrogel that incorporates MMP-sensitive crosslinkers allowing organoid-secreted MMPs to degrade the rigid PEG structure to promote organoid growth. Cleavages sites for the prokaryotic enzyme sortase were also introduced in the PEG gel to enable retrieval of the embedded grown organoids. Moreover, biochemical cues were introduced by adding a synthetic $\alpha 2\beta 1$ or $\alpha 5\beta 1$ integrin-binding peptide based on collagen (GFOGER), which was found critical for successful culture of the endometrial organoids, although they remained smaller compared to those grown in Matrigel (17). Organoids embedded in this synthetic matrix were hormone-responsive with both motile ciliated and secretory epithelial cells present (16). Organoid morphology was dependent on matrix stiffness; stiffer scaffolds ($E = \sim 2,000$ Pa and $6,000$ Pa) led to crenelated organoids whereas softer matrices ($E = \sim 300$ Pa) yielded rounded organoids similar to Matrigel-grown organoids¹. Addition of a short fibronectin-based synthetic peptide (PHSRN-K-RGD) supported the survival of endometrial stromal cells in a composite epithelial organoid-stromal cell culture (16). Moreover, it improved stromal cell distribution and morphological spreading as compared to PEG-GFOGER alone. This fully defined hydrogel system allowed to co-culture primary endometrial epithelial and stromal cells for two weeks, while natural hydrogels degraded within this timeframe. Such more stable hydrogel would enable to simulate the menstrual cycle *in vitro* for a longer and thus more physiological relevant time period to explore biological and pathological cell interactions and processes (16). As a downside, PEG-based hydrogels consist of flexible networks with only elastic mechanical properties. Viscoelastic semiflexible materials, such as natural hydrogels and primary tissue, exhibit additional important mechanical properties (e.g. stress-stiffening behavior) which can influence epithelial cell growth and patterning (50, 54). The purely elastic PEG-based hydrogels may therefore affect organoids' cellular and tissue phenotype mimicry differently than natural materials.

The list of synthetic hydrogels, displaying divergent biomechanical properties, is rapidly expanding, thereby providing interesting opportunities toward re-tuned endometrial organoid culture. For example, a light-sensitive synthetic polyvinyl-based hydrogel can guide cell invasion at microscale by *in situ* controlled photopolymerization of the cell-laden hydrogel (55). Such hydrogel could in the future be used to recapitulate ECM remodeling as occurring in both physiological (e.g. menstrual cycle) and pathological conditions (e.g. endometrial cancer and endometriotic lesions) (23, 30, 31). Still other promising hydrogels have been developed, formed by non-covalent interactions such as

¹ Not yet published following completed peer review and thus still to be interpreted with caution.

hydrogen bonds and hydrophobic interactions. Examples are the thermosensitive poly(N-isopropylacrylamide)- (56) and polyisocyanide-based matrices (57). These thermoreversible hydrogels offer the practical advantage over covalently crosslinked hydrogels of allowing gentle cell recovery by simply cooling the sample on ice, similar to the process used with Matrigel which also does not require enzymatic digestion. Hence, these materials will be more user-friendly and facilitate the transition away from Matrigel.

Challenges and future perspectives

Endometrium-derived organoid models provide powerful *in vitro* tools to decipher endometrial biology and disease. To date, 3D organoids are mainly grown in BM extract (typically Matrigel), a one-size-fits-all ECM surrogate which enables efficient organoid development and growth from various tissues, in both healthy and diseased conditions. However, this BM extract poses several challenges, particularly its poor definition, animal and tumor origin. Standardization and clinical translation urge for a better (fully) defined matrix which can support the efficient growth of organoids while reliably recapitulating the characteristics of the original tissue epithelium. Along this line, biochemical and biomechanical properties of the native tissue should be recapitulated as closely as possible. Although more defined ECM component matrices (such as collagen hydrogels) or endometrial dECM-based gels may be a step forward, they still suffer from batch-to-batch variability and mostly non-human origin. Thus, developing fully synthetic and tunable biomimetic hydrogels is necessary to tailor the matrix to the *in vivo* counterpart, encompassing important parameters such as biocompatibility, stiffness, viscoelasticity and porosity. The PEG-based hydrogel discussed above provides a first step but still lacks important biomechanical properties and the fiber architecture of the *in vivo* tissue ECM. New synthetic hydrogels, fully defined and tunable, are needed to advance standardized (while still efficient) organoid culturing from endometrial epithelium. Such better-defined hydrogels will also enable to investigate the effect of individually added ECM components on organoid formation, growth and phenotype, and on how ECM alterations influence endometrial cell behavior (e.g. pathology, response to hormones). A further challenge includes the simulation of the dynamic endometrial ECM composition and remodeling as occurring throughout the menstrual cycle and in pathological conditions. Hydrogels that provide spatial manipulation during organoid growth can offer a solution. Another challenge will be to identify defined matrices for further advanced mimicry of the endometrium, i.e. that support the reliable growth and culture of not only the epithelial cells but also other components of the endometrial tissue such as stromal, endothelial and immune cells. The co-culture of epithelial and stromal cells in assembloids, as achieved in collagen and PEG-based matrices, provides a first step toward this ambitious goal. Also immune cells and associated inflammatory processes play an important role in the endometrial processes of menstruation (58) and implantation (59), as well as in pathologies (e.g. endometriosis) (59). Including immune cells into organoid cultures would be another important advancement in endometrial 3D modeling. To study

inflammatory processes, immunomodulatory factors (cytokines) are often supplemented to the organoid medium to mimic the presence of immune cells. One study reported the co-culture of endometrial organoids and primary bone marrow-derived neutrophils to study the primary immune response after *Chlamydia* infection (60). *Chlamydia*-secreted effectors restricted neutrophil recruitment to infected organoids (60). Co-culture with other immune cell types present in the endometrium, such as macrophages and uterine natural killer cells, is needed for further enhancing the physiological relevance of the models and enables additional research in innate and adaptive immune responses (58). Interesting evolutions within the tissue engineering field can also be applied to better reconstitute the complex make-up and architecture of the endometrium, including bioprinting approaches (although still very challenging) in which appropriate hydrogels are used as bio-ink, and microfluidic technologies containing different endometrial cell types in interconnected microchambers to recapitulate cell-cell and cell-matrix interplay (1). As an asset, different types of hydrogels, each best fitting a specific cell type, can be used in the individual chambers, thereby avoiding the need for a single hydrogel that fits all cell types.

In conclusion, the identification and application of more defined hydrogels will allow to advance endometrial organoid modeling toward more reproducible and standardized applications including genetic and drug screenings. The current development of a range of defined hydrogels can provide researchers to choose the most fitting matrix to answer their research question into endometrium (patho)biology.

Author contributions

SDV and HV designed the review. SDV and CMC collected all the information and wrote the manuscript. HV and SR co-wrote and critically revised the manuscript, and HV finalized it. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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