

Establishment of mouse endometrial assembloids with luminal epithelial-like structures *in vitro*

Xiao-Yan Qiu^{1, #}, Jing-Yi Tu^{1,2,3, #}, Jie Yang^{2,3, #}, Lang Li¹, Da Wang^{2,3}, Man-Qing Long^{2,3}, Ji-Wen Tian^{2,3}, Yong-Ju Zhao^{1, *}, Tian-Qing Li^{2,3, *}

¹ College of Animal Science and Technology, Southwest University; Chongqing Key Laboratory of Herbivore Science, Chongqing 400715, China

² State Key Laboratory of Primate Biomedical Research Institute of Primate Translational Medicine, Kunming University of Science and Technology, Kunming, Yunnan 650032, China

³ Yunnan Key Laboratory of Primate Biomedical Research, Kunming, Yunnan 650500, China

ABSTRACT

Embryo implantation requires coordinated interactions between the embryo and the maternal endometrium. Endometrial organoids (EOs) have emerged as promising *in vitro* models for studying this process. At present, however, mouse-derived EOs (mEOs) are predominantly composed of a single epithelial cell type and lack a functional luminal epithelium (LE), cellular diversity, and hormonally responsive architecture, limiting their capacity to recapitulate the dynamic receptive phase of the endometrium. In this study, a robust three-dimensional mouse endometrial assembloid model was established by co-culturing endometrial gland-like organoids (GLOs) with primary endometrial stromal cells (ESCs) under air-liquid interface (ALI) conditions. Optimization of the GLO culture was achieved by fine-tuning the concentrations of Wnt3a and R-Spondin1, while stromal viability and functionality were enhanced by supplementation with hydrocortisone, L-ascorbic acid, and ITS-X. Comparative analysis demonstrated that ALI conditions significantly enhanced epithelial proliferation, gland morphogenesis, and metabolic activity across both epithelial and stromal compartments. Notably, the resulting ALI-grown mouse endometrial assembloids (ALI-mEnAOs) developed a well-defined LE layer and recapitulated molecular and cellular features characteristic of both pre-receptive and receptive endometrial states *in vivo*. Compared to existing mEOs, analyses confirmed that ALI-mEnAOs closely mimic *in vivo* endometrial architecture, lineage composition, phase-specific gene expression signatures, and dynamic hormonal response. This model offers a physiologically

relevant platform for mechanistic investigation of endometrial function and embryo implantation dynamics.

Keywords: Mouse endometrial assembloids; Luminal epithelium structure; Air-liquid interface; Endometrial receptivity

INTRODUCTION

The endometrium is a dynamic and highly specialized tissue composed of multiple cell populations, including luminal epithelium (LE), glandular epithelium (GE), endometrial stromal cells (ESCs), smooth muscle cells, endothelial cells, and immune cells. Coordinated interactions among these constituents are essential for establishing uterine receptivity and enabling embryo implantation. LE and GE both originate from a common lineage of endometrial epithelial cells (EECs) (Yang et al., 2021). Collectively, these cellular components establish the structural and signaling landscape necessary to support embryo implantation and early developmental progression (Bauersachs & Wolf, 2015; Siriwardena & Boroviak, 2022). Impaired implantation represents a critical barrier to reproductive success, contributing to early pregnancy failure in humans and reduced fertility in domestic animals (Ababneh & Troedsson, 2013; Muter et al., 2023; Sandra et al., 2017; Waclawik et al., 2017). Successful implantation occurs only when the endometrium transitions into a receptive phase, characterized by a highly orchestrated sequence of molecular and morphological changes (Sandra, 2016). Current investigations into implantation mechanisms predominantly rely on time-point sampling of rodent endometrial tissue, which limits the ability to observe the dynamic changes occurring in both the embryo and maternal

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Copyright ©2026 Editorial Office of Zoological Research, Kunming Institute of Zoology, Chinese Academy of Sciences

Received: 29 August 2025; Accepted: 15 September 2025; Online: 16 September 2025

Foundation items: This work was supported by the National Key R&D Program of China (2022YFA1103100), Key Project of Guizhou Provincial Science and Technology Plan (Qiankehe Support(2022)Key027), and National Natural Science Foundation of China (31772564, 32372834, 32130034, 32072778)

[#]Authors contributed equally to this work

^{*}Corresponding authors, E-mail: zyjswu168@163.com; litq@ipbr.cn

environment during early implantation (Jiang et al., 2022; Yang et al., 2021). In human studies, ethical and logistical challenges further restrict access to peri-implantation tissues, while *in vivo* approaches in livestock remain cost-intensive and technically complex. These constraints highlight the need for physiologically relevant *in vitro* models that recapitulate endometrial architecture and function during the implantation window. Such models would enable direct visualization of intercellular interactions during early implantation stages and facilitate mechanistic analyses of early implantation events, providing a powerful platform for advancing reproductive biology and improving fertility outcomes.

The endometrium plays a central role in mediating embryo implantation and is closely associated with various reproductive pathologies. Disruptions in its cyclical processes, including shedding, regeneration, and structural remodeling, are implicated in the pathogenesis of conditions, such as thin endometrium, implantation failure, pregnancy-related complications, and endometriosis, each exerting a profound impact on mammalian reproductive health (Roy & Matzuk, 2011). Although advances have been made in elucidating certain pathological pathways, the molecular mechanisms underlying many endometrial disorders remain insufficiently defined. Progress in this area depends on the development of appropriate *in vivo* and *in vitro* models that accurately recapitulate both physiological and pathological features of endometrial function. Rodents remain widely employed for *in vivo* studies of endometrial biology and function, primarily through the establishment of disease models and tissue collection for mechanistic research (Lin et al., 2023; Mao et al., 2024; Wu et al., 2023). However, these models require technically demanding procedures and raise concerns regarding translational relevance. Furthermore, existing *in vitro* systems often fail to accurately capture the cellular complexity, structural organization, and dynamic signaling environment of the native endometrium, thereby limiting their utility for modeling disease processes. These limitations underscore the critical need for more suitable *in vitro* platforms capable of faithfully representing endometrial architecture, function, and pathology, thereby enabling a deeper investigation into the cellular and molecular basis of reproductive disease.

Three-dimensional (3D) cultured organoids have emerged as advanced *in vitro* platforms that more accurately reproduce native tissue architecture, cellular heterogeneity, and physiological responses. These systems closely replicate interactions between cells and their surrounding extracellular matrix (ECM), offering a microenvironment that captures critical aspects of *in vivo* tissue dynamics (Lancaster & Knoblich, 2014; Rossi et al., 2018; Xinari et al., 2015). Organoids derived from mouse and human endometrial epithelial cells have been successfully established and exhibit hormonal responsiveness to estrogen and progesterone (Boretto et al., 2017), validating their relevance for reproductive studies. Building on this foundation, an increasing number of co-culture models integrating organoids with ESCs have been developed to investigate key reproductive processes such as embryo implantation and decidualization (Gnecco et al., 2023; Rawlings et al., 2021; Wiwatpanit et al., 2020). Despite these advances, current endometrial assembloid models are largely limited to GE and ESCs, with the absence of LE remaining a critical constraint. To overcome this limitation, Tian et al. (2023) engineered a

human endometrial assembloid system incorporating LE, GE, and ESCs through optimized ECM composition and adoption of air-liquid interface (ALI) culture methods (Pruniéras et al., 1983; Whitcutt et al., 1988). These assembloids effectively recapitulated the cellular complexity of the *in vivo* endometrium and responded to hormonal induction, reflecting changes associated with the menstrual cycle (Tian et al., 2023). Although these organoids represent a significant advancement, the underlying spatiotemporal mechanisms that coordinate stromal-epithelial crosstalk during endometrial remodeling and implantation remain to be elucidated.

Efforts to investigate endometrial function using human-derived assembloids have been constrained by limited access to viable human embryos and fundamental discrepancies between embryo surrogates—such as trophoblast organoids or blastoids—and authentic embryonic structures, which compromise physiological relevance. In domestic animals, the high costs and technical demands of assembloid construction and embryo co-culture further restrict experimental feasibility. These challenges highlight the necessity of developing tractable and scalable alternatives for mechanistic studies. Given their widespread use in reproductive research, mice offer distinct experimental advantages, including well-characterized reproductive physiology, efficient breeding, ease of sample collection, and reproducible outcomes across experimental replicates. By integrating endometrial gland-like organoids (GLOs) with primary endometrial stromal cells (ESCs), a three-dimensional mouse endometrial assembloid (mEnAO) system was established that recapitulates the architectural and functional properties of native tissue. This model enables high-resolution interrogation of endometrial physiology, supports the study of endometrium-associated disorders, and provides a powerful platform for exploring regeneration and implantation processes under defined experimental conditions.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (6–8 weeks old) were purchased from the Animal Experiment Center of Yunnan University and were housed under a 12 h light/dark cycle (lights on from 0900h to 2100h) with unrestricted access to standard chow and water. Euthanasia was performed by cervical dislocation, and uteri were immediately collected and preserved in phosphate-buffered saline (PBS) for subsequent cell isolation at the Kunming University of Science and Technology. All procedures were approved by the Laboratory Animal Welfare Ethics Committee of Yunnan University (Approval No. YNU20241096).

To obtain uteri for phase-specific analysis, females were mated with fertile males, with mating success confirmed by the presence of a vaginal plug the following morning, designated as gestational day 1 (GD1). Uterine tissues were harvested at 0900h on GD3 (pre-receptive phase) and GD4 (receptive phase) (He et al., 2019; Yang et al., 2021). One uterine horn was used for frozen sectioning and immunostaining, while the contralateral horn was reserved for RNA sequencing (RNA-seq).

Primary cell culture from mouse endometrium

Female C57BL/6 mice (6–8 weeks old) were used for endometrial cell isolation. Uteri were dissected and chopped

into 4–5 mm fragments using surgical scissors, which were collected into a 50 mL centrifuge tube containing 10 mL of separation medium 1 (RPMI 1640 medium (Thermo Fisher Scientific, USA) supplemented with 0.25% Trypsin (Biological Industries, Israel), 0.05% DNAase I (Roche, Switzerland), and 1% penicillin-streptomycin (BasalMedia, China). Tissues were incubated at 37°C for 1–2 h, then washed and resuspended in separation medium 2 (RPMI 1640 medium containing 2 U/mL Dispase II (Sigma-Aldrich, USA), 2 mg/mL collagenase V (Sigma-Aldrich, USA), 10% fetal bovine serum (FBS, ZETA LIFE, USA), 1% penicillin-streptomycin, and 0.05% DNAase I) and incubated at 37°C for 3–4 h. The suspension was filtered through a 70 µm cell strainer (Jet Bio-Filtration, China), washed repeatedly with RPMI 1640 medium, and centrifuged at 37°C at 1000 r/min for 4 min. The resulting pellet containing endometrial stromal cells was resuspended in modified ESC medium (Dulbecco's Modified Eagle Medium (DMEM), BasalMedia, China) containing 10% FBS, 1% non-essential amino acids (NEAA, Gibco, USA), 0.05% L-ascorbic acid (Sigma-Aldrich, USA), 1% ITS-X (Gibco, USA), 50 ng/mL recombinant human EGF (Oryzogen, China), 25 ng/mL hydrocortisone (Sigma-Aldrich, USA), 2 mmol/L GlutaMAX supplement (Gibco, USA), and 10 µmol/L Y-27632 (Selleck, USA). Cells were seeded onto Petri dishes precoated with 1% human collagen IV (Sigma-Aldrich, USA) and cultured at 37°C in 5% CO₂. To induce decidualization, cells were treated for 72 h with 10 nmol/L β-estradiol (E2, Sigma-Aldrich, USA), 1 µmol/L progesterone (P4, Sigma-Aldrich, USA), and 0.5 mmol/L 8-bromoadenosine 3',5'-cyclic monophosphate (cAMP, Sigma-Aldrich, USA). Expression of decidualization markers was confirmed by quantitative real-time polymerase chain reaction (qPCR).

Captured glands retained on the cell strainer were backwashed, centrifuged (37°C, 1000 r/min, 4 min), and resuspended in pre-cooled 70% Matrigel (Corning, USA) diluted in Advanced DMEM/F12 (Gibco, USA). A 25 µL aliquot of the suspension was seeded into each well of a 48-well plate and polymerized at 37°C for 30 min. Organoids were expanded in mouse expansion medium (mEXM) consisting of Advanced DMEM/F12 supplemented with 1% penicillin-streptomycin, 2% B27 (BasalMedia, China), 1% N2 (BasalMedia, China), 2 mmol/L GlutaMAX supplement, 1 mmol/L nicotinamide (Sigma-Aldrich, USA), 1.25 mmol/L N-acetyl-L-cysteine (NAC, Sigma-Aldrich, USA), 100 ng/mL recombinant human Noggin (Novoprotein, China), 100 ng/mL recombinant human Wnt3a V3 (Novoprotein, China), 250 ng/mL recombinant human R-Spondin 1 (Novoprotein, China), 100 ng/mL recombinant human FGF-10 (Novoprotein, China), 50 ng/mL recombinant human EGF, 50 ng/mL recombinant human HGF (KX-PROTEIN, China), 500 nmol/L A83-01 (Selleck, USA), and 10 µmol/L Y-27632. Organoids were cultured at 37°C in 5% CO₂ (see Supplementary Materials). For hormonal responsiveness assays, organoids were treated with 10 nmol/L E2 for 5 days, or sequentially with 10 nmol/L E2 for 3 days followed by 1 µmol/L P4 and 0.5 mmol/L cAMP for two additional days. Organoids at passages 5–10 and ESCs at passages 1–3 were used for subsequent experiments. Endometrial cell proliferation was assessed using the BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 647 (Beyotime, China).

Establishment of mEnAOs

To optimize epithelial-to-stromal ratios, cell pellets were

resuspended in 70% Matrigel at defined ratios (1:0, 1:2, 1:4, 1:6, 1:8). Comparative assessment was then performed using three distinct ECM formulations: Matrigel alone, collagen I (bovine collagen type I, Sigma-Aldrich, USA), and a MAC mixture (Matrigel:collagen I:Advanced DMEM/F12=1:1:1(v/v)). After thorough mixing, 25 µL of each cell-matrix suspension was seeded into each well of a 48-well plate, followed by incubation at 37°C for 30 min to allow polymerization. mEXM was added, and samples were collected for evaluation after an 8-day culture period.

GLOs and ESCs were resuspended in MAC and loaded into the upper chambers of Transwell inserts (45 µL per insert; Jet Bio-Filtration, China), followed by incubation at 37°C for 30 min to achieve gelation. To eliminate residual Matrigel from isolated GLOs, organoids were mechanically dissociated using a low-adhesion pipette (Axygen, USA) and resuspended in 100 µL of mEXM. These fragments were added to the upper chamber of the Transwell insert, while 400 µL of mEXM was added to the lower chamber.

For ALI culture, medium was removed from the upper chamber. In parallel, submerged culture (SC) was maintained by retaining medium in the upper chamber. For hormonal stimulation, mEnAOs were treated with 10 nmol/L E2, 1 µmol/L P4, and 0.5 mmol/L cAMP to simulate the pre-receptive and receptive phases. All mEnAOs were harvested on day 11 for frozen sectioning, qPCR, and RNA-seq.

RNA-seq analysis

Paired-end libraries (2×150 bp) were prepared and sequenced using the Illumina NovaSeq 6000 platform (USA) by Annoroad Gene Technology (<http://www.annoroad.com/>). Read alignment was performed using HISAT2 (v.2.2.1) against the *Mus musculus* reference genome (GRCm39). Transcript assembly and quantification (FPKM and read counts) were calculated using StringTie (v.2.1.1). Differentially expressed genes (DEGs) were detected using DESeq2 (v.1.42.1) in R. Genes were considered significantly differentially expressed if adjusted *P*-values (padj) using the Benjamini-Hochberg procedure were <0.05, with an absolute log₂FoldChange≥0.5 and FPKM≥1 in at least one sample. Principal component analysis (PCA) was performed using the “prcomp” function in R (v.4.3.2) based on a gene matrix with FPKM≥1 in at least one sample. Pearson correlation coefficients were calculated using the “cor()” function in the stats package. Heatmaps were generated using the pheatmap (v.1.0.12) package in R. Functional enrichment analysis, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation, was conducted using clusterProfiler (v.4.10.1).

Statistical analysis

All statistical data are expressed as mean±standard error of the mean (SEM). Statistical comparisons were performed using GraphPad Prism v.9.0, applying one-way analysis of variance (ANOVA) or unpaired two-tailed *t*-tests as appropriate. A minimum of three independent replicates (*n*≥3) was used for all analyses. Bright-field images of GLOs were captured using a Leica inverted microscope at 4× magnification, with five random fields selected for imaging. Immunofluorescence images of GE were captured using a Nikon confocal microscope at 4× magnification. Quantification was based on counts from at least 20 images per condition, and diameters of GLOs or epithelial structures were measured using ImageJ. For each analysis, at least 150 GLOs or

epithelial clusters were included. Statistical significance was defined as follows: *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.

RESULTS

Optimization of GLO and ESC culture systems

Initially isolation of endometrial components followed the EDTA-based dissociation protocol described by Boretto et al. (2017), in which endometrial fragments were incubated in 30 mmol/L EDTA for 1 h. However, this approach yielded suboptimal results, including incomplete myometrial digestion and insufficient recovery of stromal cells. To address these limitations and support downstream applications, the dissociation protocol was modified by supplementing the separation medium with 2 U/mL Dispase II and 2 mg/mL collagenase V, and extending the digestion time to 3–4 h (Supplementary Figure S1A). This enhanced strategy significantly improved the efficiency of epithelial and stromal extraction from mouse uterus, enabling the concurrent isolation of gland-like organoids and stromal fibroblasts.

Mouse endometrial GLOs expressed canonical epithelial markers, retained long-term expansion capability, demonstrated clonal potential, and exhibited hormonal responsiveness to estrogen and progesterone (Supplementary Figures S1B–F, S2A–J). Early culture attempts employed conditions reported by Tang et al. (2023), but under these parameters, GLOs showed structural instability and frequent rupture, rather than forming cohesive spheroids (Figure 1A). Given the essential roles of Wnt3a and R-Spondin 1 in epithelial proliferation via WNT signaling, a matrix of culture conditions was designed, including 200W/250R (200 ng/mL Wnt3a, 250 ng/mL R-Spondin1), 200W/500R (200 ng/mL Wnt3a, 500 ng/mL R-Spondin1), 100W/250R (100 ng/mL Wnt3a, 250 ng/mL R-Spondin1), and 100W/500R (100 ng/mL Wnt3a, 500 ng/mL R-Spondin1) (Figure 1A). Although reduced proliferation and cell yields were noted under 100W/250R (Figure 1B–D), this condition produced the highest frequency of Type I morphologies, organoid structures closely resembling *in vivo* glands (Figure 1E–G).

Mouse ESCs expressed fibroblast markers (Supplementary Figure S1G–I) and underwent hormone-induced decidualization *in vitro* (Supplementary Figure S2K–M). Standard ESC medium (ESCM), consisting of DMEM with 10% FBS, 1% NEAA, and 0.05% L-ascorbic acid, was found to support only limited expansion. In culture, ESCs displayed enlarged, flattened morphologies that impeded serial passaging. Therefore, to enhance proliferative capacity and preserve functional phenotype, ESCM was modified with hydrocortisone, L-ascorbic acid, and ITS-X, agents with documented anti-inflammatory and antioxidant effects (Liu et al., 2017; Zhou et al., 2024). Given that ESCs reside within a collagen-rich stromal niche and actively secrete collagen *in vivo* (Spiess et al., 2007; Zorn et al., 1986), culture surfaces were pre-coated with type IV collagen, a substrate widely used to support stromal adhesion and proliferation (Bhuvanesh et al., 2019). These refinements markedly improved ESC growth dynamics and ensured sufficient cell yields for downstream applications (Figure 1H, I).

Establishment of mEnAOs using ALI culture

To determine optimal cellular composition for assembloid formation, various epithelial-to-stromal cell ratios were

evaluated. Among the tested conditions, a 1:4 ratio of EECs to ESCs most effectively supported the growth and structural organization of GLOs (Supplementary Figure S3A–H). This ratio was subsequently adopted for the generation of mEnAOs. In parallel, ECM formulations were assessed to optimize the co-culture environment. A composite MAC mixture, as previously reported (Tian et al., 2023), supported robust growth of both GLOs and ESCs while closely resembling the ECM characteristics of native endometrial tissue (Supplementary Figure S4A–G).

Building upon prior work (Tian et al., 2023), mEnAOs were established using an ALI culture strategy in combination with hormonal stimulation to model the pre-receptive and receptive phases of the mouse endometrium (Figure 2A). As a control, conventional SC conditions were used to create SC-mEnAOs. Hormonal treatment with E2 alone simulated the pre-receptive phase, while a combination of E2, P4, and cAMP mimicked the receptive phase (Figure 2B). Both ALI- and SC-derived mEnAOs developed complex tissue architectures composed of luminal epithelial-like structures (LELs), glandular epithelial-like structures (GELs), and surrounding stromal cells, resembling *in vivo* endometrial organization (Figure 2C, D). In the ALI-mEnAOs, P4 treatment induced a significant increase in GEL diameter and a reduction in cell number compared to E2 treatment alone, recapitulating *in vivo* glandular remodeling during the transition to receptivity (Figure 2E–H). SC-mEnAOs exhibited similar trends in GEL enlargement following E2+P4+cAMP treatment; however, changes in GEL number were not significant (Figure 2E, F). Direct comparison between ALI-mEnAOs and SC-mEnAOs under identical hormonal conditions revealed comparable numbers of GELs in both systems. Notably, GELs in ALI-mEnAOs consistently exhibited larger luminal diameters of GELs, suggesting that ALI conditions more effectively support glandular expansion and epithelial morphogenesis (Supplementary Figure S5A–D).

mEnAOs recapitulate *in vivo*-like LE structures

LE is known to secrete MUC1, a hallmark glycoprotein of the uterine luminal surface. Consistently, LELs within mEnAOs also exhibited MUC1 expression (Figure 3A), indicating functional similarity to native LE. In addition, LE cells can be further differentiated into adhesive epithelial and supporting subtypes, marked by expression of *olfm1* and *il17rb*, respectively (Wang et al., 2023). Following E2+P4+cAMP stimulation, ALI-mEnAOs displayed robust up-regulation of both *olfm1* and *il17rb*, whereas this transcriptional activation was not evident in SC-mEnAOs (Figure 3B). During the pre-receptive phase *in vivo*, LE adopts a polarized columnar architecture maintained by apical tight junctions under the influence of E2. In contrast, during the receptive phase, E2+P4+cAMP exposure weakens these tight junctions, resulting in a loss of polarity (Craciunas et al., 2019). Notably, ALI-mEnAOs closely recapitulated this transition. ZO-1 expression was continuous along LEL membranes following E2 treatment and markedly reduced after E2+P4+cAMP exposure, reflecting the loosening of tight junctions observed *in vivo* (Figure 3C, D). Conversely, in SC-mEnAOs, ZO-1 localization remained largely unaffected by hormonal treatment, indicating limited responsiveness under SC (Figure 3D; Supplementary Figure S5E). These findings suggest that the ALI-based culture more effectively supports the differentiation and hormonal modulation of LELs in mEnAOs.

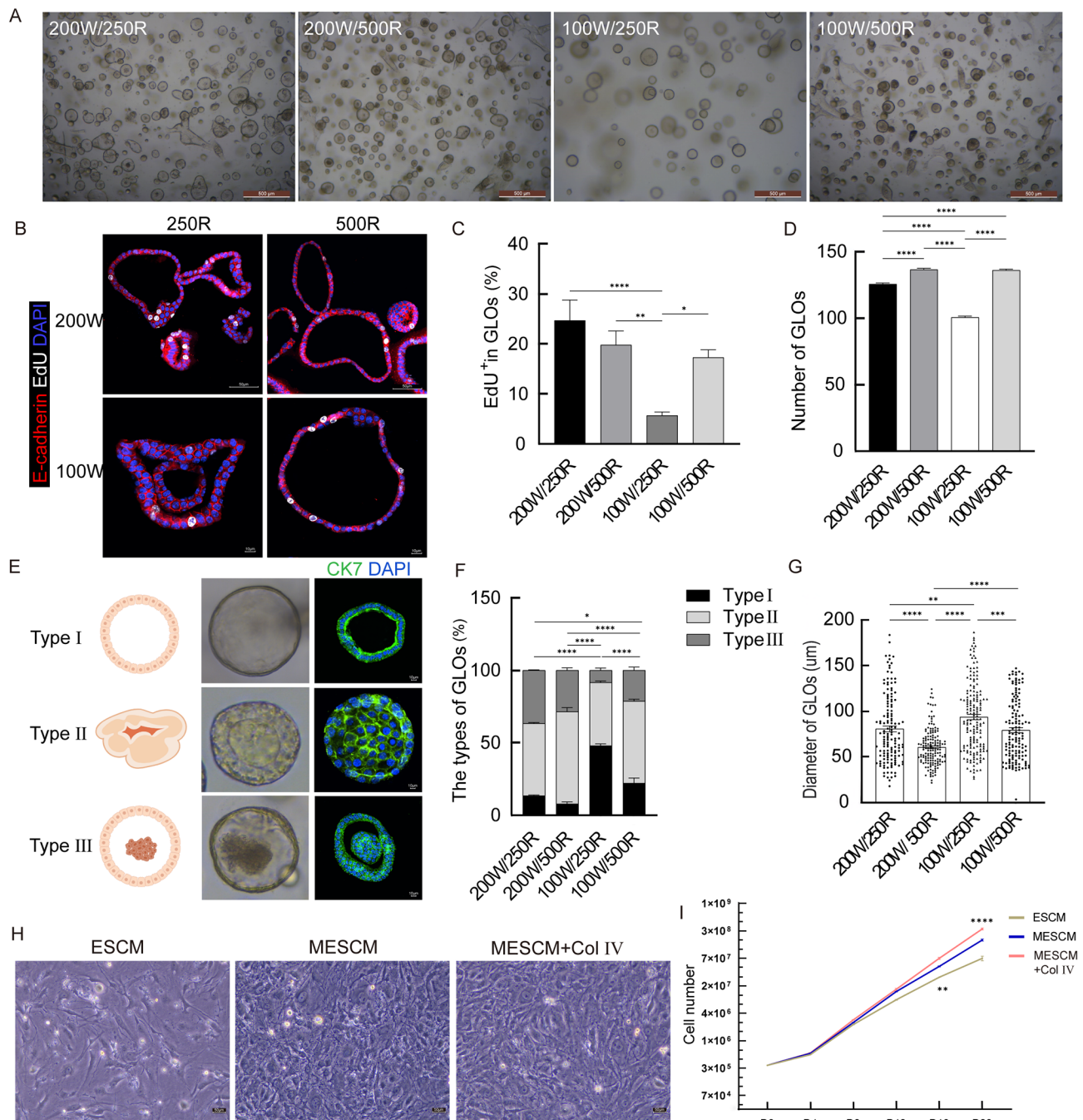


Figure 1 Effects of different culture systems on mouse endometrial gland-like organoids (GLOs)

A: Representative images of GLOs in different culture systems. Scale bars: 500 μ m. B: Immunofluorescence staining of GLOs in different culture systems. E-cadherin for epithelium; and EdU for cell proliferation. Scale bars: 50 μ m (200W/250R, 200W/500R); 10 μ m (100W/250R, 100W/500R). C: Proportion of EdU⁺ GLOs in different culture systems. D: Number of GLOs in different culture systems (per 4 $\times$ field of view). E: Representative and immunofluorescence staining images of different GLO subtypes. CK7 for epithelium. Scale bars: 10 μ m. F: Proportion of different GLO subtypes in different culture systems. G: Diameter of GLOs in different culture systems. H: Representative images of ESCs in different media. Scale bars: 50 μ m. I: Proliferation curves of ESCs in different culture systems. $n=3$ in each experiment. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$.

mEnAOs effectively simulate characteristic changes that occur during the receptive phase

The expression of estrogen (ESR) and progesterone receptors (PGR) in the mouse endometrium is tightly regulated across the estrous cycle, with dynamic, compartment-specific fluctuations in response to E2 and P4 (Tibbetts et al., 1998). To determine whether mEnAOs recapitulate these hormonal responses *in vitro*, receptor expression was evaluated under controlled culture conditions. In ALI-mEnAOs, ESR expression

was primarily localized to LELs, while PGR expression was predominantly observed in GELs (Figure 4C, F). In contrast, SC-mEnAOs exhibited broader ESR expression across LELs, GELs, and ESCs, whereas PGR induction was largely restricted to ESCs (Figure 4D, G). Quantitative comparison revealed that ALI-mEnAOs more accurately reflected the *in vivo* distribution patterns of ESR and PGR observed during the transition to receptivity (Figure 4E, H).

To confirm whether the mEnAOs exhibit gene expression

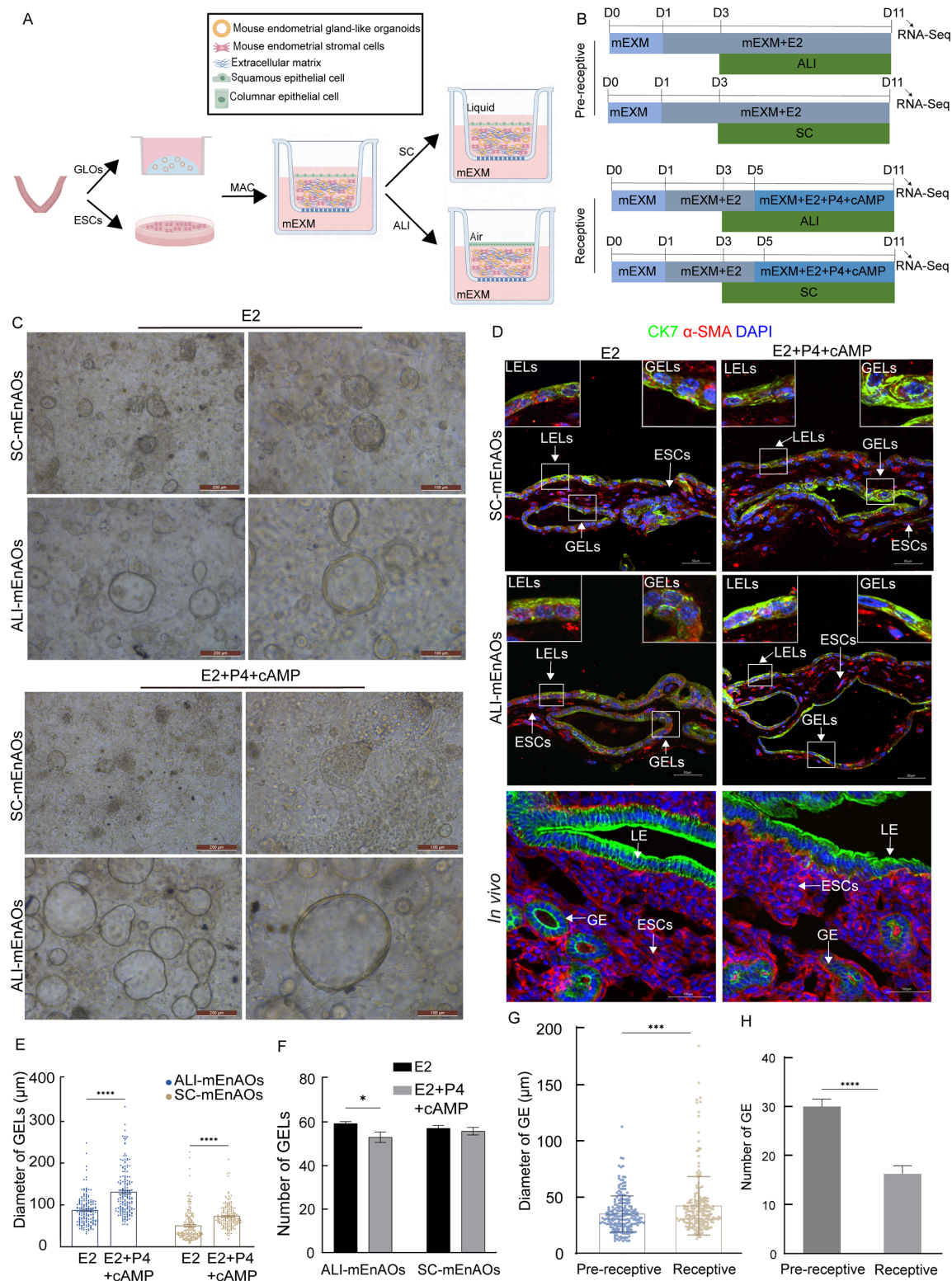


Figure 2 Establishment of mouse endometrial assembloids (mEnAOs) using air-liquid interface (ALI) culture and submerged culture (SC) systems

A: Schematic overview of ALI and SC methodologies for mEnAO construction. B: Experimental timeline for hormone treatments applied to ALI-mEnAOs and SC-mEnAOs to mimic pre-receptive and receptive phases. C: Representative bright-field images of ALI-mEnAOs and SC-mEnAOs. Scale bars: 100 μm. D: Immunofluorescence staining of ALI-mEnAOs and SC-mEnAOs. CK7 for epithelium; α-SMA for stromal cells. Scale bars: 100 μm (ALI-mEnAOs and SC-mEnAOs); 100 μm (*in vivo*). E: Quantification of GEL diameters in ALI-mEnAOs and SC-mEnAOs. F: Quantification of GEL counts in ALI-mEnAOs and SC-mEnAOs (per 4× field of view). G: Comparison of GE diameters and counts in native endometrial tissue. H: Comparison of GE counts in native endometrial tissue. GLOs, mouse endometrial gland-like organoids; ESCs, mouse endometrial stromal cells; MAC, Matrigel and collagen I; mEXM, mouse expansion medium; LE, luminal epithelium; GE, glandular epithelium; LELs, luminal epithelial-like structures; GELs, glandular epithelial-like structures. $n=3$ in each experiment. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$.

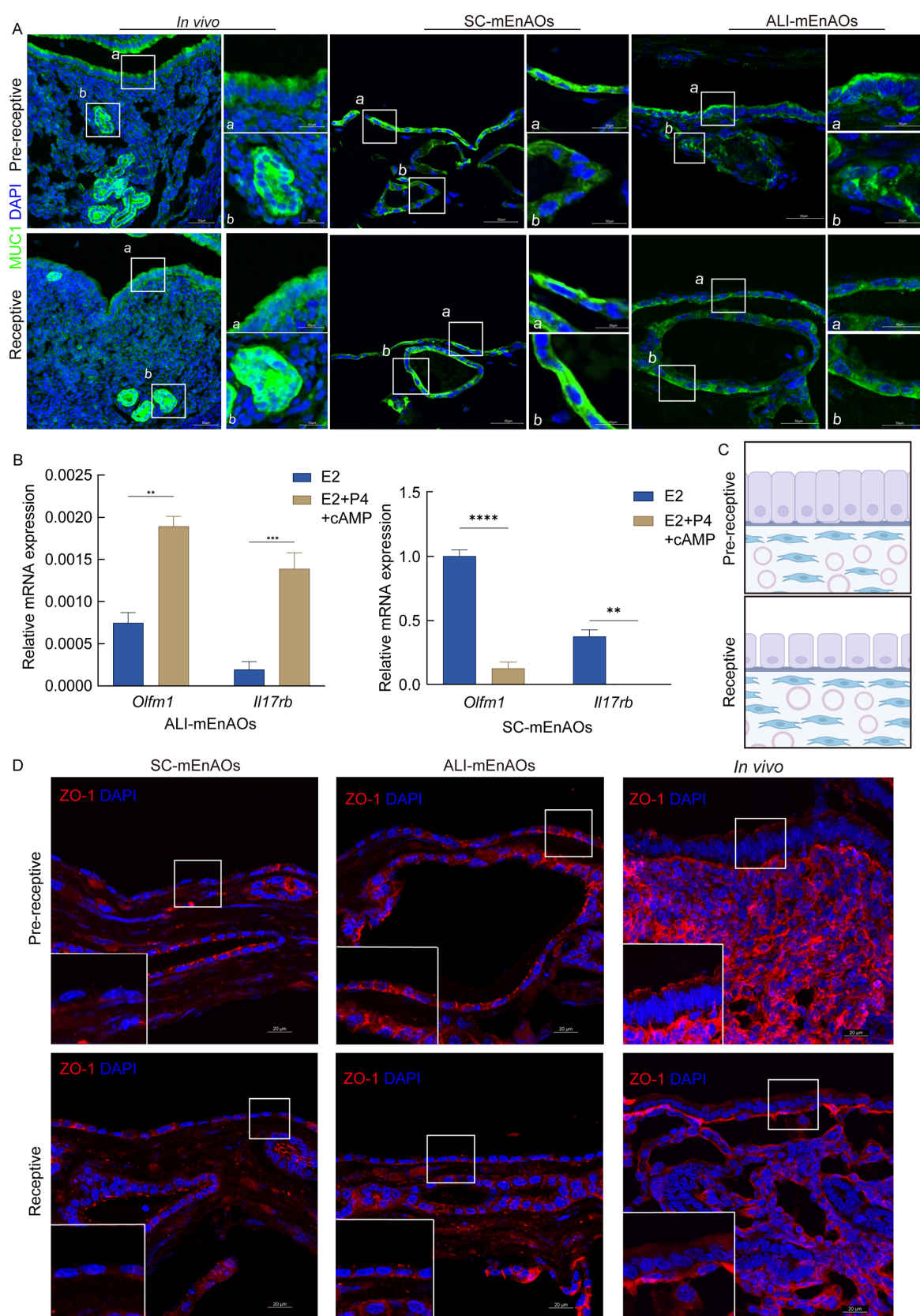


Figure 3 Characterization of luminal epithelial-like structures (LELs) in mouse endometrial assembloids (mEnAOs)

A: Immunofluorescence staining of MUC1, a luminal epithelial-secreted glycoprotein. Scale bars: 50 μ m. B: Expression levels of genes associated with LEL differentiation in mEnAOs. C: Schematic illustration of luminal epithelial polarity shifts during pre-receptive and receptive phases of the mouse endometrium. D: Immunofluorescence staining of ZO-1 in SC-mEnAOs and ALI-mEnAOs to assess epithelial polarity under hormone treatments. Scale bars: 20 μ m. $n=3$ in each experiment. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$.

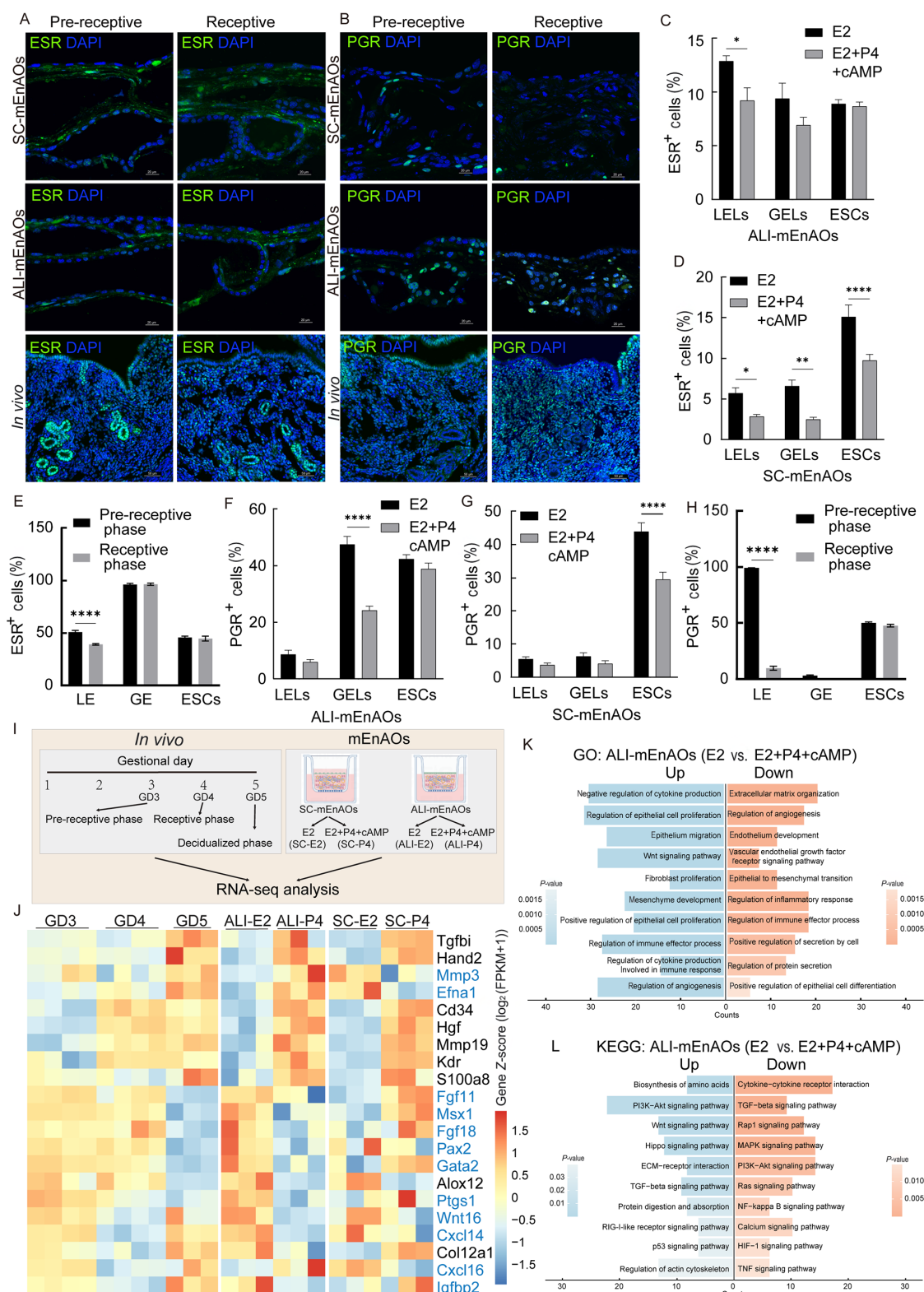


Figure 4 Effects of estrogen and progesterone on mouse endometrial assembloids (mEnAOs)

A: Immunofluorescence staining of ESR in mEnAOs and mouse endometrium. Scale bars: 20 μ m (in mEnAOs); 50 μ m (*in vivo*). B: Immunofluorescence staining of PGR in mEnAOs and mouse endometrium. Scale bars: 20 μ m (in mEnAOs); 50 μ m (*in vivo*). C–E: Quantification of ESR⁺ cells in ALI-mEnAOs (C), SC-mEnAOs (D), and *in vivo* (E). F–H: Quantification of PGR⁺ cells in ALI-mEnAOs (F), SC-mEnAOs (G), and *in vivo* (H). I: Schematic overview of experimental design and sampling timeline. J: Heatmap comparing expression of endometrial receptivity-related genes across GD3, GD4, GD5, ALI-mEnAOs (E2, E2+P4+cAMP), SC-mEnAOs (E2, E2+P4+cAMP). K: GO (biological process) terms enriched in ALI-mEnAOs under E2 or E2+P4+cAMP treatment. L: GO (biological process) terms enriched in SC-mEnAOs under E2 or E2+P4+cAMP treatment. ESR, estrogen receptor; PGR, progesterone receptor; ALI, air-liquid interface; SC, submerged culture; LELs, luminal epithelial-like structures; GELs, glandular epithelial-like structures; ESCs, mouse endometrial stromal cells. $n=3$ in each experiment. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$.

profiles similar to those observed *in vivo*, transcriptomic profiling was performed on ALI-mEnAOs, SC-mEnAOs, and uterine tissues collected from pregnant mice on GD3 (pre-receptive phase), GD4 (receptive phase), and GD5 (decidualized phase) (He et al., 2019; Yang et al., 2021). ESCs in mEnAOs were treated with E2+P4+cAMP to induce decidualization, allowing for direct comparison with GD5 uteri (Figure 4I; Supplementary Figure S5F–J). Global gene expression analysis revealed that ALI-mEnAOs clustered closely with *in vivo* endometrial tissues, exhibiting a high degree of transcriptional similarity (Supplementary Figure S5K). In ALI-mEnAOs, treatment with E2 up-regulated key endometrial receptivity-related genes, including *Msx1*, *Pax2*, *Gata2*, *Alox12*, *Ptgs1*, and *Igfbp2*, as well as chemokines *Cxcl14* and *Cxcl16*, closely resembling the GD3 uterine profile. Upon P4 stimulation, expression of receptivity-related genes *Tgfb1*, *Efn1*, *Kdr*, and *S100a8* was induced, alongside decidualization markers *Hand2* and *Mmp3*, closely resembling those observed at GD4 and GD5. In contrast, SC-mEnAOs failed to reproduce these gene expression dynamics, highlighting the superior fidelity of the ALI-based platform (Figure 4J).

Subsequently, GO analysis of DEGs in ALI-mEnAOs under different hormonal treatments revealed phase-specific biological processes. Notably, E2-treated ALI-mEnAOs were enriched in pathways associated with EEC and ESC proliferation, including regulation of epithelial cell proliferation, epithelium migration, fibroblast proliferation, and mesenchyme development. Conversely, E2+P4+cAMP-treated ALI-mEnAOs were enriched in pathways related to endometrial receptivity, including ECM organization, epithelial-to-mesenchymal transition, and positive regulation of secretion by cells (Figure 4K). KEGG pathway analysis further supported these findings. Under E2 treatment, ALI-mEnAOs were enriched in genes associated with the PI3K-AKT, Wnt, and Hippo signaling pathways, reflecting epithelial-stromal interactions characteristic of the pre-receptive phase. Similarly, under E2+P4+cAMP treatment, ALI-mEnAOs were enriched in genes related to cytokine-cytokine receptor interaction, Rap1 signaling, and MAPK signaling, indicating metabolic changes linked to epithelial-stromal interactions during the receptive phase (Figure 4L). Collectively, these results demonstrate that ALI-mEnAOs exhibit hormonally responsive gene expression profiles that closely mirror *in vivo* endometrial transitions and establish them as a reliable *in vitro* model for studying uterine receptivity.

Effects of ALI and SC systems on the molecular and functional landscape of mEnAOs

To evaluate how ALI versus SC conditions influence cellular behavior within mEnAOs, transcriptomic profiles were compared between ALI-mEnAOs and SC-mEnAOs under identical hormonal treatments (Figure 5A). Following exposure to E2, ALI-mEnAOs exhibited significant transcriptional remodeling, with 3 351 genes up-regulated and 2 961 genes down-regulated compared to SC-mEnAOs (Figure 5B). Subsequent GO enrichment analysis of these DEGs revealed that ALI-mEnAOs were enriched in biological processes related to gland development, glycerolipid metabolic process, and regulation of epithelial cell proliferation. In contrast, SC-mEnAOs were enriched in biological processes associated with negative regulation of the cell cycle, negative regulation of protein modification, and intrinsic apoptotic signaling

(Figure 5D). Notably, ALI-mEnAOs showed lower expression of regulators such as *Pik3cb*, *Cdkn1b*, and *Zfp36l2*, while genes including *Edn1*, *Gpx1*, and *Ceacam1* were up-regulated, suggesting enhanced epithelial and metabolic activity (Figure 5F). KEGG pathway analysis further revealed that ALI-mEnAOs were significantly enriched in genes involved in cytokine-cytokine receptor interaction, lysosomes, and TNF signaling, indicating metabolic changes in cells under ALI conditions. Conversely, SC-mEnAOs were enriched in genes related to ribosomes, HIF-1 signaling, and RNA degradation, reflecting metabolic changes in cells under SC conditions (Figure 5H). These results highlight divergent transcriptional and metabolic responses driven by the physical culture environment under E2 treatment.

Under E2+P4+cAMP treatment, similar trends were observed. ALI-mEnAOs exhibited 2 965 up-regulated genes and 2 978 down-regulated genes compared to SC-mEnAOs (Figure 5C). GO analysis revealed that ALI-mEnAOs were predominantly enriched in terms associated with cytokine-mediated signaling, glycerolipid metabolic process, and canonical NF- κ B signal transduction. In contrast, SC-mEnAOs were enriched in terms related to negative regulation of the cell cycle, chromatin remodeling, and chromosome segregation (Figure 5E). Notably, *Pidd1*, *Mdm2*, and *Cdkn1a*, associated with the enriched pathways identified in ALI-mEnAOs, exhibited a downward trend in expression, while *Cd74*, *Cln3*, and *Bcat2* were up-regulated (Figure 5G). KEGG pathway analysis further showed that ALI-mEnAOs were enriched in genes involved in cytokine-mediated signaling, regulation of innate immune response, and glycerolipid metabolism, suggesting distinct metabolic adaptations under ALI culture. In contrast, SC-mEnAOs were enriched in genes associated with negative regulation of the cell cycle, chromatin remodeling, and chromosome segregation, reflecting an alternative transcriptional and metabolic state associated with SC culture (Figure 5I). These results underscore the divergent functional and metabolic programs activated by ALI and SC culture systems in response to E2+P4+cAMP treatment.

DISCUSSION

The endometrium plays a central role in reproductive health, yet the pathophysiological basis of many endometrium-associated disorders remains poorly understood. This dynamic tissue undergoes cyclical remodeling driven by estrogen and progesterone, while the LE serves as the primary barrier between the embryo and maternal environment and plays a crucial role in implantation processes (Siriwardena & Boroviak, 2022). In the present study, mEnAOs were successfully generated, recapitulating key architectural features including LE-like structures. Hormonal stimulation induced transcriptional and morphological shifts consistent with the transition to endometrial receptivity, highlighting the value of the model for investigating hormone-driven differentiation and potential embryo-endometrium interactions. This platform provides a versatile and physiologically relevant framework for studying endometrial biology across species, including translational applications in humans and domestic animals.

Several studies have reported on methods for establishing mouse endometrial epithelial organoids (Boretto et al., 2017; Tang et al., 2023), yet reproducibility and yield remain limited. Technical difficulties encountered during endometrial isolation hindered enzymatic digestion, prompting the implementation

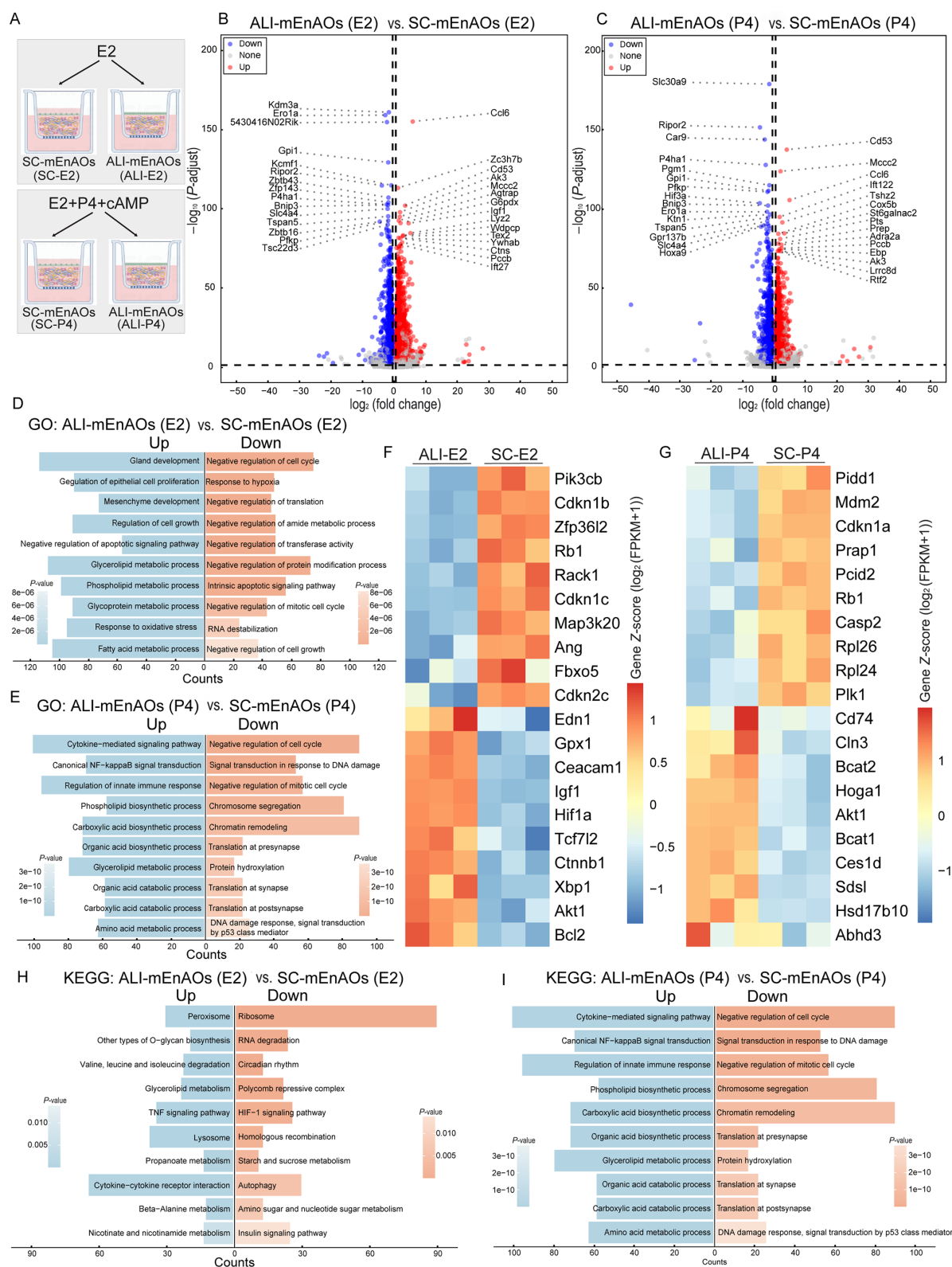


Figure 5 Transcriptomic comparison of air-liquid interface (ALI) and submerged culture (SC) systems in mouse endometrial assembloids (mEnAOs)

A: Experimental workflow summarizing the comparative analysis of ALI-mEnAOs and SC-mEnAOs. B: Volcano plot of differentially expressed genes (DEGs) between ALI-mEnAOs and SC-mEnAOs following E2 treatment. C: Volcano plot of DEGs between ALI-mEnAOs and SC-mEnAOs following E2+P4+cAMP treatment. D: Representative GO (biological process) terms enriched in mEnAOs following E2 treatment. E: Representative GO (biological process) terms enriched in mEnAOs following E2+P4+cAMP treatment. F: Heatmap showing expression profiles of representative genes in mEnAOs following E2 treatment. G: Heatmap showing expression profiles of representative genes in mEnAOs following E2+P4+cAMP treatment. H: KEGG pathway enrichment analysis of DEGs in mEnAOs following E2 treatment. I: KEGG pathway enrichment analysis of DEGs in mEnAOs following E2+P4+cAMP treatment.

of a two-step digestion strategy. This approach facilitated the efficient retrieval of both GLOs and ESCs, streamlining the workflow while improving cell recovery. Additionally, the culture conditions originally proposed by Tang et al. (2023) were suboptimal in this context, as GLOs tended to rupture rather than aggregate into organoids. By titrating Wnt3a and R-Spondin1, it was determined that concentrations of 200W/250R (200 ng/mL Wnt3a, 250 ng/mL R-Spondin1) were more conducive to GLO growth and stability. ESC culture presented additional challenges, particularly with regard to long-term expansion. To overcome this limitation, dishes were coated with type IV collagen, and the culture medium was supplemented with hydrocortisone, L-ascorbic acid, and ITS-X based on the methodology of Liu et al. (2017). These refinements markedly enhanced ESC proliferation, enabling robust downstream applications.

The establishment of endometrial receptivity is essential for endometrial function. In mice, the pre-receptive phase spans days 1 to 3 of pregnancy, during which ovarian estrogen drives endometrial proliferation. On day 4, a moderate estrogen surge, coupled with rising progesterone, initiates the transition toward receptivity. By day 5, the uterus shifts to a non-receptive state, inhibiting implantation (Wang & Dey, 2006). To recapitulate these *in vivo* transitions, mEnAOs were treated with E2, the primary hormone driving endometrial proliferation, to model the proliferative, pre-receptive phase, and with E2+P4+cAMP to simulate the receptive phase, based on established roles of progesterone and cAMP in modulating gene networks and signaling pathways associated with receptivity (De et al., 2017; Zhao et al., 2012). The effects of ALI and SC methods were compared under both hormonal conditions. A key limitation of prior studies, such as that by Tian et al. (2023), was the application of hormone treatments before the onset of ALI culture, confounding interpretation of whether transcriptional and phenotypic changes reflected hormone exposure, ALI culture, or both. By initiating ALI culture prior to hormone exposure, the experimental design isolated hormone-dependent effects and enabled a more accurate evaluation of hormone-induced cellular responses. Using this sequential approach, ALI-mEnAOs were shown to reproducibly develop LE-like structures and exhibit appropriate hormone responsiveness. In contrast, SC-mEnAOs exhibited disorganized epithelial layers with impaired structural polarity and reduced expression of critical genes, such as *Olfm1* and *Il17rb*, indicating compromised LE differentiation. This disruption likely impaired hormonal responsiveness to E2+P4+cAMP stimulation. In ALI-mEnAOs, PGR expression declined across all epithelial populations, consistent with *in vivo* trends; however, LE and GE showed distinct spatial expression patterns. Unlike the *in vivo* endometrium, where LE forms prior to glandular development, LELs in ALI-mEnAOs arose from pre-established glandular organoids, potentially altering their responsiveness to estrogen and progesterone (Brody & Cunha, 1989). *In vivo*, PGR regulation by estrogen is dynamic and context-dependent, governed by systemic cues, whereas *in vitro* exposure is static and uniformly distributed, likely contributing to the divergent PGR patterns observed (Tibbetts et al., 1998). These discrepancies underscore inherent limitations of *in vitro* systems in fully recapitulating endometrial complexity. Nonetheless, the ALI platform more effectively supported GLO expansion and the formation of polarized LE-like structures, with gene expression signatures more closely aligning with the receptive *in vivo*

endometrium. Comparative transcriptomic analysis between ALI-mEnAOs and SC-mEnAOs revealed that ALI culture promoted pathways associated with epithelial cell proliferation, gland development, and metabolic activity in both GLOs and ESCs. The ALI setup, characterized by apical air exposure and basal immersion in liquid medium, provides an asymmetric physicochemical environment that supports apical-basal polarity and columnar epithelial architecture (Stuart et al., 2018; Suzuki & Ohno, 2006). Oxygen gradients generated in ALI culture may trigger localized hypoxia, stabilizing HIF-1 α and promoting the secretion of factors that stimulate angiogenesis or ECM synthesis in interstitial fibroblasts (Semenza, 2012; Stuart et al., 2018). In contrast, HIF signaling was suppressed under SC conditions. Due to limitations in bulk RNA-seq, a more comprehensive delineation of these responses was not achieved. Future application of single-cell sequencing is warranted to resolve cell-type-specific responses and spatial organization. Further optimization is required to enhance the fidelity of ALI-mEnAOs in recapitulating *in vivo* endometrial biology. Nevertheless, these assembloids offer a promising *in vitro* platform for dissecting hormone-driven differentiation and investigating mechanisms underlying endometrial function.

Endometrial stromal cells differentiate into decidual cells that support nutrient exchange, energy transfer, and placental development during early embryogenesis (Mori et al., 2016). In mice, stromal decidualization is strictly embryo-dependent and initiated only after an embryo traverses the LE and gains access to the uterine stroma. This requirement contrasts with the human endometrium, in which decidualization proceeds spontaneously under hormonal influence (Garcia-Flores et al., 2023; Robertson et al., 2022). As the mEnAOs contain both epithelial and stromal compartments, direct recapitulation of embryo-triggered decidualization remains technically challenging. To approximate this process *in vitro*, combined E2+P4+cAMP treatment was applied to simulate stromal cell decidualization and establish a receptive-like state within the assembloids. Under these conditions, ALI-mEnAOs exhibited enrichment of pathways associated with regulation of inflammatory response and immune effector processes, indicating acquisition of immunomodulatory features characteristic of decidualized stroma, consistent with recent observations *in vivo* (Yang et al., 2023). These transcriptional signatures underscore the complex interactions among immune, epithelial, and stromal cells during the establishment of endometrial receptivity. Unlike humans and rodents, embryos of domestic species such as pigs, cattle, and sheep adhere to the LE without invading the endometrial stroma, and decidualization of stromal cells has not been observed. Despite this fundamental difference, the presence of stromal cells and their response to estrogen and progesterone play a significant role in endometrial functions in these animals (Bazer et al., 2015; Bazer & Johnson, 2014; Stenhouse et al., 2022).

Recent advances have extended endometrial epithelial organoid technology to domestic species, including horses and pigs, broadening its translational and agricultural relevance (Thompson et al., 2020, 2022; Saadeldin et al., 2024). Porcine epithelial organoids have been co-cultured with parthenogenetically activated embryos, supporting trophoblast adhesion and outgrowth, thereby establishing their potential as *in vitro* models for peri-implantation studies (Saadeldin et al., 2024). Given the high costs and logistical constraints of

in vivo experimentation in livestock, *in vitro* systems provide scalable platforms for investigating reproductive disorders in economically significant species.

Although this study used mouse-derived assembloids, the hormone responsiveness of LE, GE, and ESCs, as well as the observed epithelial-stromal interactions, provide a theoretical foundation for investigating endometrial functions in domestic mammals.

However, further refinement of the MAC system remains essential to better mimic *in vivo* physiology. Integration of immune components is particularly critical, given the central role of immune cells in modulating endometrial receptivity and implantation. Embryo implantation represents the defining functional test of endometrial competence. Therefore, future efforts must focus on optimizing co-culture strategies using mEnAOs to recapitulate this process *in vitro*. In addition, transcriptomic analysis based on bulk RNA-seq captures average gene expression across heterogeneous cells, limiting resolution of cell type-specific dynamics. Single-cell RNA-seq will be instrumental for identifying regulatory networks, enabling high-resolution comparisons between *in vivo* tissues and organoid-derived populations. Integration with emerging technologies such as 3D printing, microfluidics, and live-cell imaging may further advance research and therapeutic strategies for reproductive disorders.

CONCLUSION

In this study, mEnAOs with LE-like structures were successfully established *in vitro* by co-culturing gland-like organoids and stromal cells using ALI. These assembloids recapitulated key structural, cellular, and transcriptional features of the receptive endometrium, offering a reliable platform for investigating endometrial functions and embryo implantation in mammals.

DATA AVAILABILITY

The single-cell sequencing data generated in this study have been deposited in the Genome Sequence Archive (GSA) (<https://www.scidb.cn/en/c/zoores>) under CRA030657, Science Data Bank (doi: 10.57760/sciencedb.j00139.00271), and NCBI (<https://www.ncbi.nlm.nih.gov/>) under BioProjectID PRJNA1336449.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

T.Q.L. designed the research. X.Y.Q., J.Y.T., J.Y., M.Q.L., and J.W.T. performed the research. D.W. and L.L. analyzed the data. X.Y.Q., J.Y.T., J.Y., Y.J.Z., and T.Q.L. wrote and revised the paper. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We gratefully acknowledge all study participants and individuals who contributed to this study. We also thank www.biorender.com for illustrative components.

REFERENCES

Ababneh MM, Troedsson MHT. 2013. Endometrial phospholipase A2 activity during the oestrous cycle and early pregnancy in mares. *Reproduction in Domestic Animals*, **48**(1): 46–52.

Bauersachs S, Wolf E. 2015. Uterine responses to the preattachment

embryo in domestic ungulates: recognition of pregnancy and preparation for implantation. *Annual Review of Animal Biosciences*, **3**(1): 489–511.

Bazer FW, Johnson GA. 2014. Pig blastocyst-uterine interactions. *Differentiation*, **87**(1–2): 52–65.

Bazer FW, Ying W, Wang XQ, et al. 2015. The many faces of interferon tau. *Amino Acids*, **47**(3): 449–460.

Bhuvanesh T, Machatschek R, Lysyakova L, et al. 2019. Collagen type-IV Langmuir and Langmuir-Schäfer layers as model biointerfaces to direct stem cell adhesion. *Biomedical Materials*, **14**(2): 024101.

Boretto M, Cox B, Noben M, et al. 2017. Development of organoids from mouse and human endometrium showing endometrial epithelium physiology and long-term expandability. *Development*, **144**(10): 1775–1786.

Brody JR, Cunha GR. 1989. Histologic, morphometric, and immunocytochemical analysis of myometrial development in rats and mice: I. Normal development. *The American Journal of Anatomy*, **186**(1): 1–20.

Craciunas L, Gallos I, Chu J, et al. 2019. Conventional and modern markers of endometrial receptivity: a systematic review and meta-analysis. *Human Reproduction Update*, **25**(2): 202–223.

De Clercq K, Hennes A, Vriens J. 2017. Isolation of mouse endometrial epithelial and stromal cells for *in vitro* decidualization. *Journal of Visualized Experiments*, (121): 55168.

Garcia-Flores V, Romero R, Peyvandipour A, et al. 2023. A single-cell atlas of murine reproductive tissues during preterm labor. *Cell Reports*, **42**(1): 111846.

Gnecco JS, Brown A, Buttrey K, et al. 2023. Organoid co-culture model of the human endometrium in a fully synthetic extracellular matrix enables the study of epithelial-stromal crosstalk. *Med*, **4**(8): 554–579. e9.

He JP, Zhao M, Zhang WQ, et al. 2019. Identification of gene expression changes associated with uterine receptivity in mice. *Frontiers in Physiology*, **10**: 125.

Jiang LH, Cao DD, Yeung WSB, et al. 2022. Single-cell RNA-sequencing reveals interactions between endometrial stromal cells, epithelial cells, and lymphocytes during mouse embryo implantation. *International Journal of Molecular Sciences*, **24**(1): 213.

Lancaster MA, Knoblich JA. 2014. Organogenesis in a dish: modeling development and disease using organoid technologies. *Science*, **345**(6194): 1247125.

Lin SC, Li WN, Lin SC, et al. 2023. Targeting YAP1 ameliorates progesterone resistance in endometriosis. *Human Reproduction*, **38**(6): 1124–1134.

Liu XF, Krawczyk E, Supryniewicz FA, et al. 2017. Conditional reprogramming and long-term expansion of normal and tumor cells from human biospecimens. *Nature Protocols*, **12**(2): 439–451.

Mao CY, Liu XS, Guo SW. 2024. Medlazine improves endometrial repair and reduces simulated menstrual bleeding in mice with induced adenomyosis. *American Journal of Obstetrics and Gynecology*, **231**(1): 113. e1–113. e13.

Mori M, Bogdan A, Balassa T, et al. 2016. The decidua—the maternal bed embracing the embryo—maintains the pregnancy. *Seminars in Immunopathology*, **38**(6): 635–649.

Muter J, Lynch VJ, McCoy RC, et al. 2023. Human embryo implantation. *Development*, **150**(10): dev201507.

Pruniéras M, Régnier M, Woodley D. 1983. Methods for cultivation of keratinocytes with an air-liquid interface. *The Journal of Investigative Dermatology*, **81**(1 Suppl): 28s–33s.

Rawlings TM, Makwana K, Taylor DM, et al. 2021. Modelling the impact of decidual senescence on embryo implantation in human endometrial assembloids. *eLife*, **10**: e69603.

Robertson SA, Moldenhauer LM, Green ES, et al. 2022. Immune determinants of endometrial receptivity: a biological perspective. *Fertility and Sterility*, **117**(6): 1107–1120.

Rossi G, Manfrin A, Lutolf MP. 2018. Progress and potential in organoid

- research. *Nature Reviews Genetics*, **19**(11): 671–687.
- Roy A, Matzuk MM. 2011. Reproductive tract function and dysfunction in women. *Nature Reviews Endocrinology*, **7**(9): 517–525.
- Saadeldin IM, Han A, Bang S, et al. 2024. Generation of porcine endometrial organoids and their use as a model for enhancing embryonic attachment and elongation. *Reproduction*, **167**(2): e230429.
- Sandra O. 2016. Hormonal control of implantation. *Annales d'Endocrinologie*, **77**(2): 63–66.
- Sandra O, Charpigny G, Galio L, et al. 2017. Preattachment embryos of domestic animals: insights into development and paracrine secretions. *Annual Review of Animal Biosciences*, **5**: 205–228.
- Semenza GL. 2012. Hypoxia-inducible factors in physiology and medicine. *Cell*, **148**(3): 399–408.
- Siriwardena D, Boroviak TE. 2022. Evolutionary divergence of embryo implantation in primates. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **377**(1865): 20210256.
- Spieß K, Teodoro WR, Zorn TMT. 2007. Distribution of collagen types I, III, and V in pregnant mouse endometrium. *Connective Tissue Research*, **48**(2): 99–108.
- Stenhouse C, Seo H, Wu GY, et al. 2022. Insights into the regulation of implantation and placentation in humans, rodents, sheep, and pigs. *Advances in Experimental Medicine and Biology*, **1354**: 25–48.
- Stuart JA, Fonseca J, Moradi F, et al. 2018. How supraphysiological oxygen levels in standard cell culture affect oxygen-consuming reactions. *Oxidative Medicine and Cellular Longevity*, **2018**(1): 8238459.
- Suzuki A, Ohno S. 2006. The PAR-aPKC system: lessons in polarity. *Journal of Cell Science*, **119**(Pt 6): 979–987.
- Tang SN, Parks SE, Liao ZA, et al. 2023. Establishing 3D endometrial organoids from the mouse uterus. *Journal of Visualized Experiments*, (191): 10.3791/64448.
- Thompson RE, Johnson AK, Dini P, et al. 2020. Hormone-responsive organoids from domestic mare and endangered Przewalski's horse endometrium. *Reproduction*, **160**(6): 819–831.
- Thompson RE, Meyers MA, Pukazhenthi BS, et al. 2022. Evaluation of growth, viability, and structural integrity of equine endometrial organoids following cryopreservation. *Cryobiology*, **104**: 56–62.
- Tian JW, Yang J, Chen TW, et al. 2023. Generation of human endometrial assembloids with a luminal epithelium using air–liquid interface culture methods. *Advanced Science*, **10**(30): 2301868.
- Tibbetts TA, Mendoza-Meneses M, O'Malley BW, et al. 1998. Mutual and intercompartmental regulation of estrogen receptor and progesterone receptor expression in the mouse uterus. *Biology of Reproduction*, **59**(5): 1143–1152.
- Waclawik A, Kaczmarek MM, Blitek A, et al. 2017. Embryo - maternal dialogue during pregnancy establishment and implantation in the pig. *Molecular Reproduction and Development*, **84**(9): 842–855.
- Wang HB, Dey SK. 2006. Roadmap to embryo implantation: clues from mouse models. *Nature Reviews Genetics*, **7**(3): 185–199.
- Wang HQ, Liu Y, Li D, et al. 2023. Maternal and embryonic signals cause functional differentiation of luminal epithelial cells and receptivity establishment. *Developmental Cell*, **58**(21): 2376–2392. e6.
- Whitcutt MJ, Adler KB, Wu R. 1988. A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. *In vitro Cellular & Developmental Biology*, **24**(5): 420–428.
- Wiwatpanit T, Murphy AR, Lu ZX, et al. 2020. Scaffold-free endometrial organoids respond to excess androgens associated with polycystic ovarian syndrome. *The Journal of Clinical Endocrinology & Metabolism*, **105**(3): 769–780.
- Wu XY, Yang XW, Tian YC, et al. 2023. Bisphenol B and bisphenol AF exposure enhances uterine diseases risks in mouse. *Environment International*, **173**: 107858.
- Xinaris C, Brizi V, Remuzzi G. 2015. Organoid models and applications in biomedical research. *Nephron*, **130**(3): 191–199.
- Yang M, Ong J, Meng FJ, et al. 2023. Spatiotemporal insight into early pregnancy governed by immune-featured stromal cells. *Cell*, **186**(20): 4271–4288. e24.
- Yang Y, Zhu QY, Liu JL. 2021. Deciphering mouse uterine receptivity for embryo implantation at single - cell resolution. *Cell Proliferation*, **54**(11): e13128.
- Zhao KQ, Lin HY, Zhu C, et al. 2012. Maternal Smad3 deficiency compromises decidualization in mice. *Journal of Cellular Biochemistry*, **113**(10): 3266–3275.
- Zhou ZL, Wu XM, Chen TW, et al. 2024. Restoration of functional endometrium in an intrauterine adhesion rat model with endometrial stromal cells transplantation. *Stem Cell Research & Therapy*, **15**(1): 181.
- Zorn TM, Bevilacqua EM, Abrahamsohn PA. 1986. Collagen remodeling during decidualization in the mouse. *Cell and Tissue Research*, **244**(2): 443–448.