Human receptive endometrial organoid for deciphering the implantation window

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Abstract

Human endometrial receptivity is a critical determinant of pregnancy success; however, in vivo studies of its features and regulation are particularly challenging due to ethical restriction. Recently, the development of human endometrial organoids has provided a powerful model to examine this intricate biological process. In this study, we established a specialized human window-of-implantation (WOI) endometrial organoid system that mimics the in vivo receptive endometrium. The endometrial organoid recapitulates not only the structural attributes of glands and stroma, but also the in vivo endometrium's cell types at the single-cell level. Furthermore, the WOI endometrial organoid exhibits the features of in vivo implantation endometrium such as hormone responsiveness, secretory functions, and distinctive implantation window hallmarks, including decidualization, extracellular matrix (ECM) remodeling, pinopode formation, cilia generation and metabolism. This in vitro model also effectively demonstrates the process of proliferation-secretory transformation, ciliary epithelium differentiation, and epithelial-mesenchymal transition (EMT). As such, WOI organoids hold great promise as a robust platform to unravel the intricate mechanisms governing endometrial receptivity regulation, maternal-fetal interactions, and associated diseases, ultimately driving impactful advancements in the field.

Keywords

Endometrium, organoid, implantation window

Introduction

The human endometrium, comprising various cell types, experiences shedding (menstrual phase), regeneration (proliferative phase), and differentiation (secretory phase) under the regulation of estrogen and progesterone during the menstrual cycle (1). A brief window, called the "window-of-implantation (WOI)" or mid-secretory phase, allows for embryo implantation into the endometrium (1). The endometrium at the embryo implantation site contains diverse cells, including ciliated epithelial cells, decidualized stromal cells and immune cells. Cyclic endometrial changes akin to those in humans are exclusive to apes, Old World monkeys, molossid bats, and spiny mice, but not mice (1), rendering typical mouse models insufficient for accurately simulating human endometrium. Existing endometrial cell lines, such as Ishikawa and HEEC cells, consist solely of a single cell type, thus failing to reproduce the intricate physiological structure and function of the endometrium.

Replicating and reconstructing human organs has become essential for exploring tissue physiology and function. Organoid, a self-assembled 3D structure, closely resemble in vivo tissue or organ (2). They offer high expansibility, phenotypic, and functional properties, emerging as powerful tools for investigating tissue physiology and disease (3). In 2017, the first long-term and hormone-responsive human endometrial organoid was established using adult stem cells from endometrial biopsy(2). Based on this, Margherita Y. Turco, et al developed noninvasive methods to construct endometrial organoids from menstrual flow(4). Apart from adult stem cells, pluripotent stem cells were also induced to endometrial stromal fibroblasts and epithelium, and then cocultured to form organoids(5, 6), which offered vigorous proliferative capacity but lacked immune cells and other components of the microenvironment. In efforts to mimic human endometrial cell types in vitro, the endometrial organoids progressed from epithelial organoids(7), to assemblies of epithelial and stromal cells(8, 9) and then to stem cell-laden 3D artificial endometrium(10, 11), which were solely closer but not completely identical to the endometrium. Additionally, pathological endometrial organoid models have been established for conditions like endometriosis, endometrial hyperplasia, Lynch syndrome, and endometrial cancer (12). These organoids simulated endometrial morphology, hormone responsiveness, and physiological and pathological processes in vitro (3, 7, 13-15), facilitating the study of physiological phenomena (13, 16, 17), pathogenic mechanisms(14)

and drug screening(12).

Although various regulators have been suggested to be involved in WOI, we are far from understanding how embryo implantation occurs during the WOI due to ethical limitations and lack of in vitro receptive endometrial model. Transforming from proliferative to receptive endometrium involves dynamic changes like decidualization, epithelial-mesenchymal transition (EMT) and ciliated epithelial development (1, 18), which have not been reconstructed in any in vitro model to date. Here, we successfully established a receptive endometrial organoid system regulated by hormones and reflecting the in vivo WOI endometrial features, providing a platform for studying physiological and pathological endometrium and maternal-fetal interactions during the peri-implantation period.

Results

Endometrial organoids possess endometrial morphology and function

To establish endometrial organoids in vitro, prereceptive endometrium from reproductive-age women was dissociated into single cells or small cell masses, which were embedded with Matrigel in expansion medium (ExM). We devised a "window-of-implantation (WOI)" organoid by supplementing the culture system with prolactin (PRL), human chorionic gonadotropin (hCG), and human placental lactogen (hPL), alongside E2, MPA, and cAMP. In contrast, endometrial organoids maintained in ExM constituted the "control (CTRL)" group, while organoids treated with E2 for two days, followed by E2, MPA, and cAMP for an additional six days, induced the secretory phase as previously reported(7), and were designated the "secretory (SEC)" group (Fig. 1A). Theoretically, the WOI organoids are initially in the secretory phase, thus, possessing characteristics of the SEC organoid, more importantly, they should exhibit the traits of mid-secretory phase. The endometrial organoid, consisting of vesicle-like glands, fibrous stromal cells and other surrounding cells, developed into a 3D structure with the support of Matrigel (Fig. 1B~1C, Video S1~S4). As the organoids grew and differentiated, the endometrial glands enlarged, epithelial cells adopted a paving stone arrangement, and stromal cells formed an extensive network (Fig. 1C). The glandular epithelium marker E-cadherin, stromal cell marker vimentin, and endometrial gland marker FOXA2 were expressed in the cultured endometrial organoid, which resembled the endometrium morphologically (Fig. 1D). Moreover, the endometrial organoids exhibited substantial expression of the proliferation marker Ki67, while the apoptosis marker cleaved caspase-3 was undetectable, signifying the organoids' robust proliferative capacity (Fig. 1E).

Functionally, endometrial organoids successfully secreted glycogen into the lumen, analogous to in vivo endometrial activity, thereby providing nourishment for embryo implantation (Fig. 1D). The CTRL organoids exhibit minimal glycogen secretion, whereas both SEC and WOI organoids display substantial glycogen secretion into the glandular lumen (Fig.S1A). Furthermore, the endometrium undergoes cyclical alterations under regulation by estrogen (E2) and progesterone in vivo. Following hormonal stimulation, WOI organoids exhibited slower growth than SEC and CTRL organoids, while CTRL organoids maintained robust proliferative activity (Fig. 1B). In our

organoids, after treatment with E2 for two days and subsequently with medroxyprogesterone acetate (MPA) and cAMP for fourteen days, the organoids demonstrated significantly increased expression of progesterone receptor (PR A/B) and a slight elevation in estrogen receptor α (ERα) relative to the CTRL group (Fig. S1B), suggesting the sensitivity to hormone treatment. These results demonstrate that our organoids exhibited comparable morphology, secretory function, and hormone responsiveness to the in vivo endometrium.

Developing receptive endometrial organoids in vitro mimicking the implantation-window endometrium

Single-cell transcriptomics analysis with reference to CellMarker, PanglaoDB, Human Cell Atlas, Human Cell Landscape, and scRNASeqDB, and previous endometrium related studies(1, 7, 9, 19) revealed the presence of epithelium, stromal cells and immune cells in WOI organoids (Fig. 2A, Fig. S3B~S3C). With respect to glandular and luminal epithelium, secretory epithelium, LGR5 epithelium, ciliated epithelium and EMT-derived stromal cells, the WOI organoids closely resemble the mid-secretory endometrium (Fig.2A, Fig.S3C~S3D) according to comparisons of the scRNA-seq data of our organoids and mid-secretory endometrium described by Stephen R. Quake in 2020 (1). We have captured the morphology of immune and stromal cells using 3D clearing staining and light sheet microscopy imaging, with vimentin marking stromal cells, CD44 designating immune cells and FOXA2 identifying glands (Fig.2B~2C). Thus, we sought to confirm the presence of immune cells and investigate the proportion of T cells and macrophages in the endometrial organoids using flow cytometry. The gating strategy for T cells and macrophages was shown in Fig. 2D. White blood cells (WBC) were determined as $CD45^+$ cells, while T cells and macrophages were defined as $CD45^+CD3^+$ cells and $CD45^+CD68^+CD11b^+$ cells, respectively.

Given that the mid-secretory phase is when the implantation window opens and significant transcriptional changes occur, we focused on comparing gene expression and transcription regulation during this phase (Fig.2E~2F), referring to Stephen R. Quake 2020 for mid-secretory phase scRNA-seq data(1). Pathways including mitochondrial energy metabolism, cell adhesion, cytoskeleton regulation, and epithelial cell growth were all upregulated in both the WOI organoid and mid secretory endometrium compared with that in the CTRL and SEC organoids (Fig. 2E).

Analysis of key transcription factors (TFs) involved in implantation revealed similarities between WOI organoids and mid secretory epithelium when compared to the CTRL and SEC organoids, including hypoxia response (such as previously reported HIF1A(20)), embryo implantation (such as FBLN1(21)), lipid metabolism (such as VMP1(22)), cell migration, and cell junction (such as TJP1(23)) (Fig. 2F). Moreover, markers of endometrial decidualization (such as S100A10(24)), EMT (such as FAT1(25) and FOXF2(26)), and receptivity (such as NEAT1(27), SERPINB9(28), SOX17(29) and SOX4(30)) were expressed at comparable level in EMT-derived stromal cells in both groups (Fig. 2F). We then conducted the endometrial receptivity test (ERT) to assess the receptive state of the endometrium(31) and endometrial organoids (Fig. 2G). Hormone-treated organoids derived from pre-receptive endometrium entered the receptivity phase, suggesting the successful formation of the implantation window in vitro. Collectively, these results demonstrate that the developed WOI organoids closely resemble the in vivo endometrium during the implantation window in terms of cell composition, function, transcriptional regulation, and endometrial receptivity.

Receptive endometrial organoids recapitulate WOI-associated biological characteristics

We analyzed the transcriptome and proteome profiles of WOI organoids compared to those of CTRL or SEC organoids to assess WOI associated biological characteristics (Fig. 3A, 3E, Fig. S2A~S2D). WOI organoids exhibited a strong hormone response, as demonstrated by PGR at the transcriptome level (Fig. 3B). Combined analysis of the transcriptome and proteome of the WOI organoids demonstrated 179 upregulated genes/proteins compared with those in the CTRL group (Fig. S2E), and most of these genes/proteins are involved in the estrogen response (Fig. S2F). Furthermore, the progesterone response level tested by PRA/B immunostaining was highest in the WOI group, meanwhile estrogen responsive protein OLFM4 was also upregulated (Fig. 3C). FOXO1 is a critical marker of endometrial receptivity depending on PGR signaling. We found WOI organoids showed a significantly elevated FOXO1 compared with the CTRL organoids (Fig.3D, Video S5), indicating that progesterone signaling was involved in WOI organoid establishment. The above results demonstrated that the WOI organoids responded strongly to estrogen and progesterone.

The growth and development, assembly and movement of cilia, a characteristic endometrial

structure, are dynamically regulated by the hormones during the menstrual cycle, which are essential for the establishment of the endometrial implantation window and embryo implantation. Cilia assembly(marked by acetyl-α-tubulin(13)) and motile cilia (marked by TPPP3 and ODF2(32)) appeared dominant in the WOI organoids (Fig. 3F~3G, Fig.S2I). Moreover, the expression of tubulin, integrin and components of the PI3K-Akt pathway, which function to generate pinopodes and respond to invasion, was also upregulated in the WOI organoids (Fig. 3F, Fig.S2J). Electron microscopy confirmed the existence of pinopodes, glycogen particles, microvilli and cilia in the endometrial organoids (Fig. 3H). The pinopodes of WOI organoids arranged more densely on the luminal side of epithelium than that of CTRL organoids, which contributes the embryo adhesion. The endometrial epithelium is composed of ciliated cells and non-ciliated cells with microvilli. The microvilli of WOI organoids were densely packed, while cilia were only observed in the WOI organoids. In addition, the WOI organoids had much more glycogen particles than the CTRL group. These structures marked the establishment of endometrial receptivity and benefited for the embryo adhesion to the endometrium.

Other characteristics of the implantation window involved decreased cell proliferation (marked by CDH5 and MEIS1), increased cell differentiation (marked by FGFBP1 and TFF1), extracellular matrix (ECM) remodeling (marked by PDGFB and IGFBP4(33)) and reduced cell adhesion (marked by EFNA5, LAMA3 and RHO GTPase) (Fig. 3B, Fig. S2G). Besides, energy metabolism (represented by SLC25A1(34)) and hypoxia response (represented by HIF1α(20)) were also characteristically enhanced in WOI organoids (Fig. 3B, Fig. S2F~S2H), which maintained the receptive state of endometrial organoids.

Structural cells construct WOI with functionally dynamic changes

Based on the overall WOI associated biological characteristics at the organoid level, we further examined the functionally dynamic changes in structural cells during the WOI. Although the differences between the WOI and SEC organoids were slight at the organoid level, the WOI organoids displayed more thoroughly differentiation to the mid-secretory endometrium than the SEC organoids at the single-cell level.

In terms of energy metabolism, the WOI organoids exhibited upregulation of monocarboxylic acid

and lipid metabolism and hypoxia response. Likewise, the secretory epithelium, critical for the implantation window, accumulated more in the SEC and WOI groups, contributing to cellular metabolic processes and HIF-1 signaling pathway response to hypoxia at the single-cell level (Fig. 4A, Fig. S3E). WOI organoid secretory epithelium exhibited enhanced peptide metabolism and mitochondrial energy metabolism compared to the SEC group, supporting endometrial decidualization and embryo implantation (Fig. 4B). Moreover, increased secretory epithelium and decreased proliferative epithelium in the SEC and WOI organoids suggested the transformation from proliferation to secretory phase, which is an obvious feature of the implantation window (Fig. 4A). Proliferative epithelium differentiates into secretory epithelium under the regulation of branch nodal genes between state 5 and state 6, such as the GAS5, RPL and RPS families, as revealed by single-cell trajectory (Fig. 4C, Fig. S4A~B, Fig. S4G). The WOI organoids showed more thoroughly differentiation from proliferative to secretory epithelium in relation to the SEC organoids (Fig. 4C, Fig. S4A). Overall, the WOI organoids indicated that proliferative epithelium transformed to secretory epithelium with increased energy metabolism and hypoxia response during the implantation window.

Given that the WOI organoids exhibited increased cilia assembly and motile cilia, the transformation and function of the ciliated epithelium gained attention. Single-cell transcriptome analysis revealed hormone treatment increased ciliated epithelium and decreased unciliated epithelium in SEC and WOI groups by altering the expression of key genes (Fig. 4A). These crucial genes, including KRT19, MALAT1, RPL21 and RPS2, expressed divaricately at the fork of differentiation from ciliated cells toward ciliated or unciliated cells (Fig. 4D, Fig. S4C~D, Fig. S4H). Ciliated epithelium was enriched in protein binding, cilium organization, and assembly genes, while unciliated epithelium was enriched in actin cytoskeleton and translation genes (Fig. S3E). The WOI organoids' ciliated epithelium regulated vasculature development and displayed higher transcriptional activity than the SEC group (Fig. S3F). Ciliated epithelium interacts with immune cells and secretory epithelium, modulating angiogenesis via NRP1-VEGFA and NRP2-VEGFA(35) in all groups, with NRP2 highly expressed in WOI organoids (Fig. 4F, Fig. S2K). The WOI ciliated epithelium showed enhanced invasion ability via CD74-COPA(36), CD74-APP(36) and ROR2-WNT5A(37) (Fig. 4F). Thus, WOI organoids revealed accumulated ciliated epithelium's role in preparing the implantation

window.

The WOI organoids displayed upregulated cell differentiation not only at organoid level but also at cellular level, which is represented by EMT. EMT is a common and crucial biological event in the endometrium during the implantation window(38). Synthetic analysis of the transcriptome and proteome revealed increased EMT occurrence in WOI organoids (Fig. S2F). EMT-derived stromal cells, exhibiting gene expression patterns characteristic of both epithelial and stromal cells, are the indication of EMT and more abundant in the SEC and WOI groups (Fig. 4A). Pseudotime trajectory indicated proliferative epithelium transformed into EMT-derived stromal cells, regulated by key genes such as DOC2B, FXYD3, and LPCAT3 (Fig. 4E, Fig. S4E~F, Fig. S4I). The WOI organoids exhibited more thoroughly differentiation to EMT-derived stromal cells than the SEC organoids (Fig. 4E). EMT-derived stromal cells showed alterations in protein binding, cell cycle, organelle organization, and reproduction, with more enhanced lamellipodium-mediated cell migration, cell junction and cytoskeleton regulation in the WOI organoids than the SEC organoids (Fig. S3E~S3F). EMT-derived stromal cells and epithelium cooperated in transitioning from proliferation phase to implantation window (Fig.4G). We found that NRP1 and SLC7A1 were highly expressed by EMT-derived stromal cells, and their receptors (SEMA3A and CSF1) were more upregulated in the epithelium of WOI group than SEC group (Fig.4G). NRP1-SEMA3A has been reported to promote vascularization and responds to hypoxia(39). SLC7A1(40) is the key arginine transporter, and CSF1(41) is a kind of pro-survival cytokines, both of which support receptivity establishment, embryo implantation and development. However, angiogenesis-related and TNF-related pathways were downregulated in WOI organoids (Fig.4G). The functions of NRP1-VEGFB and ADRB2-VEGFB in angiogenesis are not yet clear and require further exploration. Elevated TNF has been reported connected to stromal cell demise and pregnancy failure(42). Compared with common stromal cells, EMT-derived stromal cells communicate slightly differently with epithelial or immune cells. CD44 and CD46, which are involved in cell adhesion(43) and immunoregulation(44), are highly expressed in stromal cells and EMT-derived stromal cells, respectively, and bind separately with SPP1 and JAG1 in epithelial and stromal cells (Fig. 4G, Fig. S3G). In general, WOI organoids demonstrated EMT's role in mediating endometrial transformation towards the implantation window.

Discussion

In our study, we constructed the WOI endometrial organoids, and observed the remarkable resemblance in structure and function to the in vivo endometrium. The organoids consist of three primary types of endometrial cells, specifically epithelial, stromal, and immune cells, with epithelial cells assembling into glands and surrounded by immune cells and stromal cells. This similarity in cellular composition and tissue architecture to the endometrium lays the foundation for simulating receptive endometrium.

Although previous studies treated endometrial organoid with E2, P4, and cAMP to induce a transition to the secretory phase in the endometrium, the methodology to accurately simulate the implantation window or mid-secretory endometrium remains elusive and warrants further investigation. Previous research suggested that placental signals could further promote the differentiation of endometrial organoids (2), and PRL, hCG and HPL have been implicated in processes such as decidualization, implantation, immunoregulation, and angiogenesis (45-47). Detailly, PRL, synthesized by the adenohypophysis, endometrium, and myometrium, plays a vital role in implantation, immunoregulation, and angiogenesis, with the secretory endometrium producing PRL in response to MPA and E2, leading to ciliated cell formation and stromal cell decidualization (2, 45). HCG, secreted by trophoblasts in early pregnancy, influences decidual cells (46) and improves endometrial thickness and receptivity(48). The infusion of hCG increases the levels of key factors associated with endometrial receptivity, such as endocytosis proteins, hypoxia-inducible factor 1 (HIF1), chemokines, and glycodelin (49). HPL contributes to the development and function of uterine glands(47). Consequently, the additional supplementation of PRL, hCG and HPL in our system augmented hormone responsiveness and receptivity, while promoting cell differentiation, ultimately yielding a model more representative of the receptive endometrium.

Here, our WOI organoids display specific features of in vivo receptive endometrium. They show similarly upregulated energy and lipid metabolism to the in vivo implantation window. Enhanced energy metabolism involves monocarboxylic acid metabolism and mitochondrial oxidative phosphorylation. Monocarboxylic acids, exemplified by lactate, pyruvate, and ketone bodies, are vital metabolites in most mammalian cells. During the implantation window, elevated lactate levels

mobilize endometrial monocarboxylic acid metabolism and function as a pregnancy-related signal, stimulating ATP secretion in the epithelium. Subsequently, ATP, the primary product of energy metabolism, induces neighboring epithelial cells to release IL8, promoting decidualization of stromal cells (50). Lipid metabolism, responsible for energy storage, signal transduction, cell proliferation, apoptosis, and membrane trafficking, plays a crucial role in endometrial receptivity and implantation, although its mechanisms remain unclear (51-53). Thus, the WOI organoids build a metabolic framework for simulating in vivo implantation window.

Functional alterations in secretory epithelium and EMT-derived stromal cells at the single-cell level also occur during the implantation window and warrant attention. The secretory epithelium responds to hypoxia through the HIF1 signaling pathway during the implantation window. HIF1A maintains oxygen homeostasis by increasing VEGF levels throughout the menstrual cycle, while HIF2A promotes angiogenesis for embryo implantation (20, 54). Angiogenesis and vascular remodeling support functional endometrial layer repair, endometrial thickening, receptivity, and embryo implantation. Importantly, EMT, a signature transition to the receptive endometrium, occurs in the proliferative epithelium and EMT-derived stromal cells and is involved in opening the epithelial layer of endometrium(38), during which epithelial cells lose polarity and cell-to-cell junctions, stromal cells acquire the migratory and invasive abilities (55). These implantation window associated cellular characteristics are reproduced in the WOI organoids, which confirms that they are currently the closest model to the in vivo state. The WOI organoids will allow us to explore the underlying mechanisms and advance the field of embryo implantation research.

However, our WOI endometrial organoids also have some limitations. We observed a decreased abundance of immune cells, primarily T cells characteristically expressing TSPYL2, RSRP1 and CD44, in WOI organoids, suggesting a possible immunoregulation in preparation for embryo implantation. But the immune cell composition of organoids varies from that of the in vivo endometrium. Although the organoids contain epithelial, stromal and immune cells, making them the closest model to the in vivo endometrium currently available, it is undeniable that they cannot perfectly replicate the in vivo state. The in vivo endometrium consists of functional and basal layers

with more abundant cell subtypes than organoids due to the superior regulation by systemic hormones and nutrients. Moreover, the endometrial organoids lack the mechanical properties of the myometrium that impact in vivo endometrium (56). We are looking forward to constructing the whole uterus in vitro with stem cell induction, 3D printing technology, and microfluidic systems.

In summary, we developed a receptive endometrial organoid that mimics the features of in vivo implantation-window endometrium features (Fig. 5). Our human endometrial organoid comprises epithelium, stromal cells, and immune cells, ensuring hormone responsiveness and endometrial secretion function while recapitulating aspects such as decidualization, ECM remodeling, pinopode formation, cilia generation, EMT and metabolism. This receptive endometrial organoid serves as a platform to investigate endometrial physiology, maternal-fetal interactions, and functional or organic diseases, with potential for functional applications and clinical translation.

Materials and Methods

Establishment of endometrial organoids

All experiments involving human subjects followed medical ethical principles and the Declaration of Helsinki, and were approved by the Ethics Committee of Qilu Hospital of Shandong University. Informed consents were obtained from patients. Females of reproductive age who received hysterectomy for benign diseases were selected for this study. Clinical information of patients providing endometrial tissue was listed in Dataset S1. Experimental procedures are presented in detail in the Supporting Information.

Hormone treatment of endometrial organoids

The hormone regimen for inducing endometrial secretory phase, as described by Thomas E. Spencer et al (7), consisted of estradiol for 2 days followed by a combination of estradiol (Sigma E2758), medroxyprogesterone acetate (Selleck S2567) and N6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (cAMP) (Sigma D0627) for 6 days.

To simulate the endometrium of implantation window, we developed a model by incorporating various pregnancy-related hormones. The hormone regimen included estradiol for the first two days, followed by a combination of estradiol, medroxyprogesterone acetate, cAMP, Human Chorionic Gonadotropin (HCG) (Livzon Pharmaceutical Group Inc), Human Placental Lactogen (HPL) (R&D Systems 5757-PL), and prolactin (Peprotech 100-07) for 6 days. The CTRL group was cultured in ExM without the addition of hormones. (Fig. 1A)

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Author contributions: Y. Zhang, R. Zhao and H. Zhao designed the experiments. Y. Zhang and R. Zhao performed the experiments, analyzed and interpreted data. Y. Zhang wrote the manuscript. R. Zhao, Y. Li, B. Liu, and H. Zhao revised the manuscript. P. Liu provided human endometrium samples. C.Yang, J. Song, T. Li, C. Yin, M. Lu, Z. Hou and C. Zhang provided technical support. H. Zhao, K. Wu and Z.-J.C conceived the project and supervised research. All authors revised and approved the manuscript.

The authors declare no competing interest.

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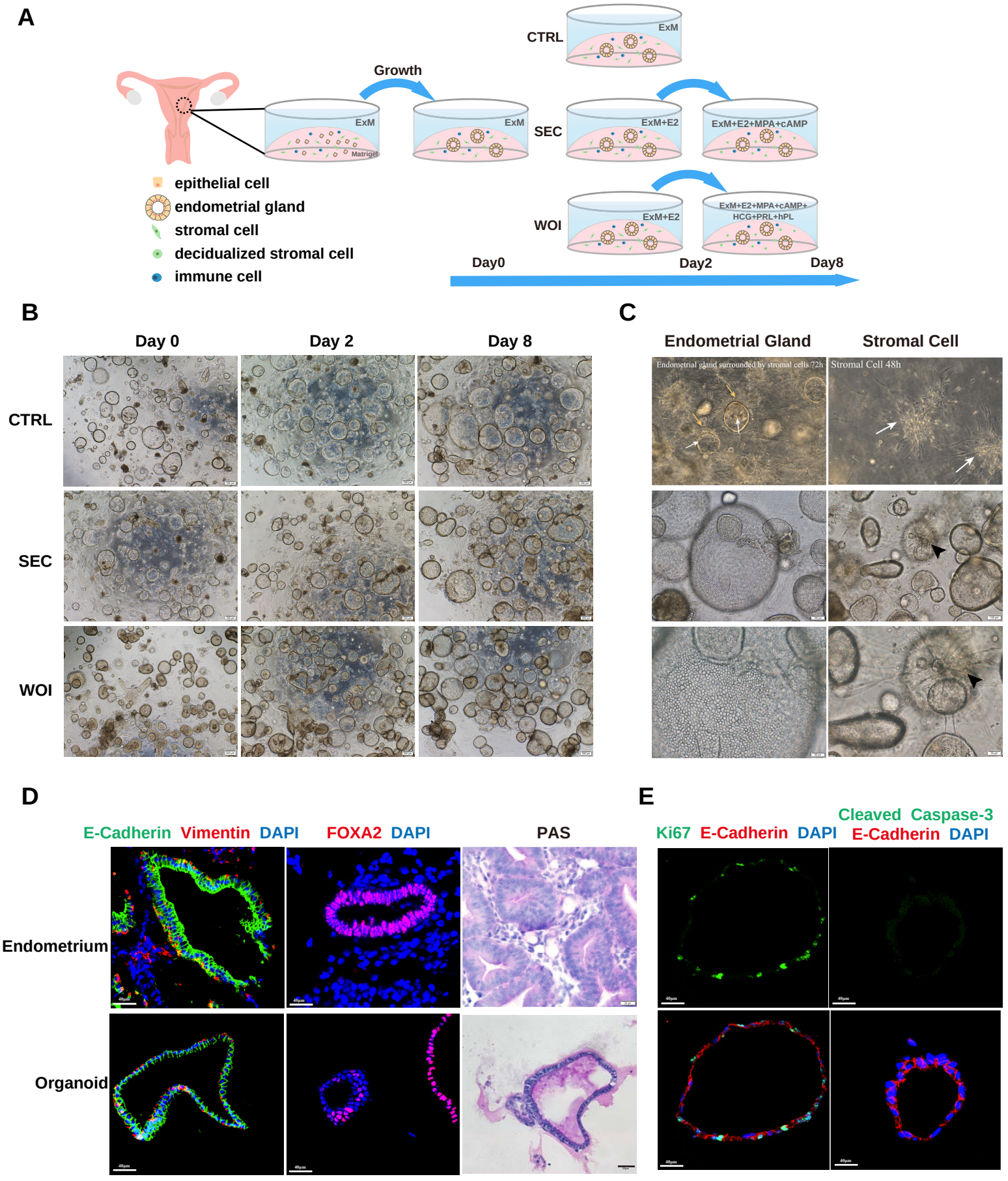
A
CTRL
ExM
Growth
ExM
Matrigel
ExM
SEC
ExM+E2
ExM+E2+MPA+cAMP
WOI
ExM+E2
ExM+E2+MPA+cAMP+
HCG+PRL+hPL
epithelial cell
endometrial gland
stromal cell
decidualized stromal cell
immune cell
Day0
Day2
Day8
B
Day 0
Day 2
Day 8
CTRL
SEC
WOI
C
Endometrial Gland
Stromal Cell
Endometrial gland surrounded by stromal cells 72h
Stromal Cell 48h
D
E-Cadherin Vimentin DAPI
FOXA2 DAPI
PAS
Endometrium
Organoid
E
Ki67 E-Cadherin DAPI
Cleaved Caspase-3
E-Cadherin DAPI

Fig 1 Endometrial organoids possess endometrial morphology and function

(A) Human endometrial organoids constructed from adult stem cells were treated with expansion medium (ExM) (CTRL) or subjected to hormonal stimulation. Timeline of endometrial organoid cultured by ExM (CTRL), ovarian steroid hormones simulating secretory phase (SEC), ovarian steroid hormones combining PRL and placental hormones to mimic the window of implantation (WOI).

(B) Endometrial organoids in the CTRL, SEC and WOI groups displayed similar growth patterns during the culture period. Scale bar = 200 μm.

(C) Screenshot of video S1 showing endometrial glands gradually developing into a vesicular shape, and the surrounding stromal cells arranging in a fibrous pattern in the endometrial organoids (100x, up-left). Screenshot of video S2 displaying the stromal cells growing in fibrous pattern and forming an extensive network in the endometrial organoids (200x, up-right). (The yellow arrows indicate stromal cells and the white arrows indicate endometrial glands). The epithelial cells arrange like paving stones (middle and down, left). Stromal cells formed an extensive network (middle and down, right). The arrowhead indicates stromal cells. Scale bar = 100 μm (middle), Scale bar = 50 μm (down).

(D) Validation of epithelial, stromal cell and endometrial gland markers (E-cadherin, vimentin and FOXA2, respectively) with immunofluorescence (IF) in the endometrium in vivo and endometrial organoids in vitro. Nuclei were counterstained with DAPI. Scale bar = 40 μm. of endometrium in vivo and endometrial organoid in vitro. Scale bar = 20 μm.

(E) IF analysis of proliferation and apoptosis indicated by Ki67 and cleaved caspase-3, respectively. Nuclei were counterstained with DAPI. Scale bar = 40 μm.

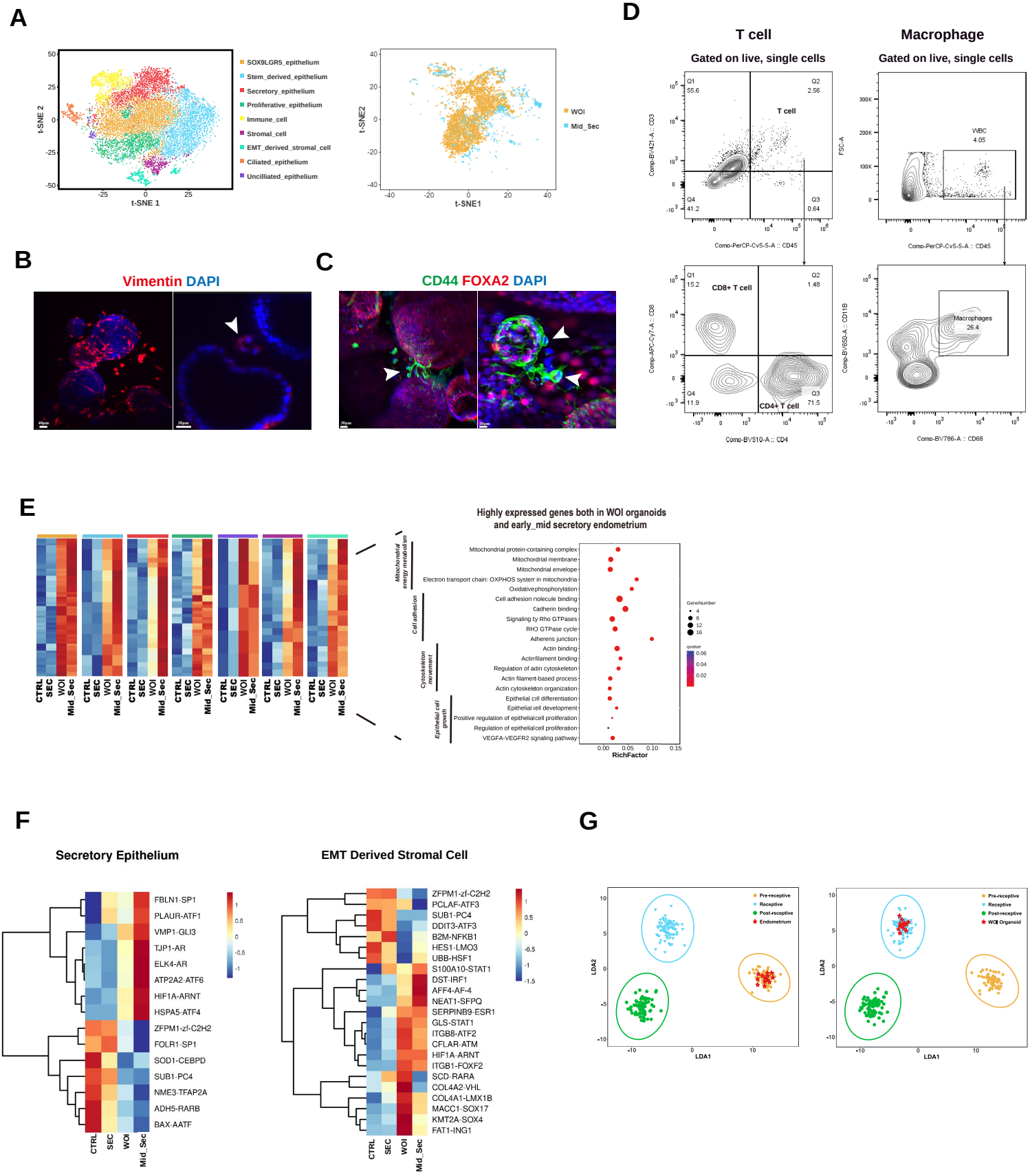
A
t-SNE 2
t-SNE 1
SOX9LGR5_epithelium
Stem_derived_epithelium
Secretory_epithelium
Proliferative_epithelium
Immune_cell
Stromal_cell
EMT_derived_stromal_cell
Ciliated_epithelium
Uncilliated_epithelium
t-SNE2
t-SNE1
WOI
Mid_Sec
B
Vimentin DAPI
C
CD44 FOXA2 DAPI
D
T cell
Macrophage
Gated on live, single cells
Gated on live, single cells
T cell
CD8+ T cell
CD4+ T cell
Macrophages
Comp-PerCP-Cy5-5-A :: CD45
Comp-BV421-A :: CD3
Comp-APC-Cy7-A :: CD8
Comp-BV510-A :: CD4
Comp-BV650-A :: CD11B
Comp-BV786-A :: CD68
FSC-A
E
Highly expressed genes both in WOI organoids
and early_mid secretory endometrium
CTRL
SEC
WOI
Mid_Sec
Mitochondrial energy metabolism
Cell adhesion
Cytoskeleton movement
Epithelial cell growth
Mitochondrial protein-containing complex
Mitochondrial membrane
Mitochondrial envelope
Electron transport chain: OXPHOS system in mitochondria
Oxidative phosphorylation
Cell adhesion molecule binding
Cadherin binding
Signaling by Rho GTPases
RHO GTPase cycle
Adherens junction
Actin binding
Actin filament binding
Regulation of actin cytoskeleton
Actin filament-based process
Actin cytoskeleton organization
Epithelial cell differentiation
Epithelial cell development
Positive regulation of epithelial cell proliferation
Regulation of epithelial cell proliferation
VEGFA-VEGFR2 signaling pathway
RichFactor
GeneNumber
qvalue
F
Secretory Epithelium
EMT Derived Stromal Cell
FBLN1-SP1
PLAUR-ATF1
VMP1-GLI3
TJP1-AR
ELK4-AR
ATP2A2-ATF6
HIF1A-ARNT
HSPA5-ATF4
ZFPM1-zf-C2H2
FOLR1-SP1
SOD1-CEBPD
SUB1-PC4
NME3-TFAP2A
ADH5-RARB
BAX-AATF
ZFPM1-zf-C2H2
PCLAF-ATF3
SUB1-PC4
DDIT3-ATF3
B2M-NFKB1
HES1-LMO3
UBB-HSF1
S100A10-STAT1
DST-IRF1
AFF4-AF-4
NEAT1-SFPQ
SERPINB9-ESR1
GLS-STAT1
ITGB8-ATF2
CFLAR-ATM
HIF1A-ARNT
ITGB1-FOXF2
SCD-RARA
COL4A2-VHL
COL4A1-LMX1B
MACC1-SOX17
KMT2A-SOX4
FAT1-ING1
G
Pre-receptive
Receptive
Post-receptive
Endometrium
WOI Organoid
LDA1
LDA2

Fig 2 Developing receptive endometrial organoids in vitro mimicking the implantation-window endometrium

(A) T-SNE plot of scRNA-seq data from three individual endometrial organoids of the CTRL, SEC and WOI groups (left). T-SNE plot of combined scRNA-seq data from the three kinds of organoids and mid-secretory endometrium (right).

(B) Exhibition of stromal cell marked by vimentin through combination of organoid clearing, IF and light sheet microscopy. Nuclei were counterstained with DAPI. The arrowhead indicates stromal cells. Scale bar = 40 μm (left), Scale bar = 30 μm (right).

(C) Exhibition of immune cell marked by CD44 and endometrial gland marked by FOXA2 through combination of organoid clearing, IF and light sheet microscopy. Nuclei were counterstained with DAPI. The arrowhead indicates immune cells. Scale bar = 30 μm (left), Scale bar = 10 μm (right).

(D) Flow cytometric analysis of T cells and macrophages in the endometrial organoid. Gating strategy used for determining white blood cells ($CD45^+$ cells), T cells ($CD45^+CD3^+$ cells) and macrophages ($CD45^+CD68^+CD11b^+$ cells).

(E) Heatmap and bubble diagram illustrating highly expressed genes as well as GO functions enriched in both organoids during the WOI and mid secretory endometrial tissue in terms of SOX9+LGR5+ epithelium, stem-derived epithelium, secretory epithelium, proliferative epithelium, unciliated epithelium, stromal cells and EMT-derived stromal cells. The color of heatmap represents log-transformed fold change of gene expression.

(F) Heatmaps showing differentially expressed TFs of endometrial organoids and endometrium in the secretory epithelium (left) and EMT-derived stromal cells (right). The color represents log-transformed fold change of gene expression.

(G) Endometrial receptivity evaluation of endometrium and their derived WOI organoids through ERT. Asterisks indicate individual samples.

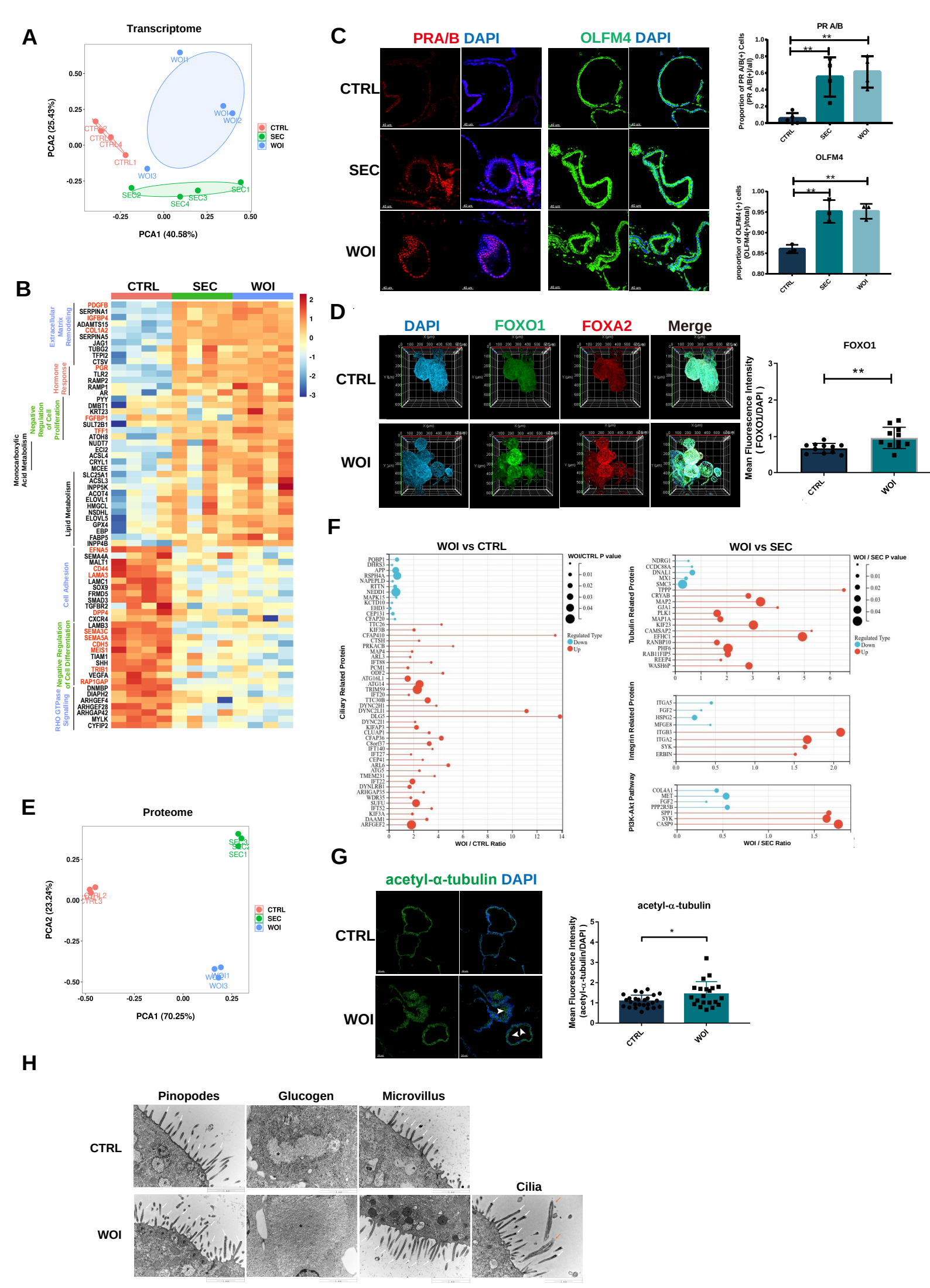
A
Transcriptome
PCA2 (25.43%)
PCA1 (40.58%)
CTRL
SEC
WOI
B
CTRL
SEC
WOI
Extracellular Matrix Remodeling
Hormone Response
Negative Regulation of Cell Proliferation
Monocarboxylic Acid Metabolism
Lipid Metabolism
Cell Adhesion
Negative Regulation of Cell Differentiation
RHO GTPase Signalling
C
PRA/B DAPI
OLFM4 DAPI
CTRL
SEC
WOI
PR A/B
Proportion of PR A/B(+) Cells (PR A/B(+)/all)
OLFM4
proportion of OLFM4 (+) cells (OLFM4(+)/total)
D
DAPI
FOXO1
FOXA2
Merge
CTRL
WOI
FOXO1
Mean Fluorescence Intensity (FOXO1/DAPI)
E
Proteome
PCA2 (23.24%)
PCA1 (70.25%)
CTRL
SEC
WOI
F
WOI vs CTRL
WOI/CTRL P value
Regulated Type
Down
Up
Ciliary Related Protein
WOI / CTRL Ratio
WOI vs SEC
WOI / SEC P value
Tubulin Related Protein
Integrin Related Protein
PI3K-Akt Pathway
WOI / SEC Ratio
G
acetyl-α-tubulin DAPI
CTRL
WOI
acetyl-α-tubulin
Mean Fluorescence Intensity (acetyl-α-tubulin/DAPI)
H
Pinopodes
Glucogen
Microvillus
Cilia
CTRL
WOI

Fig 3 Receptive endometrial organoid exhibits the implantation window at the transcription and protein levels

(A) Principal component analysis (PCA) plot computed with differentially expressed genes in the bulk transcriptome of endometrial organoids belonging to the CTRL, SEC and WOI groups.

(B) Heatmap showing that enrichment of differentially expressed genes for the terms of extracellular matrix remodeling, hormone response, negative regulation of cell proliferation, monocarboxylic acid metabolism, lipid metabolism, cell adhesion, negative regulation of cell differentiation and RHO GTPase signaling. The color represents log-transformed fold change of gene expression.

(C) Responsiveness to progesterone and estrogen was evaluated by IF to PRA/B and OLFM4 with IF respectively. Scale bar = 40 μm, **$P \leq 0.005$.

(D) Exhibition of implantation marker (FOXO1) and endometrial gland marker (FOXA2) through combination of organoid clearing, IF and light sheet microscopy. Nuclei were counterstained with DAPI. **$P \leq 0.005$.

(E) PCA plot computed with differentially expressed proteins in the microproteomics of endometrial organoids belonging to the CTRL, SEC and WOI groups.

(F) Dot bar diagram exhibiting protein expression levels related to cilia in the CTRL and WOI groups (left). Dot bar diagram displaying tubulin, integrin and PI3K-AKT pathway-related protein expression levels in the SEC and WOI groups (right).

(G) IF analysis of cilia assembly marked by acetyl-α-tubulin. Nuclei were counterstained with DAPI. The arrowhead indicates cilia. Scale bar = 50 μm. *$P \leq 0.05$.

(H) Electron micrograph of the CTRL (top) and WOI (bottom) endometrial organoid showing pinopodes (P), glycogen granule (asterisk), microvilli (white arrows) and cilia (orange arrows). Scale bar = 1 μm.

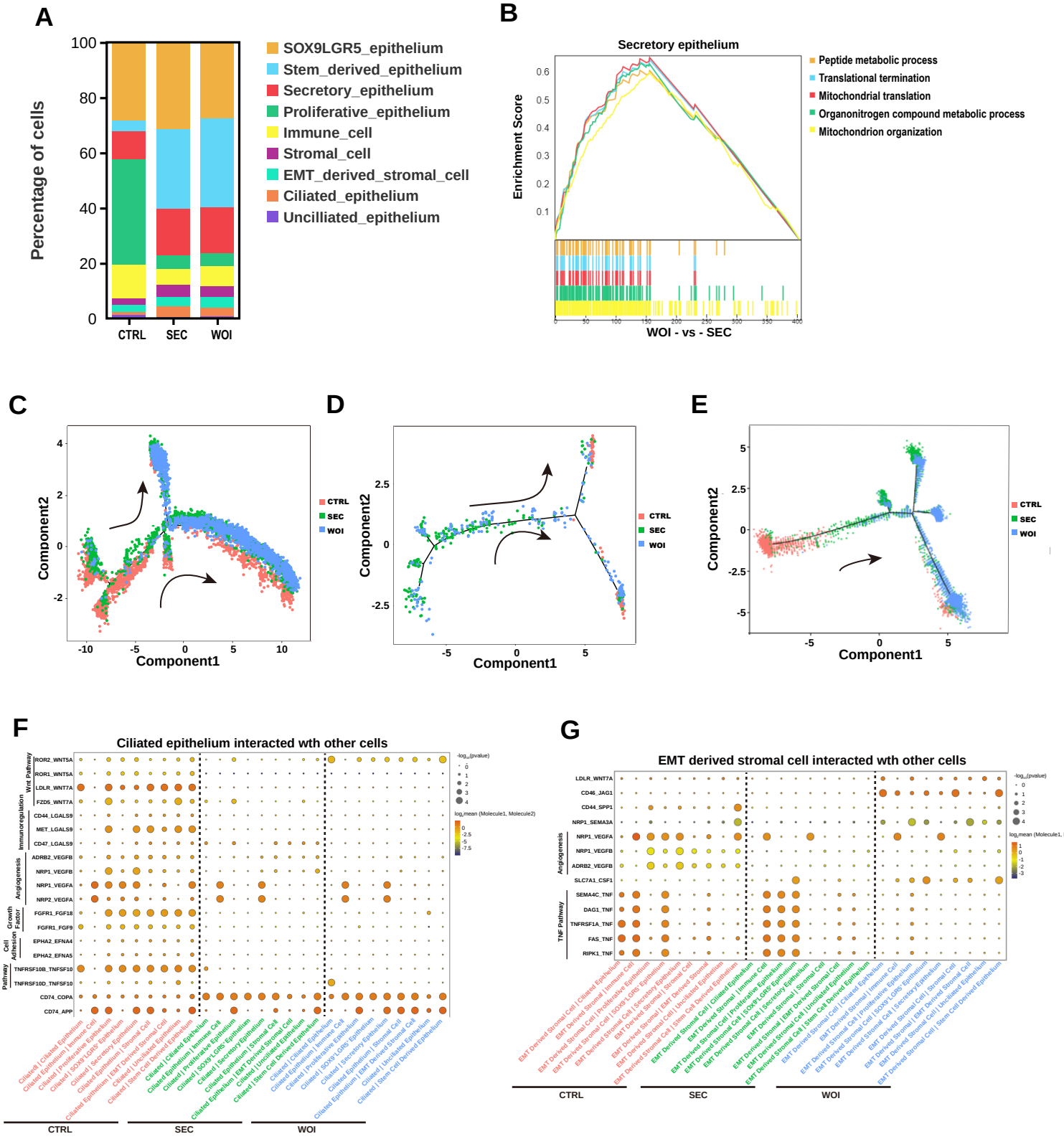
A
Percentage of cells
100
80
60
40
20
0
CTRL
SEC
WOI
SOX9LGR5_epithelium
Stem_derived_epithelium
Secretory_epithelium
Proliferative_epithelium
Immune_cell
Stromal_cell
EMT_derived_stromal_cell
Ciliated_epithelium
Uncilliated_epithelium
B
Secretory epithelium
Enrichment Score
0.6
0.5
0.4
0.3
0.2
0.1
Peptide metabolic process
Translational termination
Mitochondrial translation
Organonitrogen compound metabolic process
Mitochondrion organization
WOI - vs - SEC
C
Component2
Component1
CTRL
SEC
WOI
D
Component2
Component1
CTRL
SEC
WOI
E
Component2
Component1
CTRL
SEC
WOI
F
Ciliated epithelium interacted wth other cells
Wnt Pathway
Immunoregulation
Angiogenesis
Growth Factor
Cell Adhesion
TNF Pathway
ROR2_WNT5A
RORI_WNT5A
LDLR_WNT7A
FZD5_WNT7A
CD44_LGALS9
MET_LGALS9
CD47_LGALS9
ADRB2_VEGFB
NRP1_VEGFB
NRP1_VEGFA
NRP2_VEGFA
FGFR1_FGF18
FGFR1_FGF9
EPHA2_EFNA4
EPHA2_EFNA5
TNFRSF10B_TNFSF10
TNFRSF10D_TNFSF10
CD74_COPA
CD74_APP
CTRL
SEC
WOI
G
EMT derived stromal cell interacted wth other cells
Angiogenesis
TNF Pathway
LDLR_WNT7A
CD46_JAG1
CD44_SPP1
NRP1_SEMA3A
NRP1_VEGFA
NRP1_VEGFB
ADRB2_VEGFB
SLC7A1_CSF1
SEMA4C_TNF
DAG1_TNF
TNFRSF1A_TNF
FAS_TNF
RIPK1_TNF
CTRL
SEC
WOI

Fig 4 Receptive endometrial organoid exhibits the implantation window at single cell level

(A) Bar graph exhibiting various percentages of each cell type in the three groups.

(B) Gene set enrichment analysis (GSEA) between the SEC and WOI groups for the secretory epithelium.

(C-E) Pseudotime trajectory showing the transformation between proliferative and secretory epithelium (C), ciliated and unciliated epithelium (D), proliferative epithelium and EMT-derived stromal cells (E) in the CTRL, SEC and WOI groups. Arrows indicate the direction of the pseudotime trajectory.

(F-G) Dot plots demonstrating the Cellphone DB analysis of relevant receptors and ligands of ciliated epithelium (F) and EMT-derived stromal cells (G) with other cell types. The size of the dot represents the level of significance. The color of the dot indicates the mean of the average expression level of interacting molecule 1 in ciliated epithelium or EMT-derived stromal cells and molecule 2 in other cell types.

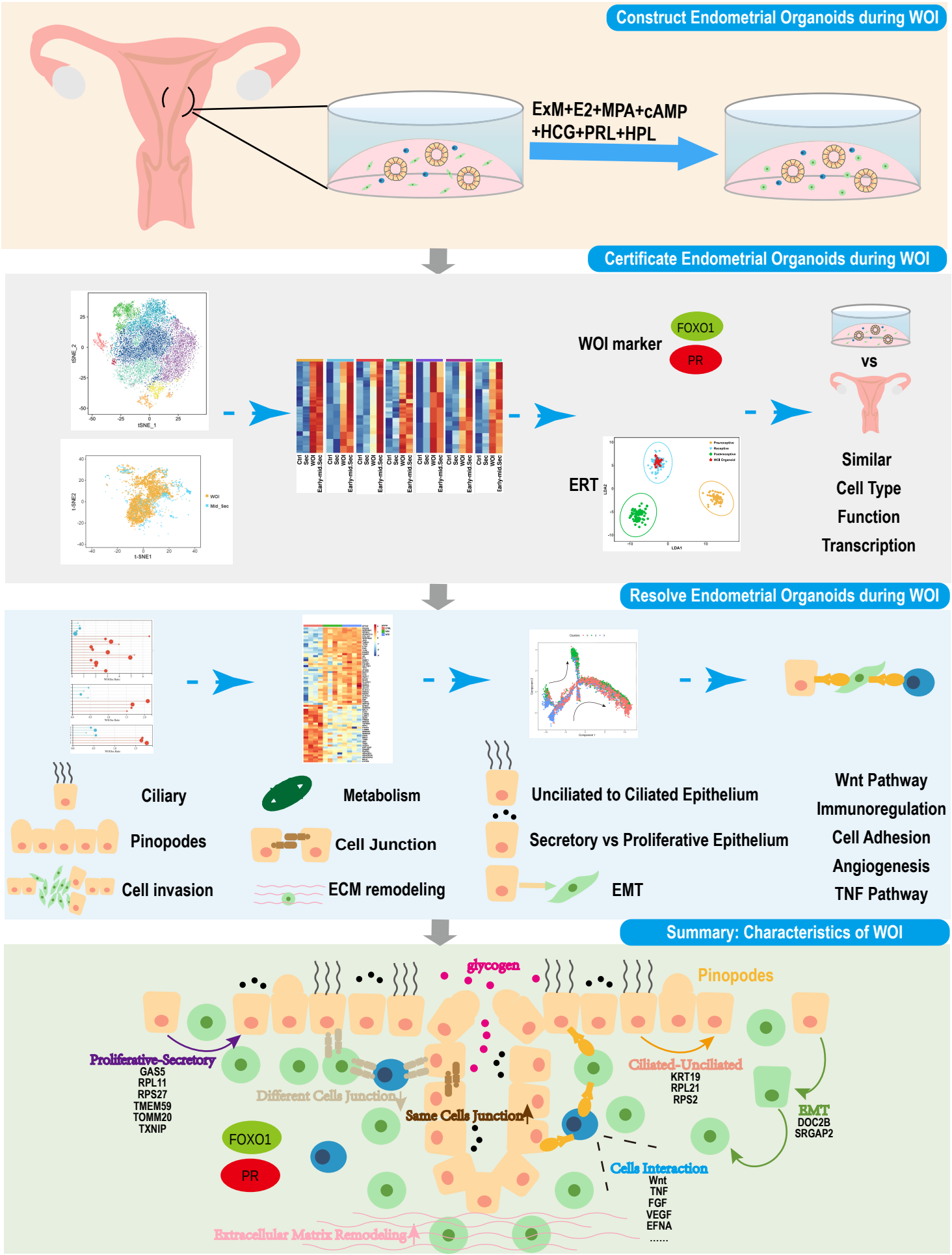
Construct Endometrial Organoids during WOI
ExM+E2+MPA+cAMP
+HCG+PRL+HPL
Certificate Endometrial Organoids during WOI
WOI marker
FOXO1
PR
ERT
vs
Similar
Cell Type
Function
Transcription
Resolve Endometrial Organoids during WOI
Ciliary
Pinopodes
Cell invasion
Metabolism
Cell Junction
ECM remodeling
Unciliated to Ciliated Epithelium
Secretory vs Proliferative Epithelium
EMT
Wnt Pathway
Immunoregulation
Cell Adhesion
Angiogenesis
TNF Pathway
Summary: Characteristics of WOI
glycogen
Pinopodes
Proliferative-Secretory
GAS5
RPL11
RPS27
TMEM59
TOMM20
TXNIP
Different Cells Junction
Same Cells Junction
Ciliated-Unciliated
KRT19
RPL21
RPS2
EMT
DOC2B
SRGAP2
FOXO1
PR
Cells Interaction
Wnt
TNF
FGF
VEGF
EFNA
.....
Extracellular Matrix Remodeling

Fig.5 Schematic diagram displaying the establishment and validation of receptive endometrial organoids, and summarizing the characteristic biological events of endometrial implantation window.