



# Endometrial membrane organoids from human embryonic stem cell combined with the 3D Matrigel for endometrium regeneration in asherman syndrome

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## ABSTRACT

Asherman's syndrome (AS), a leading cause of uterine infertility worldwide, is characterized by scarring of the uterine surfaces lacking endometrial epithelial cells, which prevents endometrial regeneration. Current research on cell therapy for AS focuses on mesenchymal and adult stem cells from the endometrium. However, insufficient number, lack of purity, and rapid senescence of endometrial epithelial progenitor cells (EPCs) during experimental processes restrict their use in cell therapies. In this study, we induced human embryonic stem cells-9 (H9-ESC) into EEPs by optimizing the induction factors from the definitive endoderm. EEPs, which act as endometrial epithelial cells, accompanied by human endometrial stromal cells provide a niche environment for the development of endometrial membrane organoids (EMOs) in an *in vitro* 3D culture model. To investigate the function of EMOs, we transplanted tissue-engineered constructs with EMOs into an *in vivo* rat AS model. The implantation of EMOs into the damaged endometrium facilitates endometrial regeneration and angiogenesis. Implanting EMOs developed from human embryonic stem cells into the endometrium might prove useful for "endometrial re-engineering" in the treatment of Asherman's syndrome.

## 1. Introduction

Asherman's syndrome (AS), characterized by obliteration of the uterine cavity with adhesions, is a leading cause of uterine infertility worldwide [1]. Periodic abdominal pain, abnormal menstruation, and subfertility, including recurrent pregnancy loss, are common manifestations of AS. Treatments for AS include hysteroscopic lysis of adhesions, hormonal therapy, and insertion of physical barriers [2]. The primary pathophysiology is damage to the basilar layer of the endometrium, with loss of endometrial stem cells, following surgical curettage. Strategies to regenerate the damaged endometrial tissue via transplantation of endometrial epithelial progenitor cells (EPCs) could be more effective in the treatment of AS than the current therapies. Adult endometrial stem cells [3], mesenchymal stem cells [4], bone marrow-derived mesenchymal stem cells [5] (BMDSC), and human umbilical cord

Wharton's jelly derived mesenchymal stem cells [6] have potential therapeutic value as they can cross lineage barriers to differentiate into specialized cell types of some organs, including the endometrium [7,8]. However, insufficient yield, lack of purity, and rapid senescence during experimental procedures restrict their use in clinical practice. EEPs derived from human embryonic stem cells are relatively easy to obtain and hence can be prospectively used for the treatment of AS. The generation of a human female reproductive tract epithelium from human embryonic stem cells has been reported [9], although definitive endometrial epithelial progenitor cells and endometrial tissues have not been derived from human embryonic stem cells.

During embryogenesis, the definitive endoderm gives rise to the epithelial lining of the respiratory tract [10], digestive tract, liver [11], pancreas [12], thyroid [13], and thymus [14]. EEPs have an epithelial lineage, and are derived from epiblast cells undergoing

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epithelial-mesenchymal transition (EMT) in the primitive streak, which alter the expression of E-cadherin proteins on their cell surface [15]. The molecular pathways leading to the specification and terminal differentiation of EEPs from human embryonic stem cells during embryogenesis are still unclear. The process of adult stem cell differentiation involve the following: 1) multipotency, 2) asymmetric cell division, 3) quiescence, 4) self-renewal, 5) undifferentiated state, and 6) *in vivo* reconstitution of an adult stem cell system via long-term repopulation [16]. Significantly, a specific “niche” is required for each type of adult stem cell to perform its stem cell activity. However, stem cell niches are often difficult to reproduce *in vitro* [17]. Specific markers of the precursor cells are another key factor in their differentiation. Many human EEP markers demonstrate stem cell origin, but the characterization of their endometrial specificity has proved difficult. Recent studies indicate that FOXA2 is a specific endometrial epithelial gland marker [18], and that SOX17 is a key player in human endometrial receptivity and embryo implantation [19]. Furthermore, the genome sequence landscape suggests that FOXA2 and SOX17 become transcriptional enhancers in endometrial cancer [20].

The results of stem cell treatment of AS in animal models and clinical trials are inconsistent. AS mouse models have been developed by traumatizing the lumens of both uterine horns. Bone marrow-derived mesenchymal stem cells (BMDSCs) are recruited to the endometrium in response to injury. Fertility improves after BMDSC transplantation in AS mice, demonstrating the functional role of these cells in uterine repair [21]. In a rat model of partial, full-thickness uterine excision, the collagen/BMDSC system also increased the proliferation of the endometrium [5]. Hyaluronic acid hydrogel integrated with the mesenchymal stem cell secretome produced endometrial regeneration in a rat model of AS [22]. However, in a bone marrow transplant (BMT) mouse model, BMDSCs could engraft to the endometrium, but only to the stromal compartment. Only a portion of stromal cells, but not endometrial or epithelial cells, originate from the bone marrow [23]. Moreover, in an irradiated BMT mouse model, no bone marrow-derived stroma, epithelium, or endothelium was observed in the endometrium [24]. In clinical trials, transplantation of endometrial angiogenic stem cells isolated from autologous adult stem cells [25], autologous mononuclear stem cells [26], collagen scaffolds with autologous bone marrow mononuclear cells [27], and menstrual blood-derived stem cells [28] have been reported to increase the endometrial thickness resulting in menstruation or pregnancy. Nevertheless, the choice of the source of epithelial cells and functional cells of the endometrium for transplantation still poses a challenge. Further efforts to create robust EEPs and an endometrium on biomaterials or scaffolds to deliver therapeutic cells to the site of tissue injury are necessary. Organoids are a self-organizing 3D culture system, made of progenitor and differentiated cells that are analogous to natural tissues. Human organoids have been derived from tissue-resident adult epithelial cells from the gut, liver, pancreas, prostate, and fallopian tubes [29–35]. The organoids simulate the features of uterine glands *in vitro*, and are able to respond to hormonal signals, produce endometrial secretions, and differentiate into ciliated cells. Since human endometrial glands were first established *in vitro* [34], endometrium-like organoids have been developed from the mouse endometrium and human endometrium [35,36]. However, reconstructing the human endometrium in traditional culture systems is limited by the inability to reproduce a functional endometrial barrier that is comparable to the normal human endometrium.

In this study, FOXA2 and SOX17 were used as definitive markers of endometrial glandular epithelial progenitors. The human embryonic stem cell-9 line (H9-ESC) was differentiated into EEPs and EMOs. To monitor differentiation, GFP-labeled H9-ESCs (H9-ESC-GFP+) were used for tracing. Human endometrial stromal cells were used to create an *in vitro* niche environment, and H9-ESC-derived EEPs were seeded in a modified 3D model to generate endometrial membrane organoids (EMOs). EMOs were implanted into the injured endometrium and its regenerative potential was assessed in rat models of AS.

## 2. Materials and methods

### 2.1. Culture of hESCs

The NIH-registered H9-ESC line was isolated and established at the University of Wisconsin, and the H9-ESC-GFP+ cell line was established in a stem cell lab at the National University of Singapore [37]. The experiments using H9-ESC and H9-ESC-GFP+ cells that were performed at the National University of Singapore were approved by the Stem Cell Oversight Committee (MOU #04W093, SLA #05-W074). Subsequently, H9-ESC and H9-ESC-GFP+ cells were sub-cultured on 1:30 Matrigel (BD Biosciences, #354277) in complete mTeSR1 medium (StemCell Technologies) and incubated at 37 °C with 5% CO<sub>2</sub>. Finally, H9-ESC retained their undifferentiated state and consistently expressed GFP.

### 2.2. Establishment of endometrial organoids

To model the fetal endometrium, we established a modified EMO 3D culture on Matrigel (BD Biosciences, #354277) to optimize the culture system *in vitro* and for comparison with the 3D culture, as described previously [38]. The human endometrial stromal cell line (ATCC, CRL-4003) was cultured in DMEM/F12 (Gibco, 12634010) supplemented with 10% FBS (Sigma-Aldrich, F8318) in culture flasks. To mimic the intrauterine growth space, a model group was coated with Matrigel on five sides. In brief, eight-well chamber slides (Ibidi, Germany) were coated with 40 µL of Matrigel on each side for 30 min at 37 °C and, subsequently,  $2 \times 10^5$  cells of the human endometrial stroma cell line (ATCC, CRL-4003) were seeded and mixed with confluent  $2 \times 10^5$  EEPs, and human endometrial stroma cells were used to provide niche cells. The control group conditions were as described by Nguyen et al. [38]. Briefly,  $2 \times 10^5$  EEPs were mixed with Matrigel (1:1) in 200 µL of a stromal cell suspension and were seeded on the five sides of the slides, including the bottom layer. The epithelial/stromal-Matrigel mixtures were set at 37 °C for 30 min before culture medium supplemented with 10% FBS, 2 mM GlutaMAX (Life Technologies, 35050-061), and 50 ng/mL epidermal growth factor (EGF, Gibco, PHG0311L) was added. Estrogen (100 nM; E2, Sigma-Aldrich, E-060) was added to each well for 6 days, followed by 6 days of estrogen replacement with 100 nM E2 + 1 µM progesterin (P4, Sigma-Aldrich, P-069) and 6 days of the original medium without any hormone supplement. The culture medium was changed every two days. Differentiation of H9-ESC-GFP+ cells into EMOs was monitored using a microscope (OLYMPUS IX53).

### 2.3. Murine model of AS and EMO implantation

All animal experiments were approved by the Animal Ethics Committee of Zhejiang University (15251). Sprague Dawley (SD) female rats (8-week-old) were used to establish an AS injury model [21]. In brief, after administration of chloral hydrate, a small incision was made in each uterine horn at the utero-tubal junction and the horn was traumatized in a standardized fashion using a 27 Gauge needle inserted two-thirds of the way through the lumen, rotated, and withdrawn four times. After establishing the injury, different treatments were conducted in each group (n = 10) via direct injection into the uterine cavity through the previous incision: 1) control group: left untreated; 2) model group: right side of the untreated uterus after molding; 3) EPPC/Matrigel group (Model + EEP group): the right side of the uterus received only an EPPC-loaded Matrigel solution, while the left side was untreated; 4) EMO/Matrigel group (Model + EMO group): the right side of the uterus received an EMO-loaded Matrigel solution (cells were detached using dispase; protein concentration: 10 mg/mL), while the left side was untreated. The incisions on the uterus and abdomen were closed using sutures. Cohorts of rats with endometrial injury were sacrificed two weeks after receiving intrauterine EMO/Matrigel injections. The uterine horns were collected, sectioned longitudinally, and stained with hematoxylin and eosin to evaluate the histological evidence

of fibrosis. Immunofluorescence analysis was performed using vimentin, CK19, and CD34 antibodies.

### 3. Results

#### 3.1. H9-ESCs give rise to endometrial epithelial progenitor cells (EEPCs)

H9-ESC-GFP<sup>+</sup> and H9-ESC were differentiated using a two-stage, single-cell method. First, H9-ESCs were differentiated into definitive endoderm epithelial cells (H9-ESC-DECs). H9-ESC-GFP<sup>+</sup> cells underwent the same process to trace H9-ESC differentiation throughout the experiment. Subsequently, H9-ESC-DECs were differentiated into EEPCs. At stage 1, serial subculture and round-shaped endoderm epithelial cells with a homogeneous morphology were observed (Fig. 1B). After five passages, the cells showed no signs of senescence. H9-ESC-DECs were analyzed on day 0 (D0), D5, D6, and D7. The phenotype of H9-ESC-DECs was characterized using qRT-PCR and flow cytometry. The qRT-PCR analysis of H9-ESC-DECs demonstrated the downregulation of transcripts related to pluripotency (OCT4 and NANOG) and definitive endoderm (GSC and CXCR4) lineage, accompanied by the upregulation of an epithelial, lineage-associated transcript (E-cadherin), but also showed the downregulation of a mesenchymal lineage-associated transcript (Bglap). FOXA2- and SOX17-related endoderm lineage was upregulated on D5, D6, and D7. A slight downregulation of FOXA2 with a slight upregulation of SOX17 on D7 was observed in H9-ESC-DECs compared to that on D5. There was no statistical difference between the FOXA2 and SOX17 expression levels in H9-ESC-DECs at stage 1 on D5 and D7 (Fig. 1E). FOXA2 and SOX17 were not expressed at stage 1 on D0. According to our previous studies, FOXA2 and SOX17 were not expressed in the ESC stage, when cells were not differentiated [11,39]. A total of  $20 \times 10^6$  H9-ESC-DECs were analyzed using flow cytometry, and the results indicated a strong expression of CXCR4 (98.3%) and E-cadherin (92.4%) on D5, and a reduced expression of CXCR4 (95.9%) and E-cadherin (92.0%) on D6 and of CXCR4 (51.2%) and E-cadherin (88.7%) on D7 (Fig. 1F). Cells on D5 were collected for endometrial epithelial progenitor cell differentiation without sorting.

The critical point to define the optimal conditions is to maximize the induction of the expression of FOXA2 and SOX17, and to minimize the induction of genes representing other tissues. At stage 2, to optimize the differentiation medium for EEPC, M1, M2, and M3 culture media were compared based on the maintenance of the endometrial epithelial progenitor cells. The EEPC differentiation ability of H9-ESC-DECs was characterized after three days using qRT-PCR and immunofluorescence. The estrogen receptor (ESR) was expressed in all three groups. qRT-PCR analysis revealed that FOXA2 was expressed in hESC-EEPCs in the M1 of the EGF growth factor group, but not in the M2 without growth factor, and in M3 with full epithelial growth factor. Non-endometrial, epithelial lineage markers, including AFP (hepatocyte) and NKX2.1 (lung and thyroid), were not expressed in the M1 group but were expressed in the M2 and M3 groups. Non-endometrial epithelial lineage markers, including CDX2 (intestinal lineage) and PDX1 (endocrinal pancreas and stomach lineage), were expressed in all three groups. To mimic the endometrial conditions, E2 was added to the H9-ESC-DECs in the M1, M2, and M3 groups at different concentrations (0 nM, 100 nM, or 1  $\mu$ M) for 3 days. qRT-PCR analysis of cells exposed to E2 demonstrated a significant upregulation of FOXA2 transcripts in all three groups at D3. Furthermore, an E2 concentration of 100 nM was the optimal concentration for inducing FOXA2 expression, but there was no significant effect on the ESR expression in all three groups. E2 promotes non-endometrial epithelial gene transcription at specific concentrations. For NKX2.1, CDX1, and PDX1, an E2 concentration of 100 nM was optimal for promoting low transcription levels of these genes, with the exception of AFP in the M2 group, in which E2 promoted high levels of AFP transcription (Fig. 2).

Flow cytometry analysis of EEPCs indicated that the EEPC purity was

98%, accompanied by a strong expression of CD184 (98.3%) and CD324 (97%) when the cells were maintained in M1 conditions for three days without sorting (Fig. 3A). qRT-PCR analysis demonstrated a significant upregulation of FOXA2 and SOX17 transcripts, which is associated with endometrial epithelial progenitor differentiation (Fig. 3B). The expression of FOXA2 and SOX17 was positive, but vimentin (Fig. 3C) was negative in the immunofluorescent staining analysis, demonstrating the homogeneity of endometrial epithelial progenitor cells.

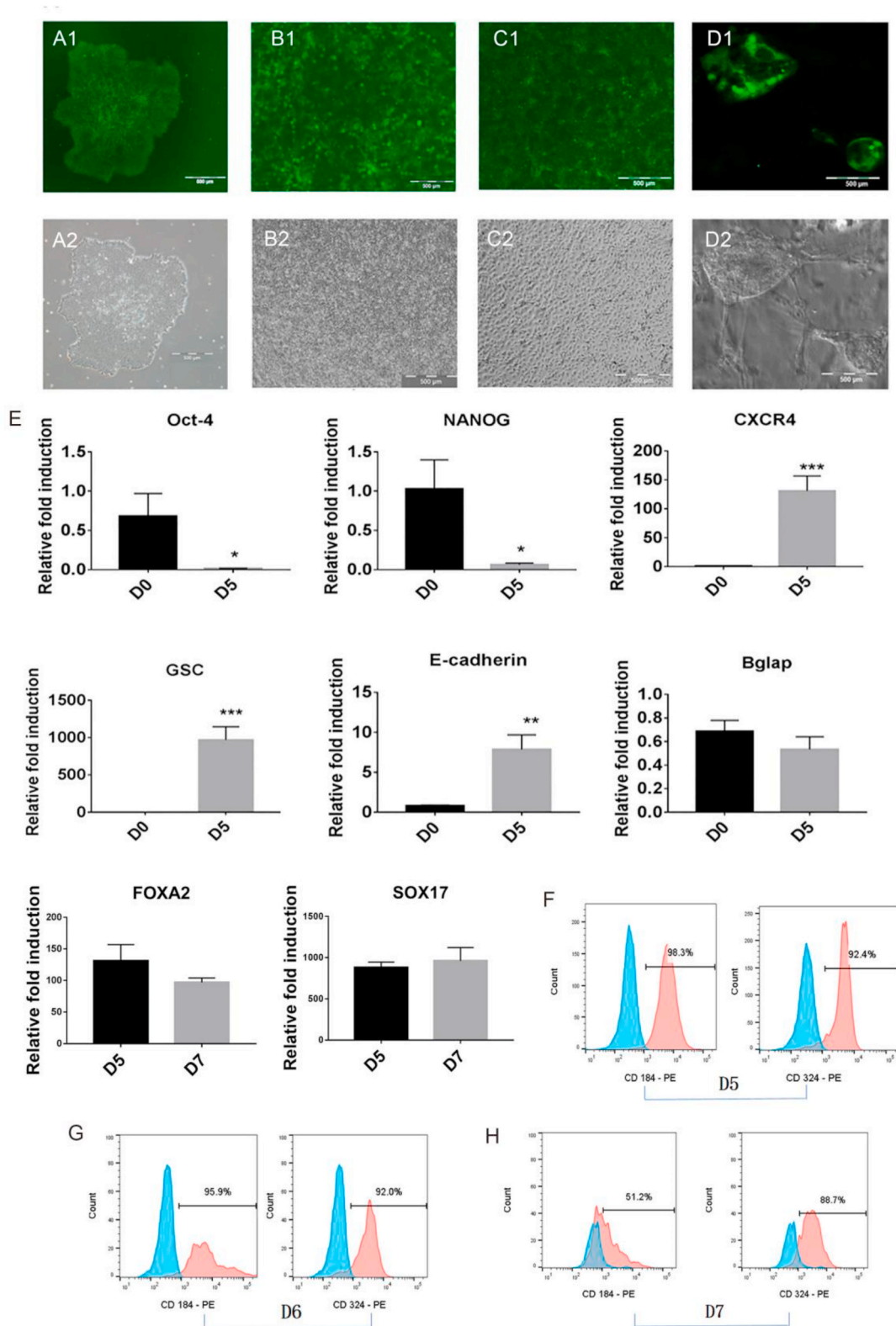
As shown in Fig. 1C, there was no significant difference in the morphology of EEPCs treated with different media, as assessed using microscopy (Fig. 3D). The M1 group treated with 100 nM E2 was identified as an optimal environment for EEPC differentiation in organoid cultures. Therefore, the developed protocol for culturing the cells under enhanced conditions in M1 led to a significant increase in the generation of EEPCs. From the perspective of scaling up the production of these cells, the optimal conditions involve reducing the culture time to eight days, including five days for definitive endoderm differentiation and three days for EEPC differentiation. EGF is a key inducer that promotes the differentiation of endoderm cells into EEPCs.

#### 3.2. Characterization of human endometrial stromal cell-EEPC loaded 3D culture

Basement membranes are continuous sheets of specialized extracellular matrixes that can be found at both the dermal-epidermal junction and the base of all lumen-lining epithelia throughout the digestive, respiratory, and urinary tracts that support the parenchyma of the endocrine and exocrine glands. The major components of BD Matrigel™ are laminin, collagen IV, entactin, and heparan sulfate proteoglycan, DMEM, and 50  $\mu$ g/mL gentamycin. Matrigel has been reported to be an optimized surface for stem cell research [40,41]. In these experiments, we coated the five sides of the chamber, except for the top, with Matrigel, and stromal cells mixed with EEPCs were seeded on the five sides, which mimics the presence of cells in the uterus and their contact with each other (Fig. 4B). Human endometrial stromal cell-EEPC-loaded Matrigel exhibited the desired “stickiness and stiffness.” To further observe the detailed morphological structure of the cell-loaded Matrigel, scanning electron microscopy (SEM) and confocal laser scanning microscopy were utilized. The results revealed that Matrigel possesses a compact construction, with fiber scaffolds (Fig. 4A), which allows it to achieve a sustained, long-term release of human endometrial stromal cell-EEPCs. These morphological and physical traits render Matrigel a suitable, sustained-release platform for intrauterine administration of human endometrial stromal cell-EEPCs. To determine the single-cell connection between stromal cells and EEPCs, EEPCs were seeded on stromal cells. EEPCs mono-cultured on stromal cells in the 3D Matrigel model were round with no extending processes, but the stromal cells showed a connective fiber skeleton arrangement for membrane extension (Fig. 4C). To evaluate the monolayer thickness of the EMO, the height values of the monolayer membrane structure were measured using confocal IMARIS8.1 analysis. The average EMO thickness was 100  $\mu$ m (Fig. 4C). In the mixed 3D culture, spheroids and EMOs formed immediately, and the membrane began to thicken and spread close to the nearby EMO. The EMOs flourished simultaneously at different stages in 3D culture (Fig. 4D).

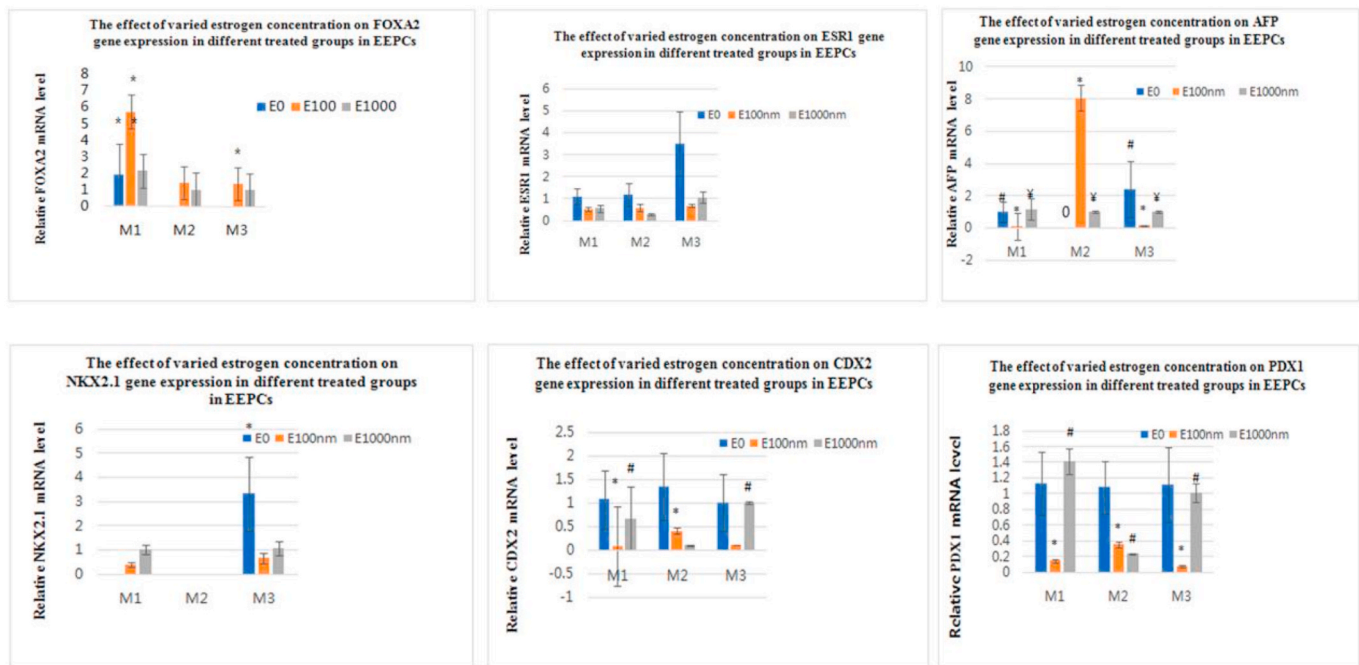
#### 3.3. EMO established from EEPCs in vitro

Reconstructing the human endometrium in traditional culture systems is limited by the inability to reproduce a functional endometrial barrier comparable to that of the normal human endometrium. In this study, to compare the proliferation of EEPCs grown in the M1, M2, and M3 groups, three kinds of EEPCs from three differentiation media were seeded on the modified 3D model to generate EMO. Cell attachment began 1 h after co-culture. On day 3 post-seeding, dense glandular spheroids were formed (Fig. 5A). On day 7 post-seeding, a mature



**Fig. 1. Differentiation of H9-ESCs-GFP<sup>+</sup> and H9-ESCs into endoderm epithelial cells.** (A) Differentiation of H9-ESCs-GFP<sup>+</sup> into EMO. The fluorescent (green) and phase contrast (gray) photomicrographs demonstrate the differentiation of H9-ESCs-GFP<sup>+</sup> (A1, A2) into definitive endoderm cells (B1, B2), EEPs (C1, C2), and EMO (D1, D2). Scale bar: 500 μm. (E) Characterization of H9-ESCs-DEC using real-time PCR for pluripotency (OCT4 and NANOG), endoderm (GSC and CXCR4), epithelial (E-cadherin), and mesenchymal (Bglap) lineage-associated transcription factors and endometrial progenitor epithelial markers (FOXA2 and SOX17). The transcript levels were normalized to the β-Actin levels and to H9-ESCs. Values represent the mean ± SD of three experiments (\**p* < 0.05 vs. H9-ESCs control). Flow cytometry was used to analyze the expression of endoderm epithelial markers CXCR4 and E-cadherin on D5 (F), D6 (G), and D7 (H).





**Fig. 2. Optimized medium affects the gene expression of endoderm epithelial progenitor cells.** Characterization of EEPC differentiation using qRT-PCR by analyzing (A) an epithelial lineage-associated marker (FOXA2), (B) an endometrial marker (ESR), (C) a hepatocyte marker (AFP), (D) a lung and thyroid marker (NKX2.1), (E) an intestinal marker (CDX2), and (F) a marker of endocrine cells in the pancreas and stomach (PDX1). The transcript levels were normalized to the  $\beta$ -actin levels expressed by cells at different E2 concentrations. Values represent the mean  $\pm$  SD of three experiments (\*, #, ¥  $p < 0.05$  vs. the respective controls). EEPCs were differentiated for 3 days as a part of three unique treatment groups based on the differences in media (M1, M2, and M3). M2 without EGF were used as controls.

membrane-like network formed and then continued to develop up to day 21 in the EEPCs derived from M1 and human endometrial stromal cell-EEPC co-culture group, while the control group began to exhibit partial networks on day 3 post-seeding.

The resulting EMO in the 3D model group exhibited a fully covered structure when compared with that in the control group, with more glandular spheroids at first, that then spread to the connective net scaffolds. The EMO in the model group tended to cluster in areas where the two sides intersected. After 4 days, the EMO membranes in the control group became constricted and fragile. However, in the model group, the membranes of EMO were continuous and spread to fully cover the bottom of the well until day 21 (Fig. 5A). Any differences in the EEPCs did not translate to observable structural differences in the EMO (Fig. 5F). All EMO tended to cluster under any culture conditions. The EEPCs derived from M3 contained more growth factors compared to the EEPCs derived from M1 in the 2D condition, which, after cross-irrigation, did not translate into observable differences in our final composite EMO. In the control group, the number of EMOs decreased rapidly. The density of the EMOs obtained was increasingly higher when grown from EEPC cultured in M1, M3, and M2 (Fig. 5F). Meanwhile, the thickness of the EMO incorporated in the human mature endometrial base layer control was  $\sim 1000 \mu\text{m}$ , which is higher than that found in our EMO. After observing the presence of different protein components in the immunofluorescence staining images, we concluded that the staining of the EMO components was positive, expressing both estrogen receptor (ESR) and progesterone receptor (PGR) (Fig. 5B).

### 3.4. Endometrial properties of EMO on 3D Matrigel

To mimic the response of organoids to hormones, EMO were exposed to 100 nM E2, followed by treatment with 1  $\mu\text{M}$  P4. The immunophenotypes of the endometrium from the 3D model were analyzed using immunohistochemistry. We examined whether human EMO could reproduce the menstrual cycle, particularly in the secretory phase. EMO

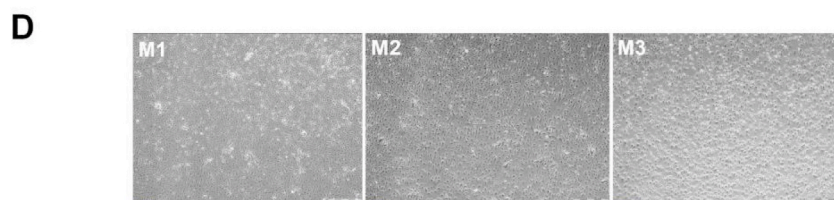
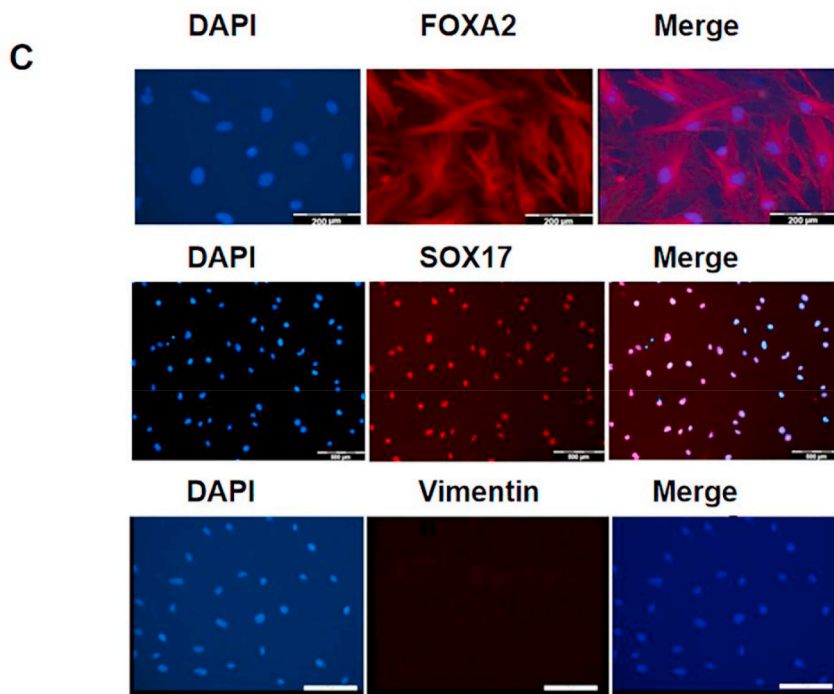
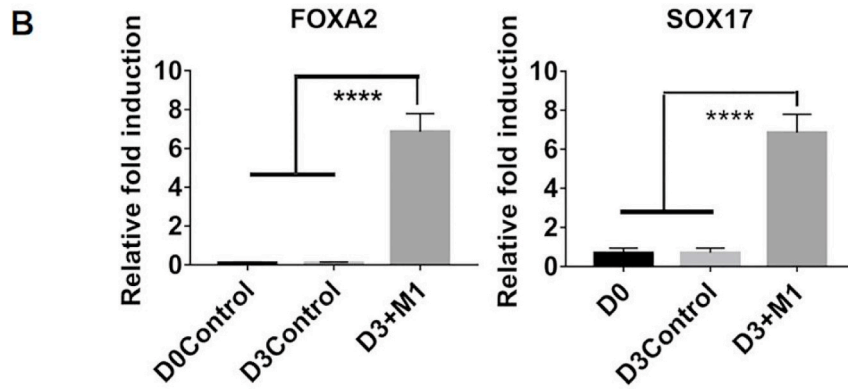
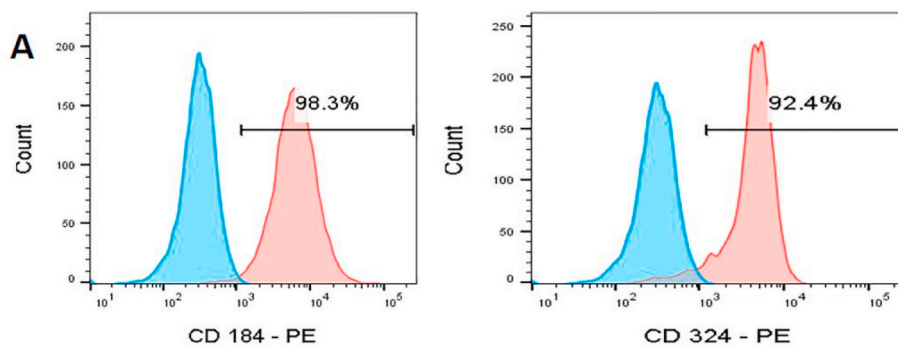
formed glandular cavities, confirming their endometrial-like structure (Fig. 5C), expressing CK19 and MUC-1, confirming their glandular origin [36], and expressed ESR and PGR [42–45], confirming their endometrial properties (Fig. 5D). EMO secreted acidic mucus, which was stained using AB, and EMO-secreted glycogen, which was stained using PAS, compared to the controls from a human adult endometrium, confirming their endometrial glandular secretory function (Fig. 5E).

### 3.5. EMO seeded- 3D Matrigel implantation improves AS recovery in vivo

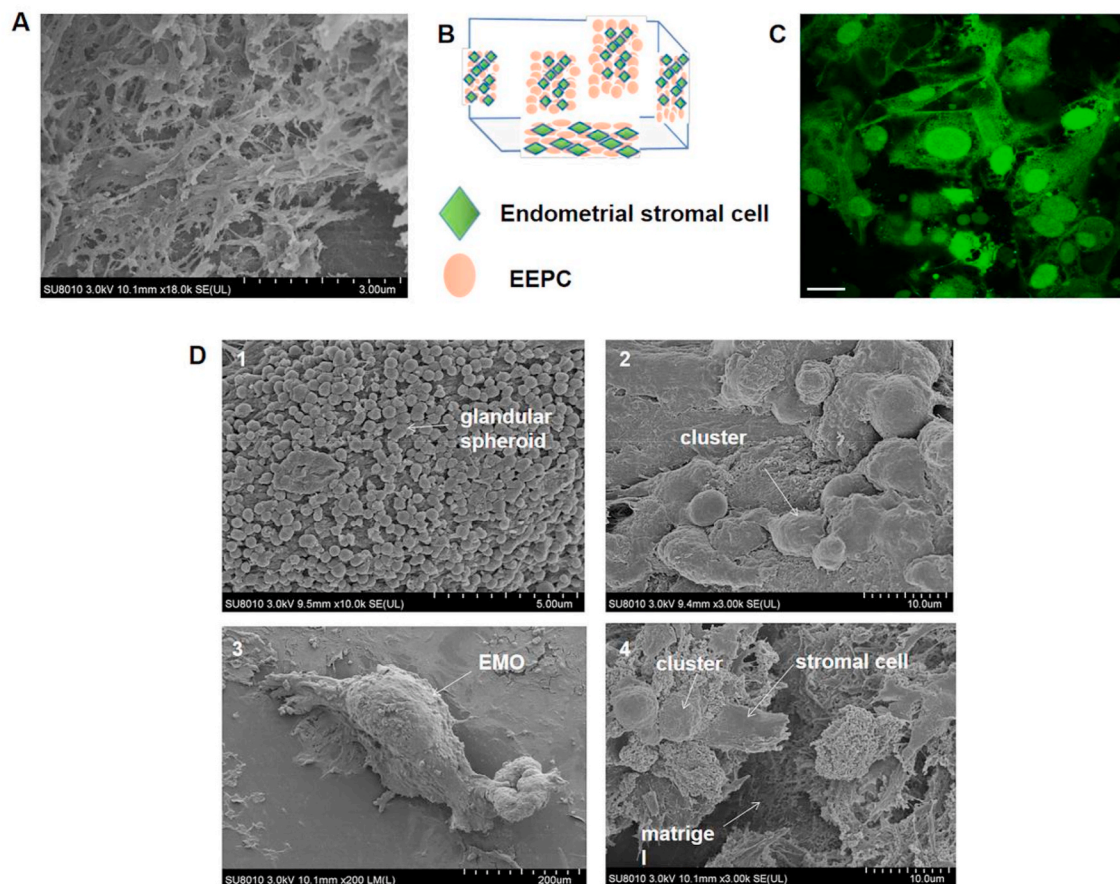
To investigate the *in vivo* therapeutic effect of EMO/gel, the rats were divided into three groups: normal uterine (control group), treated group without implantation of EMO or EEPC (model group), EEPC/Matrigel (model + EEPC group), and EMO/Matrigel (Model + EMO group). EMO was injected into the scar lesion (200  $\mu\text{L}$  on each side at a 1 mm depth). When the rats were killed and dissected at week 2 post-implantation, the Matrigel was completely degraded. The endometrial growth was observed in all the rats (10/10) in the Model + EMO group, in 7/10 rats in the Model + EEPC, and in no rats (0/10) in the model groups (Fig. 6A&B).

The endometrium in the EMO-implanting group proliferated, while the model group demonstrated obvious scarring or sparse endometrial hyperplasia (Fig. 6B). In all the traumatized rats, histological evidence of fibrosis was confirmed in the uterus. Fibrosis varied from 40% to 80% in each rat (Fig. 6C). No significant difference in fibrosis was observed between the control and the Model + EMO groups based on H&E staining.

To examine the cellular mechanisms after EMO treatment, endometrial regeneration, fiber formation, and angiogenesis were assessed by staining the uterine horn lesions for Cytokeratin-19 (CK19), vimentin, and CD34, respectively. At the injury/implantation site, the expression of CK19, vimentin, and CD34 was significantly higher in the rats treated with EMO than in those treated with EEPC/Matrigel and in the model group. CK19 was positively expressed throughout the



**Fig. 3. Identification of EEPC.** (A) Immunocharacterization of EEPC purity was analyzed using flow cytometry by checking the expression of endoderm epithelial surface markers CXCR4 (CD184) and E-cadherin (CD324) without sorting. Characterization of EEPC differentiation (B–C). (B) Characterization of EEPC differentiation using real-time RT-PCR. The transcript levels of FOXA2 and SOX17 were normalized to the respective  $\beta$ -actin levels and the control group cultured in M2. Values represent the mean  $\pm$  SD of three experiments (\* $p < 0.05$  vs. the respective controls). EEPCs were differentiated for 3 days in the presence of endometrial epithelial factors (M1) on D0 and EEPCs were differentiated for 3 days without endometrial epithelial factors, and used as controls. (C) EEPC differentiation by expressing FOXA2, SOX17, but not Vimentin, was demonstrated using immunofluorescence. Scale bar: 200  $\mu$ m. (D) EEPCs cultured in M1, M2, and M3 were observed. A large part of EEPCs were differentiated from the three media. EEPCs appeared to have an elliptical and uniform morphology with large nuclei. M1: EEPCs differentiated from M1; M2: EEPCs differentiated from M2; M3: EEPCs differentiated from M3. Scale bar: 500  $\mu$ m.



**Fig. 4. Characteristics of the 3D culture model.** (A) Representative SEM image of Matrigel indicates the compact collagen forming scaffolds. Scale bar: 3  $\mu\text{m}$ . (B) Scheme showing the 3D culture of EMO/Matrigel. Matrigel was used to coat five sides of the chamber and cells were attached onto each side. (C) 3D montage image of a GFP-labeled monolayer EMO membrane reveals a network of fibrous Matrigel and EEPs within a Z-substack monolayer ( $100 \mu\text{m} \times 100 \mu\text{m} \times 10 \mu\text{m}$  images acquired every 2  $\mu\text{m}$ ). EEPs were surrounded by the antennae of stromal cells. (D) Scanning electron microscopy illustrates the stages of *in vitro* organogenesis (1–4). The small glandular spheroids shown in (1) typically form at D3. Mid-sized organoids formed the clusters indicated in (2), typically at D7, and the large EMO in the center of (3) typically formed after 3 weeks of culture. The EMO structure is shown in (4), where the endometrial stromal cells attached onto Matrigel, the extend antennae, and the EEPs sticking to stromal cells to form clusters can be observed.

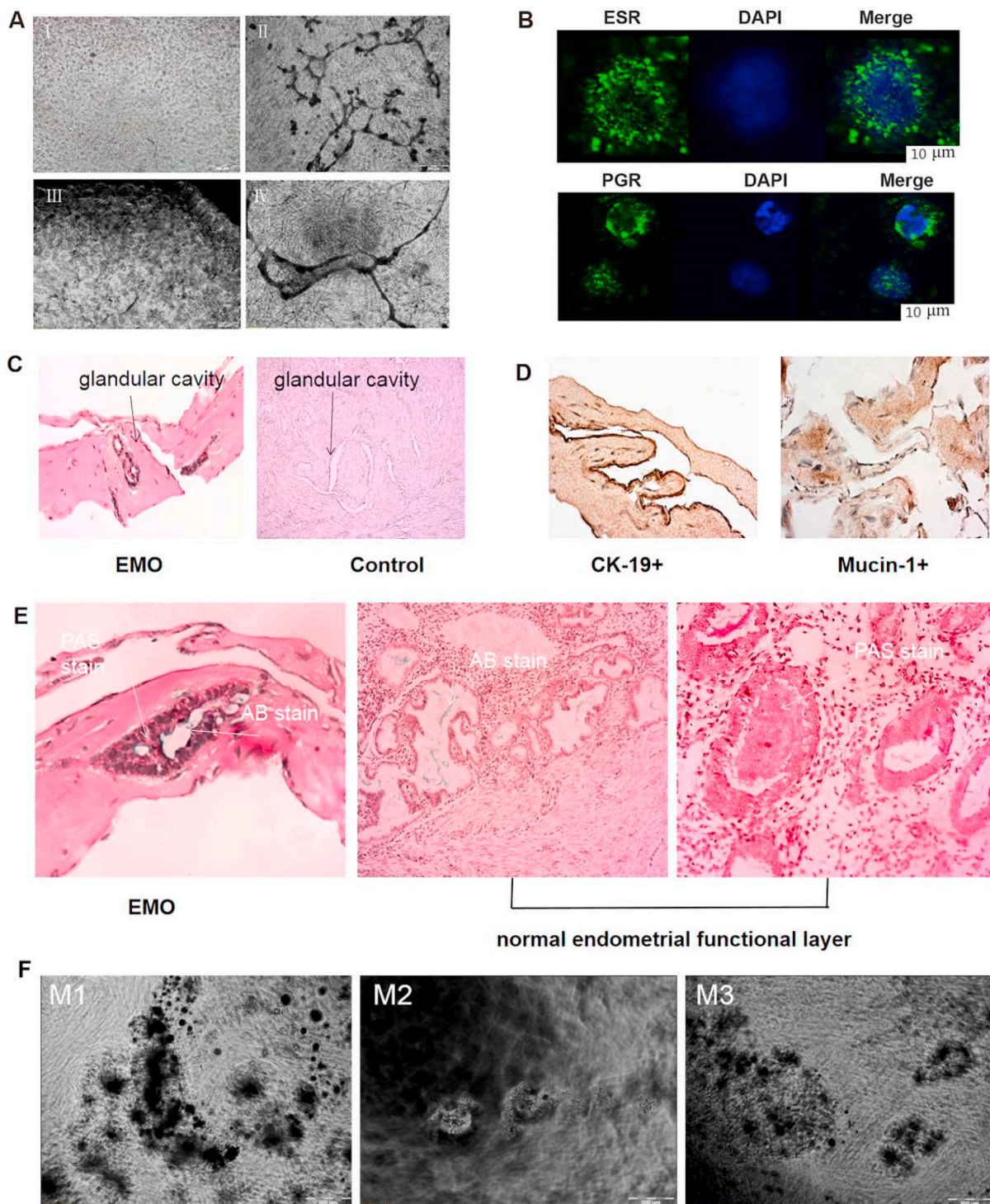
epithelial cell layer in the model + EMO group (Fig. 7A). Vimentin was expressed in the stromal cells in the basal layer in the model + EMO group (Fig. 7C). Additionally, the highest level of angiogenesis, as indicated by CD34 expression, was measured in the model + EMO group (Fig. 7B). CD34 was expressed in endothelial cells in the vascular tissue throughout the endometrial glands and the basal layer (Fig. 7B). Similar trends were observed in the expression of CK19 and vimentin, indicating more glandular and stromal cells in the model + EEP group (Fig. 7A).

#### 4. Discussion

Asherman's syndrome, which features intrauterine adhesions or fibrosis resulting from damage to the basal layer of the endometrium, is associated with infertility due to the loss of normal endometrium [46, 47]. Progenitor cells are thought to be a source of endometrial regeneration [48]. However, the identification, sufficient quantity, lack of purity, and susceptibility to aging of EEPs create challenges for their use. In this study, we induced EEP in H9-ESCs. A sufficient number of pure EEPs can be achieved by controlling H9-ESC differentiation within a defined time course and culture conditions [49]. In our protocol, we differentiated the cells *in vitro* for 8 days. First, H9-ESC were directed to the definitive endoderm lineage. This population was enriched to near homogeneity using the cell-surface receptors GSC+ [50] and CXCR4+ [11]. GSC was strongly upregulated, which was consistent with a previous report by Masahiro Yasunaga et al. [50] We

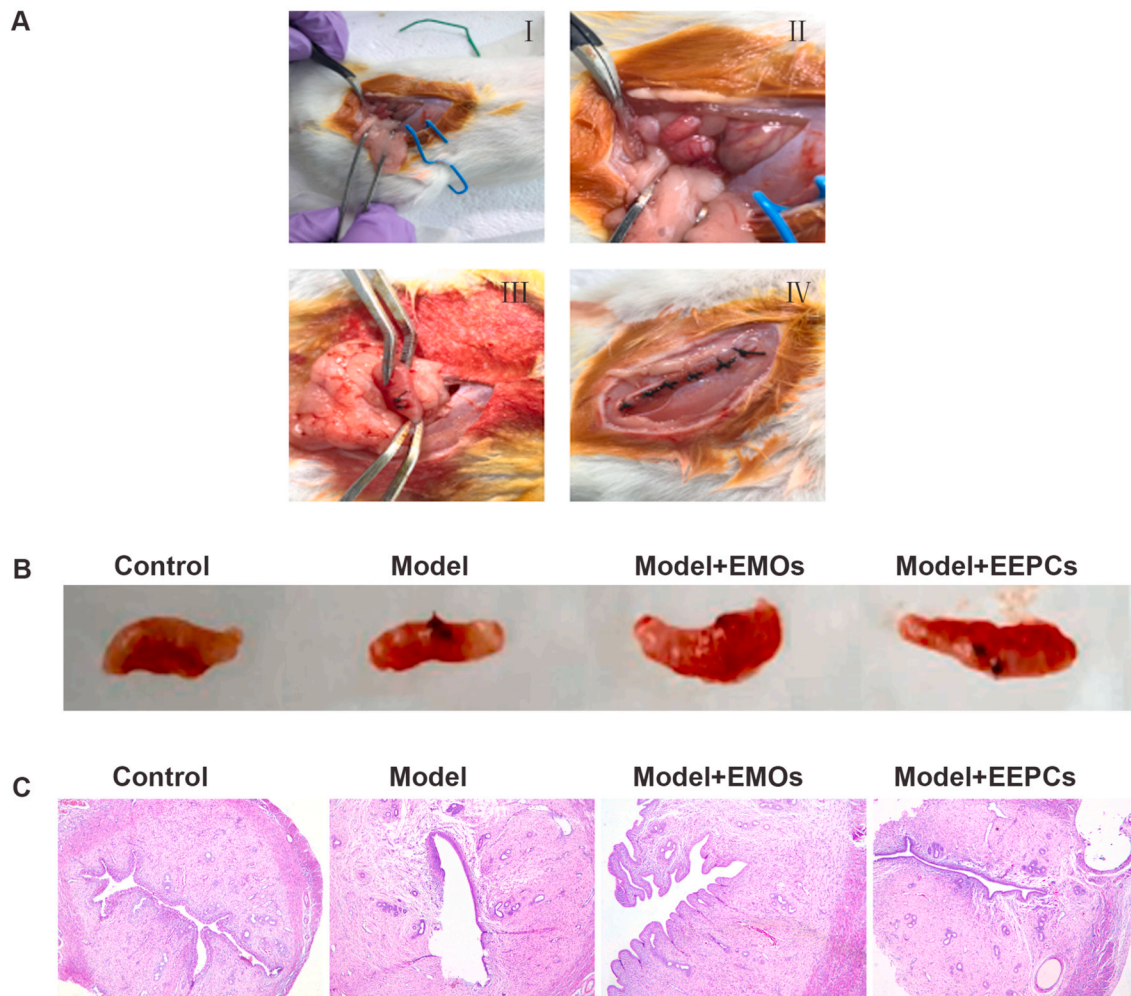
chose GSC to monitor the differentiation to definitive endoderm, as a relatively pure GSC + population of cells can be induced by stage 1 conditions [50]. Then, epithelial progenitor cells were induced to adopt a specific epithelial progenitor fate [50]. In this study, we succeeded in generating an almost pure CXCR4+/ECAD+ population on day 5 without sorting. Finally, EEP was induced in endoderm epithelial cells, identified with FOXA2 in M1. EEPs were determined to be clonogenic cells. EEP markers, such as E-cadherin, Lgr [51], N-cadherin [38], and SSEA-1 [52], have been fully characterized [53], but their endometrial specificity has not been demonstrated. A recent study on the genome-wide identification and analysis of transcriptional enhancers based on DNA methylation demonstrated that SOX17 and FOXA2 are endometrial cancer drivers [20]. In this study, SOX17 and FOXA2 were specifically expressed in endoderm epithelial progenitor cells, which distinguishes EEPs from the non-endometrial epithelial lineage. To induce epithelial lineage, human bone marrow-derived MSCs [4], umbilical cord Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) [6], human-endometrium mesenchymal stem cells (eMSCs) [54], and menstrual blood endometrial regenerative cells (ERCs) [55] have been reported to generate epithelial stem cells via mesenchymal-to-epithelial transition (MET). A few studies report that MSCs do not originate from pure mesoderm, questioning the long-established dogma about the differentiation of adult stem cells being restricted to tissues derived from their germ layer of origin [56]. It remains an open question as to whether MSC-derived cells incorporated into the human endometrium





**Fig. 5.** EMO developed from EEPs and EMO function responds to sex hormones (A) Colony outgrowth and organogenesis *in vitro*. Cultures are depicted 1 week after the establishment as gland fragments. (I) Initially, the gland fragments retain a spherical structure on D3 (Magnification: 100 ×). (II) These structures soon collapse as growth as a monolayer begins. (III) A large organoid which formed after 3 weeks of culture. (IV) The large glandular structure is located centrally, over the monolayer, and seems to be connected to other parts of the colony by smaller tubules. (B) The immunofluorescence of endometrium-organoids grown out of model group are shown for ESR and PGR, respectively. Scale bar: 10 μm. (C) H&E staining of the glandular cavities are shown relative to the normal human endometrium. Scale bar: 200 μm. (D) Immunohistochemical staining of the epithelium in EMO. Scale bar: 200 μm. (E) AB and PAS staining in the glandular cavities of EMO after exposure to E2 and P4 are shown relative to the normal human endometrium at a fertile age. Some cells in EMO secreted AB (blue area, arrowheads) and some cells secreted PAS (magenta area, arrowheads). Some cells in normal endometrial secretory epithelia cells secreted AB and some cells secreted PAS in the glandular cavities. Scale bars: 200 μm. (F) Images were taken on D3 post-seeding. EMO grown from EEPs differentiated from M1, M2, and M3 were observed. EMO were cultured in a 3D culture system introduced by Nguyen H et al. [38]. EMO floated in the media, formed clusters, and aggregated into fragments. The morphology of EMO grown from M1, M2, and M3 was similar. The number of EMOs obtained from M2 and M3 was lower and they were more scattered compared to those obtained from M1. M1: EMO composed of EEPs differentiated from M1; M2: EMO composed of EEPs differentiated from M2; M3: EMO composed of EEPs differentiated from M3. Scale bar: 500 μm.





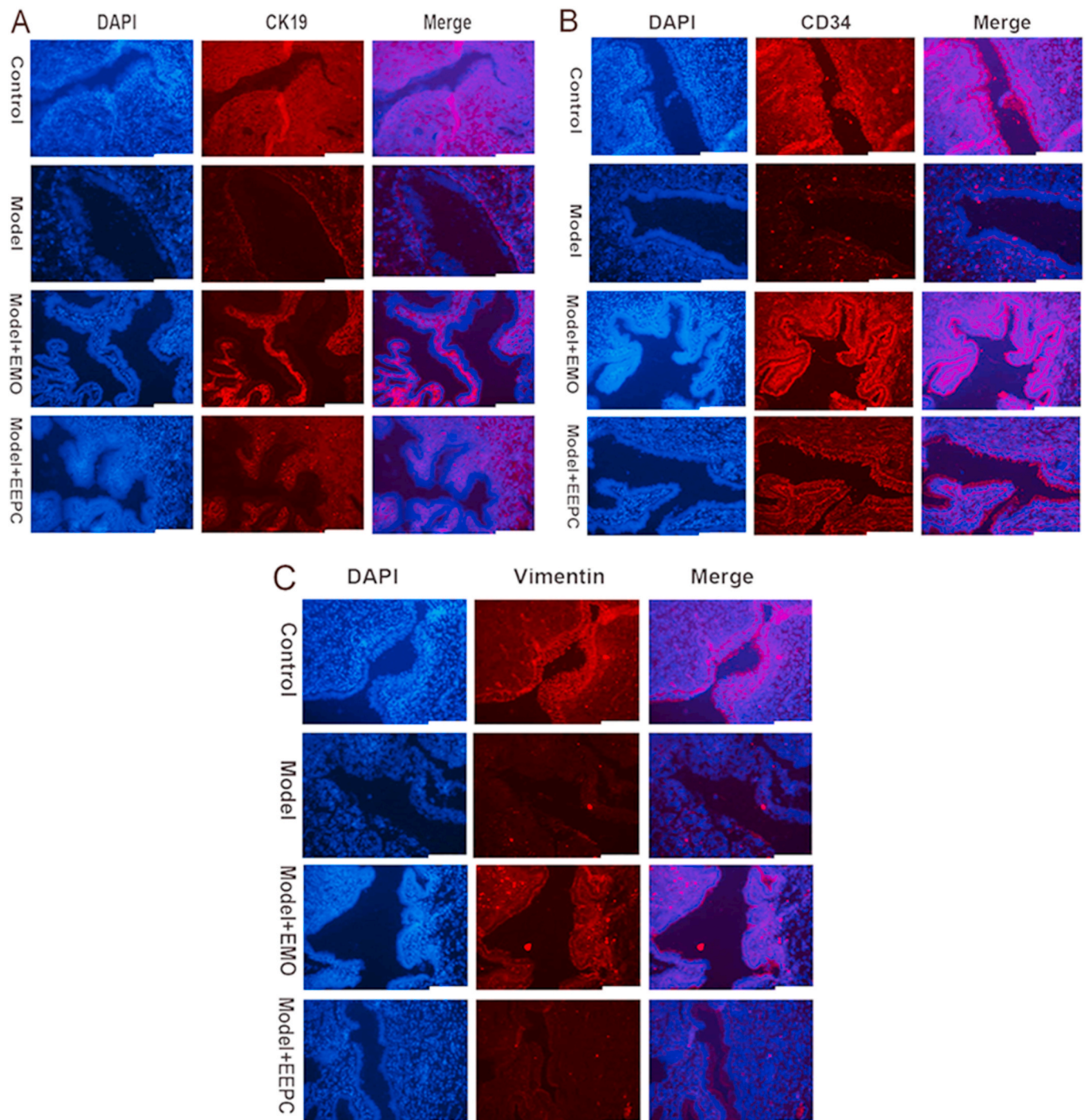
**Fig. 6. The effects of EMO/Matrigel on endometrial development.** (A) Pictures showing the procedure of inducing injury and delivering the treatment. I: incision of the rectus abdominis and uterus exposure. II: electrocoagulation injury on the right side of the uterus. III: closure of the uterine incision. IV: closure of the abdomen. (B) General view of rat AS specimens. (C) Representative H&E-stained uterine section 14 days after injury: (1) fibrosis was observed in the damaged uteri; (2) no fibrosis was seen in the uteri from EMO/Matrigel, EEPC/Matrigel and control groups. Original magnification:  $200\times$ .

are stem cells or immune cells [53]. In our study, EEPCs derived from H9-ESCs marked with SOX17 and FOXA2 originated from endoderm germ cells and were able to generate infinite, pure, specific EEPCs, which did not require isolation from adult endometrium or MET. Epithelial growth factor (EGF) is the only growth factor required for FOXA2 expression during EEPC differentiation. EGF has been established as a major effector of angiogenesis in the endometrium [57]. Our optimized medium was consistent with the growth factors of epithelial progenitors from human endometrium cultured by Carolin et al. [58]. E2 plays a role in MET, SENP1-ER $\alpha$  re-programs stromal SM22 $\alpha$ + cells into CD34<sup>+</sup> KLF4+ progenitor cells [59], and E2 induces the differentiation of WJ-MSCs into EEC-like cells [6]. We demonstrated for the first time that ESR and PGR were expressed in all epithelial progenitor cells and that E2 had no effect on ESR or PGR expression, which was consistent with the presence of ESR and PGR in epithelial-derived tissues, such as the brain [60], stomach [61], liver [62], and skin [63]. Other studies have reported that ESR and PGR are not expressed in stem/progenitor cells from hormone-responsive tissues, such as the mammary glands and uterine epithelia, and always lack ESR expression [64–68]. However, estrogen encourages epithelial progenitor cells to develop into mature glandular epithelial cells.

Subsequently, we investigated the ability of EMO to form H9-ESC-induced EEPCs and the function of EMO. We compared our 3D culture model with previously reported methods for 3D culture. In previous

methods, epithelial cells were mixed with Matrigel to form a 3D space, and the cells were isolated using Matrigel. The connections between cells were blocked. In this study, Matrigel was used to coat five sides of the chamber, the cells were attached onto the five sides, and a shared medium was present in the container (Fig. 4B). This 3D culture mimics the uterine growth and promotes intercellular signaling. This unobstructed sharing of medium is conducive to maintaining EMO.

Finally, the regenerative potential and vascular benefits of EMO constructs in a rat model of AS were investigated. The group treated with EEPC/Matrigel showed a moderate recovery and a moderate endometrial regeneration. However, the implantation of EMO constructs contributed to recovery effects on the injured tissue, accompanied by significant angiogenesis and the regeneration of the endometrium. This is probably attributable to the transcription factor effects of FOXA2 and SOX17, as demonstrated by epithelial-mesenchymal transition (EMT). The transforming growth factor beta (TGF $\beta$ ) signaling pathway induces both epithelial-mesenchymal transition and endothelial-mesenchymal transition. The overexpression of SOX17 during endothelial to hematopoietic transition (EHT) partially recapitulates the effects of TGF $\beta$  signaling activation<sup>[69]</sup>. Our results support the finding that the endothelialization of the endometrial tissue promotes the function of engineered implants. EMO implants were able to improve blood perfusion, endometrial regeneration, and reverse fibrosis in Asherman's syndrome.



**Fig. 7. Localized uterine damage regenerates the endometrium.** (A) Immunofluorescence staining of cytokeratin (red) indicates the differentiated epithelial cells. CK19 was not expressed in the model and control groups; CK19 was highly expressed in the model + EMO group and weakly expressed in the model + EEPC group. (B) CD34 (red) indicates the differentiated angiogenesis. CD34 was not expressed in the model group; CD34 was weakly expressed in the model + EEPC group and highly expressed in the model + EMO and control groups. (C) Vimentin (red) represents the differentiated stromal cells. Vimentin was highly expressed in the control group and the model + EMO group and weakly expressed in the model + EEPC group. No Vimentin was expressed in the model group. DAPI was used to stain the nuclei (blue). Original magnification:  $200\times$ .

## 5. Conclusion

In conclusion, this study establishes a method to develop EEPCs from H9-ESCs *in vitro*. H9-ESC-DECs were able to continuously produce pure EEPCs that express FOXA2 and SOX17. Endometrial epithelial progenitor cells can form endometrial organoids. This work demonstrated the regenerative potential of bioengineered, endometrial, and EMO-

embedded constructs in an AS rat model. Meanwhile, our results suggest the superiority of harnessing endometrial regeneration with stem cell-embedded constructs for repairing the endometrium in patients with Asherman's syndrome.



## CRediT authorship contribution statement

**Xiuxiu Jiang:** Investigation, Methodology, Data curation, Writing – original draft, Funding acquisition. **Xingmiao Li:** Investigation, Methodology, Software. **Xiangwei Fei:** Methodology, Software. **Jiajie Shen:** Methodology, Software. **Jianhua Chen:** Formal analysis, Software. **Meijun Guo:** Investigation, Methodology. **Yangyang Li:** Project administration, Supervision, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioactmat.2021.04.006>.

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