Multi-Lineage Human Endometrial Organoids on Acellular Amniotic Membrane for Endometrium Regeneration

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Abstract

Asherman's syndrome is an endometrial regeneration disorder resulting from injury to the endometrial basal layer, causing the formation of scar tissue in the uterus and cervix. This usually leads to uterine infertility, menstrual disorders, and placental abnormalities. While stem cell therapy has shown extensive progress in repairing the damaged endometrium and preventing intrauterine adhesion, issues of low engraftment rates, rapid senescence, and the risk of tumorigenesis remain to be resolved for efficient and effective application of this technology in endometrial repair. This study addressed these challenges by developing a co-culture system to generate multi-lineage endometrial organoids (MLEOs) comprising endometrial epithelium organoids (EEOs) and endometrial mesenchymal stem cells (eMSCs). The efficacy of these MLEOs was investigated by seeding them on a biocompatible scaffold, the human acellular amniotic membrane (HAAM), to create a biological graft patch, which was subsequently transplanted into an injury model of the endometrium in rats. The results indicated that the MLEOs on the HAAM patch facilitated endometrial angiogenesis, regeneration, and improved pregnancy outcomes. The MLEOs on the HAAM patch could serve as a promising strategy for treating endometrial injury and preventing Asherman's syndrome.

Keywords

Asherman's syndrome, primary prevention, endometrial epithelium organoids, endometrial mesenchymal stem cell, human acellular amniotic membrane, regeneration

Background

The endometrium is a dynamically changing tissue that can undergo shedding and re-epithelialization every month without scar formation under the influence of hormones, such as estrogen and progesterone. Like skin repair, regeneration of the injured endometrium begins with the migration of epithelial cells from exposed endometrial glands and adjacent intact endometrium to the exposed stromal component[1,2]. Asherman's syndrome, an acquired, gynecological disorder of the uterus results from injury to the endometrium basal layer primarily caused by hysteroscopic surgery and recurrent induced abortion, leading to loss of endometrial stem progenitor cells, and replacement of the injured area with pathological fibrotic tissue[3,4]. However, severe endometrial injuries are often irreparable, reducing the chance of subsequent pregnancy, and live birth rates[5]. Therefore, developing an approach for the primary prevention of uterine adhesion is urgent. In recent years, stem cell therapies have shown great potential in repairing injured endometrium, with some studies proving efficacy in clinical trials. As a new hope for endometrial regeneration, stem cell therapy might be more efficient than original prevention approaches for primary prevention of intrauterine adhesion (IUA). Therefore, developing an approach for the primary prevention of uterine adhesion is urgent.

Endometrial epithelial progenitor cells (EEPCs) have shown some promise in stem cell therapy for repairing the injured endometrium. However, the main limitation of the therapy is the low engraftment rate, which includes two factors: (1) EEPCs cannot expand long-term *in vitro*, resulting in an insufficient number of cells for transplantation and (2) deficient cell-cell interaction in the microenvironment causes a low survival rate of endometrial epithelial stem cells after transplantation to the injured area of the endometrium. The emergence of organoids has provided an alternative method to complement existing technologies to resolve this problem as they have high proliferation *in vitro*, recapitulate the microanatomy of the primary tissue, and can

maintain genotype stability[6]. Current studies have indicated that endometrial epithelium organoids (EEOs) can regenerate injured endometrium[7,8]. However, tissue regeneration requires coordinated and dynamic remodeling of stem and progenitor cells within the surrounding niche. We proposed that combining organoids with mesenchymal cells could enhance the organoid-repairing efficiency of the injured endometrium.

Mesenchymal stem cells (MSCs) have become a potential cell for stem cell therapy due to their self-renewal, low immunogenicity, and immune regulatory function. It can be harvested from various tissues, including bone marrow[9,10], umbilical cord blood[11], amniotic membrane[12], and endometrium[13]. Endometrial mesenchymal stem cells (eMSCs), one of the stem progenitor cells in the endometrium, may provide an appropriate microenvironment for repairing the endometrial cell niche.

In most mammals, the developing fetus is enclosed by an amniotic membrane or amnion. The human acellular amniotic membrane (HAAM) preserves extracellular matrix (ECM) components, including abundant collagen, laminin, fibronectin, elastin, hyaluronic acid, fibroblast growth, and transforming growth factors[14]. It provides low immunogenicity and good biocompatibility, making it an excellent biological scaffold. Over the last decades, researchers have focused on the biomedical applications of HAAM, such as the development of a simple sheet for skin or cornea repair, amnion nanocomposite, and hydrogels for high-throughput regeneration of muscle, cartilage, and tendon[15].

In our study, we developed multi-lineage endometrial organoids (MLEOs) with EEOs and eMSCs to investigate their efficiency in repairing injured endometrium and decelerating apoptosis. We then seeded these organoids on HAAM, which was termed MLEOs on HAAM (MLEO-HAAM). Our results indicated that the MLEO-HAAM patch could serve as a promising approach to repair the injured endometrium, which had undergone mechanical abrasions to the basal layer, and could improve pregnancy outcomes.

Results

MLEOs Contribute to the Regeneration of Damaged Endometrium

As endometrial mesenchymal cells supported the stem niche, we suspected that MLEOs could repair endometrial damage. To test this hypothesis, we established a MLEO system in which endometrial mesenchymal and epithelial cells were co-cultured in Matrigel as shown in the schematic diagram in Fig. 1A. Organoids and mesenchymal cells showed sufficient expansion, as illustrated in Fig. 1B. Confocal microscopy confirmed that these MLEOs were positive for the epithelial cell marker EpCAM, while the fibroblast-like cells were positive for the fibroblast cell marker Vimentin (Fig. 1C).

Mesenchymal Cells Support the Growth and Self-Renew of EEOs

Mesenchymal cells could serve as a stem cell niche that supports the proper functioning and self-renewal of epithelial stem cells. To test this hypothesis in the endometrium, we utilized a 3D Matrigel culture system that allowed for the growth and self-renewal of EEO. Organoids were unable to proliferate or self-renew when Rspo1 and Noggin were withdrawn. However, when EEOs were co-cultured with endometrial mesenchymal cells, they spontaneously formed self-organizing organoids in the absence of Rspo1 and Noggin (Fig. 2A). Organoids in the co-culture group exhibited more Ki67+ cells (a marker associated with proliferation) and Sox9+ cells (a marker associated with stemness) as observed by immunofluorescence (Fig. 2B). Real-time polymerase chain reaction (RT-PCR) analysis demonstrated that organoids co-cultured with mesenchymal cells expressed higher levels of stemness-associated genes (Axin2 and Sox9) compared with the control group (Fig. 2C). The proportion of stage-specific embryonic antigen 1 (SSEA-1+) cells, considered as a marker for EEPCs, was measured using flow cytometry. The results

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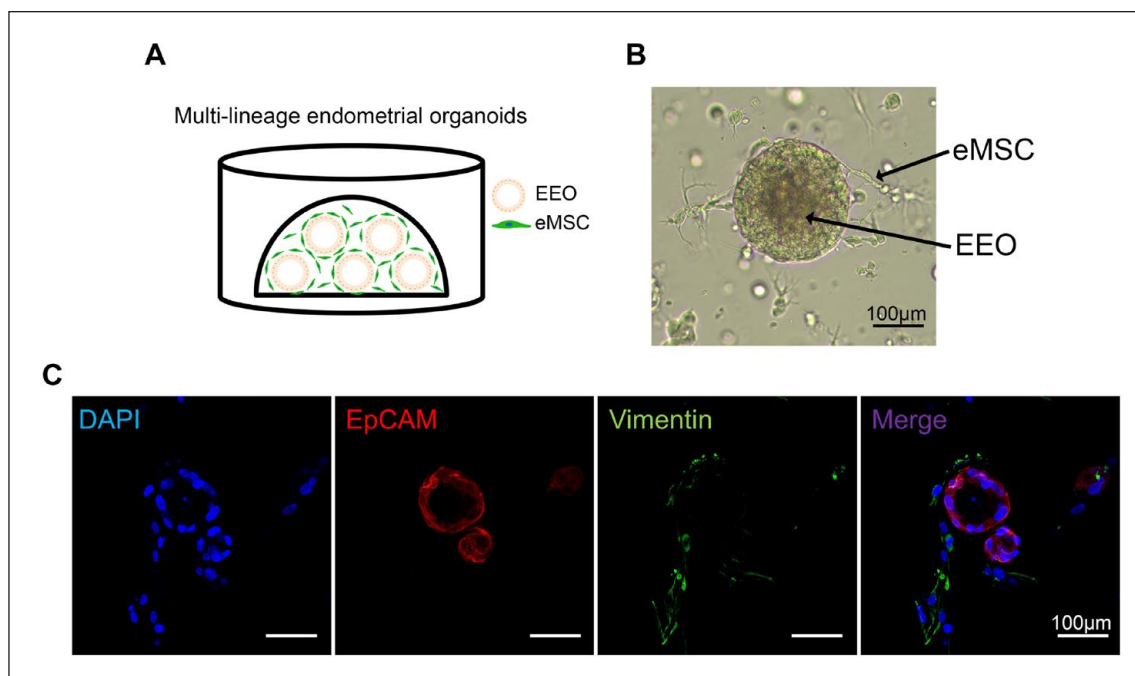Email: chenxiaojun1366@fckyy.org.cn

Figure 1. The formation of multi-lineage endometrial organoids. (A) Schematic diagram of the MLEOs consisting of EEO and eMSCs embedded within the Matrigel dome. (B) A representative bright field image of EEO-eMSC MLEOs. Scale bar: 100 μm. (C) Immunofluorescence images of the MLEOs stained with antibodies against EpCAM (red), and Vimentin (mesenchymal cell marker, green). Scale bar: 100 μm. DAPI: 4′,6-diamidino-2-phenylindole; MLEO: multi-lineage endometrial organoid; EEO: endometrial epithelium organoid; eMSCs: endometrial mesenchymal stem cells; EpCAM: endometrial epithelial cell marker.

showed that the proportion of SSEA-1+ cells increased in organoids co-cultured with mesenchymal cells (Fig. 2D). These findings suggest that endometrial mesenchymal cells provide a stem cell niche that enables the growth and self-renewal of EMOs.

To evaluate the regenerative capabilities of MLEOs in injured endometrium, we established endometrial-injured rat models by performing endometrial abrasion curettage with a curette. Two weeks after surgery, we transplanted the MLEOs into the injured uterine and collected the uterine tissue (Fig. 3A). Histological analysis of uterine tissue stained with hematoxylin and eosin (H&E) showed that the endometrial thicknesses and the number of endometrial glands in all three cell-treatment groups had improved considerably as compared with the model group. In particular, the MLEO-treated group showed the most significant improvement (Fig. 3B–E). Masson's trichrome staining was used to evaluate the fibrotic phenotype, and the fibrotic area ratio was significantly lower in the MLEO group than in the other groups (organoid group and mesenchymal cell group) (Fig. 3F).

To assess the angiogenesis capacity of the different treatment groups, uterine tissue was stained with CD31 (an endothelial cell marker) (Fig. 4A). The expression of CD31 was significantly increased in the MLEO group as compared with the other cell-treatment groups (Fig. 4B). Moreover, to

further investigate the uterine receptivity of the different groups, we analyzed the expression of integrinβ3, HOXA10, BCL6[16,17] (a marker of uterine receptivity) by quantitative real-time polymerase chain reaction (qRT-PCR) (Fig. 4C; Supplementary Fig. 2). The results demonstrated that the MLEO group showed the highest expression of integrinβ3, indicating that MLEOs might improve the pregnancy outcome of the injured uterus. Taken together, these results suggest that MLEO-HAAM treatment could potentially enhance the repairment of the injured endometrium, thereby improving pregnancy outcomes.

Establishment of MLEOs on HAAM Patch

H&E staining showed that the resident cells of HAAM were removed entirely after the decellularization procedures (Supplementary Fig. 1a). We then seeded MLEOs on the HAAM to improve their survival after intrauterine transplantation (Supplementary Fig. 1b). H&E staining showed that the EEOs closely adhered to HAAM under the wrapping of eMSCs, displaying a flaky and cluster-like morphology (Supplementary Fig. 5c, d). Consistent with previous experimental results, EEOs maintained their proliferation state when co-cultured with eMSCs after seeding on the HAAM (Supplementary Fig. 5e, f).
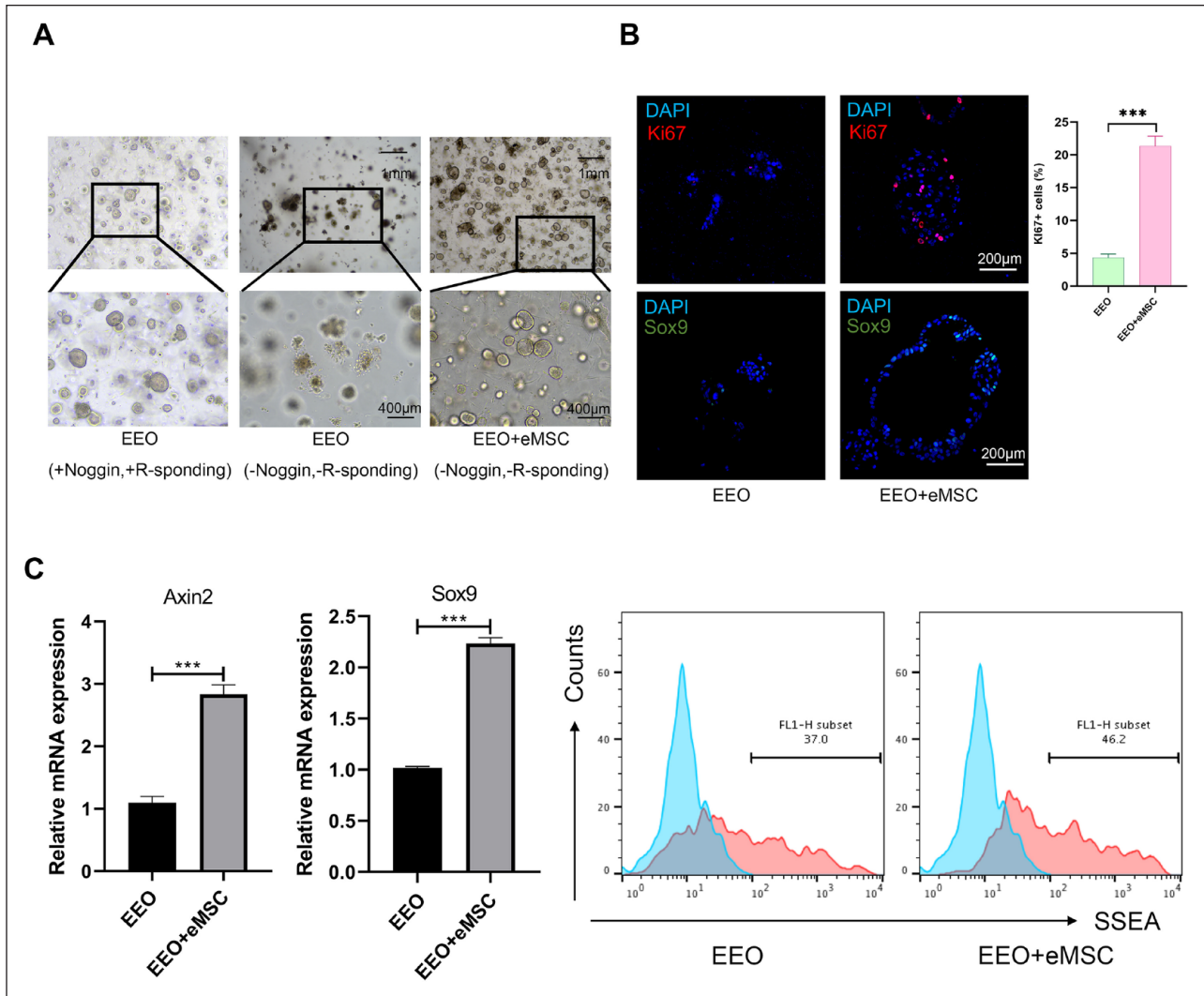
Figure 2. The eMSC maintains the stemness and proliferation of EEOs. (A) Representative bright field images of EEOs culture with Noggin and R-spondin1, EEOs and eMSCs co-culture system upon withdrawal of Noggin and R-spondin1. (B) Immunofluorescence staining of EEOs co-cultured with and without eMSCs. The expression of Ki67 (a marker of active cell proliferation, shown in red) and SOX9 (a marker of stemness, shown in green) was higher in the eMSCs+EEOs group compared with the EEOs-only group. Scale bar: 200 μm. Data are the mean ± SEM. Student's *t* test; ns, non-significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (C) Real-time PCR analyses of the endometrial progenitor stem cell markers SOX9 and Axin2. Data are the mean ± SEM. Student's *t* test, ***$P < 0.001$. (D) Flow cytometry analysis of SSEA-1+ cells. DAPI: 4′,6-diamidino-2-phenylindole; eMSC: endometrial mesenchymal stem cell; EEO: endometrial epithelium organoid; SEM: standard error of the mean; PCR: polymerase chain reaction.

MLEOs on HAAM Improve Pregnancy Outcome in Endometrium-Injured Rats

To verify the effectiveness of the MLEO-HAAM patch in repairing the injured endometrium *in vivo*, we divided the endometrial-injured rat models into three groups: model group, MLEO group, and MLEO-HAAM group. Compared with other groups, the number of implantation embryos significantly increased in the MLEOs in the HAAM-treated group (Fig. 5A, B), demonstrating that the MLEO-HAAM patch positively affects the repair of newly injured tissues, thus improving pregnancy outcomes.

Discussion

Re-epithelialization of the endometrium is an efficient process that provides scar-free healing and repair. The extreme regenerative capacity of the endometrium is based on the presence of progenitors, located in the epithelial and stromal cell compartments of the basalis layer[18,19,20]. A previous study demonstrated that intrauterine transplantation of EEOs effectively repaired the basal layer of the endometrium in an IUA mice model and conducted the intact endometrium[7]. In our study, we generated the first MLEOs on HAAM patch and performed intrauterine transplantation to the endometrium
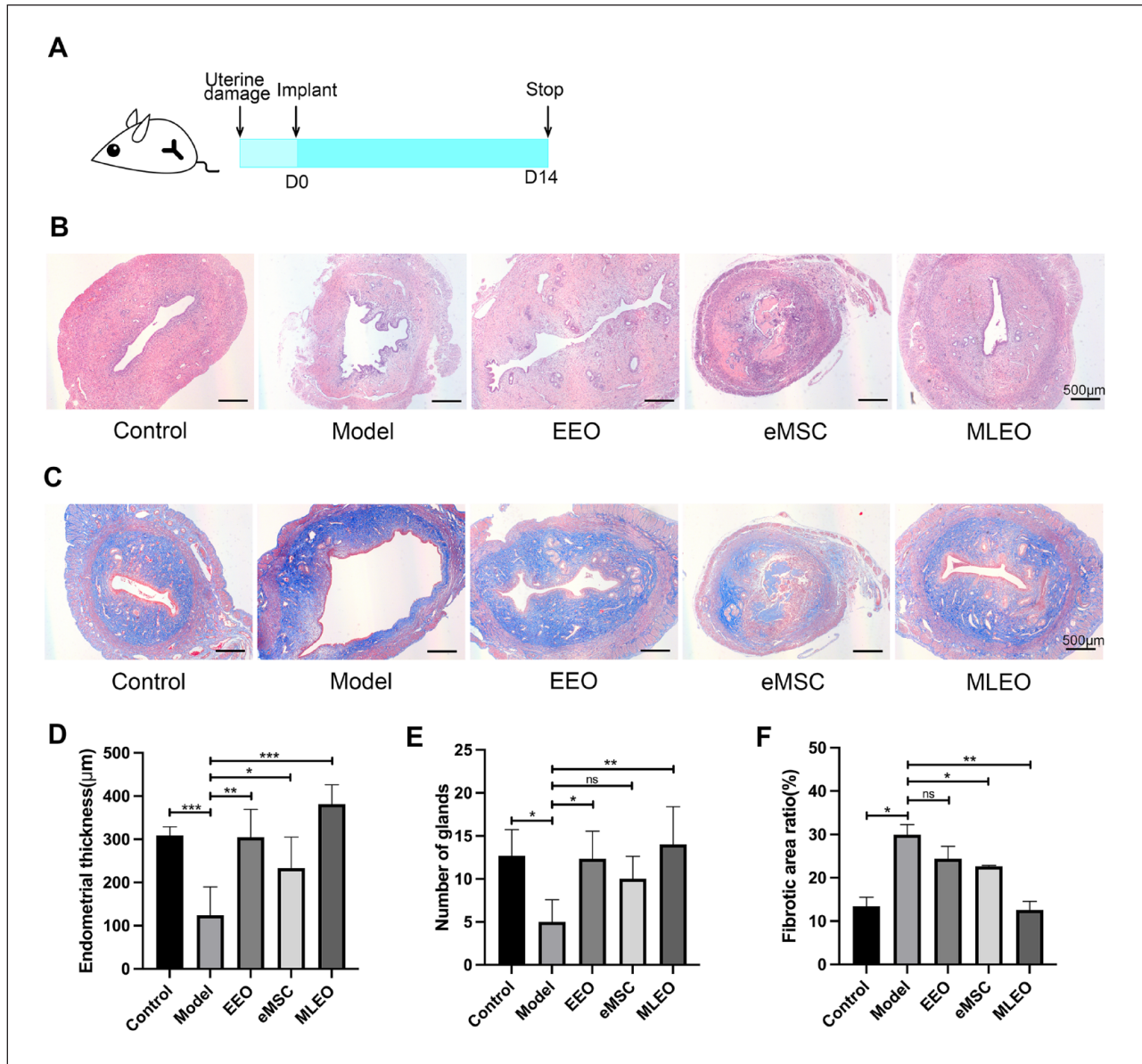
Figure 3. Efficacy of multi-lineage endometrial organoids in repairing injured endometrium. (A) Schematic diagram of intrauterine transplantation of MLEOs in endometrium-injured mice at different time points. (B) Representative H&E images of uterine sections at day 14 of injury treatment. (C) Masson trichrome-stained sections of the uterine at day 14 of injury treatment. (D) Statistical analysis of endometrial thickness after injury treatment. (E) Analysis of the number of glands. (F) Statistical analysis of fibrotic area measured in different groups. Data are the mean $\pm$ SEM. MLEO: multi-lineage endometrial organoid; H&E: hematoxylin and eosin; SEM: standard error of the mean; EEO: endometrial epithelium organoid; eMSC: endometrial mesenchymal stem cell. Student's *t* test; ns, non-significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

injury in rat models. The follow-up results showed that the complex patch has the potential to repair the endometrium and improve the outcome of pregnancy.

It was suggested that the regenerative efficacy of stem cells depended on the stem cell niche provided by adjacent cells[21]. A study indicated that Bim1-expressing cells located in the crypt base of the small intestine could give rise to LGR5-expressing cells after ablating adjacent LGR5-expressing cells. This phenomenon suggested that a stem cell niche could transform reserve stem cells into functional stem cell populations to preserve the homeostasis of the small intestine epithelium[22]. Analogous to this study, the existence of the eMSCs imitated the microenvironment of the endometrial gland to provide the stem cell niche to EEOs, leading to increased expression of stem genes and maintained stemness of EEOs. In our study, EEOs co-cultured with eMSCs can
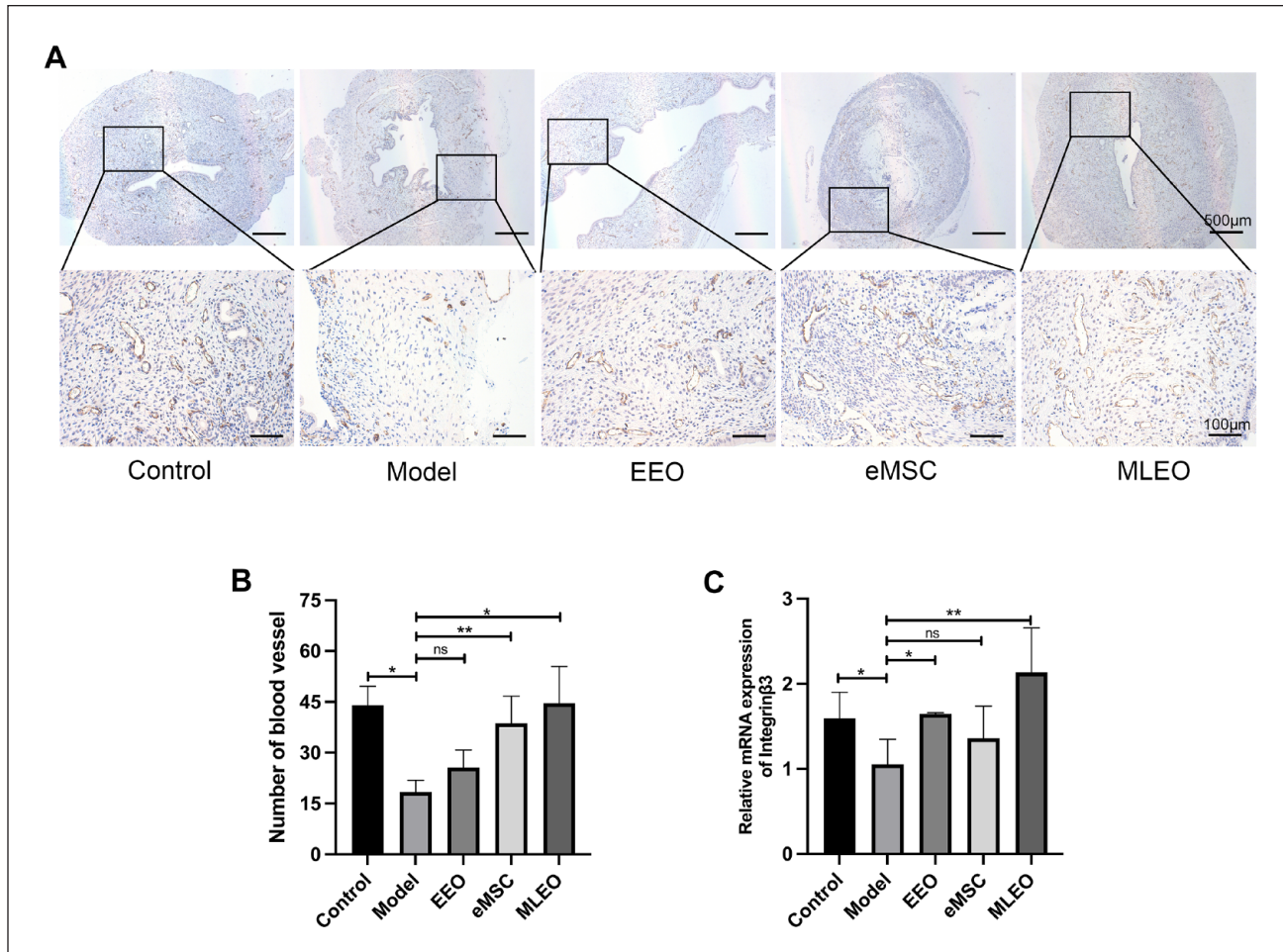
Figure 4. Multi-lineage endometrial organoids improve the angiogenesis capacity and uterine receptivity of the injured endometrium. (A) Immunohistochemical staining of CD31 (an endothelial cell marker) in different groups. Scale bar: 100 μm. (B) Quantification of CD31 expression in different groups. (C) qPCR analysis integrinβ3 expression. Data are the mean ± SEM. Student's *t* test. qPCR: quantitative polymerase chain reaction; SEM: standard error of the mean; EEO: endometrial epithelium organoid; eMSC: endometrial mesenchymal stem cell; MLEO: multi-lineage endometrial organoid; ns: non-significant. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

spontaneously form self-organizing organoids without R-spondin1 and Noggin. To our knowledge, R-spondin1 and Noggin were the main factors in maintaining the stemness of the organoids in the culture medium. Complete withdrawal of R-spondin1 and Noggin could reduce the proliferation efficiency of the organoids or enhance apoptosis[23,24]. R-spondin1 has been implicated in the activation and regulation of the Wnt signaling pathway[25]. The paracrine effect of bone marrow MSCs could promote endometrial regeneration and proliferation via the Wnt/β-catenin signaling pathway[26]. A similar effect was described in the study on the paracrine effect of human umbilical cord MSCs on the endometrium[27]. Our finding suggests that even without R-spondin1, the eMSCs might generate Wnt niches to support organoid growth and maintain the stemness of organoids.

HAAM was used as a scaffold to load the MLEOs, which comprised EEOs and eMSCs. It provided a compatible scaffold with low immunogenicity and promoted cell

proliferation, differentiation, and adhesion[28,29]. A recent report has indicated that rat hair follicle stem cells (rHFSCs) seeded on HAAM showed flaky and cluster-like morphology and could adhere and infiltrate more effectively into the wound[30], like the morphology of the MLEOs observed in this study. Another study has shown that HAAM loaded with menstrual blood stem cells can regenerate fully injured skin and restore skin function.

HAAM could serve as a dependable potential scaffold with application in regeneration therapy. The China Food and Drug Administration has approved hyaluronic-acid gel (HA-GEL) for clinical intrauterine injection to prevent postoperative IUAs. HA-GEL has been shown to have good lubrication and compatibility. However, due to its liquid nature, it could be partially lost after intrauterine injection through the dilated cervix, and only a small portion remains in the uterine cavity. As a semi-solid film structure, HAAM is not easily lost as it can fit into the shape of the uterine
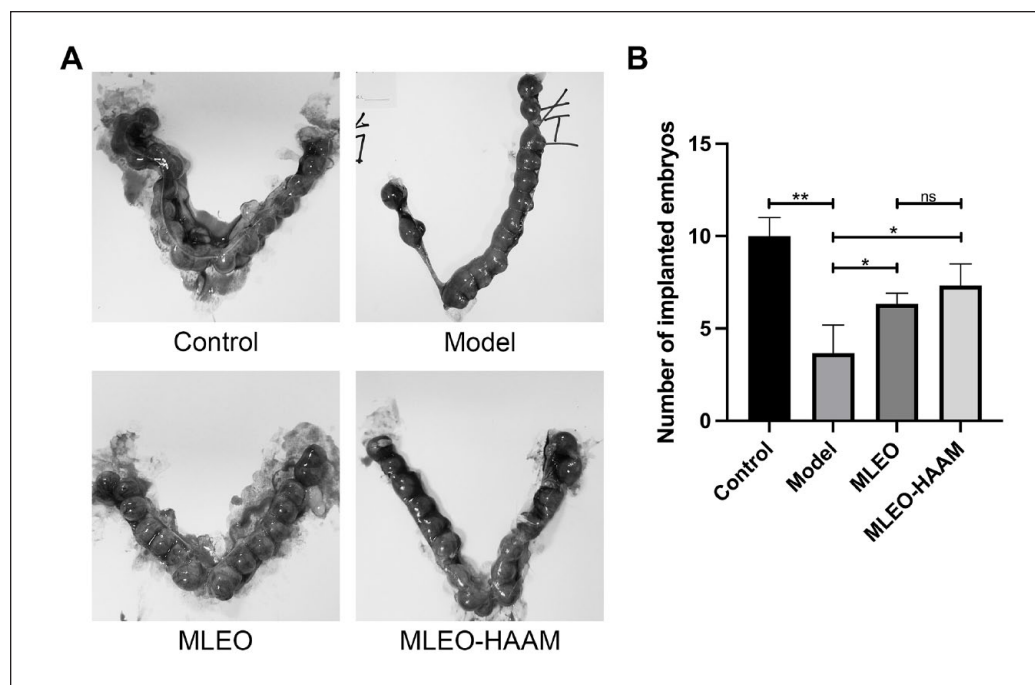
Figure 5. Multi-lineage endometrial organoids on HAAM improved pregnancy outcome in rats. (A) Representative of the number of implanted embryos following different treatments. (B) Comparison of embryo implantation in rats after treatment. HAAM: human acellular amniotic membrane; MLEO: multi-lineage endometrial organoid.

endometrium. Moreover, HAAM provides a sufficient surface for the expansion of EEOs and eMSCs to achieve better repair of the injured endometrium.

There are some limitations in this study. First, organoids are cultured in Matrigel™[31], which is a heterogeneous, complex mixture of ECM proteins, proteoglycans, and growth factors secreted by Engelbreth–Holm–Swarm mouse sarcoma cells. Thus, this tumor-derived matrix has limited clinical translational potential and a safer hydrogel is needed to replace Matrigel™ in future study. Second, further investigations would be required to confirm the applications of this novel repair system since we did not conduct long-term follow-ups on the endometrial-injured mouse model after treatment with the MLEOs on the HAAM patch to investigate spontaneous IUA rates. Further studies would be required to understand the histological changes in the endometrium during implantation.

Conclusion

Altogether, the MLEOs were shown to promote endometrial regeneration, inhibit fibrosis, and effectively improve pregnancy outcomes. The MLEO-HAAM patch has the potential to be a primary prevention method for IUA. Future studies could focus on the regenerative function of the MLEO-HAAM patches in other IUA animal models to further confirm *in vivo* endometrial regeneration.

Method

Organoids and Mesenchymal Cell Culture From Endometrial Biopsies

Endometrial biopsies were collected from premenopausal women undergoing routine hysterectomies for benign uterine conditions following the institutional review board-approved protocol of the Obstetrics and Gynecology Hospital of Fudan University. Endometrial biopsies were collected and transported from the operating room to the laboratory on ice. The tissue was then placed in a 6 cm sterile culture dish and extensively rinsed in $Ca^{2}+/Mg^{2}+$-free phosphate-buffered saline (PBS; Gibco). The endometrium was excised from the uterine tissues, minced into small pieces, and dissociated in a tissue dissociation medium (bioGenous Biotechnologies). The tissue was incubated at 37°C with gentle agitation for 40–50 min, and the extent of digestion was monitored every 15 min. The digestion was terminated by adding 10% fetal bovine serum (Wisent), and the supernatant was passed through a 100 μm strainer and then a 40 μm strainer to obtain the mesenchymal component. The pellet was resuspended and cultured in Dulbecco's modified eagle medium (DMEM)/F12 (Gibco) with 10% fetal bovine and 1% penicillin/streptomycin. The 40 μm strainer was turned upside down and washed with DMEM/F12 supplemented with 1% penicillin/streptomycin to harvest the endometrial component. After centrifugation, the cells were then

pelleted, resuspended in organoid culture ECM (bioGenous Biotechnologies), and seeded in 24-well plates as 40 μl droplets for organoid culture in the endometrial culture medium.

Separation of eMSCs by Flow Cytometry

Endometrial stromal cell suspensions (1×10^7 cells/ml) were first blocked in 3% bovine serum albumin (BSA) for 15 min at 4°C, washed with PBS, and then stained with PE-SUSD2 antibodies for 30 min. Flow cytometry analysis was performed on a BD FACSAria Fusion (BD Biosciences), and the data were analyzed using FlowJo 7.6.3. Cells were selected for fluorescence-activated cell sorting (FACS) by electronic gating according to their forward versus side scatter profile.

Preparation of HAAM Scaffolds

Fresh human amniotic membrane was rinsed with PBS and transferred to a 50 ml sterile centrifuge tube containing an equal volume of 1% Triton X-100 solution. The mixture was then incubated at 37°C at 120 rpm for 24 h. Following incubation, the amniotic membrane was removed from the centrifuge tube, placed in a 15 cm sterile culture dish, and washed five times with PBS. Subsequently, 0.25% trypsin (Gibco) and 0.02% ethylenediaminetetracetic acid disodium (EDTA-2Na) solution (Sigma) were added to the membrane and then incubated at 37°C with 120 rpm for 4 h. After incubation, the membrane was washed with PBS, and a prepared HAAM scaffold was laid flat on a new 15 cm sterile cell culture dish and subjected to ultraviolet radiation for 2 h. To further sterilize the scaffold, it was soaked in a mixture of penicillin/streptomycin for approximately 1 h. The scaffold was then placed into a vacuum freeze dryer for 4 h until it was completely dry. Finally, the HAAM scaffold was cut into 3×3 cm pieces and stored at 4°C for use in subsequent experiments.

Seeding eMSCs and EEOs on the HAAM Scaffolds

Before transplantation, the freeze-dried HAAM was soaked in DMEM/F12 with 1% penicillin/streptomycin for 30 min until it became soft. The soft HAAM was then transferred to a six-well plate and 2 ml of suspension mixture containing EEOs conducted by 2×10^5 epithelial cells and 1×10^5 eMSCs was added. The MLEO-HAAM patch was incubated in a 5% CO_2 incubator at 37°C for 3 days. After 3 days, the complex was transplanted to the injured endometrium and allowed to strengthen the contact between the HAAM and organoid complex.

H&E Staining

The tissue or organoids slide was rehydrated by passing it through different gradients of alcohol solutions and finally with water. Hematoxylin solution (Servicebio) was added to stain the cell nuclei, followed by decolorization with 1% hydrogen chloride. Subsequently, the slide was stained with Eosin (Servicebio), followed by decolorization in 90% ethanol. The sections were mounted using a drop of mounting medium and a coverslip. Dehydration was achieved by dipping the slide in different gradients of alcohols and then xylene.

Immunofluorescence Staining

To prepare the tissue or organoid section for immunofluorescence staining, it was rehydrated by passing it through a series of alcohol solutions of different gradients and finally with water. The slide was then soaked in 0.01 M sodium citrate buffer and transferred to a pressure cooker for 30 min. Once the sodium citrate buffer had cooled down, the section was washed with PBS twice and blocked with 3% BSA for 1 h at room temperature. Next, the section was incubated with primary antibodies at 4°C overnight. After primary antibody incubation, the section was incubated with secondary donkey anti-rabbit or anti-mouse antibody for 1 h at room temperature. Finally, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime) to identify the nuclei. Images were captured with a confocal microscope (Olympus). The following primary and secondary antibodies were used: anti-Axin2 (Abcam, 1:100), anti-Sox9 (Abcam, 1:200), anti-Ki-67 (Abcam, 1:250), anti-EpCAM (Protech, 1:100), anti-Vimentin (Abcam, 1:200).

Masson's Trichrome Staining

The deparaffinized and hydrated section was transferred to distilled water and then soaked in Weigert's iron hematoxylin (Sigma) for 5 min. The section was briefly washed in running water and rinsed in two changes of distilled water. Next, the section was placed in 1% hydrogen ethanol for 5 s, followed by washing in running tap water for 30 s. The section was then covered with ponceau acid fuchsin (Sigma) staining solution for 5 min. After staining, the glass slide was cleaned with 0.2% glacial acetic acid aqueous solution to remove any redundant red color. The slide was then covered with a phosphomolybdic-phosphotungstic acid solution for 5 min, spun until there was no redundant water, and then covered with an aniline blue solution for 10 min. After staining, the slide was rinsed in distilled water and soaked in an acetic acid solution for 10 s. Finally, the stained slides were dehydrated by dipping them in graded alcohols and then xylene.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from cells using the RNAprep pure Micro Kit (TIANGEN), and 5× FastKing-RT SuperMix

(TIANGEN) was used to reverse-transcribe RNA into cDNA. Quantitative polymerase chain reaction (qPCR) was performed using $2\times$ SYBR Green qPCR master mix (Bimake), and the expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated using the $2-\Delta\Delta Ct$ method. The following primers were used for RT-PCRs: Gapdh, forward: 5'-AACGGATTTGGTCGTATTG-3', reverse: 5'-GGAAGATGGTGATGGGATT-3'; Axin2, forward: 5'-TGTTGACAGTGGATACAGGTCC-3', reverse: 5'-TGTTTCTTACTGCCCACACG-3'; Sox9, forward: 5'-GGCAAGCTCTGGAGACTTCTG-3', reverse: 5'-CCCGTTCTTCACCGACTTCC-3'; integrinβ3, forward: 5'-GAGTGCTCTGAGGAGGATTACCG-3', reverse: 5'-TGCAGTAGTAGCCAGTCCAGTCC-3'; HOXA10, forward: 5' -CTCCTACTCCTCCAACCTGC-3', reverse: 5'-GTTCCTGCCCACCGTGCTAT-3';BCL6,forward:'-TAAGACTGGACCGAGGTTGT-3', reverse:5'-CTCGCCTTCCCAGAAATGAA-3'.

Endometrial Injury Model and MLEOs on HAAM Complex Transplantation

Female Sprague–Dawley rats (6–8 weeks old) were obtained from the experimental animal center of Fudan University and were housed under controlled conditions, with a feeding temperature of 20–26°C, the humidity of 50%–60%, and a 12 h light/dark cycle. 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-bent gauge needle inserted two thirds of the way through the lumen and curettage for four times. After establishing the injury, different treatments were conducted in each group ($n = 10$) via direct injection or transplantation into the uterine cavity through the previous incision: (1) control group: untreated, (2) model group: right side of the untreated uterus after molding, (3) MLEOs group: the uterus received an eMSCs-EEOs-loaded Matrigel solution, and (4) MLEO-HAAM group: the organoid complex seeding surface of the patch was attached to the surface of the injured endometrium, and the whole patch was expanded with two small sizes gauge needle. The adherence of the whole patch to the injured endometrium was double-checked and then fixed by the absorbable suture on four corners.

Rats' Mating

On the 14th day of following treatment, we carefully selected three of 10 rats from each group as successfully mated rats, maintaining a balanced proportion of female and male rats in a 1:1 ratio. Upon confirmation of pregnancy, we terminate the gestation in E13, to obtain uterine tissue for subsequent analysis and investigation.

Author Contributions

BZ and XC designed and supervised the study; YX, SC, QW, MC, and XH performed the experiments and analyzed the data; SC, EED, BZ, and XC wrote the manuscript.

Availability of Data and Materials

The data sets used and/or analyzed during the current study are available from the corresponding author on request.

Ethics Approval

All animal breeding and experimental procedures were performed following the relevant guidelines and regulations of the Institutional Animal Care and Use Committee of the Obstetrics and Gynecology Hospital of Fudan University (No.2021-182).

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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