

Original Article

Umbilical cord-derived mesenchymal stem cell transplantation in vaginal replacement *in vitro* and in a rat model

Yiqun Zhang^{1,2,3}, Zhongyi Zhu^{1,2,3}, Keqin Hua^{1,2,3}, Liangqing Yao^{1,2,3}, Yongjun Liu⁴, Jingxin Ding^{1,2,3}

¹Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, P. R. China; ²Shanghai Medical College, Fudan University, Shanghai, P. R. China; ³Shanghai Key Laboratory of Female Reproductive Endocrine-Related Diseases, Fudan University, Shanghai, P. R. China; ⁴Alliancells Institute of Stem Cells and Translational Regenerative Medicine, Tianjin 300308, P. R. China

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Abstract: Cell transplantation strategies represent a potential therapeutic approach towards repair of congenital vaginal agenesis. In this study, the efficacy and mechanisms of action of treatment with human umbilical cord-derived mesenchymal stem cells (UC-MSCs) on vaginal regeneration was explored. UC-MSC transplantation alone, small intestinal submucosal (SIS) grafting alone, and a combination of UC-MSC transplantation/SIS grafting were performed with a vaginal defect rat model. Histological analyses of tissue sections were subsequently performed. UC-MSCs promoted the recovery of keratinizing squamous epithelium and papillae to nearly the same levels as in normal tissue. Of the treatments tested, UC-MSC transplantation showed optimal performance in inhibiting collagen deposition and accelerating the synthesis of elastin to maintain tissue elasticity. UC-MSC treatment also increased Cyclin D1, Ki67, and CD31 expression as assessed by immunohistochemistry. We also investigated the effects of UC-MSC secretions on keratinocytes in a co-culture model. UC-MSCs significantly stimulated vaginal tissue repair by promoting vaginal epithelium regeneration via paracrine factors but not by exploiting their keratinocyte differentiation potential. Further, UC-MSCs facilitated epithelial cell viability and promoted cell cycle progression via the AKT/GSK3 β /Cyclin D1 pathway. These results indicate that UC-MSC transplantation is a feasible approach for vaginal tissue regeneration.

Keywords: Vaginal regeneration, human umbilical cord-derived mesenchymal stem cells, paracrine factors, keratinocytes, tissue repair, AKT/GSK3 β /Cyclin D1 pathway

Introduction

Congenital vaginal agenesis is a birth defect with an incidence that ranges from 1 in 4,000 to 1 in 10,000 [1]. The most common form of vaginal agenesis is Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome, an aplasia or dysplasia of the Mullerian duct that causes partial vaginal agenesis [2]. Difficulty with vaginal intercourse and infertility that result from vaginal agenesis frequently lead to personality problems in patients [3].

Vaginoplasty generates a functional vagina using grafts from oral mucosa [4], lower abdominal skin [2], or the pelvic peritoneum [5, 6] and can improve the quality of life for women with vaginal absence. Disadvantages of this proce-

dure include surgical complexity, remnant scars at the donor site [7], incorrect vascularization, graft rejection, fibrosis, and contraction of the neovagina [8]. Several studies have described successful reconstructions of the vagina using tissue-engineering techniques, such as reconstruction with an acellular dermal matrix [9, 10] and small intestinal submucosal (SIS) grafting [11]. However, these treatments require patients to wear a vaginal mold for three to six months to achieve squamous epithelialization of the neovagina [12]. Recent studies investigating vaginal recovery have used a combination of autologous cells and a biological scaffold [13, 14]. However, sources for clinical autogenous tissues for vaginal reconstruction is limited and this can consequently increase the risk of infection and bleeding.

UC-MSCs for vaginal replacement

Mesenchymal stem cells (MSCs) are easy to harvest and can be used to accelerate the native wound repair process. MSCs are therefore a reliable and widely used source for cell implantation [15, 16]. Human umbilical cord (UC) tissue can also be obtained non-invasively, possesses a significant proliferation capacity, and exerts strong immunomodulatory activity via a paracrine mechanism. The latter properties are important for tissue engineering [16-18]; however, there have been relatively few studies evaluating the use of UC-MSCs for vaginal reconstruction.

Here, we performed UC-MSC transplantation alone, SIS grafting alone, and a combination of UC-MSC transplantation/SIS grafting in defective vaginas of female rats immediately following partial vaginectomy. The results indicate that UC-MSCs alone are sufficient to stimulate repair of the vaginal mucosa in rats and that UC-MSCs promote viability and cell cycle of epithelial cells through activation of the AKT/GSK-3 β /Cyclin D1 signaling pathway.

Materials and methods

Rats

All animal experiments were approved by the animal ethics committee of Fudan University Medical School and all protocols were performed in accordance with the Guidelines for the Ethical Treatment of Experimental Animals (Ministry of Science and Technology, China, revised 2006). Adult female Sprague-Dawley rats weighing 240 to 260 g were supplied with food and water in a constant-temperature room under a 12 h-12 h light-dark cycle.

Culture of UC-MSCs

UC-MSCs were obtained from the Alliances Institute of Stem Cells and Translational Regenerative Medicine (Tianjin, China) and used from passages 2 to 8 in all experiments. UC-MSCs were cultured in DMEM/F12 (1:1) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% glutamine, and 10 ng/ml epidermal growth factor.

Phenotype analysis of UC-MSCs using flow cytometry

UC-MSCs were incubated with monoclonal antibodies against the following proteins: CD11b, CD19, CD34, CD45, CD73, CD90, CD105, and

HLA-DR. Standard isotype controls were performed along with all studied markers. The UC-MSCs were then analyzed using flow cytometry.

Osteogenic and adipogenic differentiation

The osteogenic differentiation of UC-M can improve the quality of life for women with vaginal absence SCs were confirmed with alizarin red S staining after incubation with complete osteogenesis differentiation medium (ThermoFisher Scientific, Waltham, MA, USA) for 14-17 days. The adipogenic differentiation of UC-MSCs was confirmed with Oil Red O staining after incubation with complete adipogenesis differentiation medium (ThermoFisher Scientific, Waltham, MA, USA) for 21 days.

Culture and identification of rat keratinocytes

Rat vaginal tissues were cut and floated dermis-side down in dispase II (Sigma, St. Louis, USA) and digested into single-cell suspensions. The cells were maintained in a keratinocyte serum-free medium (Defined Keratinocyte-SFM, Invitrogen, Carlsbad, CA). Mouse monoclonal antibodies against rat AE1/AE3 and cytokeratin 14 were used to confirm the keratinocyte phenotypes.

Surgical procedures

To perform the partial vaginectomy, the vaginal epithelium was cut along the exterior entrance to the upper vagina. Either UC-MSC suspension or PBS (control) was injected into the subcutaneous tissue at multiple points.

Histological staining

The vaginal sections were stained with hematoxylin-eosin (HE), Masson trichrome, and Weigert-Van Gieson stain. For immunohistochemical analysis, the tissues were incubated with mouse anti-CD31 (1:50, Abcam, Hong Kong, China), anti-Ki67 (1:100, Abcam, Hong Kong, China) and anti-Cyclin D1 (1:50, CST, Massachusetts, USA) antibodies followed by incubation with secondary labeled antibodies. Expression levels of Ki-67 and Cyclin D1 in neovagina were reported as ratios of the number of brown-colored cells to the total number of vaginal epithelial cells multiplied by 100. The mean CD31+ vessel densities (each microvessel comprised fewer than six endothelial cells) was assessed in sections of the central area of the vagina.

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UC-MSC labeling with CM-Dil

UC-MSCs were labeled with CM-Dil fluorescent dye (Invitrogen, Carlsbad, CA) at the working concentration of 2.5 µg/ml through incubation for 5 min at 37°C and then for 15 min on ice. The cells were then isolated by centrifugation prior to resuspension in PBS at 1×10^8 cells/ml.

Immunofluorescent staining

On Day 21 after the transplantation, we assessed the survival of UC-MSCs by identifying the red fluorescent cells in the neovaginal sections. The differentiation of the CM-Dil-stained UC-MSCs in the neovagina was detected by performing fluorescence immunohistochemistry staining by incubation with a monoclonal mouse AE1/AE3 antibody (1:50, Abcam, Hong Kong, China) followed by incubation with an anti-mouse Alexa488-labeled secondary antibody.

Co-culture of UC-MSCs and rat primary keratinocytes

5×10^4 rat primary keratinocytes were placed in the lower chambers of Transwell inserts and 2×10^4 UC-MSCs were seeded onto the upper chambers. Rat primary keratinocyte monocultures at the same density as what was used in the lower chambers of the Transwell inserts were used as controls. All cells were cultured with a 1:1 ratio of Defined Keratinocyte-SFM to MSC complete medium.

Cell proliferation assay

The keratinocytes and indirectly co-cultured keratinocytes/MSCs were maintained under the culture conditions described above for 0, 12, 24, 48, and 72 h. At these time points, cell proliferation was evaluated with a CCK8 (Dojindo Laboratories, Kumamoto, Japan) assay kit following the manufacturer's instructions. Absorbance was quantified at 450 nm using a microplate reader (F-2500 Fluorescence Spectrophotometer, HITACHI).

Cell apoptosis assay

The effect of UC-MSCs on keratinocyte apoptosis was analyzed via an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) and flow cytometry. Primary keratinocytes indirectly co-cultured with UC-MSCs for

72 h constituted the experimental group. Mono-cultured keratinocytes were used as the control group.

Cell cycle analysis

Following incubation for 72 hours, the co-cultured and mono-cultured keratinocytes were treated with PI/RNase Staining Buffer (BD Biosciences, San Jose, CA, USA) and analyzed using a FACS Calibur flow cytometer.

Western blot

Following incubation for 72 hours, the co-cultured and mono-cultured keratinocytes were lysed in radioimmunoprecipitation (RIPA) buffer with protease inhibitor cocktail and PhosSTOP (Roche). Mono-cultured keratinocytes were used as the control group. The total amount of protein extracted was determined using a BCA quantitation kit (Beyotime, Beijing, China) following the manufacturer's instructions. Proteins were subjected to SDS-PAGE electrophoresis with a 4-12% gradient in polyacrylamide concentration and then transferred to a PVDF membrane with primary antibodies for AKT, p-AKT, GSK3β, p-GSK3β, Cyclin D1, and GAPDH. Horseradish peroxidase (HRP)-linked secondary antibodies (Abcam, Hong Kong, China) and enhanced chemiluminescence (ECL)-based detection reagents (Millipore Corp., Billerica, MA, USA) were subsequently added.

Statistical analysis

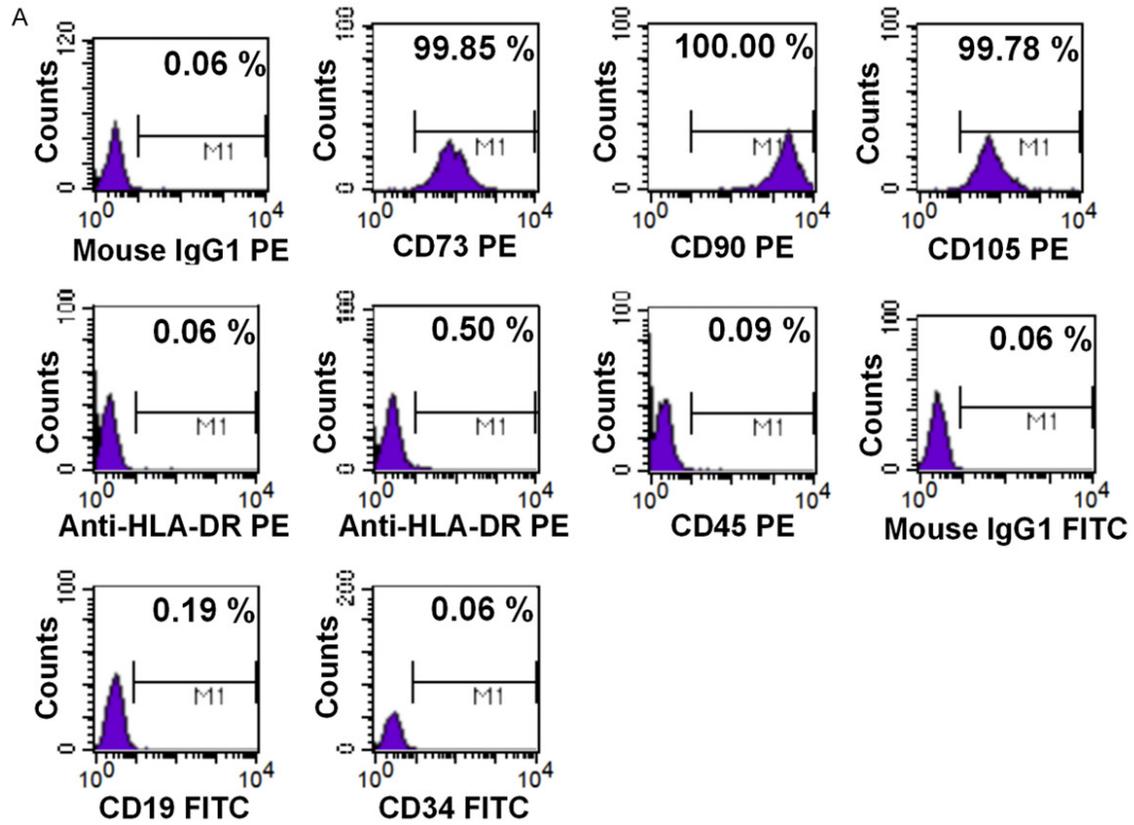
All statistical analyses were performed using GraphPad Prism 5.0. The data are expressed as the mean ± standard deviation (SD). Student's t test was performed for the quantitative variance analysis between two groups; variance (ANOVA) and a Tukey's test were applied to compare values among multiple groups. A *P*-value < 0.05 was considered significant. NS indicated no difference. All experiments were repeated three times.

Results

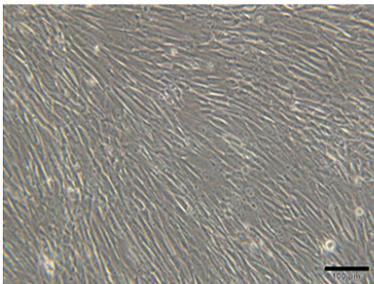
Characteristics of cultured UC-MSCs and rat primary keratinocytes

The cultured UC-MSCs expressed mesenchymal stem cell markers (i.e., CD73, CD90, and CD105) but did not express hematopoietic markers (i.e., HLA-DR, CD11b, CD19, CD34, and CD45), as demonstrated via flow cytome-

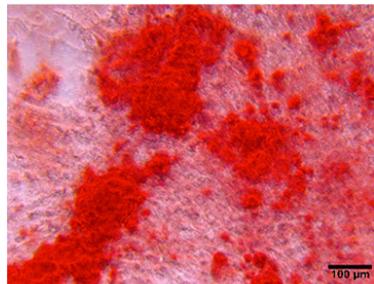
UC-MSCs for vaginal replacement



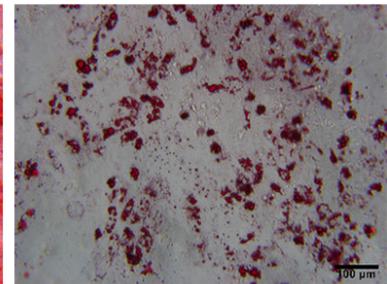
B UC-MSCs



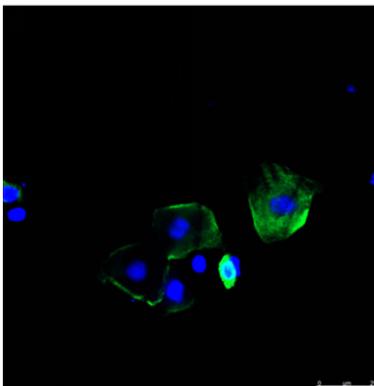
C Osteogenic



D Adipogenic



E AE1/AE3



F CK14

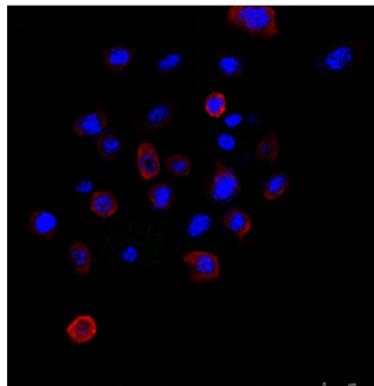


Figure 1. Characteristics of UC-MSCs and keratinocytes. (A) Cell surface markers of UC-MSCs were assessed using flow cytometry. UC-MSCs expressed CD73, CD90, and CD105, but not HLA-DR, CD11b, CD19, CD34, and CD45. (B) Image of UC-MSCs at the fourth passage. UC-MSCs showed a typical fibroblast-like appearance. (C) Osteogenic differentiation of UC-MSCs was confirmed by the presence of calcifying nodules stained with alizarin red S. (D) Ad-

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ipogenic differentiation of UC-MSCs was confirmed by the presence of lipid droplets stained with Oil Red O. (E, F) Confocal microscopy images of primary rat keratinocytes expressing AE1/AE3 (green) and CK14 (red). Scale bars in (B-D) 100 μm , (E and F) 25 μm .

try (**Figure 1A**). Confluent cell populations were homogeneous with fibroblastic, spindle, and rhomboid morphologies (**Figure 1B**). The osteogenic and adipogenic differentiation potentials of the UC-MSCs were confirmed using alizarin red S (**Figure 1C**) and Oil Red O (**Figure 1D**) staining, respectively. Primary rat keratinocytes showed a typical cobblestone-like appearance and were found to express AE1/AE3 and keratin 14 (**Figure 1E** and **1F**).

UC-MSC transplantation in a rat model stimulates vaginal tissue repair by reducing fibrosis and promoting vaginal epithelium regeneration and angiogenesis

Animal experiments and histological analyses were used to assess the efficacy of UC-MSCs in vaginal replacement. Forty rats were randomly assigned to five groups: 1) the “intact control” group received sham operations and no treatment; 2) the “negative control” group underwent partial vaginectomies to induce vaginal defects, received 100 μl PBS, and wore vaginal molds; 3) the “SIS” group underwent the same vaginectomies as the negative control group, rolling SIS grafts (1 cm \times 1.2 cm, Cook) were implanted during the surgery, and the SIS meshes were fixed on the outer surfaces of the vaginal molds with absorbable sutures; 4) the “UC-MSCs” group underwent the same vaginectomies as the negative control group and were injected with UC-MSCs (1×10^7 cells per rat) immediately after surgery at the site of the surgical resection; and 5) the “UC-MSC/SIS” group underwent the same vaginectomies as the negative control group, SIS scaffolds were implanted during the surgery, and UC-MSCs were injected at the same concentration as the UC-MSC group. The intact control group displayed a normal vaginal morphology, and the negative control group displayed a morphology similar to that observed in an MRKH model. PBS and the UC-MSC suspension injections were made into the subcutaneous tissue.

As shown in **Figure 2A**, treatment with UC-MSCs alone resulted in enhanced epithelial growth, the appearance of dermal papillae, hallmarks of healing, and a nearly normal vaginal morpho-

logy. As assessed with the intact control group, the normal vaginal mucosa is comprised of a keratinized stratified squamous epithelium and dermal tissue. The SIS and UC-MSC/SIS groups showed a restoration of certain papillae and increased epithelium thickness. In contrast, in the negative control group, which best resembled MRKH, the epithelium was found to be highly irregular and thin/absent and papillae in the connective tissue were not observed.

Relative to the negative control group, the UC-MSC, SIS, and UC-MSC/SIS groups displayed significantly fewer collagen fiber bundles (**Figure 2B**). Compared to the intact control group, collagen content was significantly higher in the negative control, SIS, and UC-MSC/SIS groups but nearly the same in the UC-MSC group. Thus, the UC-MSC, SIS, and UC-MSC/SIS treatments all reduced collagen deposition; however, only UC-MSC treatment alone effectively decreased fibrosis and reduced tissue damage.

As indicated in **Figure 2C**, based upon Weigert-Van-Gieson Staining, the elastin fiber-fiber content was significantly higher after UC-MSC administration than that observed in the negative control group. Further, in the UC-MSC group, the elastin fibers were configured in a wavy pattern, forming an extensive elastin network under the epithelium. In contrast, the number of elastin fibers did not differ among the SIS, UC-MSC/SIS, and negative control groups, and the broken elastin fibers resulted in the typical appearance of tissue damage. Compared with that in the intact group, the content of resilient fiber in the negative control group after partial vaginal resection was decreased. Thus, only UC-MSC treatment alone promoted the growth of elastin fibers *in vivo*.

As shown in **Figure 3A**, although slight increases in Ki67 were observed in the SIS and UC-MSC/SIS groups than in the negative control group, the greatest increase occurred in the UC-MSC group. The expression of Ki67 did not differ between the negative and intact control groups. Therefore, only UC-MSC treatment alone promoted epithelial cell proliferation *in vivo*.

UC-MSCs for vaginal replacement

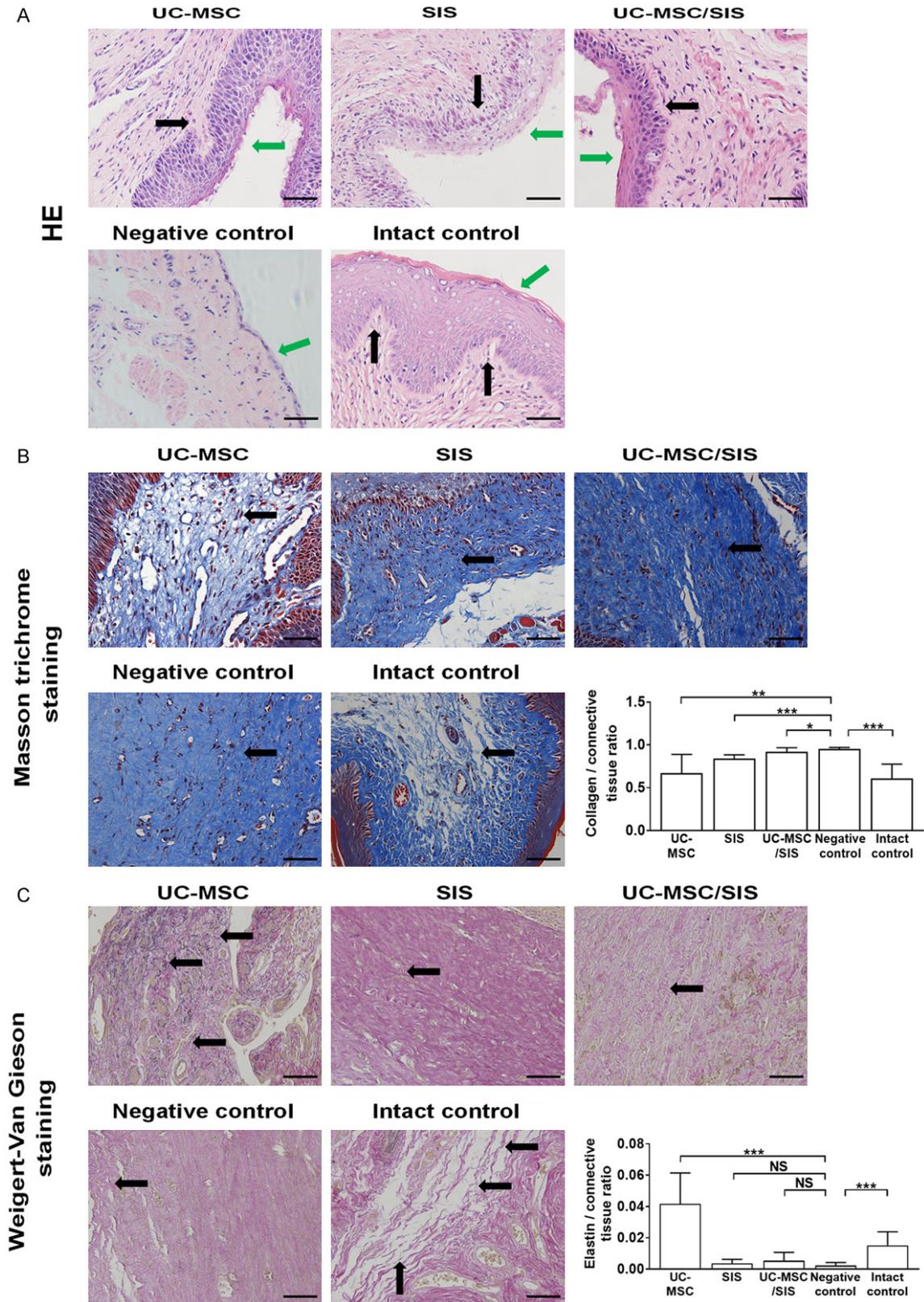


Figure 2. Reparative effects of UC-MSCs on the vagina around the site of implantation in rats 21 days after surgery. A. Representative neovaginal sections stained with H&E. Green arrow = epithelium, black arrow = dermal papillae. Dermal papillae, a fingerlike projection, is the uppermost layer of the dermis. It intertwines with the epidermis and is composed of loose collagen fibers. B. Representative neovaginal sections stained with Masson's trichrome. Red

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areas = smooth muscle cells or epithelium, blue areas = collagen, blue-black = nuclei. Lower right: Quantitative analysis of collagen area normalized to the total connective area. C. Representative neovaginal sections stained with Weigert-Van Gieson. Red = collagen, black arrow = elastin. Lower right: Quantitative analysis of elastin area normalized to the total connective area. Scale bars: 50 μ m. All values are expressed as mean \pm SD and compared by variance (ANOVA) followed by Tukey's post hoc test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

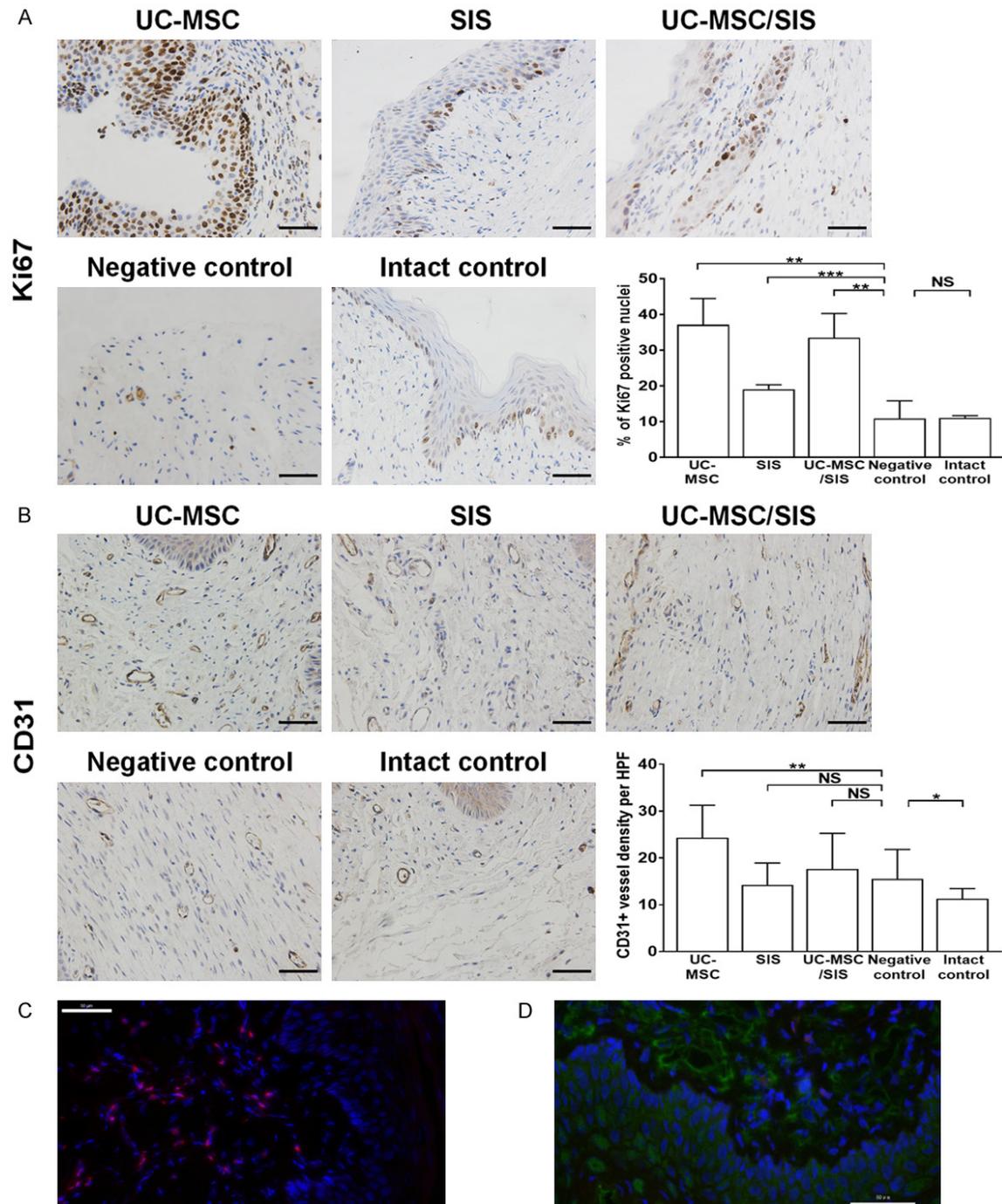


Figure 3. Reporative effects of UC-MSCs on the vagina around the site of implantation in rats 21 days after surgery (n = 8 per group). A. Representative neovaginal sections stained with Ki67. Brown = Ki67. Lower right: Quantification of the percentage of Ki67-positive vaginal epithelial cells. B. Representative neovaginal sections stained with CD31. Brown = CD31. Lower right: Quantification of the mean CD31+ vessel density per HPF (microvessels were those comprising fewer than six endothelial cells). Scale bars: 50 μ m. All values are expressed as mean \pm SD and compared by variance (ANOVA) and a Tukey's test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. C. CM-Dil-labeled UC-

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MSCs were observed engrafted in the dermal region of neovaginal sections by fluorescent staining. Red = CM-Dil, blue = DAPI. D. Fluorescent staining of undifferentiated UC-MSCs in neovaginal sections. Red = CM-Dil, green = AE1/AE3, blue = DAPI.

Compared with the negative control group, only the UC-MSC group yielded significantly higher CD31 expression (**Figure 3B**). Taken together, the results suggest that optimal reparative effects were observed after UC-MSC administration, as evidenced by reduced fibrosis and enhanced epithelial regeneration and angiogenesis.

UC-MSCs induce repairing process of vagina through the paracrine pathways

In the dermal tissues of recipient rats, CM-Dil-stained UC-MSCs were found 21 days after transplantation (**Figure 3C**). There was no visible overlap of UC-MSCs (red) and keratinocytes (green), indicating that the UC-MSCs did not directly convert into keratinocytes (**Figure 3D**). UC-MSCs seeded in the subepithelial connective tissue of the neovaginas survived but failed to differentiate into epithelial cells, implying that UC-MSCs exert trophic effects on the epithelium rather than by undergoing true differentiation.

UC-MSCs facilitate epithelial cell viability and influence the cell cycle in vitro, likely by releasing paracrine factors

The CCK8 assay revealed that the conditioned medium from UC-MSCs promoted epithelial cell viability (**Figure 4A**). Cell cycle analysis showed that UC-MSCs increased the percentage of keratinocytes in the S and G2/M phases and decreased the percentage in the G0/G1 phase (**Figure 4B** and **4C**). The apoptosis assessment indicated that UC-MSC exposure had no significant effects on the apoptosis of keratinocytes (**Figure 4D** and **4E**). UC-MSC factors significantly upregulated p-AKT, p-GSK3 β , and Cyclin D1 expression in epithelial cells (**Figure 4F**). Cyclin D1 was significantly increased in the new epithelia of UC-MSC group compared to that of the negative control (**Figure 4G**) group. Thus, the UC-MSC microenvironment markedly enhanced keratinocyte viability through activation of the AKT/GSK3 β /Cyclin D1 signaling pathway.

Discussion

The experiments presented here-in demonstrate that UC-MSCs have the potential to pro-

mote vaginal tissue repair in patients diagnosed with congenital vaginal agenesis. During wound healing, the efficacy of UC-MSC treatment alone was greater than that of SIS grafting alone and a combination of SIS grafting and UC-MSC treatment. UC-MSC transplantation resulted in several reparative effects, including reduced fibrosis, increased neovascularization, and a normalized vaginal epithelium appearance (**Figures 2** and **3**). This result is consistent with previous studies that used UC-MSC transplantation into wounds to accelerate wound closure in excisional full-thickness skin murine models [19, 20]. SIS grafting alone and the combination of SIS and UC-MSCs only mildly stimulated vaginal tissue repair. Several studies have shown that stem cell/SIS implants can regenerate the vaginal epithelium more rapidly than SIS alone, suggesting that stem cells/SIS is a promising method in treating vaginal prolapse [14, 21]. However, based on the present study, UC-MSCs alone have improved efficacy over both SIS grafting alone and UC-MSC/SIS combination treatment in vaginal reconstruction. In addition, SIS grafting has been shown to cause chronic inflammation accompanied by collagen deposition [22, 23] and to confer a high risk of infection in contaminated surgical settings [24].

In vivo tracking supported the concept that the repairing effects of UC-MSCs are mediated by paracrine signaling rather than by cell replacement and differentiation (**Figure 3C** and **3D**). Stem cells have previously been shown to enhance survival, increase proliferation, and decrease apoptosis of corneal epithelial cells that were chemically induced by stanniocalcin (STC-1), an antiapoptotic molecule [25]. Stem cells are recruited to wounds and synthesize proangiogenic cytokines that promote endothelial and epithelial cells growth [26]. Our co-culture model of UC-MSCs and epithelial cells mimicked early epithelial development in the body. According to previous studies, the heterotypic mesenchyme is related to the modulation of epithelial proliferation, apoptosis, and original differentiation [27]. In the present study, UC-MSC secretions were shown to extend the regenerative capacity of keratinocytes by accelerating the cell cycle (**Figure 4**) via activation of the

UC-MSCs for vaginal replacement

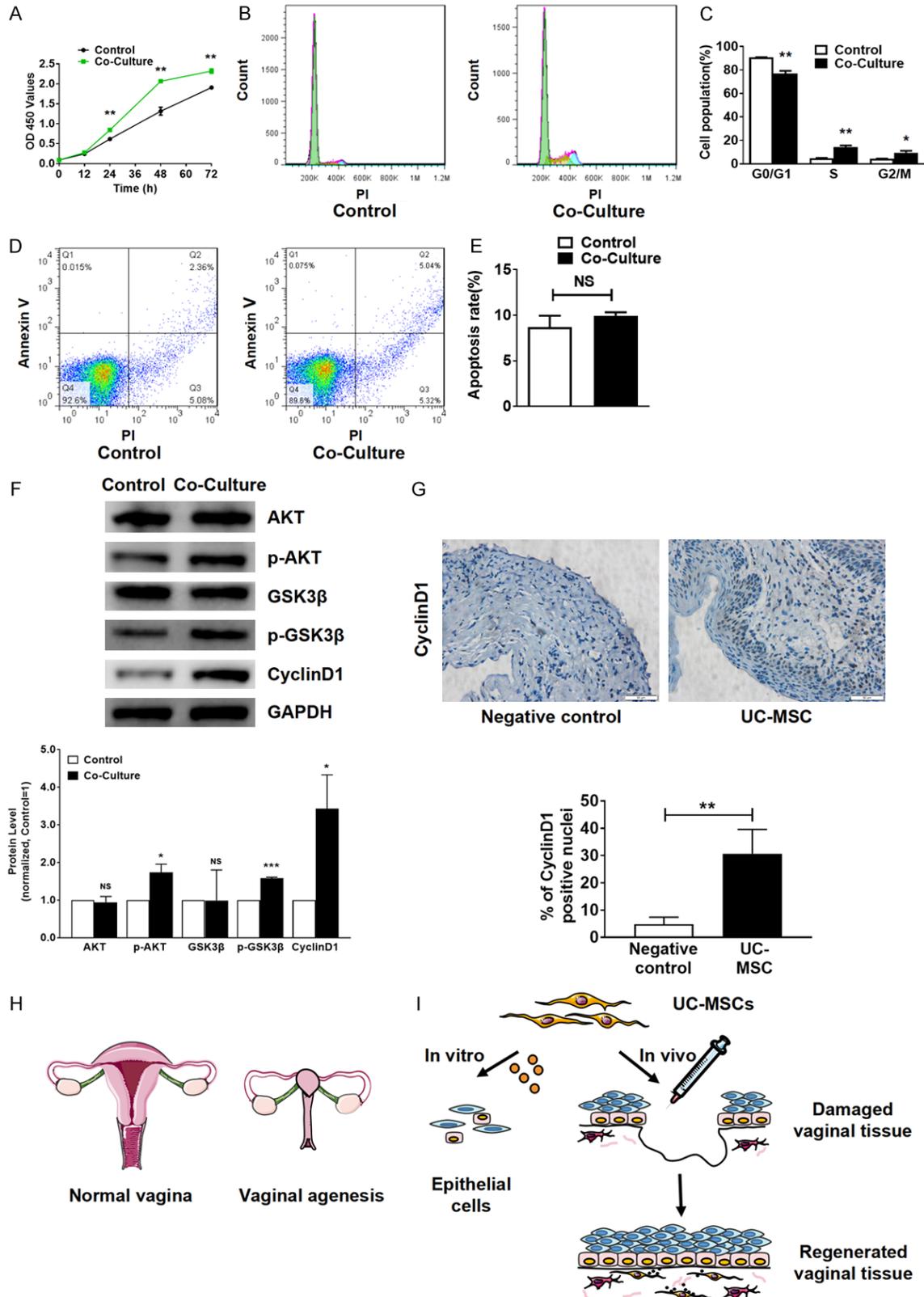


Figure 4. UC-MSC-mediated promotion of keratinocyte proliferation and regulation of cell cycle progression. A. CCK8 assays of control keratinocytes and those co-cultured with UC-MSCs at the indicated time points. B. Representative cell cycle distribution as determined by FACS for control and co-cultured keratinocytes cultured for 72 h. C. Quantification of cells in each cell cycle phase for keratinocytes cultured for 72 h. D. Representative cell apoptosis results

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for keratinocytes cultured for 72 h. E. Quantification of the cell apoptosis rate for keratinocytes cultured for 72 h. F. Western blot analysis of Akt, p-Akt, GSK3 β , p-GS3 β , and Cyclin D1 levels in keratinocytes cultured for 72 h. G. IHC analysis of cyclin D1 expression in the neovaginas of the negative control and UC-MSC groups. Scale bars: 50 μ m, n = 8 per group. H. Diagrams of the normal vagina and MRKH syndrome (congenital absence of uterus and vagina). I. Graphic summary of the present study's results. UC-MSCs improved vaginal tissue regeneration *in vitro* mainly through the release of paracrine factors, corresponding to cytokines, and in a rat model. All values are expressed as mean \pm SD and compared by Student's *t* test. All experiments were repeated three times. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

AKT/GSK3 β /Cyclin D1 pathway. Further, UC-MSCs were shown to be involved in epithelial homeostasis via the regulation of the dynamic and continuous transition of keratinocytes from a non-proliferating to a proliferating state.

UC-MSCs showed advantages over cells previously tested for use in vaginal replacement. For examples, unlike bone marrow MSCs and autologous vaginal tissue, UC-MSCs have greater accessibility and fewer ethical constraints. Consequently, establishing a stem cell bank for future clinical applications is feasible. Furthermore, we showed that treatment with UC-MSCs alone had enhanced therapeutic efficacy compared to treatment with UC-MSCs in combination with SIS grafting. By eliminating the need for SIS grafting, medical costs and difficulties in application could be reduced.

This is the first reported study investigating the use of human UC-MSCs in women or animal models with vaginal agenesis (**Figure 4H and 4I**). Our data demonstrates the efficacy of these cells in facilitating vaginal tissue healing through paracrine mechanisms. Future studies should focus on defining the paracrine factors, such as cytokines or MSC-derived extracellular vesicles, involved in the therapeutic potential of MSCs in vaginal tissue healing. The present study provides new evidence supporting the use of UC-MSCs in vaginal reconstruction.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Yongjun Liu, Alliancells Institute of Stem Cells and Translational Regenerative Medicine, Tianjin 300308, P. R. China. Tel: +86 18920692888; E-mail: Yongjunliu323@gmail.com; Jingxin Ding, Department of Gynecology, The Obstetrics and Gynecology Hospital of Fudan University, 419 Fang Xie Road, Shanghai 200011, P. R. China. Tel: +86 13918206746; Fax: +86 21 634-55050; E-mail: djxdd@fudan.edu.cn

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