

Original Article

Transplantation of menstrual blood-derived mesenchymal stem cells (MbMSCs) promotes the regeneration of mechanical injured endometrium

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Abstract: Purpose: The ultimate cause of intrauterine adhesions (IUAs) is the substantial destruction of the endometrium, which makes the regeneration of endometrium difficult. The purpose of this study was to observe menstrual blood-derived mesenchymal stem cells (MbMSCs)'s effect on the endometrial regeneration with different methods of transplantation. We also studied whether MbMSCs transfected with the FGF2 gene can improve the regenerative effect. Methods: 75 female SD rats with endometrium removed were used as IUA models. These IUA models were divided into 5 groups: group A was the IUA control, group B received a scaffold transplant, group C received a scaffold+MbMSC transplant, group D received a scaffold+FGF2-MbMSC transplant, and group E received FGF2-MbMSCs injected via the tail vein. After the intervention, 5 rats from each group were sacrificed on the 7th day and the 28th day respectively. The distribution of MbMSCs in endometrium was traced using enhanced green fluorescent protein. The endometrial morphology, number of endometrial glands, area of endometrial fibrosis and immunohistochemistry (IHC) of Ki67, VEGF, and CD31 were evaluated. In addition, the fertility of all groups was tested. Results: On the 7th day after transplantation, enhanced green fluorescent protein showed that there were more MbMSCs in the uterine cavity of group D than that of group E. The endometrial morphology of groups A and B was atrophic and thinned with a high proportion of fibrosis in the endometrium. The endometrium of groups C, D and E was thickened, contained more glands, exhibited reduced fibrosis, and had increased expression of Ki67, VEGF and CD31. The endometrial regenerative effect from high to low was D > C > E with significant differences between each two groups. The fertility test verified the regenerative effect. Conclusions: These results suggest that the injection of MbMSCs into the tail vein was an effective way to stimulate endometrial regeneration, but the effect was not so well as the intrauterine transplant of MbMSCs with scaffold. The FGF2-transfected MbMSCs exhibited enhanced regenerative effect.

Keywords: Intrauterine adhesions (IUAs), endometrial regeneration, menstrual blood-derived mesenchymal stem cells (MbMSCs), FGF2 transfect, scaffold

Introduction

Asherman's syndrome, also called intrauterine adhesion (IUA), is characterized by partial or total adhesion/occlusion of the cavity, fibrosis of the uterine cavity due to mechanical destruction of the endometrium and/or infection with the symptoms of amenorrhea/hypomenorrhea, infertility, recurrent pregnancy loss and/or abnormal placentation [1]. Currently, the clinical treatment for IUAs is transcervical hysteroscopic intrauterine adhesiolysis, which can

restore the uterine cavity to a large extent [2]. Some methods such as intrauterine devices, balloon stents, and sodium hyaluronate have been reported to prevent the recurrence of adhesions [2]. Nevertheless, patients with moderate or severe intrauterine adhesions have a very high rate of recurrence, which is reported to be as high as 62.5% [3]. Because the rate of recurrence is so high, the pregnancy prognosis of patients with IUAs is poor [1-3]. The ultimate cause of IUA is the substantial destruction of the endometrium, which makes regeneration of

active endometrial tissue difficult. We have established a mechanical injury-induced IUA model that is similar to the pathogenic factors of clinical IUA [4, 5]. In this study, we investigated the therapeutic effect and possible mechanism of MbMSCs in vivo. In addition, several studies have reported that MSCs may contribute to endometrial regeneration [6-9], but the relative effectiveness of different methods of transplanting stem cells has not been reported. Therefore, the purpose of this study was to observe the effect of MbMSCs on endometrial regeneration in an IUA model with different methods of transplantation. As FGF2 is important for the regeneration and repair of tissue damage, we also studied whether MbMSCs that were transfected with the FGF2 gene could improve the regenerative effect.

Materials and methods

Animals and experimental protocol

Sexually mature 6- to 8-week-old female and male SD rats were purchased from Silaike Corporation (Shanghai, China). All rats were housed in a room at 20°C with a 12 h light:12 h dark cycle and provided access to food and water ad libitum. All animals were treated according to a protocol approved by the institutional ethics review board of the Ob/Gyn Hospital of Fudan University, Shanghai. All the IUA rat models were established as described previously [4, 5]. Briefly, after being anesthetized by a pentobarbital injection and placed in the supine position, the rats were shaved and disinfected, an incision was made in the abdominal area, the uterine horns were incised and the endometrium was completely removed with a pair of scissors. The uteri were then sutured and gently replaced in the pelvic cavity.

In total, 75 female rats were established as severe IUA models and were divided into 5 groups with 15 rats in each group (group A, IUA control; group B, scaffold (Zhenghai Biotechnology, Shandong, China); group C, scaffold+MbMSCs; group D, scaffold+FGF2-MbMSCs; and group E, FGF2-MbMSCs by injection). After the intervention, 5 rats from each group were sacrificed on the 7th day and the 28th day. The uterus of the rats was removed and several cross sections were taken, fixed in 4% formaldehyde, and then embedded in paraffin; the central tissue of the uterus was taken for con-

tinuous sectioning. The remaining rats were used for fertility testing.

Isolation and identification of MbMSCs

Menstrual blood samples from healthy female volunteers aged 18 to 35 years were collected using a DivaCup (Canada) during the second or third day of menstruation. The collected menstrual blood was mixed with a cell preservation solution containing 1% penicillin, 1% streptomycin, 4% 0.25 mg/ml amphotericin B, and 2% EDTA-Na₂ and was then stored in a 4°C refrigerator. The MbMSCs were isolated within 48 hours by the Biaomo biotechnology limited. Briefly, the collected blood samples were added to a centrifuge tube containing 6 ml of lymphocyte separation solution (density 1.077 g/ml) and then centrifuged at 1600 rpm for 15 minutes. The middle white layer was then collected and transferred to a sterile centrifuge tube; PBS was added, and the sample was centrifuged twice. An appropriate amount of complete medium (α -MEM medium+1% penicillin+10% FBS) (Sigma aldrich, Australia) was then added. The supernatant was mixed and transferred into a cell culture flask and then incubated in a 37°C incubator under 5% CO₂. The culture medium was changed every 3 day. When the cells reached 80%-90% confluence, trypsin digestion and subculture were performed. Cell morphology and growth were observed under an inverted microscope.

The MbMSCs were identified by their ability to differentiate into osteoblasts, adipocytes or chondroblasts and by the expression of the cell surface markers CD29, CD44, CD73, CD90, CD105, CD146, CD34, CD45, and HLA-DR (Invitrogen, USA) using flow cytometry.

FGF2-MbMSC transfection

Using recombinant gene technology, the GV-287 vector (genechem, China) was cut by the BamHI/AgeI enzyme, and the amplified FGF2 gene fragment was then inserted. The constructed lentivirus carrying the FGF2 gene was then transfected to MbMSCs. The recombinant MbMSCs with the FGF2 gene is abbreviated as FGF2-MbMSCs in the process that follows.

Preparation of the FGF2-MbMSC scaffold

The FGF2-MbMSC scaffold was constructed as follows: after digestion with trypsin, a single-

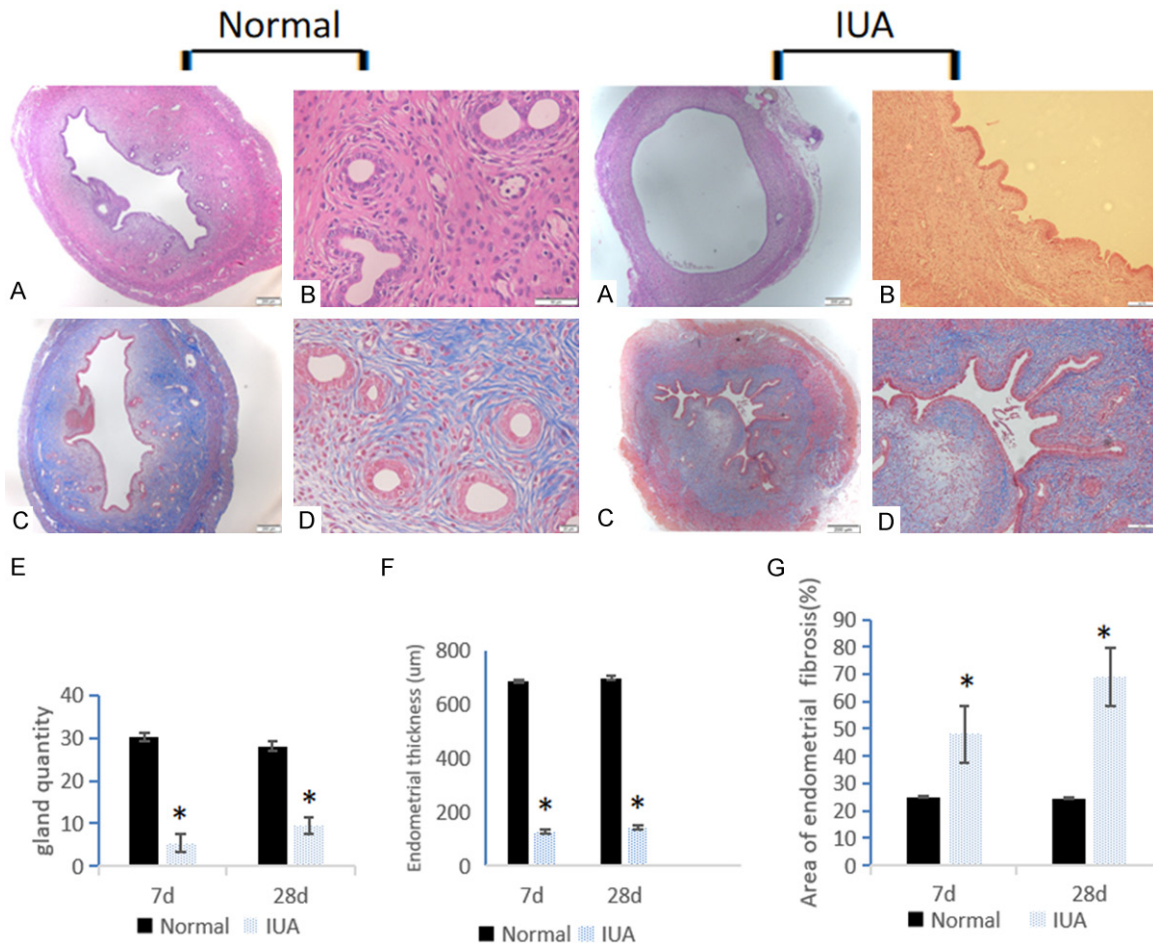


Figure 1. The micro view of IUA rat model. A. HE staining of rat uterine after 7 days; the IUA uterine cavity disappeared and gland quantity decreased sharply. Neutrophil cells and red blood cells also filled in IUA endometrium. Scale bar = 200 µm. B. HE staining of rat uterine after 7 days at high magnification. Scale bar = 100 µm. C. Massion staining of rat uterine after 7 days. Scale bar = 200 µm. D. Massion staining of rat uterine after 7 days at high magnification. Scale bar = 100 µm. E. Gland quantity decreased significantly in the 7 d and 28 d IUA group compared with that of normal groups, $P < 0.01$. F. Endometrial thickness decreased significantly in the 7 d and 28 d IUA group compared with that of normal groups, $P < 0.01$. G. Area of endometrial fibrosis increased significantly in the 7 d and 28 d IUA group compared with that of normal groups, $P < 0.01$.

cell suspension was made using well-grown P4-5 FGF2-MbMSCs with an 80-90% FGF2 fusion rate. The suspension was then centrifuged at 1000 rpm for 3 minutes, and the FGF2-MbMSC suspension was adjusted to a density of 5×10^7 /ml. The 4 cm × 6 cm collagen scaffold with pores of 20-200 µm in diameter [10] (Zhenghai Biotechnology Company, Shandong, China) was cut to 2.5 cm × 0.5 cm and rinsed with xeno-free MSC culture medium (MesenCult™ MSC Basal Medium, Stem cell Technologies, Vancouver, Canada) in a 12-well plate, which was placed in humid air containing 5% CO₂ at 37°C for 1 h before transplantation. The loose surface of the scaffold was oriented up to facilitate the reception of MbMSCs

onto the loose surface. After 1 hour, excess medium was aspirated, and a suspension of 5×10^7 FGF2-MbMSCs was dripped uniformly onto the scaffold. The scaffold was then incubated under 5% CO₂ at 37°C for 1 h to ensure that the MbMSCs adhered to the scaffold. Finally, the FGF2-MbMSCs were transferred to the rat IUA model within 3 hours. At 7 d and 28 d after transplantation, 5 rats from each of the 5 groups were sacrificed for analysis (see **Figure 1**).

Histological analysis

The entire uterus was dissected, fixed in 10% buffered formalin overnight, dehydrated in gra-

ded alcohols and embedded in paraffin. Transverse sections (5 μ m) of the uterus were taken. Hematoxylin-eosin staining was applied to observe the tissue structure, and Masson's trichrome staining was used to highlight the collagen under light microscopy.

Immunohistochemistry

After deparaffinization, dehydration and antigen retrieval, the slides were immersed in 3% hydrogen peroxide to block endogenous peroxidase activity and were then blocked in goat serum for 1 hour. The slides were then incubated overnight at 4°C with the primary antibodies against VEGF (ab2349, Abcam, Cambridge), CD31 (ab28364, Abcam) and Ki67 (AB-9260, Millipore, Billerica). Biotinylated goat anti-mouse or goat anti-rabbit secondary antibodies (ab97051, Abcam) were applied for 60 min at room temperature. The slides were later stained with 3,3-diaminobenzidine (DAB) at room temperature, lightly counterstained with hematoxylin, dehydrated and covered with glass cover slips. The quantification of immunoreactivity was performed using Image-Pro Plus 6.0; 3-5 fields were randomly selected from each slide to determine the mean optical density (MOD). Image-Pro Plus 6.0 (media cybernetics, USA) was used to evaluate Ki67-positive cells. The number of capillary vessels and proliferation cells were counted and averaged from at least 3 randomly selected fields at a magnification of 400 \times .

Hematoxylin & eosin (HE) staining

The uteri were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. The slides were first deparaffinized and rehydrated and then stained with hematoxylin and eosin (ab-245880, Abcam). We visualized the entire image of the pathological uterus and recorded the gland quantity to predict IUA.

Masson staining

Masson staining was used to detect endometrial fibrosis. The deparaffinized and rehydrated slides were incubated with a Masson staining mixture for 5 min and then stained with a phosphomolybdic acid-aniline blue solution for 6.5 min. The area of blue-stained collagen fibers relative to the total field of view was calculated using Image-Pro Plus 6.0.

PCR and quantitative real-time PCR (qRT-PCR)

The FGF2 5'-flank was obtained from the genomic DNA of U937 cells by PCR. The sequence was compared with the published FGF2 5'-flank sequence (ENSEMBL database), and promoter fragments were subcloned into the pGL3-basic reporter vector (Promega). Other constructs were generated with mutation of identified Hox binding sites in the FGF2 promoter. Reporter constructs were also generated using the pGL3-promoter vector (with a minimal promoter and reporter) and three copies of the proximal or distal Hox binding cis elements from the FGF2 promoter.

The excised uterine tissues were ground using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The purity and concentration of the RNA were assessed and then the RNA was reverse transcribed to cDNA. Quantitative real-time PCR using 384-well optical plates was performed in a SYBR green (Takara, Japan) format with a 10 μ L template. Each group included 6 samples, and each sample was run in triplicate. We used the $2^{-\Delta\Delta CT}$ method to analyze relative gene expression.

Enzyme linked immunosorbent assay (ELISA)

The supernatant of FGF2-MbMSCs were immediately processed and frozen at -70°C. The FGF2 ELISA was performed according to the manufacturer's instructions (ab246531, Abcam); samples were diluted 1:20 and supernatant 1:10 using the diluent supplied. The FGF2 ELISA unit was calculated by the formula [(test sample optical density (OD) value-mean of negative control performed in triplicate OD) \times 100 / (Mean OD of weak positive control performed in duplicate-mean OD of negative control performed in triplicate)].

Fertility testing

To study fertility after the different types of MbMSC transplantation, the transplanted IUA female rats were bred with male rats at 28 d after transplantation, and then 21 d later, the female rats were isolated from the male rats. The change in body weight and abdominal shape of the female rats were recorded each day. During the middle and late pregnancy periods, the females were laparotomized, and the

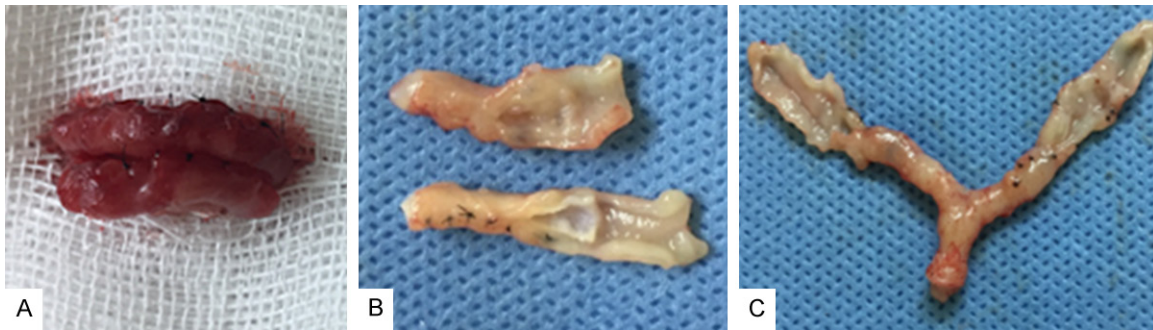


Figure 2. A. Shows the moment after transplantation of MbMSCs+scaffold to the IUA model rat and suturing of uterine. B. Shows the 7th day after MbMSCs+scaffold transplantation: the scaffold can be seen after the incision of uterus. C. Shows 28th days after MbMSCs+scaffold transplantation: the scaffold was mostly been absorbed and can't be easily seen.

pregnancy rate and number of fetuses in each uterine horn were recorded.

Statistical analysis

The data were analyzed using SPSS 20.0. All measurement data are shown as the mean \pm SD. A t-test and one-way analysis of variance were used to analyze the data from the different groups, followed by a Bonferroni test for post hoc comparisons. Spearman's rank correlation coefficient was used to evaluate the correlations between variables. $P < 0.05$ was defined as indicative of statistical significance.

Results

Confirmation of the severe IUA model in rats

On the 7th day after IUA modeling, hydrops was observed in the uterine horns. HE and Masson staining showed that the uterine cavities were completely blocked or dropsical with few glands, and almost the entire endometrial epithelium had disappeared including both the functional and basal layers (see **Figures 1** and **2**). The stroma became edematous and extracellular matrix (ECM) collagen deposition was slightly increased. The capillaries were degraded, resulting in severe hemorrhaging, and neutrophils had infiltrated the stroma. On the 28th day after IUA modeling, there was no epithelial regeneration, neutrophils had decreased and there was more severe interstitial fibrosis.

Compared with the control group, there was a significant decrease in the endometrial thickness and the number of glands and an increa-

se in the fibrotic area on the 28th day (see **Figure 1**).

Construction of FGF2-MbMSCs and transfection of the FGF2 gene lentivirus vector into MbMSCs

PCR was used to determine the presence of FGF2-positive clones. The PCR product size of positive FGF2 clones was 676 bp, and the PCR product size of negative FGF2 clones was 136 bp (see **Figures 3** and **4**).

Validation of FGF2 gene expression in FGF2-MbMSCs

In this study, five multiplicities of infection (MOI) of lentivirus were used: MOI 15, MOI 20, MOI 25, MOI 30, and MOI 35. The FGF2 mRNA expression levels of the FGF2-MbMSCs in each group were significantly higher than that of the control group ($P < 0.0001$), indicating that the FGF2 gene was successfully transmitted to the offspring. The optimal MOI was 30.

The FGF2 level in the culture supernatant of FGF2-MbMSCs was detected using an ELISA. Compared with the blank transfection group and the MbMSC control group, the expression of FGF2 in the culture medium of the FGF2-MbMSCs was significantly increased 6 d and 11 d after transfection ($P < 0.001$), which suggests that the FGF2 gene integrated into the MbMSCs can be transferred to the progeny and stably expressed. There was no significant difference between the blank transfection group and the MbMSC control group. The expression of FGF2 in the culture medium at 11 d after transfection was also higher than the expres-

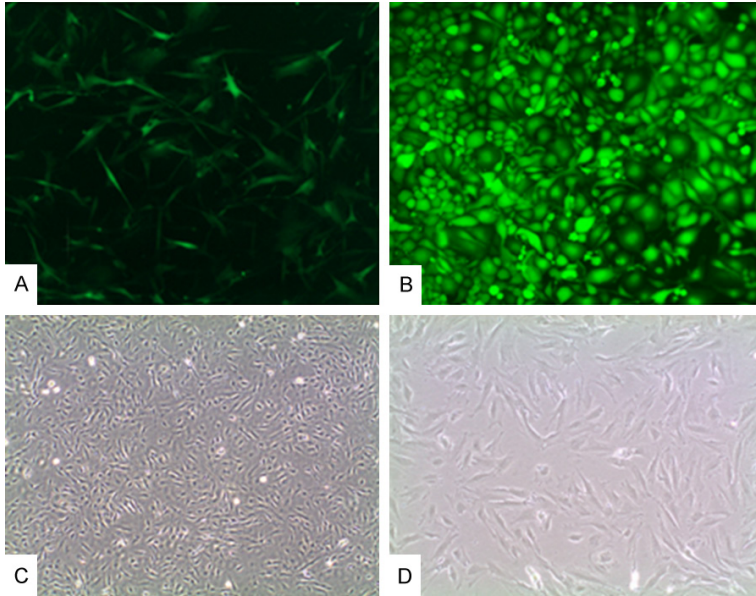


Figure 3. (A and B) show the expression of enhanced green fluorescent protein of FGF2 gene transfected to P3-MbMSCs under the fluorescence microscope: (A) shows the fluorescence expression of MbMSCs was weak on the 7th day after transfection. (B) shows that P4 FGF2 MbMSCs which were passaged from transfected P3-MbMSCs. P4 FGF2 MbMSCs had returned to spindle shape, well grown and have high fluorescence expression. (C and D) were P5 FGF2-MbMSCs, which were no different from that of non transfected MbMSCs (C was 40 times and (D) was 100 times magnification respectively).

sion level 6 d after transfection ($P < 0.05$) (see **Figure 5**).

Distribution of MbMSCs in endometrial tissue using enhanced green fluorescent protein

On the 7th day after transplantation, the distribution of MbMSCs in the endometrial tissue using enhanced green fluorescent protein showed that there were more MbMSCs, which are brighter, in the uterine cavity of group D than that of group E, indicating that transplantation via scaffold into the uterine cavity may be a more effective method than tail vein injection (see **Figure 6**).

Morphological changes in the uterus after MbMSCs transplantation

The endometrial morphology after MbMSC treatment showed that the endometrium of groups A and B was atrophic, thinned, and had a high proportion of fibrotic tissue. In contrast, the endometrium of group D was thickened with more glands and showed reduced fibrosis (see **Figure 7**).

Endometrial fibrosis after MbMSC treatment

For groups A and B, there were no significant differences in fibrosis between 7 d and 28 d after treatment ($P_A = 0.258$, $P_B = 0.263$). The proportion of fibrotic area of the other (C, D and E) groups at 28 d after treatment were lower than those at 7 d, but the difference was only significant for group C ($P_C = 0.002$). For groups D and E, there were no significant differences in fibrosis between 7 d and 28 d after treatment ($P_D = 0.051$, $P_E = 0.095$) (see **Figure 8**).

There was a significant difference in fibrosis among the five groups on the 7th day after treatment ($P_{7dABCDE} < 0.001$, $F = 145.73$). There was also a significant difference among the five groups on the 28th day after treatment ($P_{28dABCDE} < 0.001$, $F = 127.19$). Seven

days after treatment, the rank order of treatments in regards to the fibrotic area of the endometrium was $A > B > E > C > D$. Except between groups A and B ($P = 0.124$), there were significant differences between all the other groups ($P_{BE}, P_{EC}, P_{CD} < 0.001$). Twenty-eight days after treatment, the rank order of the treatments in regards to the fibrotic area of the endometrium was $A > B > E > C > D$; except between groups C and D ($P_{7dCD} = 0.241$), there were significant differences between the other groups ($P_{28dAB}, P_{28dBE}, P_{28dEC} < 0.001$).

Endometrial thickness after MbMSC treatment

For groups A and B, there were no significant differences in endometrial thickness between the 7th and 28th days after treatment ($P_A = 0.123$, $P_B = 0.278$). The endometrial thickness of the other (C, D and E) groups on the 28th day after treatment were all significantly thicker than those on the 7th day ($P_C, P_D < 0.001$, $P_E = 0.005$) (see **Figure 8**).

There was a significant difference in endometrial thickness among the five groups at 7 d as

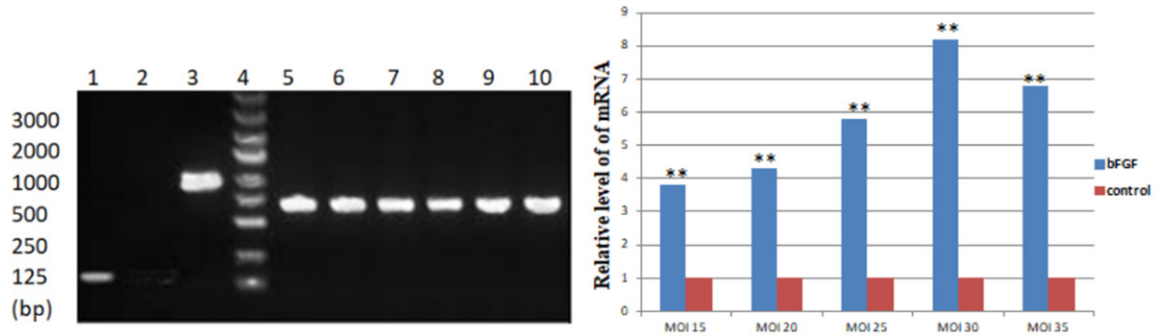


Figure 4. The electrophoretic map of PCR TM4-TOPO-FGF2 recombinant plasmid was as above: 1: negative control (blank vector- control group); 2: negative control (ddH₂O); 3: positive control (GAPDH); 4: pseudopositive particle enzymolysis marker; 5-10: FGF2 enzymolysis positive clone plasmid 1-6. The figure right shows the expression of FGF2 mRNA in FGF2-MbMSCs infected with different MOI was significantly higher than that of the control group.

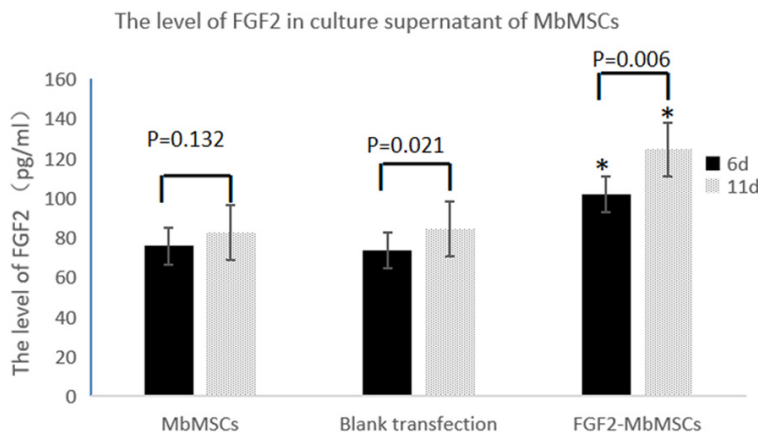


Figure 5. The expression of FGF2 in the culture medium of the MbMSCs was significantly increased 6 d and 11 d after transfection (* $P < 0.001$) when compared with the blank transfection group and the MbMSC control group. This suggests that the FGF2 gene integrated into the MbMSCs can be transferred to the progeny and stably expressed. There was no significant difference between the blank transfection group and the MbMSC control group. The expression of FGF2 in the culture medium at 11th d after transfection was higher than the expression level 6th d (all $P < 0.05$).

well as 28 d after treatment. There was no significant difference in endometrial thickness between groups A and group B ($P_{7dAB} = 0.302$, $P_{28dAB} = 0.415$), whereas the rank of the other groups in regards to endometrial thickness was $D > C > E$ with significant differences between all groups at both 7 d and 28 d (P_{EC} , $P_{CD} < 0.001$).

The number of endometrial glands after MbMSC treatment

There was no significant difference in the number of endometrial glands between 7 d and 28 d for group A ($P_A = 0.089$). The numbers of

endometrial glands in the other (B, C, D and E) groups at 28 d after treatment were significantly higher than those at 7 d.

There were significant differences in the number of endometrial glands among the five groups at both 7 d and 28 d after treatment ($P < 0.001$). The number of glands was lowest in groups A and B, and there was no significant difference between these two groups ($P_{7dAB} = 0.663$). The rank order of the number of glands in the other three groups was $D > C > E$; there was no significant difference between groups C and E ($P_{7dCE} = 0.084$), but the number of glands in group D was greater

than that in group C ($P_{7dCD} = 0.044$). The rank order of the number of glands at 28 d was $D > C > E > B > A$; group D had more glands than group C, but the difference was not significant ($P_{28dCD} = 0.131$). There were significant differences in the number of endometrial glands between the other groups ($P_{28dAB} = 0.011$, P_{BE} , $P_{28dCE} < 0.001$).

Expression of CD31, VEGF, and Ki67 in the endometrium after MbMSC transplantation

Endometrial microvessel density (MVD) after MbMSC treatment: A significant difference in MVD was detected via the expression of CD31

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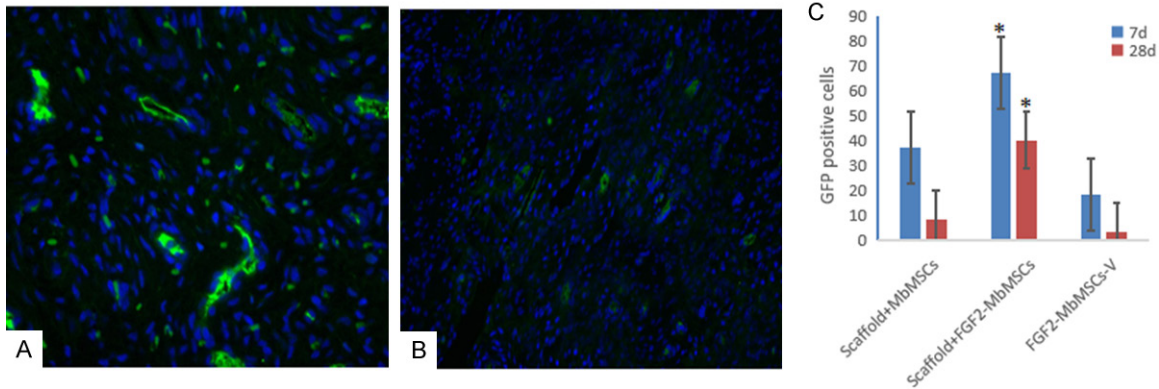


Figure 6. As shown in the figure, enhanced green fluorescent protein traced the distribution of MbMSCs in endometrial tissue (7 days after treatment). (A) shows FGF2-MbMSCs directly injected into uterine cavity through scaffold and (B) shows FGF2-MbMSCs injected into tail vein. It can be seen that FGF2-MbMSCs directly injected into the uterine cavity with scaffold are more and brighter compared with the cells injected into the tail vein. (C) shows the number of GFP cells of scaffold+FGF2-MbMSCs group is more than scaffold+MbMSCs group, which is more than FGF2-MbMSCs-V group both on 7th day and 28th day.

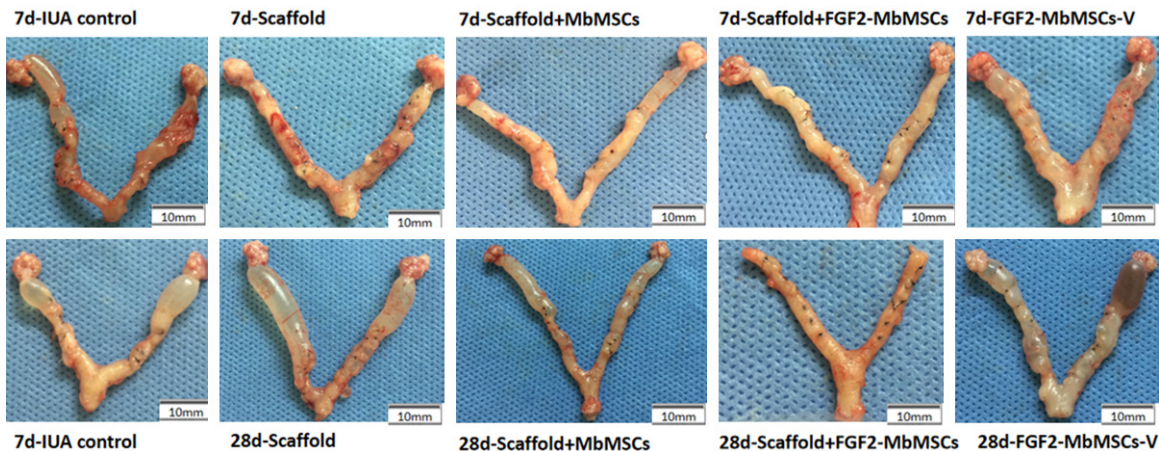


Figure 7. The Uterine contour picture of 5 groups at 7 and 28 days after MbMSCs transplantation. The upper row were pictures at 7th day and the lower row were pictures at 28th day. On 7th days after transplantation, group A which is control group had hydrous dilatation in part of the uterine cavity and atrophy in some other part of the uterine cavity. There was no significant difference in groups B, C, and D with the inspection of the naked eye, while Group E had edema. On the 28th day after transplantation, group A showed that part of the uterine cavity was more dilated by hydronephrosis, and some part atrophied more obviously. Hydronephrosis was the most serious. In group B. Hydronephrosis seemed to be alleviated in group C. The appearance of the uterus was nearly recovered in group D which is the treated by FGF2 transfected MbMSCs+scaffold. In Group E which is treated by injecting of FGF2 transfected MbMSCs into the tail vein, the appearance of the uterus was kind of recovered, and there was hydronephrosis in part of the uterine cavity.

in the endometrium among the five groups after treatment (P_{7d} , $P_{28d} < 0.001$). After treatment, the Bonferroni method was used for comparisons among the groups. The MVD in the endometrium of all groups on the 28th day after treatment were higher than those on the 7th day ($P < 0.001$), which indicates that regeneration could be maintained after 7 d.

On the 7th day after treatment, the rank order of the MVD was $C > D > E > B > A$. There was no

significant difference between groups A and B ($P_{7dAB} = 0.136$), but there were significant differences between the other groups (P_{7dBE} , P_{7dDE} , $P_{7dCD} < 0.001$). The rank order of the MVD was $D > C > E > B > A$ at 28 d after treatment, and there were significant differences between the groups (P_{28dAB} , P_{28dBE} , P_{28dEC} , $P_{28dCD} < 0.001$) (see **Figure 9**).

Expression of VEGF after MbMSC treatment: There was no significant difference in the ex-

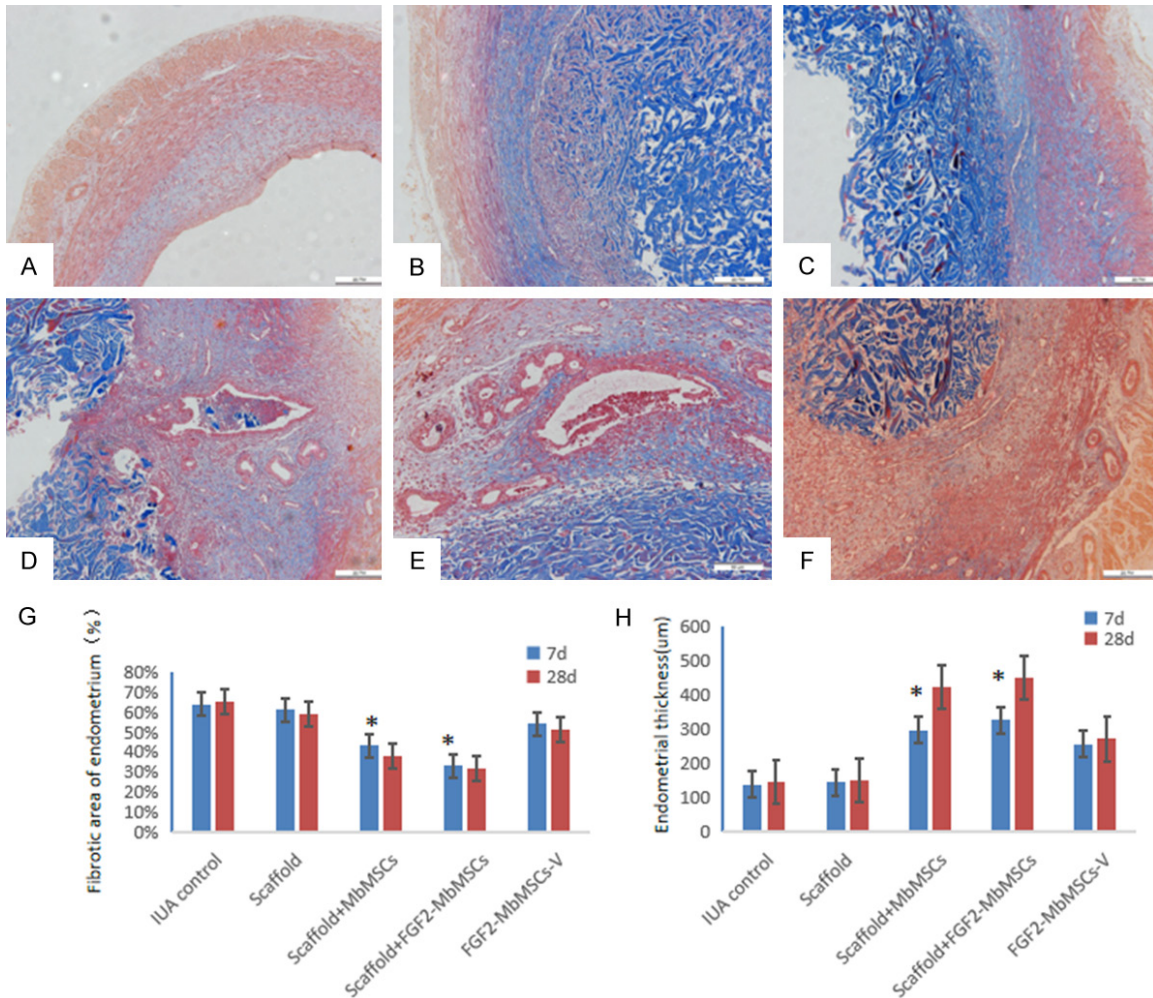


Figure 8. The endometrial morphology after MbMSCs treatment: The upper row including (A) from IUA control group and (B/C) from the scaffold control group shows endometrial atrophy, thinning, high proportion of fibrosis. The lower row including (D-F) show endometrial thickening, gland regeneration, and reduced proportion of fibrosis in the FGF2 transfected MbMSCs group. (G) Shows the fibrotic area of endometrium decreased in the scaffold+MbMSCs and scaffold+FGF2-MbMSCs group. (H) Shows the endometrial thickness increased in the scaffold+MbMSCs and scaffold+FGF2-MbMSCs group with $P < 0.01$.

pression of VEGF between days 7 and 28 in groups A and B ($P_A = 0.091$, $P_B = 0.148$). In groups C, D and E, the expression of VEGF in the endometrium on the 28th day after treatment was lower than that on the 7th day ($P_C, P_D, P_E < 0.001$).

There were significant differences in VEGF expression among the five groups ($P < 0.001$). The rank order of the level of expression was $D > C > E > B > A$, and the differences between groups were statistically significant ($P_{AB} = 0.001$, others $P < 0.001$). On the 28th day, the expression of group B was higher than that of group A ($P_{AB} = 0.009$). There was no significant differ-

ence between groups C and E ($P_{CE} = 0.092$). The highest level of expression was observed in group D, which was significantly higher than the levels of the other groups ($PCD < 0.001$) (see **Figure 9**).

Expression of Ki67 after MbMSC treatment: The expression of Ki67 by cells in the endometrium on the 28th day after treatment was lower than that on the 7th day and was statistically significant for all groups.

On the 7th day after MbMSC treatment, there was a significant difference in the expression of Ki67 among the 5 groups ($P_{7dABCDE} < 0.001$).

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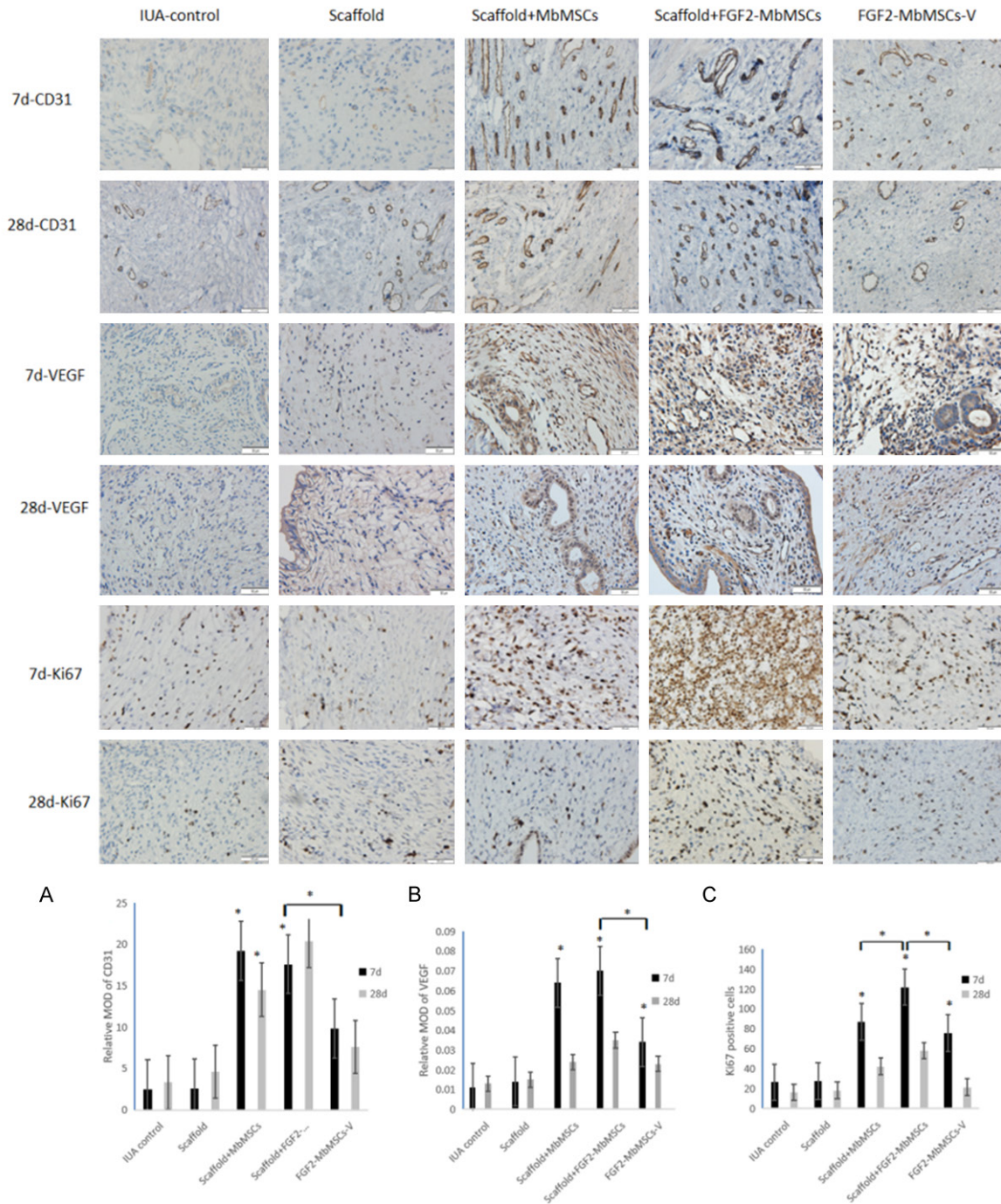


Figure 9. Representative immunostaining of CD31, VEGF and Ki67 positive cells of the endometrium on the 7th day and 28th day after treatment. Scale bar = 20 μ m. **A:** There was a significant difference expression of CD31 in endometrium among the five groups after treatment ($P < 0.001$). The expression of MVD in the endometrium of all groups on the 28th day after treatment was higher than that on the 7th day with was statistically significant difference ($P < 0.001$), which means the regeneration could be maintained after 7 days. On the 7th day after treatment, MVD was highest expressed in scaffold+MbMSCs group, then scaffold+FGF2-MbMSCs group and then FGF2-MbMSCs-V group. There was no significant statistical difference between control group and scaffold group ($P = 0.136$). There was significant difference between the other groups (all $P < 0.001$). MVD was IUA control group < Scaffold group < FGF2-MbMSCs-V < scaffold+MbMSCs < scaffold+FGF2-MbMSCs from low to high 28 days after treatment, and there was significant statistical difference between each two groups (all $P < 0.001$). **B:** There was no significant difference in the expression of VEGF between 7th day and 28th day in control group and scaffold group. In the other

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3 groups, the expression of VEGF in the endometrium on the 28th day after treatment was lower than that on the 7th day with statistically significant difference (all $P < 0.001$). There were significant differences among the five groups ($P < 0.001$). The results showed that the expression of group ABCE increased in turn, and the difference between each two was statistically significant (all $P < 0.001$). On the 28th day, the highest expression was found in scaffold+FGF2-MbMSCs group, which was statistically more than other groups ($P < 0.001$). C: On the 7th day after MbMSCs treatment, there was significant difference among the 5 groups ($P < 0.001$). Control group was the lowest in expression but there was no significant difference between control group and scaffold group ($P = 0.180$). The other 3 groups were all significantly higher than group scaffold group (all $P < 0.001$). The expression from low to high was FGF2-MbMSCs-V < scaffold+MbMSCs < scaffold+FGF2-MbMSCs with significant difference between each two groups (all $P < 0.001$). On the 28th after MbMSCs treatment, there was significant difference among the 5 groups ($P < 0.001$). The expression from low to high was control group < Scaffold group < FGF2-MbMSCs-V < scaffold+MbMSCs < scaffold+FGF2-MbMSCs with significant difference between each two groups.

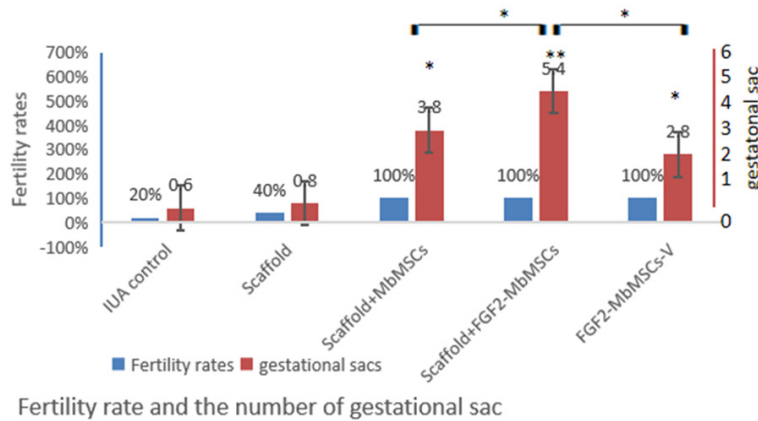


Figure 10. This figure shows the fertility rate and the number of gestational sac of 5 groups. There is no difference between control group and scaffold group. The other 3 groups had a higher number of gestational sac and fertility rate than control group and scaffold group. The number of gestational sac from high to low was scaffold+FGF2-MbMSCs > scaffold+MbMSCs > FGF2-MbMSCs-V. (* $P < 0.05$, ** $P < 0.01$).

Group A exhibited the lowest expression, but there was no significant difference between groups A and B ($P_{7dAB} = 0.180$). Groups C, D and E all had significantly higher levels of expression than group B (P_{7dBC} , P_{7dBD} , $P_{7dBE} < 0.001$). The rank order of expression was $D > C > E$ with significant differences between all groups (P_{7dCD} , $P_{7dDE} < 0.001$).

On the 28th after MbMSC treatment, there was significant difference in Ki67 expression among the 5 groups ($P_{28dABCDE} < 0.001$). The rank order of expression was $D > C > E > B > A$ with significant differences between all groups ($P_{AB} = 0.015$, P_{28dBE} , P_{28dEC} , $P_{28dCD} < 0.001$) (see **Figure 9**).

Fertility outcomes after transplantation: One rat in group A, 2 rats in group B and all rats in groups C, D and E were pregnant. The numbers of gestational sacs in the 5 groups were 0.6, 0.8, 3.8, 5.4 and 2.8, respectively. The-

re was no difference in the number of gestational sacs between groups A and B. Groups C, D and E all had higher numbers of gestational sacs and higher fertility rates than groups A and B. The rank order of the number of gestational sacs was $D > C > E$ (see **Figure 10**).

Discussion

The high recurrence rate of IUA after adhesiolysis can be attributed to the lack of active endometrial tissue, which seriously harms the prognosis of pregnancy. To seek more effective treatment for IUA, it is necessary to establish a

stable animal model of IUA. Since the 1970s, in an attempt to solve the problem of abnormal uterine bleeding, scholars have explored several IUA animal models involving the complete removal of the endometrium. These IUA models have used chemical damage (direct perfusion with formaldehyde, phenol glue or ethanol) [11], physical freezing (cryopreservation of the intrauterine endometrium with a Freon probe) [12], heat cauterization (Nd:YAG laser thermal injury) [13], induction of autogenous fibroblasts combined with polyethylene sponge implantation [14], biological bacterial infection with lipopolysaccharide (LPS) [15], mechanical injury combined with LPS infection [16], etc. All these treatments can damage the endometrium causing uterine cavity occlusion and adhesion formation. However, given that 90% of IUAs occur after intrauterine trauma, the IUA model we used in this study was created by mechanical injury by removing all visible endometrium

to establish the IUA [4]. This model simulates the clinical pathogenic factors to the greatest extent. To avoid error associated with individual operators, all the IUA models in this study were created by the same person; this person was also blind to the group treatments.

Most of the studies on stem cell therapy for IUA have used mesenchymal stem cells from bone marrow. Bone marrow-derived mesenchymal stem cells (BMMSCs) were the first mesenchymal stem cells to be discovered and have been the most extensively researched. BMMSCs have been created using autologous stem cells, which have the advantage of avoiding immune rejection. However, there are limitations; the bone marrow isolation process is painful, risky and generates adverse psychological effects for the patients. Additionally, there are individual differences in the proliferation, survival, differentiation and paracrine abilities of BMMSCs in vitro, which may be affected by patient age. Compared with the limitations of BMMSCs [6-9], MbMSCs have shown great potential for noninvasive origination and stable differentiation beyond 30 generations in vitro [17].

In addition, there are various methods of stem cell transplantation. The experimental models of BMMSCs for heart failure have used intravenous, coronary and myocardial injections [18, 19]. In the limited clinical research on the use of stem cells to treat IUAs, the methods used have included local intrauterine injection [20], subendometrial injection [21], intrauterine spiral artery injection [22], etc. Studies that have researched BMMSCs for the treatment of IUA have used intrauterine transplantation [23] and tail vein injection [24, 25] and have shown that BMMSCs may contribute to the regeneration of the endometrium. However, to date, there is no study comparing the effects of transplantation methods. Our study not only researched the role of MbMSCs on regeneration but also compared different methods. We found that MbMSCs introduced via tail vein injection promoted endometrial regeneration in both the morphological and functional analyses, but the effect was not as strong as intrauterine transplantation with a scaffold.

The reason for this difference may be explained as follows: when injected into the tail vein, MbMSCs flow back to the atrium along with the blood circulation, and then are pumped out

through the ventricles and distributed to all organs of the body. During blood circulation, MbMSCs may be engulfed as allogeneic cells by a large number of megaphagocytes in the body, and therefore the number of MbMSCs that reach the endometrium is quite limited; this was confirmed by fluorescence tracing. Fluorescence microscopy showed that MbMSCs directly transplanted into the intrauterine environment with a scaffold resulted in cells that were much brighter than those from vein injection.

In addition to intravenous injection, two clinical trials have reported transplantation by local subendometrial injection [26] and uterine spiral internal artery injection [22]. The former technique requires ultrasonic monitoring, multiple intrauterine injections, and the injection depth is difficult to control. The latter requires femoral artery catheterization, which may be risky, and the stem cells will be partially lost through blood circulation. Given that the patients with IUA require hysteroscopy for diagnosis and to separate tissues, transplanting MbMSCs into the intrauterine cavity with a scaffold not only facilitates the promotion of endometrial regeneration by stem cells, it also serves as a physical barrier post-hysterolysis. The biological component of the scaffold is collagen, which may be compatible with the organism, and the stem cells planted on the scaffold membrane can grow into the collagen pores. This scaffold could gradually degrade until it is completely absorbed.

In addition, stem cells exhibit chemotaxis to the site of inflammation. In this study, rats had IUA injury in the uterine horns with no inflammation at other sites. After endometrial injury, a variety of inflammatory factors and chemokines may be expressed in the uterus that play a role in the migration of stem cells to the endometrial site to participate in the repair [27]. In clinical settings, if there is inflammation in other parts of the body after the stem cells are injected into the vein, the effect on the endometrium will be further decreased. Therefore, in this study, although the intravenous injection of stem cells was effective, it is not the best option.

FGF2 can promote mitosis and the proliferation of vascular endothelial cells, smooth muscle cells and other cells, thus promoting the formation of new blood vessels and contributing to

the regeneration and repair of tissue damage [28]. FGF2 can also inhibit the fibrosis process. However, the half-life of FGF2 in vivo is only 3-10 min [29]. To promote the survival and regeneration of MbMSCs and the endometrium, FGF2 was transfected into MbMSCs to secrete exogenous FGF2 into the uterine cavity. Our study found that the proliferation of MbMSCs transfected with the FGF2 gene is temporarily suppressed for a week, and cell proliferation then accelerates and plays a role in the continuous secretion of FGF2. Compared with MbMSCs alone, FGF2-MbMSCs enhanced repair.

In conclusion, we found that the injection of MbMSCs into the tail vein was an effective treatment for endometrial regeneration, but the effect was not as strong as with transplantation of MbMSCs into the intrauterine cavity with a scaffold; furthermore, FGF2-transfected MbMSCs had an enhanced regenerative effect.

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Disclosure of conflict of interest

None.

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