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Transplantation of umbilical cord–derived mesenchymal stem cells on a collagen scaffold improves ovarian function in a premature ovarian failure model of mice

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Abstract

Premature ovarian failure (POF) is a refractory disease; one of the most important goals of treatment is to improve fertility. In the study, collagen scaffold loaded with human umbilical cord–derived mesenchymal stem cells (collagen/UC-MSCs) transplantation in POF mice preserved ovarian function, as supported by increased estrogen (E_2) and anti-Mullerian hormone (AMH) levels, increased ovarian volume, and an increased number of antral follicles. Immunohistochemistry results of Ki67 indicated transplantation of collagen/UC-MSCs promoted granulosa cell proliferation, which is crucial to oocyte maturation and follicular development. Additionally, transplantation of collagen/UC-MSCs significantly promoted ovarian angiogenesis with the increased expression of CD31. In general, collagen/UC-MSCs transplantation probably is an effective therapeutic strategy of POF.

Keywords UC-MSCs · Collagen scaffold · POF · Ovarian function

Introduction

Premature ovarian failure (POF) is the loss of ovarian function and affects approximately 1 in 100 women before 40 years old (Luisi et al. 2015). Many pathogenic mechanisms attributed to the POF disorders, including chromosomal, autoimmune, metabolic, and iatrogenic causes (Pelosi et al. 2015); however, underlying causes of most cases of idiopathic POF still remain elusive (Nelson 2009).

Hormone replacement therapy (HRT) can ease symptoms of estrogen deficiency. Except oocyte donation or ovarian transplantation, there is little evidence that HRT improves fertility in women with POF (Goswami and Conway 2007, Nelson 2009). In a previous study, ovarian function was reported to be restored after transplantation of ovarian tissue (Silber et al. 2005); however, it is unknown whether ovulation occurred from the grafted tissue or the tissue left in situ. Ovarian donors of a young age are rare and there are ethical issues involved with this type of treatment. In vitro activation (IVA) is one potential therapy for POF patients that have residual follicles (Suzuki et al. 2015, Kawamura et al. 2016), but the safety and efficacy of IVA need improvement (O. Yin et al. 2016). There are several types of stem cells that been used for the treatment of chemotherapy-related ovarian failure. These include human menstrual–derived stem cells (Z. Wang et al. 2017), placenta-derived mesenchymal stem cells (N. Yin et al. 2018), endometrial mesenchymal stem cells (Lai et al. 2015), human amniotic fluid stem cells (Xiao et al. 2014), and umbilical cord mesenchymal stem cells (S. Wang et al. 2013, Zhu et al. 2015, Song et al. 2016). UC-MSCs are considered to be a unique cell family since they have lower oncogenicity and faster self-renewal

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ability (S. Wang et al. 2013, Zhu et al. 2015, Song et al. 2016). In addition, they can be derived via a noninvasive collection procedure and there are no ethical issues.

The stem cell treatment has been constrained by inadequate settlement of cells in the target tissues. Stem cells can spread rapidly to the nearby organs or tissues (Suuronen et al. 2006a, b); besides, it is difficult for the retention of cells transplanted in the target organs due to many interacting factors, including inflammation, apoptosis, and ischemia injury (Moreno-Alvero et al. 2017). Application of scaffolds provided a structural support to the transplanted cells, and retaining cells in the transplanted organs showed viability. As the most abundant and easily obtained protein, collagen is widely used in various tissue engineering applications for its biocompatibility, biodegradability, and low antigenicity in animals (Ding et al. 2014). In a previous report, our study showed that collagen entrapped stem cells into scaffold network and increased the retention of stem cells in the ovary (Su et al. 2016). In this study, UC-MSCs on a collagen scaffold were transplanted into the ovaries of mice of POF, and contributed to granulosa cell proliferation, ovarian angiogenesis, and development of follicles.

Materials and Methods

UC-MSC isolation and culture Newborn umbilical cord tissue was collected from consenting donor with approval from the Clinical Research Ethics Committee at the Affiliated Drum Tower Hospital of Nanjing University Medical School. Method for UC-MSCs isolation was described as previously (Xu et al. 2017). After vessels were mechanically removed, the umbilical cord was manually dissected into small sections. The segment tissues were cultured in low-glucose Dulbecco's modified Eagle's medium (LG-DMEM; GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 IU/ml penicillin (GIBCO), and 100 μg/ml streptomycin (GIBCO). The first colony of UC-MSCs was observed after approximately 14 days. UC-MSCs at passages 3–5 were used for further experiments

Flow cytometry analysis Flow cytometry was used to analyze UC-MSC surface markers expression at passage 3. The cells were incubated for 1 h with phycoerythrin (PE)-conjugated or fluorescein isothiocyanate (FITC)-antibodies against human CD13 (eBioscience, San Diego, CA), CD29 (eBioscience), CD34 (BD Pharmingen, San Diego, CA), CD45 (eBioscience), CD73 (BD Pharmingen), CD90 (eBioscience), CD105 (eBioscience), CD146 (BD Pharmingen), and HLA-DR (eBioscience)

Differentiation of UC-MSCs In vitro differentiation potential of umbilical cord-derived mesenchymal stem cells at passage 3 was assessed by adipogenic, osteogenic, and neural-like differentiation assays. To induce adipogenesis and osteogenesis, UC-MSCs were treated with adipogenic induction medium (GIBCO) for 2 weeks and osteogenic induction medium (GIBCO) for 4 weeks. Briefly, Oil O red staining (Sigma, Steinheim, Germany) and alizarin staining (Sigma) were performed after induction to identify the differentiation of UC-MSCs. All-trans-retinoic acid (ATRA; Sigma, 10^{-7} M), a pre-induction medium containing 10 ng/ml bFGF (GIBCO) induces cells neural-like differentiation for 24 h, followed by modified MNM medium for 36 h. The signals of neuron-specific enolase (1:100, sc-292097, Santa Cruz Biotechnology, Santa Cruz, CA) and neurofilament medium polypeptide (1:100, sc-16143, Santa Cruz Biotechnology) in UC-MSCs were observed by immunofluorescence staining.

Preparation of collagen/UC-MSCs Collagen Scaffold was a gift from the State Key Laboratory of Molecular Developmental Biology (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) and was prepared according to the published methods (Su et al. 2016). Gelatinous scaffolds carried type I collagen (10 mg) dissolved in 1 ml PBS and mixed with the same volume of UC-MSCs. The transplant components were delivered on ice and mixed immediately before injection.

Preparation of the premature ovarian failure mouse model Animal care and experimental procedures were in accordance with the Animal Research Committee guidelines of Drum Tower Hospital, Nanjing University Medical School. Six-week-old female C57BL/6 mice were maintained under SPF conditions in a controlled environment of 22°C and under 12/12-h light/dark cycle with ad libitum access to food and water. Observing vaginal smears daily and only mice showing 2 consecutive normal 4-day estrus cycles were used in the study.

Adult female C57BL/6 mice were injected with CTX (40 mg/kg/d, H32020857, Jiangsu Shengdi Pharmaceutical Industry Co. Limited, Jiangsu, China) for 15 consecutive days. The mice in the control group were injected with saline.

Transplantation of collagen/UC-MSCs Mice were randomly divided into four groups depending on different treatments as follows: PBS group, collagen group, UC-MSC group, and collagen/UC-MSC group. The control group consisted of normal mice, which received no treatment. Mice were anesthetized with pentobarbital (10 mg/kg) and then shaved with decubitus position. The ovaries of POF mice were exposed via back incision. Suspensions (10 μl) were injected into the core of the ovaries. Ten-microliter PBS was injected per ovary in the PBS group. In the collagen group, 10 μl of degradable collagen was injected per ovary. For the UC-MSC group, 2×10^5 UC-MSC in 10 μl PBS was injected. In the collagen/UC-MSC group, 2×10^5 UC-MSC in 10 μl degradable collagen

was injected. Two weeks after injection, vaginal smears were obtained daily for 15 consecutive days to determine.

Estrous cycle analysis To detect estrous cycles, vaginal smears were obtained from mice by inserting a cotton-tipped swab wetted with saline into the vagina. Gently rotate the swab against the vaginal wall. Remove and wipe it onto a clean glass microscope slide. The stage of the estrous cycle was determined based on the relative quantities of leukocytes, nucleated epithelial cells, and cornified epithelial cells by examining the slides under the microscope. Vaginal smears showed mostly leukocytes and nucleated epithelial cells during diestrus. Mostly nucleated epithelial and some cornified epithelial cells appeared during proestrus. Increased number of cornified epithelial cells was presented in the stage of estrus. A large number of cornified epithelial cells and reappearing leukocytes were indicative of metestrus (Byers et al. 2012). Vaginal smears from the mice were analyzed for 15 consecutive days to follow the progression through the cycle.

Mice hormone detection Four weeks after transplantation, blood was collected from the mice during diestrus. The serum was separated using centrifugation and kept at − 80°C. The concentrations of serum follicle stimulating hormone (FSH) and E_2 were measured with ELISA kit (YANYU, Shanghai, China); the AMH was measured using a different ELISA kit (CEA228Mu, Cloud-Clone Corp., Wuhan, China).

Ovarian morphology and follicle counting The ovaries were dissected and weighted after blood collection during diestrus in mice. Ovarian index (relative weight of ovary) was expressed as a percentage of body weight (Mitropoulos et al. 1992). Ovaries were fixed overnight in 10% formalin solution, dehydrated in ascending series of ethanol, and embedded in paraffin. Sections were sliced into 3 μm; every fifth section was used as a histologic slice and stained with hematoxylin and eosin (HE). All of the sections were examined under a light microscope with magnification of 200×. The follicles were classified as previously described into primordial stage if the follicle contained an oocyte surrounded by a layer of squamous granulosa cells, primary stage if the follicle possessed an oocyte surrounded by a single layer of cuboidal granulosa cells, secondary stage if the follicle showed an oocyte surrounded by two or more layers of granulosa cells, or antral stage if the follicle possessed a single large antral space (Myers et al. 2004). Only follicles with oocyte nuclei were counted. After counting every fifth section, the number of follicles was multiplied by five.

Immunohistochemistry (IHC) Ovaries were fixed in 10% buffered-formalin overnight. After embedded and sectioned at 3 μm, sections were deparaffinized and rehydrated, and endogenous enzymes were blocked by Reagent A/B (Sangon, China) for 10 min. Tissue samples were immune labeled with anti-Ki67 antibody (Ki67, 1:400, ab16667, Abcam, Cambridge, UK) and anti-CD31 antibody (CD31, 1:200, ab28364) overnight at 4°C. The sections were then washed three times in PBS, incubated with biotin-labeled secondary antibodies and reacted with DAB reagent (Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China). Sections served as the negative control incubated in non-immune rabbit IgG. The Image-Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD) was served to measure the mean optical density (MOD) of immunostaining in follicles under a light microscope with magnification of 400×. The Image J software (version 1.51, NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/) was used to calculate the area of ovary under a magnification of 50×.

Statistical Analysis The histological measurements were obtained by two independent observers who were blinded to the origin of the section and were averaged. All assays were performed in triplicate and repeated at least three times. The data were analyzed using SPSS 19.0 (Version 19.0, IBM Corp., Armonk, NY) and presented as means ± SD. One-way analysis of variance (ANOVA) was used, followed by the post-hoc Tukey multiple comparison test to analyze data for control mice when the four POF groups were included. P value < 0.05 was considered statistically significant.

Results

Characterization and differentiation of UC-MSCs Flow cytometry analysis was employed to identify the surface markers. The UC-MSCs showed a high expression of CD13, CD29, CD73, CD90, CD105, and partially the expression of CD146, which lacked CD34, CD45, or HLA-DR expression (Fig. 1*a–i*). This result was consistent with previous studies on UC-MSCs surface markers (S. Wang et al. 2013, Zhu et al. 2015, Song et al. 2016). UC-MSCs at third passage showed a fibroblast-like appearance (Fig. 1*j*). In addition, they were able to differentiate into adipocytes, osteoblasts, and neural-like cells after cultivation in respective media (Fig. 1*k–n*), indicating that UC-MSCs had multi-lineage differentiation potential.

Estrous cycle and hormone levels of POF mice after collagen/UC-MSCs transplantation At 2 weeks after transplantation with PBS, collagen, UC-MSCs or collagen/UC-MSCs, mice in the PBS and collagen groups presented the manifestations with prolonged estrous cycles, mainly due to the increased duration of diestrus. However, estrous cycles of mice in the UC-MSC and collagen/UC-MSC groups were similar to that in the normal control (Fig. 2*a*). The average length of estrous cycle in the PBS (6.33 ± 0.61 days, $n = 6$) and collagen groups (6.08 ± 0.49 days, $n = 6$) were much longer than those in the

control (4.42 ± 0.20 days, $n = 6$, $P < 0.0001$) and collagen/UC-MSC groups (4.53 ± 0.32 days, $n = 6$, $P < 0.0001$). Furthermore, estrous cycle mean length was shorter in the UC-MSC group (4.78 ± 0.25 days, $n = 6$) than those in the PBS ($P < 0.0001$) and the collagen groups ($P < 0.001$) (Fig. 2*b*). Percentage of time in diestrus was increased, and time in estrus was reduced in PBS and collagen groups compared with those in the control, UC-MSC and collagen/UC-MSC groups. The percentages of time spent in diestrus stage in PBS and collagen groups were higher than those in other groups (control, 26.62 ± 2.57%; PBS, 52.26 ± 4.62%; collagen, 50.43 ± 3.76%; UC-MSC, 32.10 ± 4.62%; collagen/UC-MSC, 23.37 ± 3.00%; $P < 0.0001$). Meanwhile, the percentage of estrus stage of mice in control group was significantly higher than those in the PBS and collagen groups (control, 31.81 ± 2.57%; PBS, 15.91 ± 0.63%; collagen, 16.52 ± 0.51%; $P < 0.0001$). Besides, a significant increase was observed in estrus stage of the mice in UC-MSC (25.5 ± 1.84%) and collagen/UC-MSC groups (28.74 ± 1.8%) when compared to the PBS ($P < 0.01$) and collagen groups ($P < 0.001$) (Fig. 2*c*)

The levels of serum FSH, E_2, and AMH were measured to determine the endocrine factors in POF mice. At four weeks after transplantation, as compared to that in the control group (125.1 ± 4.55 pmol/l, $n = 6$), the E_2 levels decreased significantly in the PBS (91.73 ± 5.54 pmol/l, $n = 9$, $P < 0.0001$) and collagen groups (93.06 ± 2.61 pmol/l, $n = 8$, $P < 0.001$). The E_2 level in the UC-MSC group (119.6 ± 1.91 pmol/l, $n = 9$) was increased compared with the PBS and collagen groups ($P < 0.001$). Moreover, E_2 level in the collagen/UC-MSC group (111.4 ± 4.28 pmol/l, $n = 10$) was higher than those in the PBS ($P < 0.01$) and the collagen group ($P < 0.05$) (Fig. 2*d*). Furthermore, the AMH level in the collagen/UC-MSC group (620.6 ± 26.96 pg/ml, $n = 9$) was higher than those in the PBS (511.2 ± 35.54 pg/ml, $n = 9$, $P < 0.05$) and collagen groups (464.6 ± 15.01 pg/ml, $n = 8$, $P < 0.01$), comparable to control (534.7 ± 27.8 pg/ml, $n = 7$) and UC-MSC groups (537.2 ± 24.44 pg/ml, $n = 9$, $P > 0.05$) (Fig. 2*e*). However, the collagen/UC-MSC group (20.03 ± 1.54 U/l, $n = 10$) showed an increase in the level of FSH compared with those in the PBS (14.87 ± 0.48 U/l, $n = 9$, $P < 0.01$) and UC-MSC groups (15.62 ± 0.48 U/l, $n = 9$, $P < 0.05$), although comparable to those in the control (15.88 ± 1.01 U/l, $n = 6$) and the collagen groups (17.23 ± 0.73 U/l, $n = 8$) ($P > 0.05$) (Fig. 2*f*).

Ovarian morphology and follicle counting in POF mice after collagen/UC-MSCs transplantation Four weeks after transplantation, an increase in ovary size was evident in both the UC-MSC and collagen/UC-MSC groups compared to the PBS and collagen groups (Fig. 3*a*). Compared with those in the control group (40 ± 2.07 × 10^{-4} g, 41.49 ± 1.92%, $n = 14$), the ovary weight and ovary index of mice in the PBS group were lower (27.83 ± 1.88 × 10^{-4} g, 29.35 ± 2.00%, $n = 15$, $P < 0.001$). However, the ovary weight and ovary index of mice in the UC-MSC (38.18 ± 1.30 × 10^{-4} g, 38.05 ± 1.47%, $n = 11$) and the collagen/UC-MSC groups (37.32 ± 1.73 × 10^{-4} g, 38.31 ± 1.80%, $n = 11$) were obviously higher than those of mice in the PBS group ($P < 0.01$), and comparable to those in the collagen group (31.88 ± 2.0 × 10^{-4} g, 34.03 ± 2.05%, $n = 8$, $P > 0.05$) (Fig. 3*b–c*). Meanwhile, there was an increase in the body weight of mice that underwent UC-MSCs transplantation (Supplementary Fig. 1a). These results suggested that collagen/UC-MSCs or UC-MSCs transplantation could improve ovary size in POF mice.

Histological analysis revealed that ovaries from the control group displayed normal folliculogenesis and included follicles at different stages of development (Fig. 3*d–d′*). At 4 weeks after transplantation, ovaries of the mice in the PBS and collagen groups showed significant atrophy and depletion of all types of follicles at high magnification of 200×, compared with control group (Fig. 3*e–f′*). In ovaries of mice in the UC-MSC and collagen/UC-MSC groups, there were more growing follicles compared with PBS and collagen groups (Fig. 3*h–h′*).

To determine the differences of follicles in all stages, sections of ovaries from different groups were examined by H&E staining. The number of primordial follicles decreased significantly in four POF groups compared with that in the control group (control, 999.2 ± 173.6, $n = 6$; PBS, 75 ± 16.23, $n = 6$; collagen, 83.33 ± 18.15, $n = 6$; UC-MSC, 170.8 ± 17.29, $n = 6$; collagen/UC-MSC, 190 ± 34.50, $n = 6$; $P < 0.0001$). In addition, the number of primary follicles in control group (355.8 ± 15.78, $n = 6$) was higher than those in other four groups (PBS, 75 ± 17.32, $n = 6$; collagen, 95 ± 23.7, $n = 6$; UC-MSC, 169.2 ± 20.79, $n = 6$; collagen/UC-MSC, 164.2 ± 26.75, $n = 6$; $P < 0.0001$). The number of primary follicles in the UC-MSC and collagen/UC-MSC groups was significantly higher than that in the PBS group ($P < 0.05$). Besides, the control group (227.5 ± 19.44, $n = 6$) contained more secondary follicles than those in PBS (61.67 ± 11.6, $n = 6$, $P < 0.0001$), collagen (67.5 ± 7.72, $n = 6$, $P < 0.0001$), UC-MSC (120.8 ± 10.12, $n = 6$, $P < 0.001$), and collagen/UC-MSC groups (135.8 ± 17.2, $n = 6$, $P < 0.001$). Moreover, the number of secondary follicles in UC-MSC group was higher than that in the PBS group ($P < 0.05$). Similarly, the number of secondary follicles in collagen/UC-MSC group was also significantly higher than that in PBS ($P < 0.01$) and collagen groups ($P < 0.05$). The number of antral follicles in the PBS (18.33 ± 5.11, $n = 6$) and collagen groups (26.67 ± 4.94, $n = 6$) was decreased compared with that in the control group (131.7 ± 30.73, $n = 6$, $P < 0.001$), and comparable to those in UC-MSC (80 ± 9.04, $n = 6$) and collagen/UC-MSC groups (67.5 ± 12.16, $n = 6$, $P > 0.05$) (Fig. 3*i*). It suggested that UC-MSCs and collagen/UC-MSCs transplantation improved ovarian reserve.

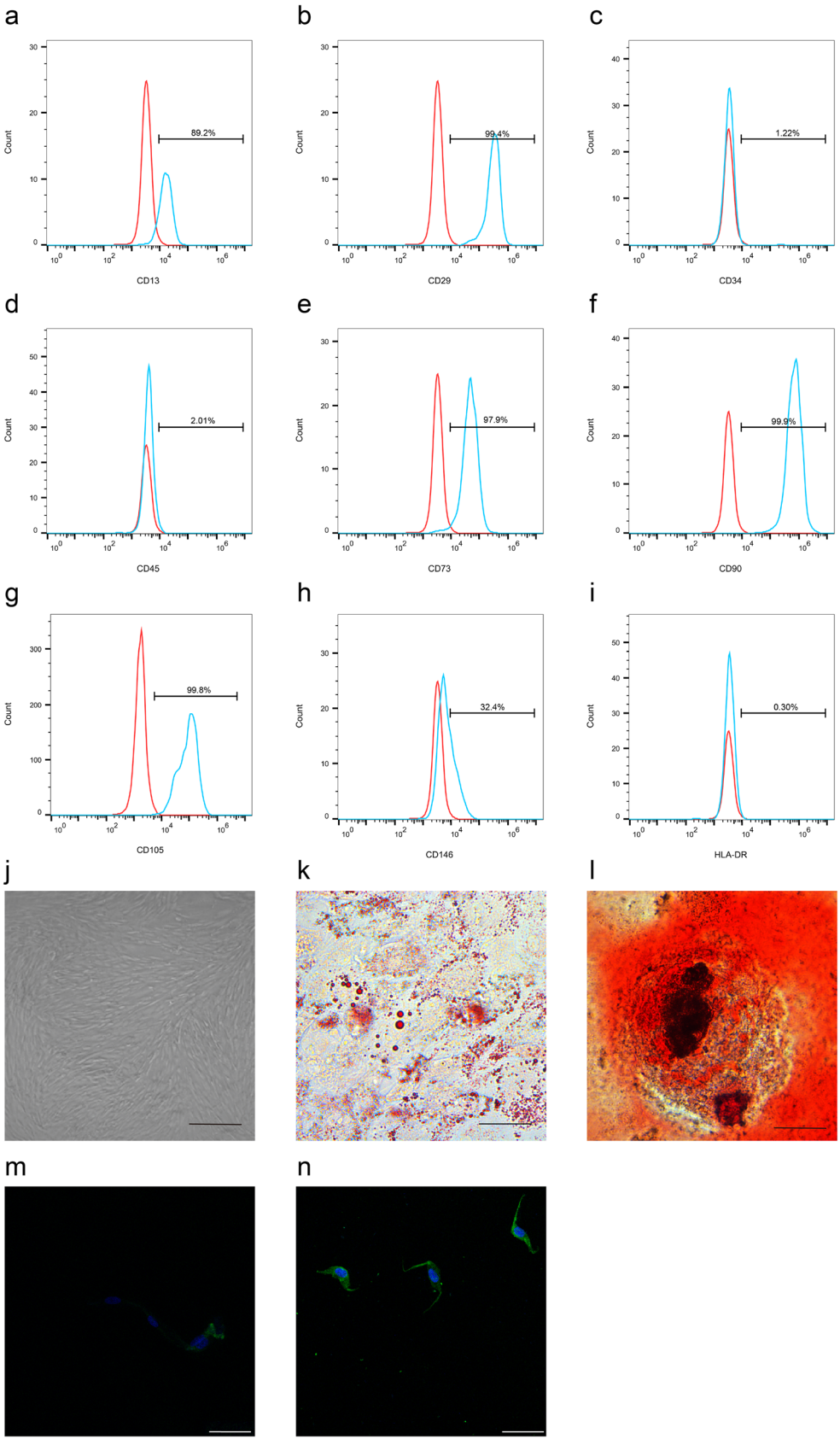

Figure 1. Characterization of umbilical cord mesenchymal stem cells (UC-MSCs). (***a–i***) Flow cytometry analysis of UC-MSCs surface markers. Cells were negative for CD34, CD45, and HLA-DR, positive for CD13, CD29, CD73, CD90, and CD105, and partially positive for CD146. (***j***) Morphology of cultured UC-MSCs. The cells at third passage showed fibroblastic morphology. *Bar*: 500 μm. (***k–n***) The differentiation of UC-MSCs. (***k***) For adipogenic differentiation, UC-MSCs were cultured in adipogenic induction medium for 14 d. The formation of lipid droplets was confirmed by Oil Red O staining. *Bar*: 50 μm. (***l***) UC-MSCs were cultured in osteogenic supplemented (OS) medium for 28 d. Calcium deposition was confirmed by Alizarin red staining. *Bar*: 50 μm. (***m***, ***n***) Differentiation of UC-MSCs to neuronal lineage after 24 h pre-induction and 36 h induction was confirmed by immunofluorescence staining of neuron-specific enolase and neurofilament medium polypeptide. *Bar*: 50 μm.

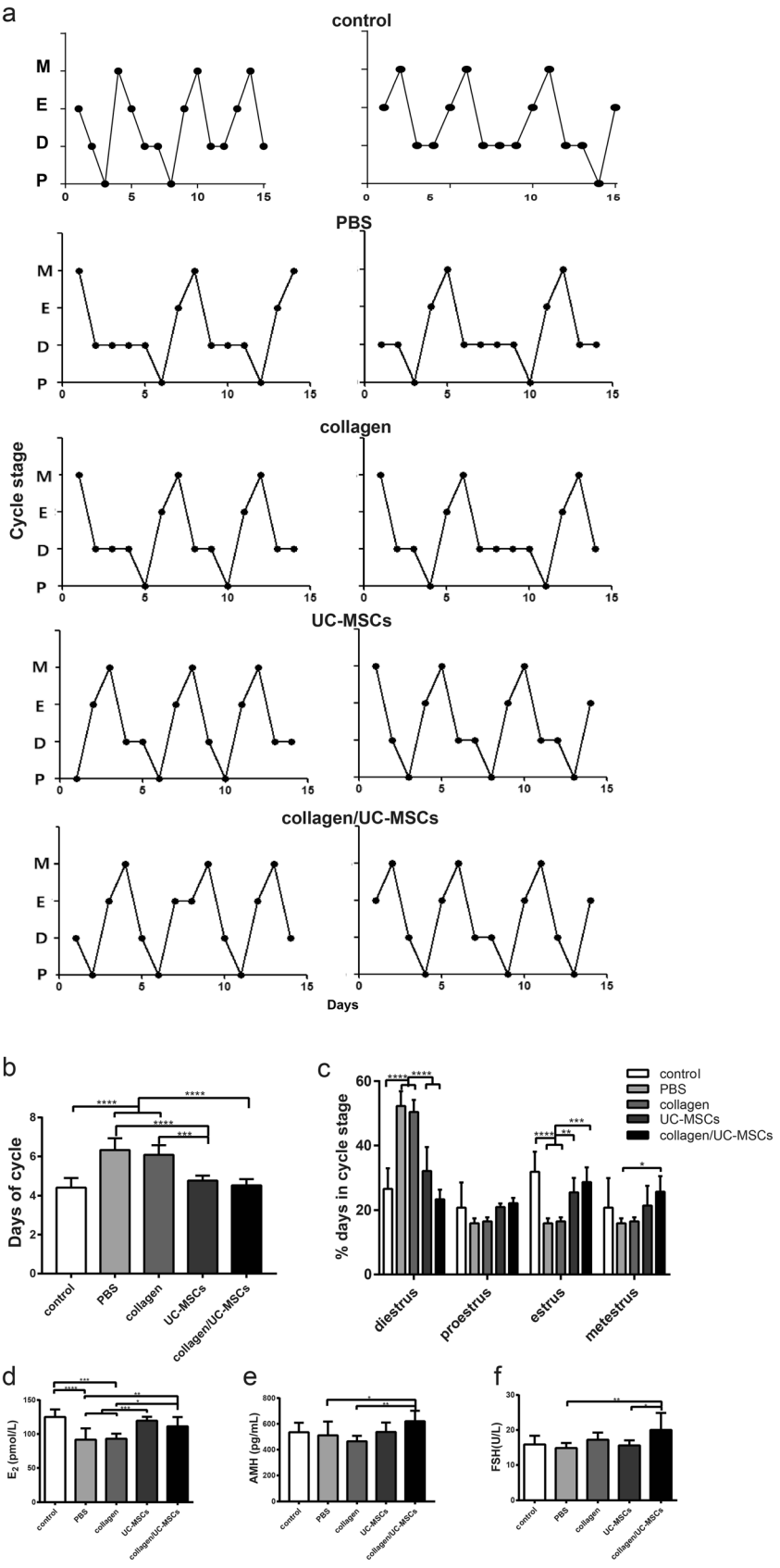

Figure 2. Estrous cycle and hormone levels of POF mice after collagen/UC-MSCs transplantation. (***a***) Representative estrous cycles of two mice received different treatments as measured by vaginal smears. M, metestrus; E, estrus; D, diestrus; P, proestrus. (***b***) Average estrous cycle length of mice in control, PBS, collagen, UC-MSC and collagen/UC-MSC groups. (***c***) Percentage of time spent in each stage during a single estrus cycle of mice among five groups. (***d–f***) The levels of E_2, AMH, and FSH were analyzed using ELISA kits.

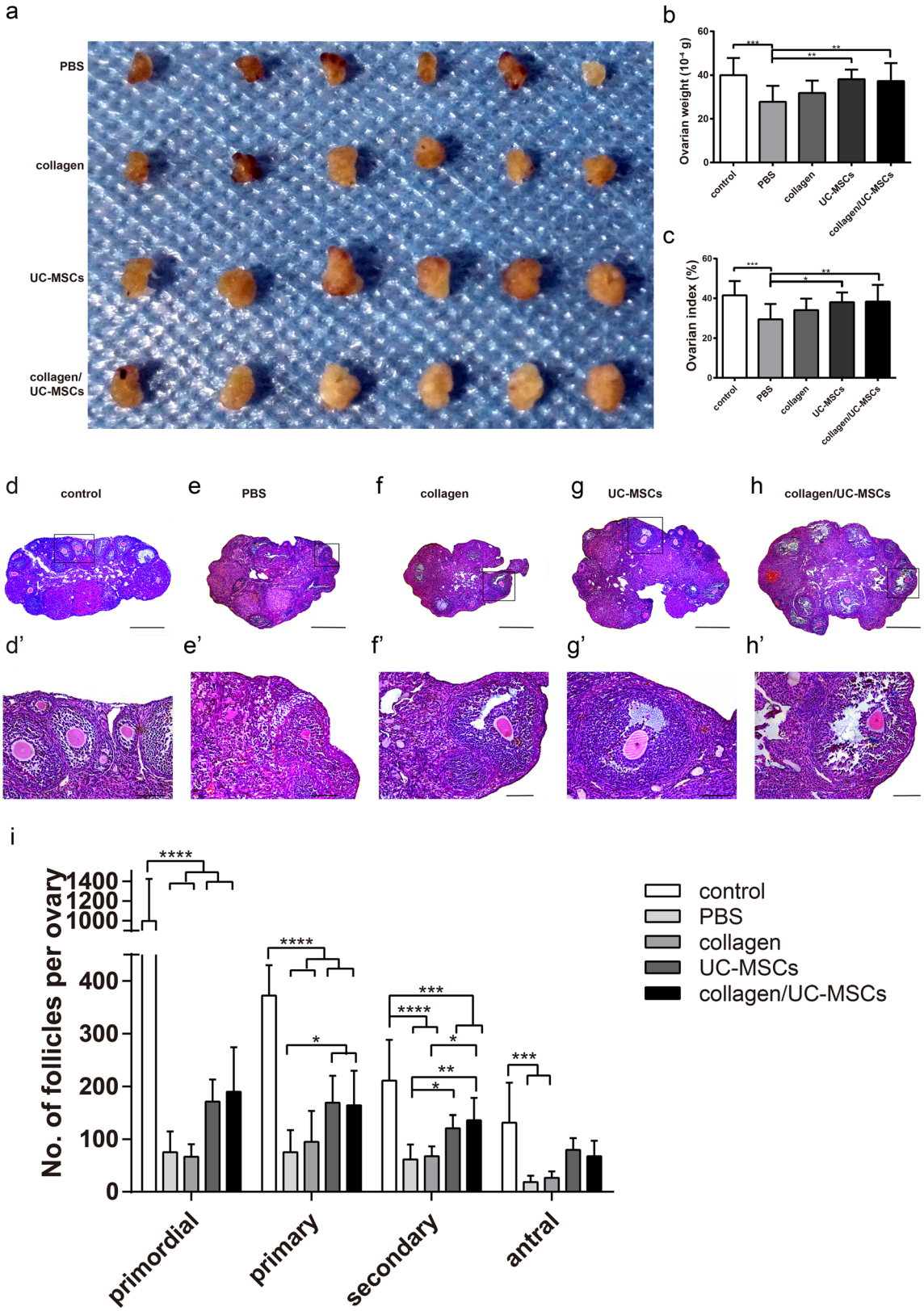

Figure 3. Ovarian morphology and follicle counting in POF mice after collagen/UC-MSCs transplantation. (***a***) The ovarian morphology of POF mice varies among four treatment groups. (***b***–***c***) The ovary weight and ovary index of mice in control, PBS, collagen, UC-MSC and collagen/UC-MSC groups. (***d***–***h′***) Histological analysis of mice ovaries. (***d***) Control group; (***e***) PBS group; (***f***) collagen group; (***g***) UC-MSC group; (***h***) collagen/UC-MSC group. *Bar*: 500 μm. (***d′***) Control group; (***e′***) PBS group; (***f′***) collagen group; (***g′***) UC-MSC group; (***h′***) collagen/UC-MSC group. *Bar*: 100 μm. (***h***) The number of follicles among five groups.

Collagen/UC-MSCs transplantation promotes granulosa cell proliferation and ovarian angiogenesis in POF mice

Immunohistochemistry results of Ki67 showed that the Ki67 expression levels in the antral follicles in the collagen/UC-MSC group and control group were higher than those in PBS group ($P < 0.05$, Fig. 4*a*–*b*). It suggested that the collagen/UC-MSCs transplantation was actively involved in granulosa cell proliferation, follicle growth, and development. Additionally, the number of vessels positive staining for CD31 in ovaries of the control group (45.53 ± 3.02, $n = 6$) was higher than PBS (21.81 ± 3.27, $n = 6$, $P < 0.001$), collagen (21.69 ± 3.75, $n = 6$, $P < 0.001$), and UC-MSC groups (26.84 ± 3.36, $n = 6$, $P < 0.01$). However, the number of vessels in the collagen/UC-MSC group (36.66 ± 3.13, $n = 6$) was higher than those of ovaries in the PBS and collagen groups ($P < 0.05$, Fig. 4*c*–*d*). It indicated that the transplantation of collagen/UC-MSCs efficiently increased blood vessels number in ovaries and promoted the follicular development.

Discussion

This study revealed collagen/UC-MSCs transplantation significantly improved ovarian functions after chemotherapy-induced ovarian injury in mice. After direct injection of UC-MSCs or collagen/UC-MSCs into ovaries, POF mice exhibited regular estrus cycles, elevated serum E_2 and AMH levels, improved follicular growth, and preserved ovarian function partially along with relieved POF symptoms.

The clinic features of POF patients include amenorrhea, hypoestrogenism (Shelling 2010), AMH deficiency, and elevated gonadotrophins (Nelson 2009). A previous study found a significant increase in serum E_2 and AMH levels and a decrease in the level of serum FSH in POF rats after UC-MSCs transplantation (Song et al. 2016). The serum E_2 level of POF mice was also found to be increased after UC-MSCs transplantation (S. Wang et al. 2013). Elevation in E_2 secreted by granulosa cell is understood to be a clinical marker for the evaluation of ovarian reserve and a consequence of the advanced follicular development (Ramalho de Carvalho et al. 2012). Moreover, AMH secreted by granulosa cell is considered to be a good and reliable parameter for the assessment of ovarian reserve (Ramalho de Carvalho et al. 2012). In this study, 4 weeks after collagen/UC-MSCs transplantation, serum E_2 and AMH levels increased in POF mice and the mice exhibited similar estrous cycles to normal control mice. These results suggested that UC-MSCs on a collagen scaffold transplantation could facilitate the proliferation of granulosa cell and improve ovarian function in mice with POF.

Histopathological changes due to POF include ovarian atrophy and follicle exhaustion (Nelson 2009). Reports have shown that mesenchymal stem cells release cytokines and exosomes which promote repair and attenuate organ fibrosis (B. Wang et al. 2016). Previous studies have indicated that UC-MSCs transplantation can compensate for ovarian atrophy in POF rats and mice (S. Wang et al. 2013, Zhu et al. 2015). In this study, the ovaries of POF mice in the PBS and collagen groups exhibited severe fibrosis; however, no obvious changes were detected in those of mice in the UC-MSC or collagen/UC-MSC groups, suggesting that UC-MSCs may secrete exosomes that have the therapeutic potential to repair or reverse organ fibrosis. Prior studies have noted that stem

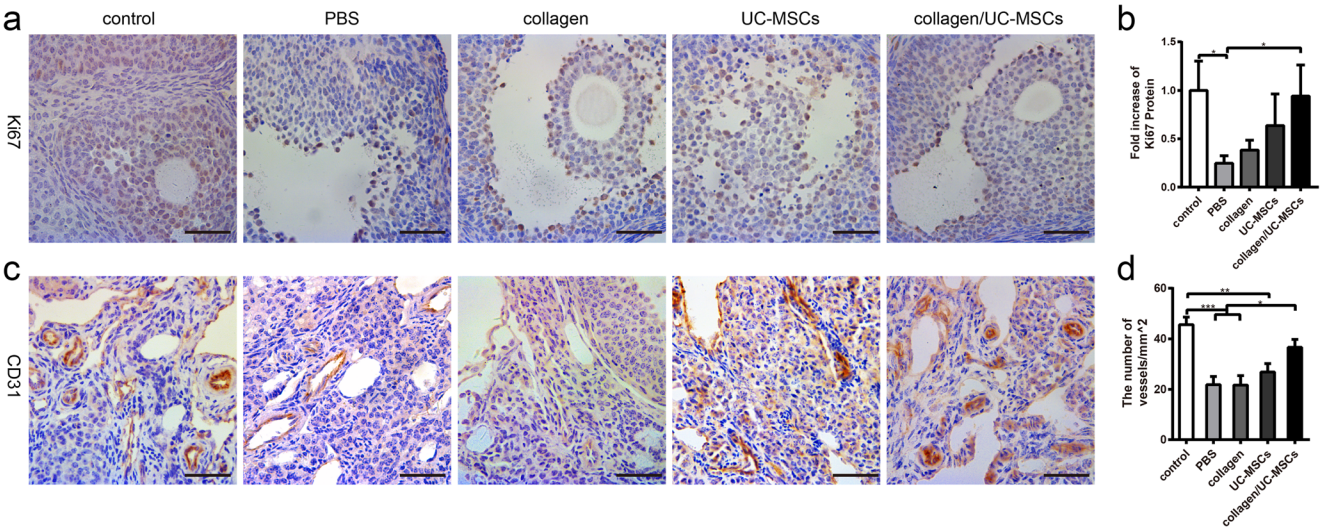

Figure 4. Immunohistochemistry staining of Ki67 for granulosa cell proliferation at 4 weeks after collagen/UC-MSCs transplantation. (***a***) Immunohistochemistry staining of Ki67 in control, PBS, collagen, UC-MSC, and collagen/UC-MSC groups. *Bar*: 50 μm. (***b***) Statistical analysis of the mean optical density of Ki67 positive area in follicles was carried out by Image-Pro Plus. (***c***) Immunohistochemistry staining of CD31 in control, PBS, collagen, UC-MSC, and collagen/UC-MSC groups. *Bar*: 50 μm. (***d***) Statistical analysis of the number of vessels per 1 mm^2 of ovaries was examined by Image J.

cells quickly spread around the target organs (Hsueh et al. 2015, Ding et al. 2018); however, the use of collagen scaffolds could increase the retention of stem cells, thus limiting stem cell distribution to the transplanted region while supporting cell attachment and proliferation (Guan et al. 2013, Guan et al. 2015). In this study, the ovaries of POF mice appeared to be enlarged after UC-MSCs or collagen/UC-MSCs transplantation, as evidenced by a higher ovarian weight and ovarian index. These results suggested that UC-MSCs on collagen scaffolds might remain biologically active and have therapeutic effects for prolonged periods. The present study evaluates the ovarian dysfunction that is associated with diminished follicles (Garris et al. 1985, Song et al. 2016). In a previous study, UC-MSCs transplantation increased the number of secondary follicles in POF rats (Song et al. 2016). In the present study, UC-MSCs or collagen/UC-MSCs transplantation increased the number of primary follicles, and secondary follicles in POF mice. The results of this study showed that ovarian function was recovered after collagen/UC-MSCs transplantation.

Follicular growth and development are closely associated with the proliferation of granulosa cell (Amos et al. 2010, Hsueh et al. 2015, B. Wang et al. 2016). A previous study found that UC-MSCs transplantation reduced granulosa cell apoptosis in POF rats and mice (S. Wang et al. 2013, Song et al. 2016). As mentioned in other studies, stem cells could also interact with collagen scaffolds to provide a 3D microenvironment leading to a higher stem cell survival rate and highly expressed VEGF, TGF-β1, FGF2, and HGF, thus promoting granulosa cell proliferation (Su et al. 2016). Furthermore, extensive vascular support is essential for normal follicular development and function of granulosa cell (Abulafia and Sherer 2000). In this study, ovaries of mice in the PBS and collagen groups exhibited decreased layers of granulosa cell in follicles, compared with those in the UC-MSC or collagen/UC-MSC groups. In addition, the expression of Ki67 in the granulosa cell of POF mice in the collagen/UC-MSC group was significantly higher than those of mice in the PBS group. The transplantation of collagen/UC-MSCs markedly increased the number of vessels of ovaries. These results indicated that collagen/UC-MSCs transplantation can be a more efficient therapy through improving proliferation of granulosa cell and promoting ovarian angiogenesis via an increased paracrine effect, resulting in follicular development and restoration of ovarian function.

However, the mechanism of interaction between collagen scaffolds and stem cells remains unknown. Studies must be performed to choose a proper density of stem cells on a collagen scaffold to allow cell to distribute evenly. Furthermore, the potential underlying mechanism of collagen scaffolds in UC-MSCs growth after transplantation also requires further investigation.

Conclusions

In summary, this is the first study where UC-MSCs on a collagen scaffold were transplanted into the ovaries of mice with POF, which contributed to granulosa cell proliferation, ovarian angiogenesis, and follicles development. Therefore, collagen/UC-MSCs transplantation may represent an ideal and promising treatment for POF.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

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