ISSN 1948-0210 (online)

World Journal of *Stem Cells*

World J Stem Cells 2020 August 26; 12(8): 706-896

Published by Baishideng Publishing Group Inc

WJSC

World Journal of Stem Cells

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ABOUT COVER

Editorial Board member of *World Journal of Stem Cells*, Dr. Perez-Campo is currently an Associate Professor in the Department of Molecular Biology at the University of Cantabria (Spain). She obtained her degree in Biological Sciences from the University of Salamanca (Spain), where she then went on to complete her PhD in 1999. Dr. Perez-Campo undertook her postdoctoral research at the Paterson Institute for Cancer Research (United Kingdom; currently known as Cancer Research UK Manchester Institute) under the supervision of Prof. Lacaud, where she remained for more than 10 years working in the field of stem cell biology. Upon returning to Spain, she joined the University of Cantabria and focused her research efforts on the molecular mechanisms that control mesenchymal stem cell (MSC) differentiation towards the osteoblastic and adipogenic lineages, and how those mechanisms are altered in osteoporosis. (L-Editor: Filipodia)

AIMS AND SCOPE

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The *WJSC* is now indexed in Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports/Science Edition, Biological Abstracts, BIOSIS Previews, PubMed, and PubMed Central. The 2020 Edition of Journal Citation Reports® cites the 2019 impact factor (IF) for *WJSC* as 3.231; IF without journal self cites: 3.128; Ranking: 18 among 29 journals in cell and tissue engineering; Quartile category: Q3; Ranking: 113 among 195 journals in cell biology; and Quartile category: Q3.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Stem Cells

ISSN
ISSN 1948-0210 (online)

LAUNCH DATE
December 31, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Carlo Ventura

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-0210/editorialboard.htm

PUBLICATION DATE
August 26, 2020

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World Journal of Stem Cells

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World J Stem Cells 2020 August 26; 12(8): 857-878

DOI: 10.4252/wjsc.v12.i8.857

ISSN 1948-0210 (online)

ORIGINAL ARTICLE

Basic Study

Human embryonic stem cell-derived mesenchymal stem cells improved premature ovarian failure

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Author contributions: Bahrehbar K and Esfandiari F were involved in collection and/or assembly of the data and manuscript writing; Bahrehbar K, Rezazadeh Valojerdi M, Baharvand H, Fathi R and Hassani SN were involved in data analysis and interpretation; Esfandiari F, Fathi R and Hassani SN were involved in conception and design; Rezazadeh Valojerdi M and Baharvand H were co-corresponding authors and were involved in conducting the experiments, manuscript proof, administrative and financial support.

Supported by Royan Institute, the National Institute for Medical Research Development, No. 963255; and the Ministry of Health and Medical Education, No. 700/147.

Institutional review board statement: This study was reviewed and approved by the

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Abstract

BACKGROUND

Premature ovarian failure (POF) affects many adult women less than 40 years of age and leads to infertility. According to previous reports, various tissue-specific stem cells can restore ovarian function and folliculogenesis in mice with chemotherapy-induced POF. Human embryonic stem cells (ES) provide an alternative source for mesenchymal stem cells (MSCs) because of their similarities in phenotype and immunomodulatory and anti-inflammatory characteristics. Embryonic stem cell-derived mesenchymal stem cells (ES-MSCs) are attractive candidates for regenerative medicine because of their high proliferation and lack of barriers for harvesting tissue-specific MSCs. However, possible therapeutic effects and underlying mechanisms of transplanted ES-MSCs on cyclophosphamide and busulfan-induced mouse ovarian damage have not been evaluated.

AIM

To evaluate ES-MSCs *vs* bone marrow-derived mesenchymal stem cells (BM-MSCs) in restoring ovarian function in a mouse model of chemotherapy-induced premature ovarian failure.

Institutional Review Board at Royan Institute.

Institutional animal care and use committee statement: All animal experiments of the study were reviewed and approved by the Institutional Ethical Committee at Royan Institute.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared according to these guidelines.

Manuscript source: Invited manuscript

Received: February 22, 2020
Peer-review started: February 22, 2020
First decision: April 29, 2020
Revised: June 1, 2020
Accepted: July 18, 2020
Article in press: July 18, 2020
Published online: August 26, 2020

P-Reviewer: Tanabe S
S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Wang LL

METHODS

Female mice received intraperitoneal injections of different doses of cyclophosphamide and busulfan to induce POF. Either human ES-MSCs or BM-MSCs were transplanted into these mice. Ten days after the mice were injected with cyclophosphamide and busulfan and 4 wk after transplantation of the ES-MSCs and/or BM-MSCs, we evaluated body weight, estrous cyclicity, follicle-stimulating hormone and estradiol hormone concentrations and follicle count were used to evaluate the POF model and cell transplantation. Moreover, terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate nick end labeling, real-time PCR, Western blot analysis and immunohistochemistry and mating was used to evaluate cell transplantation. Enzyme-linked immunosorbent assay was used to analyze vascular endothelial growth factor, insulin-like growth factor 2 and hepatocyte growth factor levels in ES-MSC condition medium in order to investigate the mechanisms that underlie their function.

RESULTS

The human ES-MSCs significantly restored hormone secretion, survival rate and reproductive function in POF mice, which was similar to the results obtained with BM-MSCs. Gene expression analysis and the terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate nick end labeling assay results indicated that the ES-MSCs and/or BM-MSCs reduced apoptosis in the follicles. Notably, the transplanted mice generated new offspring. The results of different analyses showed increases in antiapoptotic and trophic proteins and genes.

CONCLUSION

These results suggested that transplantation of human ES-MSCs were similar to BM-MSCs in that they could restore the structure of the injured ovarian tissue and its function in chemotherapy-induced damaged POF mice and rescue fertility. The possible mechanisms of human ES-MSC were related to promotion of follicular development, ovarian secretion, fertility *via* a paracrine effect and ovarian cell survival.

Key words: Premature ovarian failure; Human embryonic stem cells; Chemotherapy drugs; Mesenchymal stem cell; Bone marrow; Apoptosis

Core tip: Transplanted human embryonic stem cells are similar to bone marrow-derived mesenchymal stem cells. They can restore injured ovarian tissue structure and function in chemotherapy-induced premature ovarian failure mice and rescue fertility through the paracrine effect and ovarian cell survival.

Citation: Bahrehbar K, Rezazadeh Valojerdi M, Esfandiari F, Fathi R, Hassani SN, Baharvand H. Human embryonic stem cell-derived mesenchymal stem cells improved premature ovarian failure. *World J Stem Cells* 2020; 12(8): 857-878
URL: https://www.wjgnet.com/1948-0210/full/v12/i8/857.htm
DOI: https://dx.doi.org/10.4252/wjsc.v12.i8.857

INTRODUCTION

Premature ovarian failure (POF) disease has similar characteristics such as hypoestrogenism, elevated gonadotropin levels and infertility in animal models and in human. Some women also have symptoms such as hot flashes, night sweats, vaginal dryness, chronic anxiety, sadness and depression[1]. POF affects 1%-3% of women < 40 years of age[2]. Hot flashes, depression, anxiety, osteoporosis and sexual dysfunction are the consequences of this disease[3,4]. Although the cause of POF is often idiopathic, possible causes include autoimmune disorders, smoking, toxic chemicals, drugs and genetic defects[5-7]. Chemotherapeutics such as cyclophosphamide (Cy) and busulfan

(Bu) are the most gonadotoxic agents that lead to POF in the majority of patients[8]. Currently, ovarian protection methods, oocyte or ovarian tissue cryopreservation and embryo freezing are strategies used for fertility preservation in women diagnosed with cancer. However, these methods have serious disadvantages such as the risk of reintroducing the cancer cells, delays in cancer treatment and low success rate. Therefore, it is necessary to develop advanced therapies for women with POF[9].

Emerging evidence suggests that mesenchymal stem cells (MSCs) derived from bone marrow (BM) and other adult tissues (adipose, skin, amniotic membrane, placenta) and menstrual blood could restore ovarian function in animal models of POF[10-14]. A meta-analysis from 16 preclinical studies of animal models was conducted to assess the efficacy of stem cell transplantation. The results indicated that MSC therapy significantly improved ovarian function in cases with POF[15]. In two case studies, MSC transplantation also improved POF[15-16]. However, despite the promising results, the numbers of harvested MSCs and their *in vitro* expansion was a challenge[17]. Moreover, obtaining MSCs from bone marrow requires suitable donors and invasive procedures. The number of bone marrow-derived mesenchymal stem cells (BM-MSCs) is very limited, which greatly restricts use of BM-MSCs for clinical application[18]. The immunomodulating feature of MSCs seems to be different between species[19]. Human MSCs decrease the secretion of interferon gamma, interleukin 12 and tumor necrosis factor alpha and increase interleukin 10 secretion[20-22]. Moreover, human MSC-mediated inhibition of the T cell response could not be reversed by nitric oxide synthase inhibitor compared with mice MSCs[23]. Integrin β1 expression is important for mice MSCs migration, while C-X-C chemokine receptor type 4 expression is involved for human MSC migration to sites of tissue injury[24-25]. It has been demonstrated that 92% of MSC protein expression is similar in humans and mice[26]. MSCs represent only a small proportion of the cells in bone marrow, and their proliferation and differentiation capacity correlates inversely with age[20].

In addition to adult tissue specific MSCs, human embryonic stem cells (ES-MSCs) are an alternative source of MSCs because of their similar phenotypic characteristics that make them attractive candidates for regenerative cellular therapy[27-28]. Recently, it has been reported that ES-MSCs have higher capabilities for cell proliferation and suppression of leukocyte growth compared to MSCs from other sources[29-30]. ES-MSCs exhibited more potent anti-inflammatory properties than BM-MSCs[17,27,31-33]. The therapeutic potential of ES-MSCs has been reported in numerous animal models. When compared with BM-MSCs, these cells showed a significantly greater improvement in models of thioacetamide-induced chronic liver injury and experimental autoimmune encephalitis[30,34]. This evidence indicates that ES-MSCs may serve as better sources for clinical applications.

Human ES-MSCs can overcome the obstacles seen with harvesting MSCs from adult tissues, including lack of appropriate donors, limited numbers of cells obtained during the harvesting process, restricted *in vitro* expansion capacity and the invasiveness of the procedures. Thus, we hypothesized that ES-MSCs might restore ovarian structure and function through the paracrine mechanisms of cytokines in a POF model. To address this issue, we used a POF mouse model to evaluate the potential for transplanted ES-MSCs to restore fertility.

MATERIALS AND METHODS

Derivation of MSCs from human ES cells and BM

We isolated and cultured ES-MSCs according to our previously published protocols[17,33]. Briefly, we obtained MSCs from human ES cells by culturing these cells in basic fibroblast growth factor-free ES medium to enable embryoid body formation. The resultant embryoid bodies were plated in gelatin-coated plates and cultured in MSC medium. Spontaneous differentiation of the embryoid bodies resulted in an outgrowth of ES-MSCs. These cells were further passaged to obtain a homogenous population with spindle-shaped morphology. Passage-2 human BM-MSCs were prepared from Royan Stem Cell Bank (Tehran, Iran) and cultured in low-glucose Dulbecco's Modified Eagle Medium (Life Technologies, United States) supplemented with 10% fetal bovine serum (FBS, Life Technologies, United States) for further expansion. The medium was changed every 3 d.

Cell proliferation analysis

We cultured 1 × 10^6 cells/cm^2 in T25 cm^2 tissue culture flasks (TPP, Germany) to assess their proliferative ability. The population doubling time was calculated according to

the following formula:

Population doubling time = duration × log (2)/log (final concentration) – log (initial concentration)

Karyotype analysis

The cells were treated with 0.66 mmol/L thymidine (Sigma-Aldrich) and incubated at 37 °C for 16 h. After the cells were washed with phosphate buffered saline (PBS), they were left for 5 h and then treated with 0.15 mg/mL colcemid (Invitrogen) for 30 min. Then, the cells were exposed to 0.075 mol/L potassium chloride (Merck) and allowed to incubate at 37 °C for 16 min. After the cells were centrifuged, we removed the supernatant and resuspended the pellet in Carnoy's fixative (3:1 ratio of methanol:glacial acetic acid). The cells were dropped onto precleaned, chilled slides and standard G-band staining was performed for chromosome visualization. We screened at least 20 well-spread metaphase cells of which 10 were evaluated for chromosomal rearrangements.

Flow cytometry analysis

We sought to determine the immunophenotypes of the cultured ES-MSCs and BM-MSCs. Surface-marker expression was analyzed by flow cytometry using the following antibodies: Fluorescein isothiocyanate-conjugated human monoclonal antibodies against protein tyrosine phosphatase receptor type C and cluster of differentiation (CD) 90 (CD90); and phycoerythrin-conjugated human monoclonal antibodies against homing cell adhesion molecule, CD73, endoglin, CD11b and CD34. For flow cytometric analysis, the adherent cells were detached by using 0.25% trypsin-ethylenediaminetetraacetic acid, neutralized by FBS-containing culture medium and disaggregated into single cells by pipetting. The cells were incubated with antibodies for 30 min at 4 °C, washed twice with PBS, resuspended in 0.5 mL PBS and immediately analyzed by fluorescence-activated cell sorting Calibur flow cytometer (Becton Dickinson, United States). Analyses were performed on three independent biological samples. Data were analyzed using the FlowJo software (version 7.6.1). Supplementary Table 1 lists the antibodies used in this study.

Multilineage differentiation

Osteogenic, adipogenic and chondrogenic differentiation were verified by alizarin red, oil red O, and alcian blue staining, respectively to confirm the multipotent properties of the ES-MSCs and BM-MSCs. For osteogenesis, the cells were seeded onto 6-well plates at a density of 1×10^5 cells/cm^2. After 24 h, the medium was replaced by osteogenic differentiation medium, alpha minimum essential medium (Life Technologies, United States) supplemented with 10% FBS (Gibco, United States), 0.1 mmol/L dexamethasone (Sigma-Aldrich, United States), 10 mmol/L β-glycerophosphate (Sigma-Aldrich, United States) and 50 mmol/L ascorbic acid (Sigma-Aldrich, United States) for 2 wk. To induce adipogenesis, the cells were incubated with adipogenic differentiation medium in alpha minimum essential medium supplemented with 10% FBS, 10 mg/mL insulin (Sigma-Aldrich, United States), 1 mmol/L dexamethasone (Sigma-Aldrich, United States), 0.5 mmol/L isobutyl-methylxanthine (Sigma-Aldrich, United States) and 100 mmol/L indomethacin (Sigma-Aldrich, United States) for 3 wk. For chondrogenic differentiation, 2.5×10^5 cells were collected in a 15 mL tube and centrifuged at 350 g for 5 min. The cell pellet was subsequently cultured for 3 wk using chondrogenic induction medium (chondrogenesis differentiation kit, Gibco, United States) according to the manufacturer's instructions. Then, the pellets were fixed in 4% paraformaldehyde (Sigma Aldrich, United States) for 30 min, dehydrated in ethanol, cleared in xylene and embedded in paraffin. The paraffin-embedded cells were sectioned into 6 μm sections by using a microtome. The sections were stained with alcian blue.

Measurement of cytokine secretion

We analyzed cytokines secreted by the MSCs. Both ES-MSCs and BM-MSCs were cultured in dishes at densities of 2×10^5 cells/cm^2 each. After a 24 h culture in serum-free media, the culture media was collected and centrifuged at 2000 *g* for 5 min. The amount of cytokine expression was measured using a vascular endothelial growth factor (VEGF) Human ELISA kit (Invitrogen, United States), insulin-like growth factor 2 (IGF-2) Human ELISA kit (R&D Systems, United States) and hepatocyte growth factor (HGF) Human ELISA kit (R&D Systems, United States).

Experimental animals

All animal experiments were approved by the Institutional Ethical Committee of Royan Institute. Adult female C57BL/6 mice (6-8 wk old) were used in our study. The mice were housed under a 14-10 h light-dark cycle and had free access to food and water.

Estrous cyclicity

Vaginal smears were obtained daily. The four stages of the estrous cycle were determined as follows: Proestrus (100% intact live epithelial cells); estrus (100% cornified epithelial cells); metestrus (about 50% cornified epithelial cells and about 50% leukocytes); and diestrus (80%-100% leukocytes). The mouse estrous cycle lasts for approximately 4 d and includes the proestrus, estrus, metestrus and diestrus stages. Animals with at least two consecutive normal 4-d vaginal estrous cycles were included in the experiments. In order to validate reproductive function, we assessed the animals over 10 consecutive days of the experiment. The number of estrous cycles were checked at 8:00 am daily with a vaginal smear assay starting at 10 d after the animals were injected with Cy and Bu and 4 wk after transplantation of ES-MSCs or BM-MSCs.

Establishment of the POF model

In this study, we used chemotherapy to create the mouse model because chemotherapy is one of the major causes of POF[35]. Various reports of POF models generated in mice used from 8-30 mg/kg of Bu plus 50-200 mg/kg of Cy[36-40]. or only Cy[41-44]. However, none of the previous studies showed any significant decrease in follicle numbers during the developmental stages. Therefore, we assessed different doses of these drugs to create a POF model in our laboratory setting. Female mice were randomly divided into four treatment groups and one intact group. The treatment groups received intraperitoneal injections of different doses of Cy (Endoxan™, Germany) and Bu (Sigma-Aldrich, United States) as follows: Group 1 (POF1): 50 mg/kg Bu and 100 mg/kg Cy; group 2 (POF2): 100 mg/kg Cy for 10 consecutive days; group 3 (POF3): 200 mg/kg Cy and 50 mg/kg Bu on the 1st day followed by 50 mg/kg Cy and 5 mg/kg Bu for 9 consecutive days; and group 4 (POF4): A single injection of 20 mg/kg Bu and 200 mg/kg Cy. In order to confirm successful establishment of POF in the mouse model, we checked their body weights, estrous cyclicity, concentrations of follicle-stimulating hormone (FSH) and estradiol (E2) hormones and follicle counts. In addition, for further confirmation of POF, we also used the terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay and real-time PCR assessments, and the mice were allowed to mate 10 d after the injections.

Cell transplantation

Once the POF model was established, we randomly divided the mice into three groups. Vehicle POF mice received medium but no cell transplantation. In the ES-MSC group, POF mice were injected with 1 × 10^6 ES-MSCs. In the BM-MSC group, POF mice were injected intravenously with 1 × 10^6 BM-MSCs in 0.1 mL Dulbecco's Modified Eagle Medium. In order to evaluate the effects of the transplanted ES-MSCs and BM-MSCs, we assessed the body weights, estrous cyclicity, concentrations of FSH and E2 hormones and follicle counts in the POF mice at 4 wk after the transplantations. In addition, the mice were allowed to mate. The TUNEL assay, Western blot, immunohistochemistry and real-time PCR assessments were also performed.

Hormone assay

Blood samples were obtained from hearts of the anesthetized mice to determine serum levels of E2 and FSH. The blood samples were incubated at room temperature for 1 h, and supernatant was collected after centrifugation at 3000 rpm for 20 min. Hormone levels were determined by ELISA kits (Biotech, Shanghai, China).

Detection of apoptosis by the TUNEL assay

Cell apoptosis in the ovarian tissue was detected by the TUNEL assay. Briefly, 5 μm ovarian sections were washed twice in PBS for 5 min after deparaffinization. These sections were permeabilized by incubation in 0.1% Triton X-100 solution and 0.1% sodium citrate for 8 min. Then, the TUNEL assay was performed with an *in situ* cell death detection kit (Roche, Germany) according to the manufacturer's instructions. Counterstaining with DAPI (Sigma-Aldrich) was used to visualize the nuclei. We observed the cells under a fluorescence microscope (Olympus, Japan) for the presence

of apoptosis (green fluorescent color).

Hematoxylin and eosin staining and data quantification

The ovaries were removed and fixed in 4% paraformaldehyde (Sigma-Aldrich) for at least 24 h. The fixed ovaries were dehydrated, embedded in paraffin, serially sectioned into 6 μm sections and mounted on glass microscope slides. Routine hematoxylin and eosin staining was performed for histologic examination under a light microscope.

Follicle counting

Primordial, primary, secondary and antral follicles were counted in each of the five sections based on the method reported by Tilly[45]. Only the follicle with a nucleus was counted to avoid duplicate counting of a follicle. The follicles were classified as: Primordial (oocyte surrounded by a single layer of squamous granulosa cells); primary (intact enlarged oocyte with a visible nucleus and one layer of cuboidal granulosa cells); secondary (two or three layers of cuboidal granulosa cells without an antral space); early antral (emerging antral spaces); and preovulatory (the largest follicular types with a defined cumulus granulosa cell layer). Supplementary Tables 3 and 4 present the data for follicle counting both after chemotherapy and cell transplantation. The significance of the changes in follicle numbers in the different study groups were analyzed by two-way analysis of variance.

Gene expression analysis

Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was performed for the apoptosis genes, anti-apoptosis gene [B-cell lymphoma 2 (*Bcl2*)], apoptosis gene [cysteine-aspartic proteases 3 (*caspase 3*)], angiogenesis gene (*Vegf*), proliferation gene (*Igf-2*), granulosa marker anti-Müllerian hormone (*Amh*) and oocyte marker [growth/differentiation factor 9 (*Gdf9*)] in the intact group, POF group and both cell transplantation groups. Total RNA was isolated and purified with TRIzol reagent (Invitrogen) according to the manufacturer's protocol followed by cDNA synthesis with a cDNA synthesis kit (Fermentas). qRT-PCR reactions were performed using SYBR Green Master Mix (Applied Biosystems) and a real-time PCR system (Corbett Life Science; Rotor-Gene 6000 instrument). The samples were collected from three independent biological replicates. Supplementary Table 2 lists the primer sequences used for qRT-PCR.

Western blot analysis

The protein expression of caspase 3 in the ovaries was measured by Western blot. The mice were anesthetized, and we removed their ovaries. The proteins from the ovaries were isolated by the Q Proteome Mammalian Protein Prep kit (Merck, Germany). The total protein concentrations were measured using a standard BCA protein assay kit. The protein from each group was separated on 12% SDS-PAGE and transferred onto PVDF membranes. The blots were then incubated in blocking buffer [2% (w/v) skim milk powder in TBST] for 1 h at room temperature. Then, the membranes were incubated overnight at 4 °C with the primary antibody, anti-caspase 3 (1:2000[46]). The membranes were washed three times with TBST and incubated at room temperature for 1 h with anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:1000).

Immunohistochemistry

Expression of the granulosa cell marker (Amh) was detected by immunohistochemical staining. The ovaries were fixed in formalin and sectioned into 5 μm sections. The sections were incubated at 60 °C for 1 h, deparaffinized in xylene and rehydrated in a graded ethanol series. Then, antigen retrieval was performed by heating the sections in citrate buffer in an oven for 30 min. The sections were washed in H_2O_2 for 30 min to eliminate endogenous peroxidase activity and blocked with goat serum for 1 h at room temperature. After that, the sections were incubated overnight at 4 °C with the primary antibody, anti-Amh. After three washes with PBS for 10 min each time, the secondary antibody, streptavidin, and DAB were used for immunostaining according to the protocol from an immunostaining kit (Merck, Germany). Finally, the sections were counterstained with hematoxylin, dehydrated and mounted.

Mating trial

The mating trial was initiated 10 d after the mice were injected with Cy or 4 wk after transplantation of the ES-MSCs or BM-MSCs and continued for six weeks. Female mice were housed in the same cages with male mice for natural mating. The presence

of a copulatory plug indicated successful mating. Males were randomly rotated among the cages after each pregnancy and the numbers of offspring per litter were recorded.

Statistical analysis

All experiments were conducted in at least three independent repeats. All data are shown as mean ± standard error of the mean. One-way analysis of variance was used to determine significant differences among groups with Tukey's post-hoc test. Viability was analyzed by the *t* test. $P < 0.05$ were considered significant.

RESULTS

Derivation and characterization of ES-MSCs and BM-MSCs

Figure 1A shows the procedure used to derive MSCs from the ES cells. MSCs derived from both human ES and BM formed a homogeneous cell population with spindle-shaped morphology and a normal karyotype during long-term culture (Figure 1B and 1C). The MSCs successfully differentiated into osteogenic, adipogenic and chondrogenic lineages (Figure 1D). Flow cytometry analysis confirmed the expression of MSC-specific markers homing cell adhesion molecule, CD73, CD90 and endoglin by both human ES-MSCs and BM-MSCs; there were no detectable levels of the hematopoietic and endothelial cell markers (CD11b, CD34 and protein tyrosine phosphatase receptor type C) (Figure 1E and 1F, Supplementary Figure 1). The population doubling time assay showed significant increases in ES-MSC proliferation compared to BM-MSCs (Figure 1G; $P < 0.05$).

Establishment of a mouse model of chemotherapy-induced POF

We examined various concentrations of four different combinations of two chemotherapy drugs, Cy and Bu, in order to establish a POF model that showed the significant decreases in follicle numbers for all of the developmental stages (Figure 2A). In the intact group, the mice had regular 4-d estrous cycles; however, irregular estrous cycles were observed in the POF1, POF2, POF3 and POF4 mice. On day 5 of the treatment, the POF1, POF2 and POF3 mice were eating less and moved slowly (data not shown). The ovaries of the mice in the intact group were more reddish in color, whereas the ovaries of the mice that survived in the POF groups were pale. All of the animals were weighed before and after modeling, and we found significantly reduced body weights in the POF1 and POF2 groups (Figure 2B, Supplementary Figure 2A; $P < 0.05$). Furthermore, the size of ovaries in mice treated with the chemotherapy drugs in the POF1, POF2, POF3 and POF4 groups were smaller than ovaries from the intact mice (Supplementary Figure 2B).

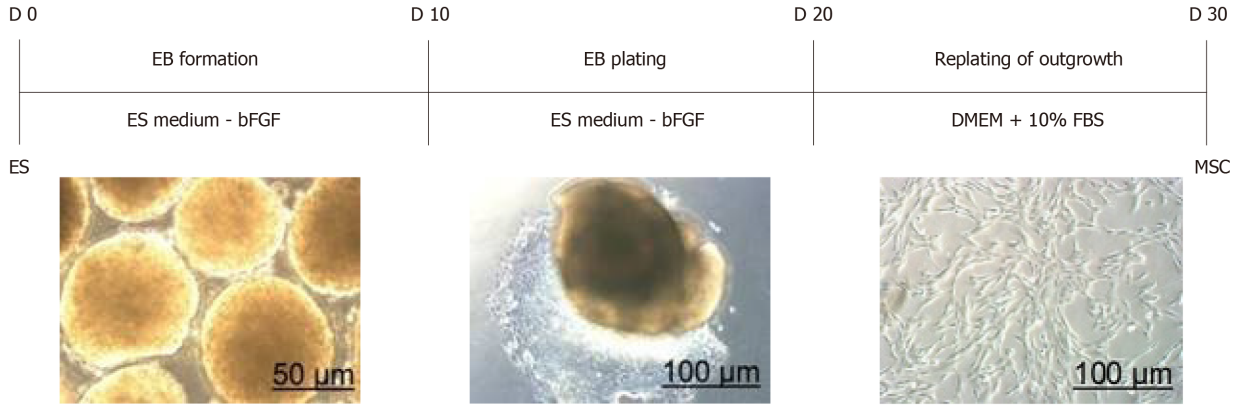
We performed hematoxylin and eosin staining to evaluate the structures of the ovaries following chemotherapy. Quantification of the follicles showed significant decreases in all of the developmental stages in the POF1 and POF2 groups (Figure 2C; Supplementary Figure 3; $P < 0.05$), while the POF3 and POF4 groups did not show significant decreases in the number of follicles in the various developmental stages.

Hormonal analysis demonstrated significant increases in serum levels of FSH and significant decreases in E2 levels in the POF1 and POF2 groups (Figure 2D and 2E; $P < 0.05$). TUNEL assay results to evaluate apoptosis in the ovaries following chemotherapy (Supplementary Figure 4A) showed a significantly increased percentage of TUNEL-positive cells in the POF1 and POF2 groups compared to the intact group (Figure 2F; $P < 0.05$). Next, we sought to determine the optimum POF model by evaluating the survival rate of the mice and the pregnancy rate following chemotherapy. We found significantly higher survival rates in the POF1 group compared to the POF2 group (Figure 2G; $P < 0.01$). However, none of the POF mice became pregnant (Supplementary Figure 4B). Therefore, we selected the POF2 model as the most appropriate model for induction of POF.

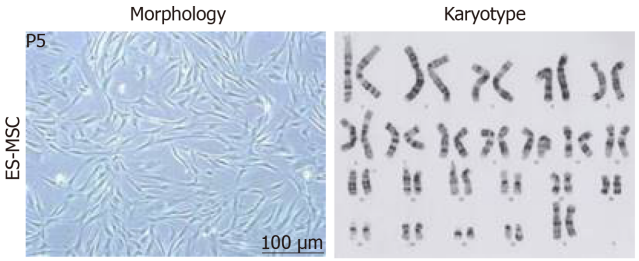
ES-MSCs and BM-MSCs improved the POF model

We explored the possibility that the MSCs could improve the POF mouse model. There were more regular estrous cycles following transplantation of both human ES-MSCs and BM-MSCs compared to the vehicle group. Moreover, the ovaries of the mice had an increased red color and were larger in size following transplantation of both ES-MSCs and BM-MSCs in comparison with the vehicle group, but they were less than the intact ovaries (Supplementary Figure 5). Body weight significantly increased 4 wk after transplantation of both ES-MSCs and/or BM-MSCs compared to the vehicle

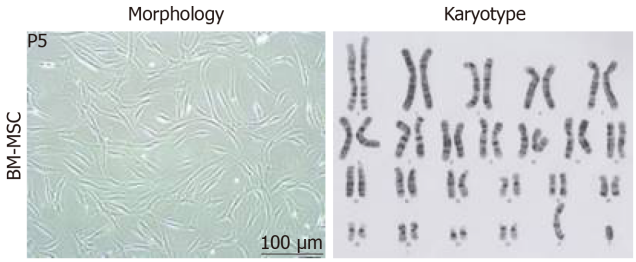
A


D 0
D 10
D 20
D 30
EB formation
EB plating
Replating of outgrowth
ES medium - bFGF
ES medium - bFGF
DMEM + 10% FBS
ES
MSC
50 μm
100 μm
100 μm

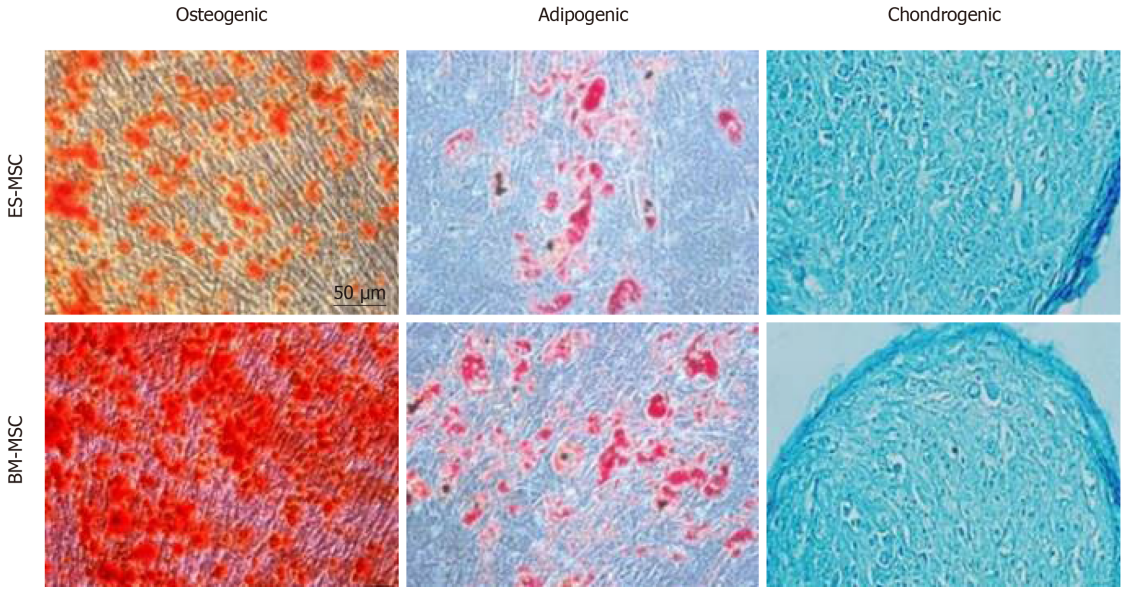
B


Morphology
Karyotype
P5
ES-MSC
100 μm

C


Morphology
Karyotype
P5
BM-MSC
100 μm

D


Osteogenic
Adipogenic
Chondrogenic
ES-MSC
BM-MSC
50 μm

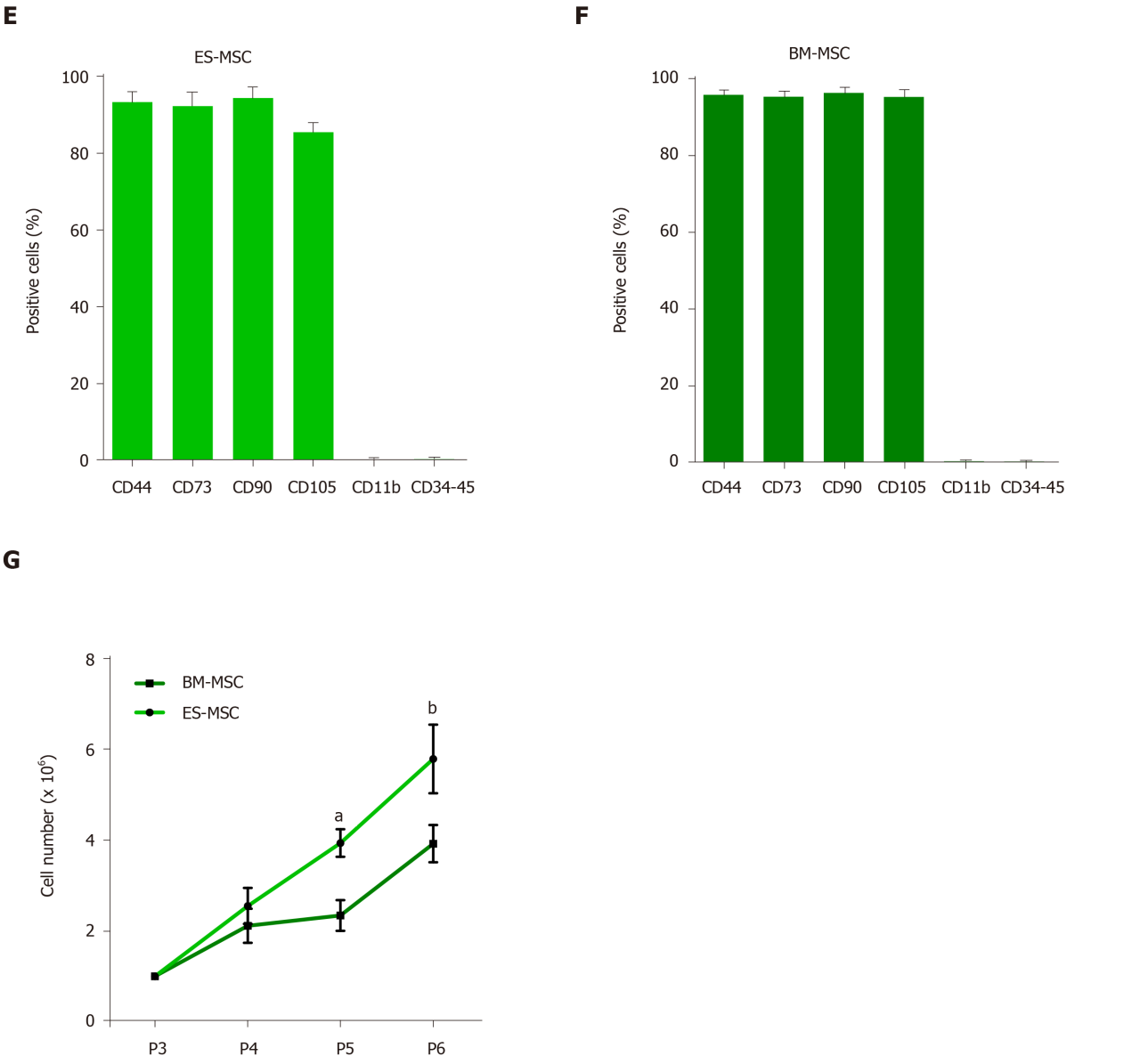


Figure 1 Derivation and identification of human embryonic stem cell-derived mesenchymal stem cells and bone marrow-derived mesenchymal stem cells. A: Schematic presentation of the procedure used to derive human mesenchymal stem cells (MSCs) from embryonic stem (ES) cells. Colonies of ES cells were enzymatically detached and cultured for 10 d in suspension to form embryoid bodies, which were then plated onto gelatin-coated tissue culture plates. After 10 d, outgrowths of the cells that sprouted from embryoid bodies were mechanically isolated by a cell scraper and subsequently expanded in mesenchymal stem cell culture medium; B and C: Morphology and karyotype of ES-MSCs and BM-MSCs. Passage-5 ES-MSCs and BM-MSCs showed a fibroblastic morphology and normal karyotype; D: Alizarin red staining after 14 d of culture in osteogenic medium indicated the osteogenic differentiation potential of ES-MSCs and BM-MSCs (P4). Oil red staining after 21 d of culture in adipogenic medium showed the adipogenic differentiation potential of ES-MSCs and BM-MSCs (P4). Alcian blue staining after 21 d of culture in chondrogenic medium showed chondrogenic differentiation potential of ES-MSCs and BM-MSCs; E, F: Flow cytometric analysis indicated that cultured ES-MSCs and BM-MSCs expressed CD44, CD90, CD73 and endoglin (CD105), but not hematopoietic lineage markers CD11b, CD34 and protein tyrosine phosphatase receptor type C (CD45); G: ES-MSCs proliferated more rapidly than BM-MSCs. Results are expressed as mean ± standard error, $^{a}P < 0.05$, $^{b}P < 0.01$; n = 3-5. ES-MSCs: Embryonic stem cell-derived mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; EBs: Embryoid bodies; bFGF: Basic fibroblast growth factor; P: Passage.

group (Figure 3A; $P < 0.05$). The survival rate significantly increased following transplantation of ES-MSCs and/or BM-MSCs (more than 60%) compared to the vehicle group (20%) (Figure 3B; $P < 0.01$).

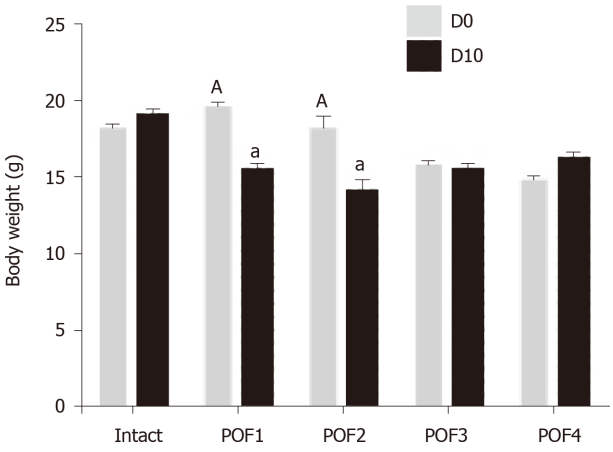
Notably, we observed significant increases in the number of follicles at all stages of development following transplantation of both ES-MSCs and BM-MSCs compared with the vehicle group (Figure 3C; $P < 0.05$). Transplantation of both ES-MSCs and BM-MSCs significantly decreased the FSH levels and increased the E2 levels compared with the vehicle group (Figure 3D and 3E; $P < 0.05$).

The results of the TUNEL assay confirmed significant decreases in apoptosis in ovaries that received the cell transplantations (Figure 4A and 4B, Supplementary Figure 6; $P < 0.05$). qRT-PCR was conducted in order to gain further insight into the

A

Group	Dose of drugs
POF1	Cy (100 mg/kg) and Bu (50 mg/kg)
POF2	Cy (100 mg/kg)
POF3	Cy (200 mg/kg) and Bu (50 mg/kg) at day 1 plus Cy (50 mg/kg) and Bu (5 mg/kg) for 9 d
POF4	Cy (200 mg/kg) and Bu (20 mg/kg)

B



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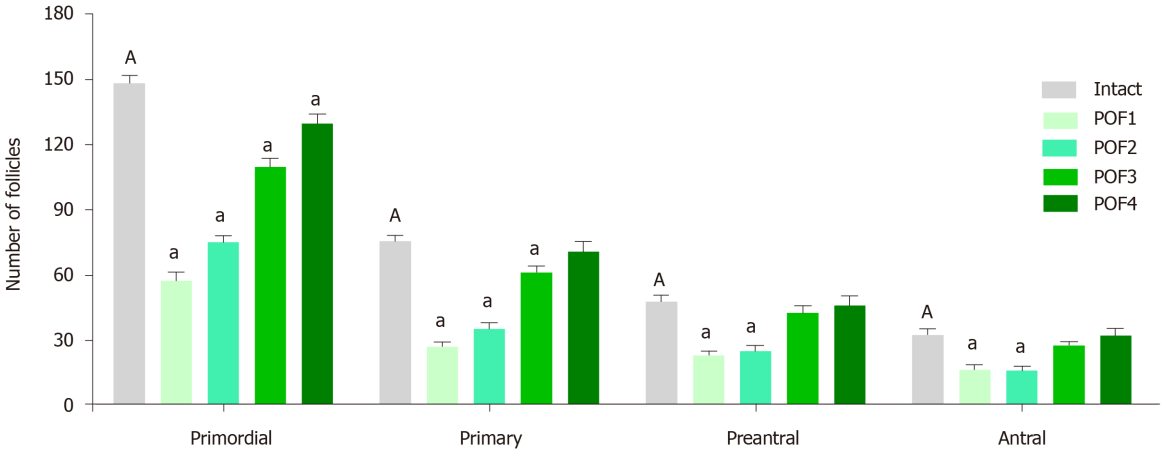

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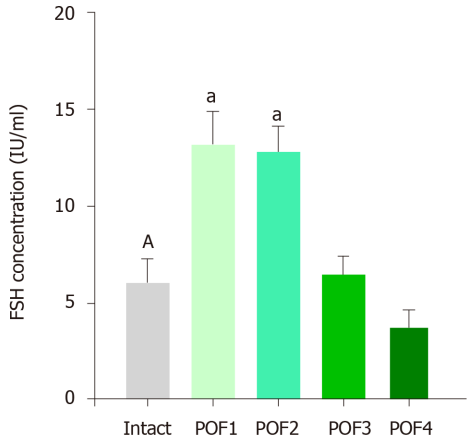

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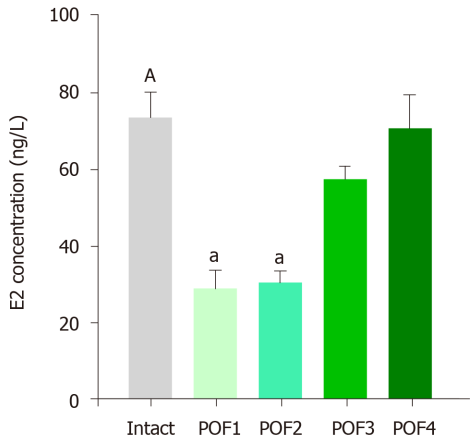


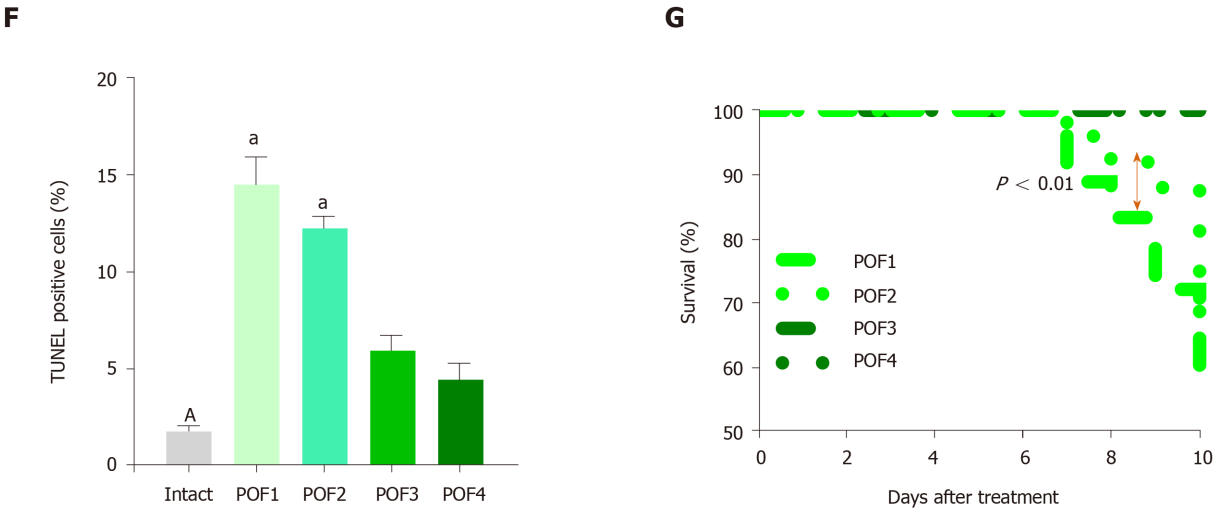


Figure 2 Establishment of a mouse model of premature ovarian failure. A: Premature ovarian failure (POF) groups were treated with different dosages of cyclophosphamide and busulfan; B: Body weight changes in the intact and POF groups after 10 d showed that the POF1 and POF2 groups had significant decreases in body weights; C: Ovarian pathology of the intact and POF groups 10 d after injection of cyclophosphamide and busulfan . Follicle count revealed that there were fewer normal follicles in the POF groups than in the intact mice. The ovaries of the intact group contained large numbers of follicles at all developmental stages, whereas the atrophic ovaries of the POF groups had fewer follicles at each stage; D, E: Serum levels of follicle stimulating hormone and estradiol 10 d after injection of cyclophosphamide and busulfan. Serum levels of follicle stimulating hormone were significantly increased in the POF1 and POF2 groups compared to those of the intact group. Serum levels of estradiol were significantly decreased in the POF1 and POF2 groups compared with the intact group; F: Apoptosis rate in the ovary. Green fluorescence indicated the presence of apoptotic cells in the POF1, POF2, POF3 and POF4 groups; G: Survival rate in the POF groups after 10 d. The survival percent showed a significant decrease in the POF1 group compared with the POF2 group. All data are presented as mean ± standard error. Small letters (a) indicate the significance ($P < 0.05$) compared to groups labeled by similar capital letters (A); $^{a}P < 0.05$ significance of experimental groups *vs* the intact group; n = 3-5. POF: Premature ovarian failure; Cy: Cyclophosphamide; Bu: Busulfan; FSH: Follicle stimulating hormone; E2: Estradiol.

effect of transplantation on the ovaries. The results showed significant downregulation of the apoptosis gene, *caspase 3*, while the anti-apoptotic gene, *Bcl2*, was significantly upregulated following cell transplantation compared with the vehicle group. In particular, the level of the angiogenesis gene (*Vegf*), proliferation gene *(Igf-2)* and granulosa marker *(Amh)* significantly increased following cell transplantation. In contrast, we observed no significant differences in the oocyte marker, *Gdf9*, following transplantation (Figure 4C; $P < 0.05$).

Cleaved- caspase 3 acts as a functional enzyme[47,48]; therefore, to further validate these results, we performed Western blot assessment of cleaved-caspase 3 protein expression. Our results showed a significant increase in the cleaved-caspase 3 protein expression level in ovaries from the vehicle group compared with the control group, whereas the cleaved-caspase 3 protein expression level decreased significantly in the ovaries after transplantation of ES-MSCs and/or BM-MSCs compared with the vehicle group (Figure 4D and 4E; $P < 0.05$). Previous studies suggested that MSCs secrete cytokines that are important for anti-apoptosis, angiogenesis, anti-inflammation, anti-fibrosis and immunoregulation, which would improve the microenvironment for promoting regeneration of injured tissues in numerous diseases[49-53]. In order to investigate the mechanism that underlies the function of these MSCs, we also analyzed VEGF, IGF-2 and HGF levels in ES-MSCs and BM-MSCs condition media by using ELISA. The results showed that in a similar manner ES-MSCs and BM-MSCs secreted VEGF, IGF-2 and HGF *in vitro* (Figure 4F). However, there were only a few GFP-labelled cells after 4 wk in the ovaries (data not shown).

Immunohistochemistry staining for Amh to confirm the changes in the granulosa cells showed decreased Amh expression in ovaries from the vehicle group compared to the intact group and increased Amh expression in ovaries from both the ES-MSCs and BM-MSCs transplantation groups compared with the vehicle group (Figure 5A).

We assessed the ability of mice that received the transplantations to conceive and give birth to offspring. The successful mating rate was investigated over 6 wk, and the presence of a copulatory plug indicated successful mating. The mice that received transplantations of both ES-MSCs (3 out of 5 mice) and/or BM-MSCs (2 out of 5 mice) became pregnant and produced live offspring, 9 pups in mice transplanted with BM-MSCs and 16 pups in mice that received ES-MSCs. None of the vehicle mice became pregnant. These results showed that ovarian functions in mice with POF were partially restored by transplantation with either ES-MSCs or BM-MSCs (Figure 5B and 5C; $P < 0.05$).

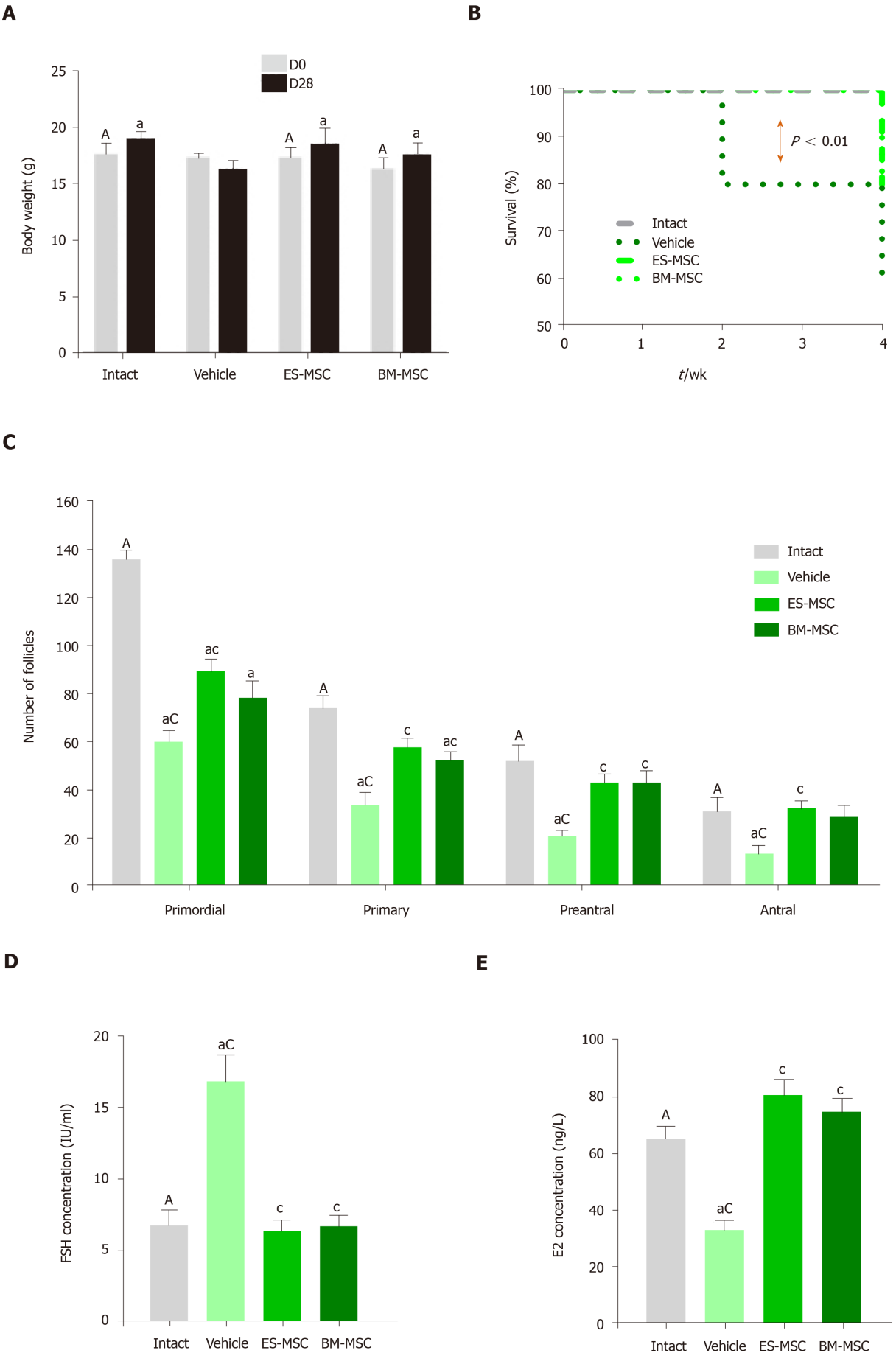


Figure 3 Effects of human embryonic stem cell-derived mesenchymal stem cells and bone marrow-derived mesenchymal stem cells

transplantation in mice with premature ovarian failure. A: Transplantation of embryonic stem cell-derived mesenchymal stem cells (ES-MSCs) and/or bone marrow-derived mesenchymal stem cells (BM-MSCs) improved body weights in mice with premature ovarian failure after 4 wk; B: Survival rate 4 wk after ES-MSCs and/or BM-MSCs transplantation. Survival rate significantly increased in both the ES-MSCs and/or BM-MSCs transplanted mice (more than 60%) compared with the vehicle group (20%); C: The follicle number increased after transplantation. The number of follicles at all stages of development in both cell transplanted groups was significantly higher than that of the vehicle mice, while it was lower than the intact mice; D, E: Both cell transplantations rescued hormone secretion in premature ovarian failure mice. Serum follicle stimulating hormone levels decreased significantly in both cell transplanted groups compared to the vehicle group. The serum estradiol level significantly recovered after both cell transplantations compared to the vehicle group. All data are presented as mean ± standard error. Small letters (a, c) indicate the significance ($P < 0.05$) compared to groups labeled by similar capital letters (A, C); $^{a}P < 0.05$ significance of experimental groups *vs* the intact group; $^{c}P < 0.05$ significance of ES-MSC and BM-MSC groups *vs* the vehicle group; n= 3-5. ES-MSCs: Embryonic stem cell-derived mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; POF: Premature ovarian failure; FSH: Follicle stimulating hormone; E2: Estradiol.

DISCUSSION

Understanding the pathogenesis of POF plays an important role in the development of effective therapeutic options for this disease. Therefore, elucidation of the mechanism for POF development is critical for the clinical treatment of POF disease[54]. The estrous cycle of female mice is similar to that of humans, although the estrous cycle of mice is shorter than that of humans[55].

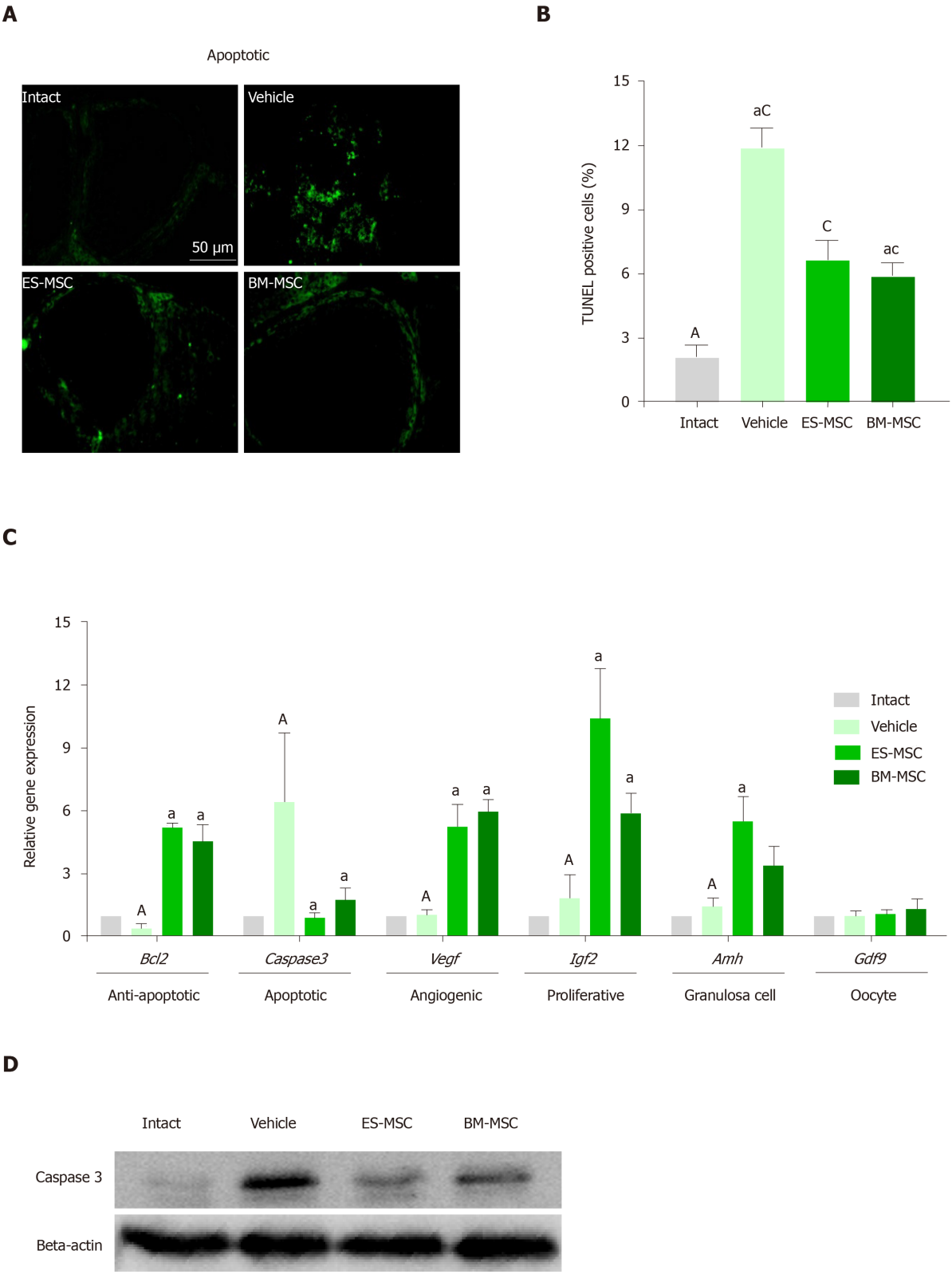
In this study, we initially established a mouse POF model by administration of Cy and Bu as the most effective chemotherapeutic drugs. The results indicated that Cy plus Bu in our established model (POF2) significantly decreased the number of follicles at various stages of development and significantly decreased ovarian size and body weight. In line with previous studies, chemotherapy increased primordial follicle recruitment, which led to significant decreases in the number of follicles at different developmental stages[35,56]. Apoptotic cells significantly increased in our established POF model, which was consistent with previous findings where chemotherapeutic drugs destroyed highly proliferating cells by activation of apoptosis[57]. We observed increased FSH levels and decreased E2 levels, which supported results of studies that showed similar patterns of hormonal changes in POF[15]. Previous studies demonstrated that chemotherapeutic drugs can cause POF in various species such as mouse, rat, rabbit and human[16,58-61]. Our results were consistent with previous reports as we showed a decrease in the number of follicles, decreased serum E2 levels, increased serum FSH levels and infertility.

MSCs features depend on both the tissue source from which they were obtained and the species. Previous studies indicated that MSCs obtained from various species and sources differ in their biological characteristics such as surface marker expression, proliferative capacity, multilineage differentiation potential and immunomodulation feature[62,63]. In this study, we investigated biological properties of ES-MSCs and BM-MSCs. We have found that ES-MSCs and BM-MSCs both expressed homing cell adhesion molecule, CD73, CD90 and endoglin, but they showed no expression of CD34, protein tyrosine phosphatase receptor type C and CD 11b, which is consistent with a previous study[31]. We indicated that ES-MSCs showed enhanced proliferation capacity compared to the BM-MSCs. On the fourth and fifth passages, there were significant differences between ES-MSCs and BM-MSCs. Previous studies have similarly reported that ES-MSCs are more proliferative compared to BM-MSCs[31,33].

In addition, we demonstrated that multilineage differentiation potential of BM-MSCs was greater than ES-MSCs. This finding was consistent with previous studies[31,33]. MSCs from different species and sources produce different cytokines. Our results were consistent with previous studies that cytokines secreted from MSCs could influence cell proliferation, differentiation, survival and tissue repair[64,65]. We observed no significant difference between ES-MSCs and BM-MSCs secreted cytokines in culture medium.

We transplanted human ES-MSCs into a mouse animal model and showed their capability in restoring ovarian function in POF. In support of transplantation of human derived MSCs to another species, previous studies have demonstrated that the transplantation of MSCs derived from various human tissues including menstrual blood, umbilical cord and amniotic fluid into animal models of POF restore ovarian function[66].

In this study, we transplanted ES-MSCs into a mouse model of POF to investigate the role of these cells and mechanisms of action for improvement of POF. Our results indicated that both ES-MSCs and BM-MSCs showed a similar trend for improvement of POF in this animal model. ES-MSCs improved ovarian structure and function in these mice as evidenced by the increased number of follicles, decreased granulosa cell apoptosis and restored FSH and E2 to near normal levels. E2 is mainly secreted by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Intact
Vehicle
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BM-MSC
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Caspase3
Vegf
Igf2
Amh
Gdf9
Anti-apoptotic
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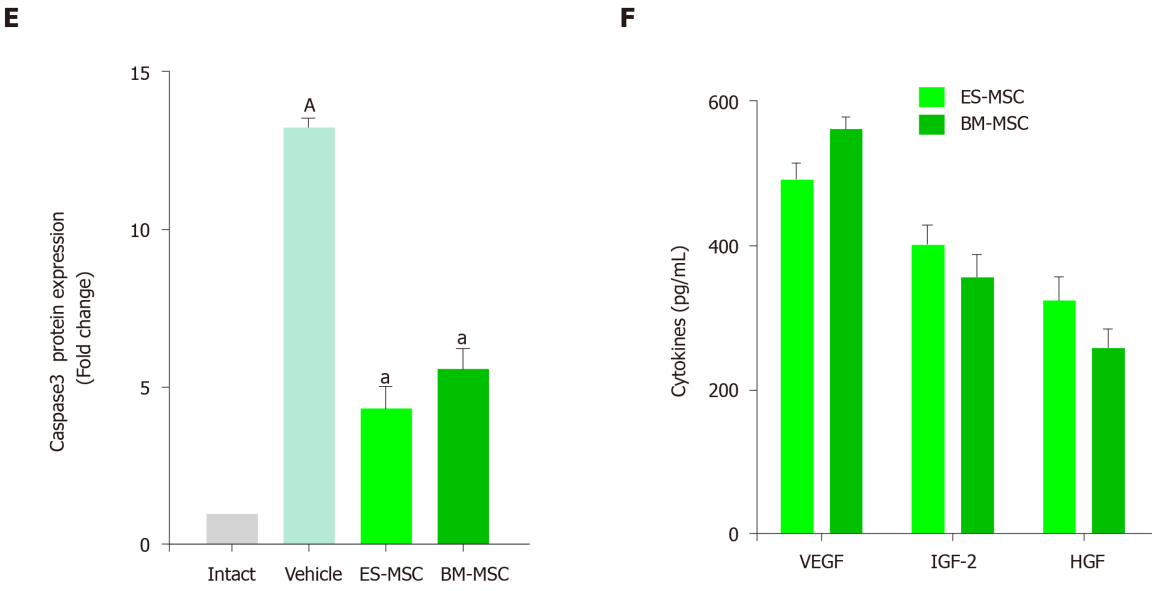


Figure 4 Human embryonic stem cell-derived mesenchymal stem cells and/or bone marrow-derived mesenchymal stem cells transplantation improved premature ovarian failure conditions. A and B: Apoptosis was reduced after both cell transplantations. The green stain color indicates terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate nick end labelling-positive cells. Data at 4 wk showed decreased levels of apoptosis following transplantation of embryonic stem cell-derived mesenchymal stem cells (ES-MSCs) and/or bone marrow-derived mesenchymal stem cells (BM-MSCs); C: Gene expression analysis showed that the expressions of B-cell lymphoma 2, vascular endothelial growth factor, insulin-like growth factor 2, anti-Müllerian hormone and growth/differentiation factor 9 significantly increased in both cell transplanted groups compared with the vehicle group, whereas cysteine-aspartic proteases 3 (*caspase 3)* significantly decreased in both cell transplanted groups compared to the vehicle group; D, E: Western blot analysis for cleaved-caspase 3 expression in the ovarian tissue. The results showed that cleaved-caspase 3 in ovarian tissue of the vehicle group significantly increased compared to the intact group. Cleaved-caspase 3 protein expression levels decreased in the ovaries from the ES-MSCs and BM-MSCs transplantation groups compared with the vehicle group; F: ELISA assessment of conditioned media of ES-MSCs and BM-MSCs for vascular endothelial growth factor, insulin-like growth factor 2 and hepatocyte growth factor. The results showed that ES-MSCs and BM-MSCs secreted vascular endothelial growth factor, insulin-like growth factor 2,and hepatocyte growth factor *in vitro*. All data are presented as mean ± standard error. Small letters (a, c) indicate the significance ($P < 0.05$) compared to groups labeled by similar capital letters (A, C), $^{a}P < 0.05$, significance of experimental groups *vs* the intact group. $^{c}P < 0.05$, significance of ES-MSC and BM-MSC groups *vs* the vehicle group. For Figure 4C: $^{a}P < 0.05$ significance of ES-MSC and BM-MSC groups *vs* the vehicle group, $n = 3$. ES-MSCs: Embryonic stem cell-derived mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; POF: Premature ovarian failure; Bcl-2: B-cell lymphoma 2; Caspase 3: Cysteine-aspartic proteases 3; Vegf: Vascular endothelial growth factor; Igf-2: Insulin-like growth factor 2; Amh: Anti-Müllerian hormone; Gdf9: Growth/differentiation factor 9.

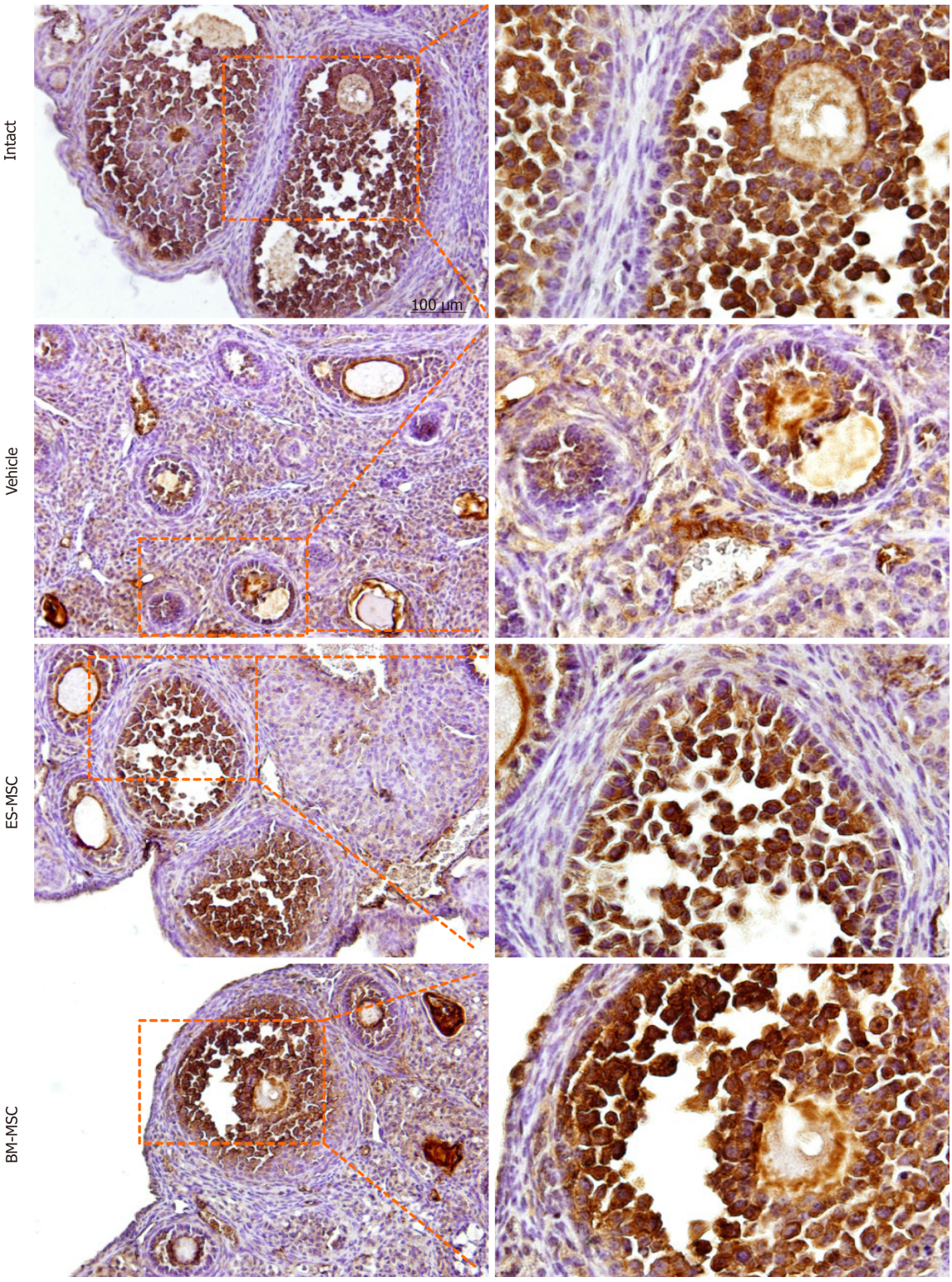
granulosa cells, which inhibit FSH secretion. Increased FSH levels could accelerate recruitment of follicles and deplete the follicular pool[14,67,68]. Increased apoptosis in the POF group might result in deceased E2 and FSH levels. Transplantation of ES-MSCs inhibited granulosa cell apoptosis and increased E2 secretion, which led to decreases in FSH; therefore, the decreased level of FSH in the ES-MSCs transplantation group resulted in an increased number of follicles.

Gene expression and Western blot results reinforced our hypothesis. There were significant increases in *Amh* expression (granulosa cell marker) and no difference in *Gdf9* expression (oocyte marker). Expression of *Bcl2* was upregulated in both of the MSC transplantation groups compared with the POF group, whereas *caspase 3* expression was downregulated in both of these groups compared with the POF group.

Immunohistochemistry results agreed with the real-time PCR results and indicated that MSCs could increase Amh expression compared with the POF mouse group. Folliculogenesis is mainly affected by interactions between the oocyte and granulosa cells[69]. Amh is expressed by granulosa cells and plays an important role in follicle growth[70,71]. We observed decreased apoptosis of granulosa cells after transplantation of the MSCs; therefore, ES-MSCs maintained the follicular niche by inhibiting apoptosis of granulosa cells. Previous findings of granulosa cell function in supporting oocytes also confirmed our results[72]. Therefore, granulosa cells support oocytes during development from the primordial state to maturation.

We observed that the ES-MSCs secreted VEGF, IGF-2 and HGF *in vitro*. VEGF, IGF-2 and HGF have an important role in inhibiting granulosa cell apoptosis, stimulating granulosa cell proliferation, inducing angiogenesis and follicle growth. VEGF promotes granulosa and endothelial cell proliferation. IGF-2 and HGF play an important role in suppressing apoptosis of granulosa cells that promote follicle maturation[73-77]. We observed decreased mRNA and protein expression of caspase 3 in both of the MSC transplantation groups compared with the POF group. The caspase

A



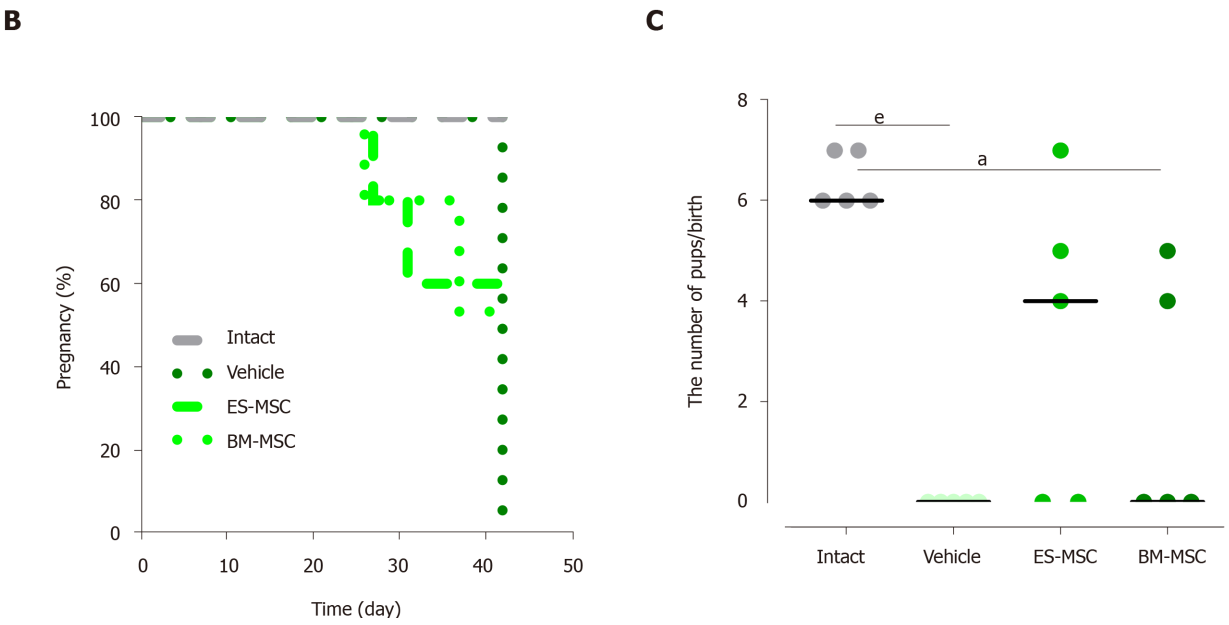


Figure 5 Human embryonic stem cell-derived mesenchymal stem cells and bone marrow-derived mesenchymal stem cells transplantation prevented follicular atresia and restored fertility in premature ovarian failure mice. A: Immunohistochemical staining showed increased anti-Müllerian hormone expression in the ovaries from the embryonic stem cell-derived mesenchymal stem cells (ES-MSCs) and bone marrow-derived mesenchymal stem cells (BM-MSCs) transplantation groups compared with the vehicle group; B, C: The percentage of pregnancies and the numbers of pups six weeks after both cell transplantations. The intact mice had a 100% (5 of 5) pregnancy rate, producing 32 live offspring. The embryonic stem cell-derived MSCs transplanted mice had a 60% (3 of 5) pregnancy rate, producing 16 live offspring. The bone marrow-derived MSCs transplanted mice had a 40% (2 of 5) pregnancy rate, producing nine live offspring. None of the premature ovarian failure mice became pregnant. Results are expressed as mean ± standard error, $^{a}P < 0.05$, $^{e}P < 0.001$, n = 3-5. ES-MSCs: Embryonic stem cell-derived mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells.

family members are important regulators of apoptosis. Caspase 3 is an effector caspase; its activation results in the final phase of cellular death[78]. Our study results suggested that ES-MSCs may have decreased caspase 3 expression in the ES-MSC transplantation group by releasing VEGF, HGF and IGF-2; therefore, these cytokines may inhibit apoptosis in granulosa cells by upregulation of *Bcl2* and downregulation of *caspase 3* in the ovaries of POF mice. Therefore, there was increased expression of the granulosa cell marker (Amh) after transplantation of the ES-MSCs. Our results might indicate that cytokines secreted by ES-MSCs reduce granulosa cell apoptosis and increase follicles by increasing E2 secretion.

We observed no significant difference in *Gdf9* expression in both MSC transplantation groups compared with POF. Gdf9 is an adult oocyte-specific marker[79,80], and this finding might indicate that transplantation of ES-MSCs restored ovarian function in POF mice *via* an indirect effect due to cytokine secretion rather than direct differentiation to oocytes.

These findings suggested that a possible mechanism by ES-MSCs and BM-MSCs restored the injured ovary by cytokine suppression of granulosa cell apoptosis and increased follicular growth. In line with our findings, the results from previous studies suggest that MSCs have an effect on restoring ovarian function by the paracrine mechanism of cytokines[81-85], which plays an important role in increased granulosa cell resistance to chemotherapeutic drugs and improves the ovarian microenvironment and follicle growth[58].

In conclusion, our results indicated that human ES-MSCs could restore ovarian structure and function in chemotherapy-induced POF mice and improve fertility. Transplantation of ES-MSCs improved the disturbed endocrine secretion system, reduced apoptosis rate in the ovaries, and improved folliculogenesis possibly through a paracrine effect and ovarian cell survival. Therefore, ES-MSCs could be a promising source for stem cell therapy in individuals with POF.

ARTICLE HIGHLIGHTS

Research background

Premature ovarian failure (POF) is characterized by amenorrhea, hypoestrogenemia, high gonadotropins and infertility in women under 40-years-old. Previous reports demonstrated that various tissue-specific stem cells could restore ovarian function and folliculogenesis in chemotherapy-induced POF mice.

Research motivation

Human embryonic stem cell-derived MSC (ES-MSC) have advantages, such as higher proliferation, more potent anti-inflammatory properties and lack of obstacles of harvesting tissue-specific MSCs that make them attractive candidates for restoring fertility in patients with POF.

Research objectives

The aim of this study was to evaluate the therapeutic efficacy of ES-MSCs in a model of chemotherapy-induced POF.

Research methods

In this study, we initially established a mouse POF model by administration of cyclophosphamide and busulfan, then we transplanted ES-MSCs and bone marrow-derived MSC (BM-MSC) into a mouse model of POF to investigate the role of these cells and mechanisms of action for improvement of POF.

Research results

The POF model established by the 100 mg/kg dose of cyclophosphamide showed significant decreases in body weight, follicle count and estradiol level but had an increased follicle-stimulating hormone level. ES-MSC and/or BM-MSC transplantation significantly improved body weight, follicle count, hormone secretion, survival rate and reproductive function in POF mice. Gene expression and Western blot analysis, terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate nick end labelling assay and immunohistochemistry indicated that the ES-MSCs or BM-MSCs reduced apoptosis in the follicles and restored fertility in chemotherapy-induced POF mice. The results of this study indicated that the effects of ES-MSCs and BM-MSCs in restoring ovarian function appear *via* the paracrine mechanisms of cytokines.

Research conclusions

Our findings demonstrated that human ES-MSCs, similar to BM-MSCs, improved ovarian function and restored fertility in a mouse POF model.

Research perspectives

Our present study results suggest that human ES-MSCs could be a promising source for stem cell therapy in individuals with POF.

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