Restoring Ovarian Function With Human Placenta-Derived Mesenchymal Stem Cells in Autoimmune-Induced Premature Ovarian Failure Mice Mediated by Treg Cells and Associated Cytokines

Na Yin¹, Wei Zhao¹, Qianqian Luo², Wendan Yuan³, Xiying Luan⁴, and Hongqin Zhang¹,⁵

Abstract
Regulatory T (Treg) cells play a key role in the regulation of autoimmunity and transplantation. Human placenta-derived mesenchymal stem cell (hPMSC) transplantation has a potential to restore ovarian dysfunction associated with premature ovarian failure (POF), while the exact function of the Treg cells in the transplantation still needs to be further investigated. In this study, hPMSCs were intravenously injected into POF mice following zona pellucida glycoprotein 3 (pZP3) treatment. Ovarian function was measured by analyzing estrous cycle, folliculogenesis, and hormone secretion, also, with the detection of apoptotic granular cells (GCs) in ovarian tissues. To determine whether immune response is involved in the regulation of ovarian function change, the population of Treg cell populations and expression of associated cytokines, for example, transforming growth factor β (TGF-β) and interferon γ (IFN-γ) were measured. After hPMSCs transplantation, the injured ovarian function is significantly improved. Also, the pZP3-treatment-induced apoptotic GCs were significantly decreased as compared with the POF mice. The transplantation of hPMSCs significantly increased the population of Treg cells which was inhibited by pZP3 treatment. The decrease in TGF-β and increase in IFN-γ in serum caused by pZP3 treatment have been reversed following hPMSCs transplantation. These findings strongly suggest that the recovery of ovarian function in POF mice is mediated via the regulation of Treg cells and production of associated cytokines following hPMSCs transplantation.

Keywords
pZP3, premature ovarian failure, human placenta-derived mesenchymal stem cell transplantation, regulatory T cells, cytokine, apoptosis

Introduction
Premature ovarian failure (POF) is a condition of the gonadal failure characterized by amenorrhea, hypoestrogenemia and hypergonadotropinemia. It is a heterogeneous disorder prevalent in 1% to 3% of women before the age of 40.¹² Classically, ovarian failure can be due to genetic, autoimmune, and environmental causes. In most cases, however, no precise cause can be identified, and these forms are referred to as idiopathic.³ Although the exact causes of POF remain unknown, the autoimmune mechanisms may play a role in approximately 4% to 30% of women with POF disorder.⁴ The immunopathology of autoimmune ovarian disease (AOD) is mostly composed of oophoritis, ovarian atrophy, and serum autoantibodies to ovarian antigens.³ These changes may be related to the complex clinical symptoms in patients diagnosed with POF. The ovarian dysfunction induced by autoimmune mechanism may be related to zona pellucida (ZP) antigens, such as ZP3. The ZP3 is an acellular matrix surrounding the developing and

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ovulated oocytes, and it serves as the major sperm receptor in fertilization. Antibodies to ZP antigen (Anti-ZP antibodies [AZP Abs]) may interfere with normal follicular development, leading to follicular depletion and amenorrhea. Studies found that the presence of ZP3 and AZPAb in serum is related to alterations in ovarian function and cause infertility by interfering with the sperm–oocyte interaction in women. Therefore, in this study we established the POF model by injecting ZP-glycoprotein 3 (pZP3) in mice to investigate a new therapy for recovering the autoimmune injured ovarian function.

Recently, stem cell therapy has been developed to treat or prevent some diseases such as diabetes, heart disease, and other conditions. The human placenta-derived mesenchymal stem cells (hPMSCs) are multipotent and nonhematopoietic progenitor cells with high differentiation and proliferation potential. The phenotype and characteristic of hPMSCs are considered to have great advantages over the MSCs isolated from other sources. In our study, it is demonstrated that the human placenta is a good source to isolate the MSCs and sufficient cells can be harvested for experimental use. Also, the isolated hPMSCs can be successfully frozen/thawed, clonally expanded in culture, and engineered to express exogenous proteins.

Although the exact mechanism of hPMSCs to protect the injured ovarian tissues is still unclear, more studies have implied that the protection may result from its inhibition on stromal cells apoptosis. It is mediated by promoting the differentiation of regulatory T cells (Treg cells, often referred to as CD4+CD25+Foxp3+T), which can produce immunomodulatory cytokines such as transforming growth factor β (TGF-β) to inhibit the immune response. The TGF-β is one of the most important growth factors that affect angiogenesis, matrix remodeling, and vessel stabilization in the mouse ovary, and it is essential for follicular development and antrum formation.

It is found that TGF-β are highly expressed in granular cells (GCs) of the preantral and small antral follicles in the ovary and have the ability to repair the damaged ovarian function. The TGF-β can inhibit the production of interferon γ (IFN-γ), which is considered an archetype inflammatory mediator. A temporary increase in the concentration of IFN-γ is found to be an essential factor for ovulation; however, IFN-γ levels that exceed normal physiologic concentrations may inhibit ovulation. These studies suggest that IFN-γ may play an important role in the development and progress of POF disorder.

In the current study, we established the POF animal model in mice by injection of pZP3 to produce autoimmune injury to ovarian and investigated the role of hPMSCs transplantation in recovering the damaged ovarian function and its mechanism. The proliferation of Treg cells and production of cytokines (eg, IFN-γ and TGF-β) are investigated on this animal model to determine whether the release of cytokines from Treg cells mediates the recovery of ovarian function following hPMSCs transplantation. The results show that the hPMSCs transplantation promotes the recovery of injured ovarian function, providing a therapeutic potential for using stem cells of hPMSCs to treat POF disorder, in the future.

Materials and Methods

Animals

Female mice (Balb/c) 7 to 8 weeks of age were obtained from Jinan Pengyue experimental animal breeding Co, Ltd (Shandong, China). All the animals were housed in animal facility and were fed a standard pellet diet with free access to water. All the experimental procedures have been approved by the Institutional Animal Care and Use Committee at the Binzhou Medical University, and the study was conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals.

The ZP3 Peptide

The ZP3 peptide was synthesized by an automatic peptide synthesizer (Hangzhou Economic & Technological Development Zone, China), and 91.5% in peptide purity as determined by high-performance liquid chromatography (HPLC) analysis. The amino acid composition was determined by amino acid analysis and the amino acid sequence of the murine ZP3330–342 peptides used in this study was NSSSSQFQIHGPR.

Isolation and Culture of HPMSCs

Human placentas were collected from pregnant women who were negative for HIV-I, hepatitis B, and hepatitis C based on the written and informed consent obtained from the study participants. The use of human tissue was approved by the institutional ethics committee. The placentas were carefully dissected, washed with phosphate-buffered saline (PBS), mechanically minced, and digested with 0.1% collagenase IV (Gibco, USA) for 30 minutes at 37°C. The 100-mm nylon membranes were used to remove undigested tissue fragments. Cells were collected and then centrifuged at 524g for 10 minutes to remove the harvest buffer. The isolated cells were resuspended in low-glucose Dulbecco modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL streptomycin sulfate, and 100 U/mL penicillin G and were cultured at 37°C in a humidified atmosphere with 5% CO₂. Cell morphology were observed under light microscope, the membrane and intracytoplasmic molecular markers of hPMSCs were examined using flow cytometry (FCM) to confirm the phenotype of hPMSCs. Following staining the cells with specific, hPMSCs surface molecule antibody with phycoerythrin-conjugated or fluorescein isothiocyanate-conjugated mouse antihuman CD29, CD44, CD105, CD166, CD34, CD45 and CD14 mAb (BD Biosciences and Invitrogen), the cells were sorted using cytometry and harvested for culture.

The POF Mice Model

To establish autoimmune-induced POF model in mice, 80 mice were randomly divided into 2 groups: control group (n = 16) and pZP3-treated group (n = 64). The PZP3-treated group was
first injected subcutaneously with 50 nmol/L of pZP3 (mouse) emulsified in complete Freund adjuvant (*Mycobacterium tuberculosis* H37RA strain, 0.16 mg/mouse; Sigma, USA) and then subcutaneously with 50 nmol/L of pZP3 (mouse) emulsified in Freund incomplete adjuvant (FIA; *Mycobacterium tuberculosis* H37RA strain, 0.16 mg/mouse; Sigma) 2 weeks later. The control group mice did not receive any treatment. One week following the treatment of pZP3 with FIA, the autoimmune response was confirmed by the presence of AZPAb. In the treatment group, the mice with AZPAb (+) were randomly divided into 3 groups: POF alone, POF + hPMSCs transplantation, and POF + PBS vehicle control. The cell suspension containing 1 × 10^6 hPMSCs of sixth passages were injected into the POF + hPMSCs group mice according to the previous studies.17–19 For comparison, the same volume of PBS was injected into the POF mice as a vehicle control.

**Measurement of AZPAb**

One week following pZP3 with FIA immunization, blood samples were collected by tail vein puncture. The AZPAb was measured by enzyme-linked immunosorbent assay (ELISA) kits (Lengton, China), according to manufacturer’s instructions, to confirm the establishment of POF animal model. In control group, the expression of AZPAb was negative. In the pZP3 immunization group, the animals with positive expression of AZPAb were selected for further experiment use.

**Examination of Estrous Cycle**

Stages of the estrous cycle were determined by examining vaginal cytology. Vaginal cells washed used saline were transferred to a glass slide, which was air-dried, fixed in methanol, and examined for histopathologic understanding (H&E). The estrous cycle was determined according to the proportions of leukocytes, nucleated epithelial cells and cornified squamous epithelial cells.

**Ovarian Follicle Counting and Morphological Analysis**

Two weeks after the hPMSC transplantation, animals were euthanized. Ovaries were collected and the tissues were fixed and stained with H&E for histopathology. The ovarian histological examination was performed using light microscopy (Olympus, Japan). The follicles were counted only on those containing an oocyte with a clearly visible nucleus. The follicles were detected and classified as primordial, primary, secondary, and atretic, according to the previously described method.20

**Immunohistochemical Staining of Caspase 3**

The cleaved caspase 3 was examined immunohistochemically using a commercially available antibody of the ovarian tissues. The staining procedure was performed using immunoperoxidase technique with paraffin sections that were prepared on adhesion microscope slides (Citoglas, China). The sections were incubated with rabbit primary polyclonal antibodies against mouse cleaved caspase 3 at 4°C overnight (1:150 dilution; Abnova, China), followed by incubation with biotinylated secondary antibodies at 37°C for 30 minutes. The reaction products were developed with diaminobenzidine as chromogen and counterstained with hematoxylin.

**Measurement of Serum hormones (Estradiol and Follicle-Stimulating Hormone) and Cytokines (IFN-γ and TGF-β)**

At the end of study, blood samples were obtained from postcava and centrifuged at 4000 r/min for 10 minutes. The serum levels of estradiol (E2) and follicle-stimulating hormone (FSH) and IFN-γ and TGF-β concentration were measured by ELISA kits (Lengton), according to manufacturer’s instructions.

**Flow Cytometry**

It is found that depletion of CD4⁺CD25⁺T cells in mice can induce autoimmune diseases, and their removal from normal naive mice can prevent autoimmune disease.21 To determine the percentage of CD4⁺CD25⁺Foxp3⁺T-cell populations in the mice, the FCM analysis was performed on isolated spleen cells using anti-mouse CD3, CD4, CD25, and Foxp3 monoclonal antibodies. Erythrocytes from the spleens were lysed in ammonium chloride buffer and the remaining living cells were washed and resuspended in PBS. Cells were incubated with Fc-block for 5 minutes at room temperature and then in a mixture of anti-mouse CD3 Allophycocyanin (APC), antimouse CD4 fluorescein isothiocyanate (FITC), antimouse CD25 peridinin-chlorophyll proteins-Cyanine5.5 (Percp-cy5.5) (eBioscience, San Diego, USA) at 4°C for 30 minutes in the dark. After cell members were ruptured, antimouse Forkhead box protein3 phycoerythrin (Foxp3 PE) (eBioscience) was added to the cell suspension and analyzed using FCM (FACSVantage diva, USA).

**Statistical Analyses**

Data were analyzed using SPSS 16.0 statistic software. The difference in AZPAb expression was analyzed by chi-square test. The other data difference among groups were compared by 1-way analysis of variance followed by least significant difference multiple comparison test, student t test. P value of <.05 was considered statistically significant.

**Results**

**Characterization of HPMSC Phenotype**

It is observed that cells isolated from human placenta began to form individual clone spheres after 7 to 10 days of inoculation, the morphology of cells appears similar to the fibroblasts as shown in Figure 1B. These adherent cells can be easily expanded in vitro by multiple trypsinization. A homogenous cell population was observed after 3 passages. Even after 10 passages, no visible morphologic changes were observed. The immunophenotyping analysis showed positive expression mesenchymal progenitors markers with CD73, CD90, and
The study conducted in animals has demonstrated that the ZP antibodies interfere with follicular development, and the presence of these antibodies can lead to follicular depletion and amenorrhea. In this study, the AZPAb was analyzed 1 week after pZP3 with FIA immunization. As shown in Table 1, mice in control group with AZPAb(+) accounted for approximately 12.50%. The number of mice with AZPAb(+) was significantly increased in pZP3-immunized group when compared to the control group (P < .01). Mice with AZPAb(−) in control group (n = 16) and with AZPAb(+) in pZP3 treatment group (n = 64) were selected to conduct the subsequent experiment.

Detection of AZPAb Following pZP3 Treatment

The study conducted in animals has demonstrated that the ZP antibodies interfere with follicular development, and the presence of these antibodies can lead to follicular depletion and amenorrhea. In this study, the AZPAb was analyzed 1 week after pZP3 with FIA immunization. As shown in Table 1, mice in control group with AZPAb(+) accounted for approximately 12.50%, while in pZP3 treatment group it was 81.25%. The number of mice with AZPAb(+) was significantly increased in pZP3-immunized group when compared to the control group (P < .01). Mice with AZPAb(−) in control group (n = 16) and with AZPAb(+) in pZP3 treatment group (n = 64) were selected to conduct the subsequent experiment.

Table 1. The Percentage of Mice With AZPAb(+) Before Cell Transplantation. *

<table>
<thead>
<tr>
<th>Group</th>
<th>AZPAb (+)</th>
<th>AZPAb (−)</th>
<th>Count</th>
<th>Positive rate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>14</td>
<td>16</td>
<td>12.50</td>
</tr>
<tr>
<td>pZP3 treatment</td>
<td>52</td>
<td>12</td>
<td>64</td>
<td>81.25</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>26</td>
<td>80</td>
<td>67.5</td>
</tr>
</tbody>
</table>

Abbreviations: AZPAb, anti–zona pellucida antibodies; pZP3, zona pellucida glycoprotein 3.
*Data are expressed as number of mice with AZPAb (−) or (+) in each group.

The HPMSC Transplantation Improves Estrous Cycles in pZP3-Induced POF Mice

Following pZP3 treatment, the estrous cycles of mice in each group were examined over a 5-week period. Normal female mice have regular estrous cycles with a duration of 4 to 6 days including proestrus for 1 day, estrus for 1 to 2 days, metestrus for 1 day, and diestrus for 1 to 2 days. However, the irregular patterns of estrous cycle in mice administered with pZP3 were observed as shown in Figure 2A. The degree of cycle abnormality (I-IV) was graded as follows: I, normal; II, regular cycles with a shortened estrus; III, irregular cycles with a prolonged diestrus and normal or prolonged estrus; and IV, no cyclicity.

Normal estrous cycles were maintained in 87.5% of mice in control group. In comparison, only 19.0% of the mice showed the regular cycle in pZP3 treatment group (Figure 2B). The numbers of mice with normal cycles in treatment group were significantly lower compared to the control group (P < .01). These results indicate that the treatment with pZP3 causes ovarian function injury, which indicates the successful establishment of POF animal model. Next, after the transplantation of hPMSCs in pZP3-induced POF mice for 2 weeks, the results show that the irregularity of estrous cycles is significantly improved in all types of abnormality as demonstrated in Figure 2B. The number of mice with regular estrous cycle (72.72%) was significantly higher as compared to the POF + PBS vehicle group without hPMSC transplantation.

Histological Examination on Ovarian Tissues Following hPMSCs Transplantation

Histological examination was conducted in all groups of mice to evaluate the ovarian tissue effects (Figure 3A-D), it was observed that the ovaries of healthy control group mice contain a large number of healthy follicles at all stages, including primordial follicles (Figure 3A-a), primary follicles (Figure 3A-b), secondary follicles (Figure 3A-c), and atretic follicles (Figure 3A-d) as indicated in Figure 3. In contrast, the ovaries of pZP3 immunization mice showed the atrophied ovaries which were mostly composed of interstitial cells in a fibrous matrix, with a reduced number of follicles at each stage of development (Figure 3B). Also, fewer follicles were found in POF mice than control group, especially for antral follicles (Figure 3I). In...
comparison, in the ovaries of mice that received hPMSCs transplantation, the number of primary follicles (12.80 ± 4.21) and secondary follicles (6.13 ± 2.17) were significantly increased compared to the POF group without transplantation (4.63 ± 0.92 and 3.80 ± 0.84, respectively, \( P < .05 \)). In addition, many blood vessels were observed in the ovaries of mice following the hPMSC transplantation, this blood vessel increase might result from the potential angiogenesis and inhibition of apoptosis effects induced by stem cell therapy.\(^{24} \) The enriched blood flow may contribute to the recovery of ovary function.

The HPMSC Transplantation Inhibits Ovarian GCs Apoptosis in pZP3-Induced POF Mice

To determine whether the ovarian GCs apoptosis is involved in the pZP3-induced ovarian injury, the cell apoptosis in ovaries was investigated by the evaluation on caspase 3 expression using immunohistochemical analysis. An increased expression of caspase 3 was observed in ovaries of pZP3-induced POF mice, the positive expression mainly occurs in the secondary follicles. In comparison, the ovaries from POF + hPMSCs group mice show mostly healthy follicles and less expression of caspase 3 activation (Figure 3E-H). These data indicate that the GC apoptosis mediates the pZP3-induced ovarian function failure and the transplantation of hPMSCs can significantly inhibit the GCs apoptosis and improve the injured ovarian function in POF mice.

The HPMSC Transplantation Increases Hormone Secretion in pZP3-Induced POF Mice

The effects of hPMSCs transplantation on the E\(_2\) and FSH hormone secretion of mice were investigated. The results in Table 2 show that the serum levels of E\(_2\) in POF group (32.88 ± 5.47 pg/mL) were significantly decreased compared to the control group (47.15 ± 4.56 pg/mL; \( P < .01 \)). Also, higher levels of FSH (8.03 ± 2.20 mIU/mL) were observed in POF mice when compared with the control group (4.59 ± 0.33 mIU/mL; \( P < .01 \)). Following the hPMSCs transplantation for 2 weeks, it was observed that the serum levels of E\(_2\) were significantly elevated and FSH secretion went down. Based upon the results from endocrine hormone changes, it suggests that the hPMSC transplantation promotes the recovery of injured ovarian function in pZP3-induced POF mice.

Effects of hPMSC Transplantation on Serum Levels of IFN-\(\gamma\) and TGF-\(\beta\) in pZP3-Induced POF Mice

Cytokines of IFN-\(\gamma\) and TGF-\(\beta\) play an important role in the regulation of immune response. To determine whether these cytokines are involved in the regulation of ovarian recovery induced by autoimmunization of pZP3, the serum levels of TGF-\(\beta\) and INF-\(\gamma\) were measured. Interestingly, it is found that the proinflammatory IFN-\(\gamma\) production is significantly higher in POF mice compared to control group as shown in Table 2. Following the hPMSC transplantation, the IFN-\(\gamma\) production induced by pZP3 was inhibited and the levels went down but still slightly higher than control group. In comparison, the anti-inflammatory TGF-\(\beta\) production was significantly decreased in pZP3-induced POF mice compared to the control group. After hPMSC transplantation, the decreased TGF-\(\beta\) levels were elevated but have not reached the levels in the control group. These data suggest that the pZP3 treatment affects the immune regulation that...
may cause the ovarian injury. The effects on cytokine release by increasing anti-inflammatory cytokine release and inhibiting proinflammatory production play an important role during the ovarian function recovery following the hPMSCs transplantation.

Table 2. Serum Levels of Hormone (E2 and FSH) and the Cytokines (IFN-γ and TGF-β) in the Studied Groups. a

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control, n = 14</th>
<th>POF, n = 17</th>
<th>POF + hPMSCs, n = 17</th>
<th>POF + PBS, n = 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH, mIU/mL</td>
<td>4.59 (0.33) b</td>
<td>8.03 (2.20) c</td>
<td>6.28 (0.49) d e</td>
<td>7.99 (0.48) c</td>
</tr>
<tr>
<td>E2, pg/mL</td>
<td>47.15 (5.66) b</td>
<td>32.88 (5.47) c</td>
<td>40.40 (3.50) d e</td>
<td>32.31 (4.98) c</td>
</tr>
<tr>
<td>IFN-γ, pg/mL</td>
<td>169.08 (4.00) b</td>
<td>216.43 (2.13) c</td>
<td>193.02 (9.67) d</td>
<td>219.38 (3.70) c</td>
</tr>
<tr>
<td>TGF-β, pg/mL</td>
<td>337.33 (6.83) b</td>
<td>254.75 (1.02) c</td>
<td>293.23 (2.23) d</td>
<td>261.70 (1.25) c</td>
</tr>
</tbody>
</table>

Abbreviations: E2, estradiol; FSH, follicle-stimulating hormone; hPMSCs, human placenta-derived mesenchymal stem cells; IFN, interferon; PBS, phosphate buffered saline; POF, premature ovarian failure; SD, standard deviation; TGF, transforming growth factor.

The data are presented as the mean ± SD. b P < .01.

c P < .05 when compared with POF group.

d P < .05 when compared with control group.

To determine whether Treg cells is involved in the recovery of ovarian function induced by hPMSC transplantation in

**Figure 3.** Histopathological examination on ovary tissues after hPMSC transplantation in POF mice (A-D) and effects of hPMSC transplantation on caspase 3 activation in pZP3-induced POF mice (E-H). Photomicrographs (×100) show hematoxylin and eosin stained ovaries from (A) control group. The following different types of ovarian follicles were observed: primordial follicle (a), primary follicle (b), secondary follicle (c), atretic follicle (d). Bar scale, 200 μm. (B) POF group. (C) POF + hPMSCs group. (D) POF + PBS group. (I) Quantitation on follicle count from ovaries in mice with or without hPMSC transplantation. Data are presented as mean ± SD. *P < .05, **P < .01 indicate the statistically significant difference between the groups with and without hPMSC transplantation. Photomicrographs (×400) show hematoxylin and DAB-stained ovaries from (E) control group. (F) POF group. (G) POF group with hPMSC transplantation. (H) POF group with PBS vehicle group. Brown in cytoplasm indicates the positive expression of caspase 3. Blue represents the cell nuclear staining. Bar scale = 50 μm. DAB indicates diaminobenzidine; hPMSCs, human placenta-derived mesenchymal stem cells; PBS, phosphate-buffered saline; POF, premature ovarian failure; pZP3, zona pellucida glycoprotein 3; SD, standard deviation.
pZP3-induced POF mice, T cells were harvested from spleens and CD25<sup>+</sup>Foxp3<sup>+</sup>/CD4<sup>+</sup> T cells were isolated and sorted by FCM. As shown in Figure 4, the percentages of CD25<sup>+</sup>Foxp3<sup>+</sup>/CD4<sup>+</sup>T significantly decreased in spleens of mice with pZP3-induced POF group compared to the control group (P < .01). Following hPMSC transplantation, the numbers of CD4<sup>+</sup> T cells with CD25<sup>+</sup> in POF mice were significantly elevated (P < .05). These data suggest that the immune regulation such as Treg cell population change might be involved in the recovery of damaged ovarian function which is caused by pZP3 autoimmunization.

**Discussion**

Stem cell transplantation has the promising potential for repairing injured tissue. It has been reported that the stem cell therapy can serve as a powerful tool in restoring fertility and pregnancy. Various types of stem cells have been investigated in POF treatment, such as adipose-derived stem cells (ADSCs), human umbilical cord blood mesenchymal stem cells (HCB-MSCs), ADSCs, and human endometrial mesenchymal stem cells (EnSCs). However, most of these studies using stem cell therapy were conducted to evaluate the ovarian recovery in mice with chemotherapy-drug-induced POF. For the POF mice induced by autoimmunization injury, it is unclear whether stem cell transplantation has the potential to recover the injured ovarian function. Therefore, the goal of our study was to determine whether MSCs isolated from human placenta can recover the pZP3-induced ovarian function injury. Furthermore, we have investigated its possible mechanism to restore the damaged ovaries.

First, we established the POF model in mice using pZP3 treatment to cause autoimmunization injury to the ovary. Autoantibody is known to induce de novo pathogenic autoimmune T-cell response, leading to severe organ-specific disease with ablation of organ function. Therefore, the positive detection of AZPAb is used in this study as a marker to demonstrate the successful establishment of POF animal model following pZP3 treatment. Through the evaluation on the expression of AZPAb, estrous cycle, ovary histopathology examination, it is demonstrated that the ovarian function is significantly damaged and the POF model is successfully established through this approach. In addition to the expression of AZPAb, the observed GC apoptosis, significant loss of healthy follicles, and disordered hormone secretion further support the ovarian function failure in the established animal model. In ovary tissues, the histological examination revealed an apparent shrinking of the ovaries with the destruction of most cortical follicles and a significant reduction in the antral follicle count (AFC). Overall,
these findings demonstrate the negative effects of pZP3 administration on the ovarian function, which are consistent with those described in the literature report.33,34 Following hPMSC transplantation for 2 weeks, it is observed that the ovarian function in POF mice is greatly improved, which includes the higher incidence of regular estrous cycle, increased healthy follicles numbers, significant elevation on E2 production, and reduced release of FSH. The decreased FSH release from the pituitary gland is a feedback due to the elevation in E2 production based upon to the pituitary–gonadal axis endocrine communication.35 These data suggest the ovarian endocrine function recovery following hPMSC transplantation. In addition to the hormone changes, the ovary histopathology examination also showed increased blood flow and less apoptosis of GCs in hPMSC transplant mice. The data from ovarian GC apoptosis showed that the apoptosis of GCs mainly occurred in the large secondary and antral follicles with no many effects on follicles in the other developmental stages. As reported, the ovarian GCs are the most important stromal cells in ovary, which surround oocyte and play a key role in folliculogenesis.36 These data indicate that the inhibition of GC apoptosis by the transplanted hPMSCs may contribute to the recovery of ovarian function and promote its folliculogenesis in POF mice. Overall, the hPMSC transplantation may alter ovarian local tissue response and its secretion of endocrine hormones during the recovery of ovarian function in POF mice.

It has been reported that the hPMSCs transplantation increased secretion of growth factors, angiogenic factors, pleiotropic cytokines, chemotactic cytokines, and extracellular matrix proteins.37 The follicular growth is systemically regulated by hormones and intraovarian regulation with cytokines, growth factors, and intracellular proteins.38 All these factors might contribute to the recovery of ovarian function mediated by hPMSCs transplantation in our experiment, but this needs to be further investigated in the future. Since there are limited reports to investigate the possible effects of immunomodulating therapy on the recovery of autoimmune-induced ovarian injury in POF disorders. In the current study, we focused on the impact of hPMSC treatment on inflammatory regulation caused by pZP3 autoimmune damage on the ovarian function. As reported, the process of follicle atresia can be initiated by the cytokine of IFN-γ, which stimulated the expression of major histocompatibility complex I (MHC I) and MHC II in the GCs, leading to the destruction of follicles.39 The IFN-γ, as well as other cytokines, plays an important role as a regulator of activating self-reactive inflammation cells that are involved in the autoimmune inflammatory response. The higher levels of serum IFN-γ detected in our study may result from the abnormal expression of CD4+T cells in POF mice. In addition, it has been reported that MSCs could promote the differentiation of Treg cells, which plays a vital role in the immunosuppression of MSCs.40 Treg cells are known to mediate the suppression of the immune response produced by immunomodulatory cytokines such as TGF-β. The TGF-β suppresses immune responses through at least 2 ways: inhibiting the function of inflammatory cells and promoting the proliferation of Treg cells. The inhibited production of TGF-β was detected in POF mice serum. Following hPMSCs transplantation, the decreased TGF-β production was reversed in POF mice. The upregulation of TGF-β might be partly due to the ability of hPMSCs to secrete this growth factor,41 or the response to the perturbations of the extracellular matrix in various situations of mechanical stress, such as tissue injury, and inflammation.42 The paracrine stimulatory effect of TGF-β in the early stages of folliculogenesis and in the regulation of the primordial follicle pool has been reported.43 Taken together, our data show that the Treg cell populations and associated cytokines are involved in the process of pZP3-induced ovarian injury and the subsequent ovarian function recovery following hPMSCs transplantation. The exact mechanisms of how the Treg cells and cytokines regulate the recovery of ovarian function following hPMSC transplantation need to be further investigated in the future study.

Conclusions
In summary, we have shown that transplantation of hPMSCs leads to recovery of injured ovarian function induced by pZP3 immunization in mice. The restoring of ovarian function is associated with the increased CD25+CD4+Treg cell populations and inflammatory regulations mediated by IFN-γ and TGF-β cytokines. These findings suggest that transplantation of hPMSCs could serve as a promising and effective therapy approach to treat POF-related diseases.

Author’s Contribution
Na Yin and Wei Zhao contributed equally to the work.

Declaration of Conflicting Interests
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References


