

RESEARCH

Open Access



# Menstrual blood derived mesenchymal stem cells combined with Bushen Tiaochong recipe improved chemotherapy-induced premature ovarian failure in mice by inhibiting GADD45b expression in the cell cycle pathway

Fengyi Guo<sup>1†</sup>, Tian Xia<sup>2†</sup>, Yedan Zhang<sup>1†</sup>, Xiaotong Ma<sup>1†</sup>, Zhongrui Yan<sup>1†</sup>, Shaohua Hao<sup>1</sup>, Yali Han<sup>1</sup>, Ruihong Ma<sup>2</sup>, Yuan Zhou<sup>3</sup> and Xue Du<sup>1\*</sup>

## Abstract

**Background:** To investigate the therapeutic effects of menstrual blood derived mesenchymal stem cells (MB-MSCs) combined with Bushen Tiaochong recipe (BSTCR) on epirubicin induced premature ovarian failure (POF) in mice.

**Methods:** Twenty-four female C57BL/6 mice of 6–8 weeks were intraperitoneally injected with epirubicin to induce POF, and then they were randomized into 4 groups of 6 mice each and treated with PBS, MB-MSCs, BSTCR, and MB-MSCs combined with BSTCR, respectively. Six mice of the same age were used as controls. Vaginal smear, TUNEL and hematoxylin-eosin staining were to observe estrous cycles, ovarian cell apoptosis and follicles. Enzyme-linked immunosorbent analysis determined serum estradiol, follicle-stimulating hormone (FSH) and anti-Müllerian hormone (AMH) levels. RT-qPCR and Western Blot analysis were to determine GADD45b, CyclinB1, CDC2 and pCDC2 expressions.

**Results:** Epirubicin treatment resulted in a decrease in the number of primordial, primary, secondary and antral follicles, an increase in the number of atretic follicles and ovarian cell apoptosis, a decrease in estradiol and AMH levels, an increase in FSH levels, and estrous cycle arrest. However, MB-MSCs combined with BSTCR rescued epirubicin induced POF through down-regulating GADD45b and pCDC2 expressions, and up-regulating CyclinB1 and CDC2 expressions. The combined treatment showed better therapeutic efficacy than BSTCR or MB-MSCs alone.

**Conclusions:** MB-MSCs combined with BSTCR improved the ovarian function of epirubicin induced POF mice, which might be related to the inhibition of GADD45b expression and the promotion of CyclinB1 and CDC2 expressions. The combined treatment had better therapeutic efficacy than BSTCR or MB-MSCs alone.

**Keywords:** Mesenchymal stem cells, Menstrual blood, Bushen Tiaochong recipe, Premature ovarian failure, Epirubicin, GADD45

\* Correspondence: [lanlandetommao@163.com](mailto:lanlandetommao@163.com)

<sup>†</sup>Fengyi Guo, Tian Xia, Yedan Zhang, Xiaotong Ma and Zhongrui Yan contributed equally to this work.

<sup>1</sup>Department of Obstetrics & Gynecology, General Hospital, Tianjin Medical University, NO.154, Anshan Road, Heping District, Tianjin 300052, People's Republic of China

Full list of author information is available at the end of the article



## Background

Chemotherapy has the potential to increase the survival of patients with malignant tumors, but concerns have also been raised about chemotherapy-induced decrease in ovarian function in young patients. Cancer patients such as breast cancer, leukemia and lymphoma are usually diagnosed at young ages and the lifetime is prolonged after chemotherapy. However, amenorrhea and infertility caused by chemotherapy has gradually been paid attention to [1–3]. Anthracycline antibiotics such as doxorubicin and epirubicin are the first-line chemotherapeutic agents for the treatment of acute leukemia, lymphoma and breast cancer. Epirubicin is a cell cycle phase non-specific anthracycline, with maximal cytotoxic effects in the S and G2 phases, the mechanism of which as an antitumour drug forms a complex with DNA by intercalation between the DNA strands, thus inhibiting replication and transcription [4]. It also suppresses the activity of topoisomerase II and upregulates the level of reactive oxygen species (ROS) to kill cancer cells [5]. It was reported that anthracycline- and taxane-based chemotherapy was associated with a 40–60% risk of treatment-induced POF in breast cancer patients under the age of 40 years [6], but the mechanism of ovarian toxicity remains unclear. Doxorubicin could induce GC apoptosis and follicle damage [7, 8]. However, the ovarian toxicity of epirubicin has rarely been reported.

Studies have reported that epirubicin induces POF in humans. The classic fluorouracil, epirubicin, and cyclophosphamide (FEC) regimen induces menopause in 60% of breast cancer patients [9]. The National Cancer Institute of Canada adjuvant trial comparing cyclophosphamide, methotrexate, and 5-fluorouracil regimen (CMF) with FEC regimen indicated that the incidence of amenorrhea was slightly higher in FEC arm (51%) in comparison with the CMF arm (42.6%) [10]. In addition, the chemotherapy of breast cancer associated with epirubicin resulted in an incidence of amenorrhea of 23.08–44.87% [11]. Thus, the point of this study was to explore the mechanisms of ovarian toxicity of epirubicin. Besides, chemotherapy induced premature ovarian failure (POF) can cause serious long-term complications such as vasomotor symptoms, osteoporosis and cardiovascular diseases [12].

The mechanism of chemotherapy induced ovarian damage may be related to the apoptosis of granulosa cells (GCs), injury to growing follicles, consumption of primordial follicles, and damage to ovarian microenvironment [13–17]. However, no effective treatment is currently available for chemotherapy induced POF. In recent years, it has been reported that mesenchymal stem cells (MSCs) have the potential to repair POF as they can be differentiated into GCs, reduce GC apoptosis, prevent follicular atresia and maintain healthy follicles, and improve the renewal of germline stem cells and ovarian microenvironment [18–

21]. We have previously found that MSCs derived from menstrual blood (MB-MSCs) could repair epirubicin induced damages to human ovarian GCs [22]. We have also found that in an epirubicin induced POF mice model, MB-MSCs could increase Estradiol and antimullerian hormone (AMH) levels and the number of primordial, primary, secondary and antral follicles and decrease the number of atretic follicles by improving the apoptosis of ovarian cells. However, the estrous cycle at 28 d of MB-MSCs transplantation showed no significant recovery. Therefore, a new strategy is needed to cooperate with MB-MSCs to improve the damaged ovarian function through a variety of ways. In traditional Chinese medicine (TCM) theory, Shen governs reproduction. Damage of Shen is thought to be the main etiology of POF. Studies have reported that Bushen, which means tonifying Shen, can improve POF [23–28]. Bushen Tiaochong recipe (BSTCR) is a traditional Chinese medicine prescription for tonifying Shen which is made up of nine kinds of Chinese herbs. It is capable of improving ovarian microenvironment and the expressions of follicle stimulating hormone receptor (FSHR) and insulin-like growth factors – 1 (IGF-1) mRNA and enhancing the efficacy of gonadotropin to GCs and the reactivity of GCs to gonadotropin. BSTCR can recover the ovarian reserve capacity via the brain derived neurotrophic factor (BDNF) pathway and improve the proliferation of ovarian GCs and the secretion of steroid hormones [23, 24]. Thus, it is expected that BSTCR may coordinate with MB-MSCs to improve the ovarian function in epirubicin induced POF mice.

In the present study, we studied the effect and the relevant mechanisms of MB-MSCs combined with BSTCR on the ovarian function after epirubicin chemotherapy in mice and compared the efficacy of the combined treatment with MB-MSCs or BSTCR treatment, respectively.

## Methods

### Cell preparation

All protocols were approved by the Ethics Committee of Tianjin Medical University, and informed written consent was obtained from all participants. MB was collected from three healthy females, and MB-MSCs were isolated, identified and cultured as described previously [29]. MB-MSCs cultured to the third generation were seeded at a density of  $2 \times 10^5$  cells/well in 6-well plates in DMEM/F12 solution containing 10% FBS. When they were grown to 60% confluence, 600  $\mu$ L of green fluorescent protein (GFP)-luciferase adenovirus suspension ( $10^7$  PFU/ml) (kindly provided by the Viral Laboratory of Hematosis Hospital of Chinese Academy of Medical Sciences) was added. Fluorescence microscopy (Zeiss, Germany) showed that MB-MSCs were successfully labeled by GFP, and then they were suspended in PBS at a density of  $5 \times 10^5$  cells/100  $\mu$ L.

### BSTCR preparation

All constituents of BSTCR (Sheng Shilong Pharmaceutical Co., Ltd., China) were decocted twice, and the two decoctions were mixed and boiled to prepare the final decoction with a concentration of 3 g crude drugs/mL.

### Establishment and grouping of animal models

Female C57BL/6 mice at 8 weeks (18–20 g) were purchased from Beijing Huafukang Biotechnology Co., LTD, (Beijing, China), and the vaginal smear showed that they had at least two consecutive normal estrous cycles of 4–5 days. All mice were fed ad libitum with sterile food and water at a controlled temperature of 21–24 °C on a 12 h light:12 h dark cycle. They were intraperitoneally injected with 0.01 mg/g epirubicin for 7 consecutive days, and vaginal smear was performed regularly each day. The animal model was successfully established if no estrous cycle was observed for 2 cycles (approximately 8 days).

Twenty-four POF mice were randomized into 4 groups of 6 mice each, which were treated with tail intravenous injection and gavage of PBS (E group); tail intravenous injection of 200 µL PBS containing  $1 \times 10^6$  GFP-labeled MB-MSCs (E + M group); 5 µL/g BSTCR gavage for 3 consecutive days (E + B group); MB-MSCs transplantation and BSTCR gavage as described above (E + M + B group), respectively. Six mice of the same age were used as controls, which were treated with intraperitoneal and tail intravenous injection and gavage of PBS. All of the experiments were repeated three times.

After MB-MSCs transplantation for 24 h, the homing of MB-MSCs was observed using a real-time imaging system in three mice in each group, and changes in estrous cycles were observed daily for 28 d in the rest. After that, they were sacrificed by cervical dislocation, and one ovary was collected for TUNEL and HE staining to observe ovarian in-situ apoptosis and follicle development, and the other ovary was collected for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western Blot analysis of GADD45b, CyclinB1, CDC2 and pCDC2 expressions.

Finally, 150 µL of peripheral blood was collected from the caudal vein for ELISA analysis of serum estradiol (E2), follicle-stimulating hormone (FSH) and anti-Müllerian hormone (AMH) levels.

### Live imaging of transplanted MB-MSCs in mice

After MB-MSCs transplantation for 24 h, mice were intraperitoneally anesthetized with 10% chloral hydrate (3 µL/g) (Kermel, China) and then intraperitoneally injected with D-fluorescein (150 µg/g) (Promega, USA). Ten minutes later, green fluorescence was observed with a live imaging system (Xenogen, USA).

### Enzyme-linked immunosorbent (ELISA) assay

After placed at room temperature for 2 h, mice blood was centrifuged at 2000 g for 20 min, and the supernatant was collected. The E2, FSH and AMH levels were measured using an ELISA kit (Cloud-Clone Corp, USA) according to the manufacturer's protocol, and the results were analyzed using a Synergy H4 Hybrid Reader (Bio Tek, USA).

### Histopathology

Ovaries were collected and fixed in 4% paraformaldehyde for 24 h. Then they were embedded in paraffin and sliced into pieces of 5 µm thick for hematoxylin and eosin staining (H&E staining).

Follicles of every stage were classified and counted according to the following criteria. A primordial follicle was defined as an oocyte surrounded by a single layer of flattened squamous pregranulosa cells. A primary follicle was defined as an oocyte surrounded by a single layer of cuboidal granulosa cells. Secondary follicles had two or more layers of cuboidal granulosa cells with no visible antrum. Follicles were defined as antral follicles if they had an antral space with a cumulus granulosa cell layer. Follicles were classified as atretic if the oocyte was degenerating (convoluted, condensed) or fragmented. [18, 25].

### Terminal deoxynucleotidyltransferase-mediated deoxy-UTP nick end labeling (TUNEL) assay

One ovary was fixed in 4% formaldehyde for 24 h and embedded in paraffin. Then, it was sliced into pieces of 5 µm thick and dewaxed for the detection of in situ apoptosis using a TUNEL kit (Yeasen, China). Five random fields per section and five sections per tissue from a mouse were examined and analyzed in each experiment.

### RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from ovarian tissue was obtained using TRIzol reagent (Life Technologies) and reverse transcribed to cDNA using ImProm-IIITM Reverse Transcription Kit (Promega, Madison, USA). Primers for GADD45b consisted of 5'-GAGGCGGCCAAACTGATGAAT-3' (forward) and 5'-CGCAGCAGA ACG ACT GGAT-3' (reverse). Primers for CyclinB1 consisted of 5'-AAGGTGCCTGTGTGTGAACC-3' (forward) and 5'-GTCAGCCCCATCATCTGCG-3' (reverse). Primers for CDC2 consisted of 5'-AGAAGGT ACTTACGGTGTGGT-3' (forward) and 5'-GAGAGATT TCCCGAATTGCAGT-3' (reverse). Primers for GAPDH consisted of 5'-AGGTCGGTGTGAACGGATTG-3' (forward) and 5'-TGTAGACCATGTAGTTGAGGTCA-3' (reverse). The mRNA expression levels were measured on a 7900 real-time PCR instrument (Applied Biosystems) with the following amplification profile: 3 min at 94 °C followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1

min. The relative expression level of mRNA was calculated using the  $2^{-\Delta\Delta C_T}$  method adjusted by GAPDH as an internal control.

### Western blot analysis

The total protein was extracted from ovarian tissue according to the manufacturer's instructions, and the total protein concentration was determined using the bicinchoninic acid assay (BCA; Solarbio, China). In each group, 10  $\mu$ L of samples were electrophoresed on a sodium dodecyl sulfate-10% polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA). The membrane was blocked in 5% defatted milk at room temperature for 2 h, and then incubated with the primary antibody against GADD45b (Santa Cruz, USA, 1:1000), CyclinB1 (Cell Signaling Technology, USA, 1:1000), CDC2 (Cell Signaling Technology, 1:1000), pCDC2 (Cell Signaling Technology, 1:1000) and  $\beta$ -actin antibody (Cell Signaling Technology, 1:1000) diluted in  $1 \times$  TBST containing 5% defatted milk overnight at 4°C, and then with HRP labeled secondary antibody (Beyotime, China, 1:1000) diluted in  $1 \times$  TBST containing 5% defatted milk at room temperature for 2 h. The bands were visualised using an enhanced chemiluminescence detection system (PerkinElmer, USA) and exposed by ImageQuant LAS 4010 Control Software (GE, USA). Fluorescence intensity was quantitated using Image J (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD). The GADD45b, CyclinB1, CDC2 and pCDC2 expressions were normalized to  $\beta$ -actin expression.

### Statistical analyses

All continuous data were expressed as mean  $\pm$  standard deviation and analyzed using SPSS 20.0 (SPSS Inc., USA). Statistical differences between two groups were analyzed by two-tailed unpaired t-test; whereas those among three or more groups were analyzed by one-way analysis of variance (ANOVA) and Tukey multiple comparison test.  $P < 0.05$  was considered statistically significant.

## Results

### MB-MSCs migrated to pelvic organs of POF mice

GFP labeled MB-MSCs were observed under a fluorescence microscope after transfection for 48 h (Fig. 1a and b) with a transfection rate of 20–30%. To elucidate the homing of MB-MSCs in vivo after tail intravenous transplantation, mice were sterilized and then screened by live imaging to track GFP (+) cells. GFP (+) cells were observed in pelvic organs 24 h after transplantation (Fig. 1c). Interestingly, stronger fluorescence was observed in E + M + B group than that in E + M group (Fig. 1d), which indicated that BSTCR might enhance the migration of MB-

MSCs in mice. However, more evidence is needed to verify this result.

### The combined treatment increased body weight of POF mic

Mice showed hair loss, loss of appetite, and poor reactivity to external stimuli three days after injection with epirubicin. Body weight was monitored daily, and those mice injected with epirubicin had a significantly lower body weight than control mice ( $P < 0.01$ ) (Fig. 2a).

MB-MSCs transplantation was given once upon the POF model was established successfully. Body weights in E + M group and E + M + B group were significantly increased from 14d onward after cell transplantation ( $P < 0.05$ , Fig. 2b). However, no significant difference was observed between E + B group and E group ( $P > 0.05$ , Fig. 2b). Besides, there was no significant difference between E + M group and E + M + B group ( $P > 0.05$ , Fig. 2b).

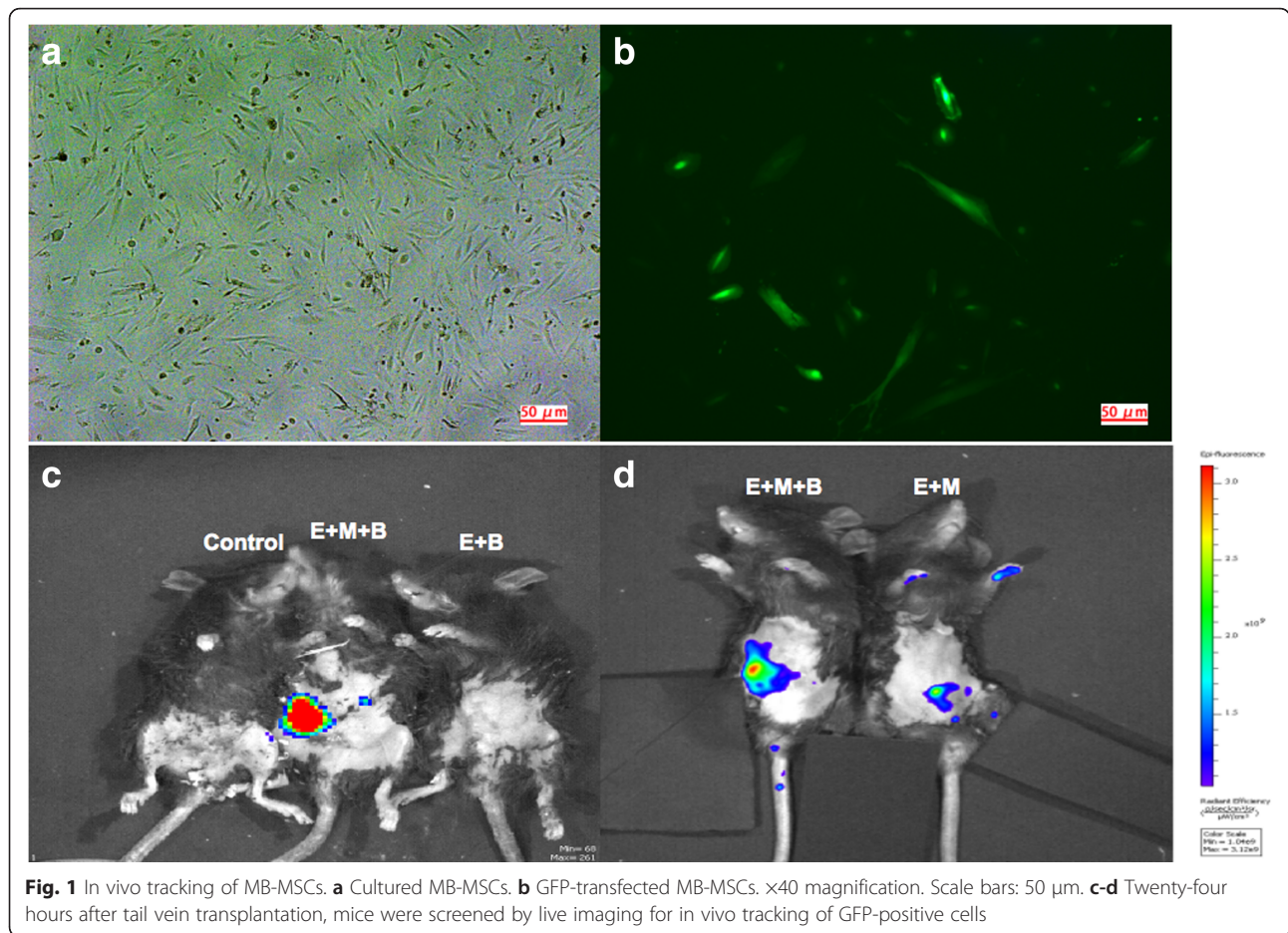
### The combined treatment improved estrous cycles of POF mice

The estrous cycle of mice can be divided into proestrus, oestrus, metestrus and diestrus. Compared with the control group, epirubicin treatment resulted in a longer estrous cycle, so that the estrous cycle was arrested in diestrus or proestrus after 7 d (Fig. 3a). Mice were observed for 2–3 consecutive estrous cycles (approximately 14 d), and it was found that those mice treated with epirubicin remained in diestrus or proestrus, indicating that epirubicin-induced POF mice model has been successfully established.

After 14 days of MB-MSCs transplantation, the estrous cycle was gradually recovered in E + M + B group and started cyclicity from 20 d onward. However, mice in other groups still showed no recovery on 28 d. The estrous cycles of mice in E group, E + M group and E + B group were still absent and remained in diestrus or proestrus (Fig. 3b).

### The combined treatment improved sex hormones secretion in POF mice

Serum sex hormone levels were significantly different among groups. The levels of E2 and AMH of mice were significantly decreased after epirubicin injection ( $P < 0.01$ , Fig. 4a and b), while the level of FSH was significantly increased ( $P < 0.01$ , Fig. 4c). The levels of E2 and AMH in E + B group, E + M + B group and E + M group were significantly increased compared to E group ( $P < 0.01$ , Fig. 4a and b), while the level of FSH in E + B group, E + M + B group and E + M group was significantly lower than that in E group, respectively ( $P < 0.01$ , Fig. 4c). In addition, the levels of sex hormones in E + M + B group were almost significantly different from E + M group and from E + B group, respectively (E2:  $P > 0.05$  v.s. E + M group;  $P < 0.01$  v.s. E + B group. AMH:  $P < 0.05$  v.s. E + M group;  $P < 0.01$  v.s. E +



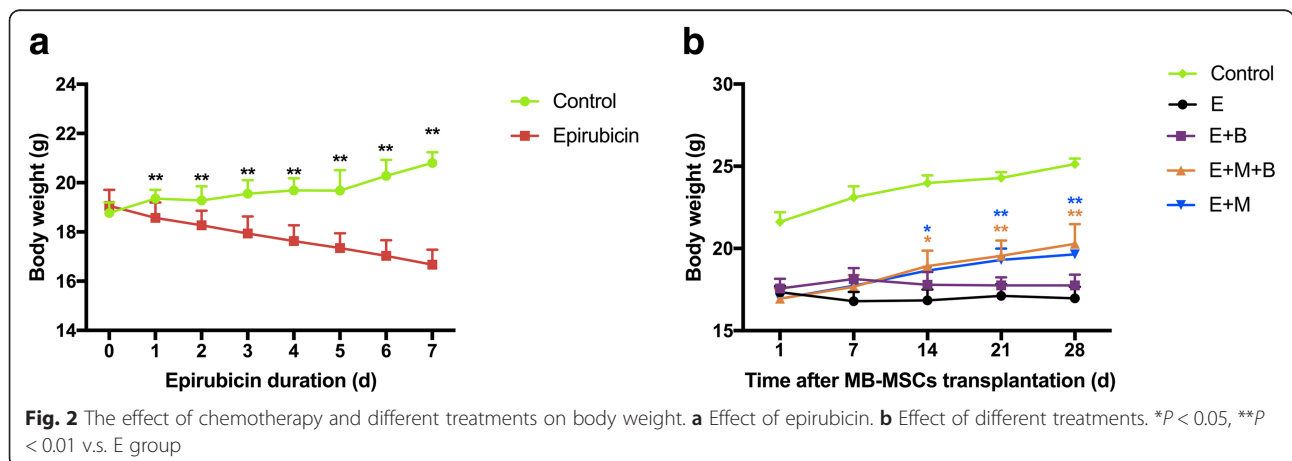
B group. FSH:  $P < 0.01$  v.s. E + M group;  $P < 0.01$  v.s. E + B group).

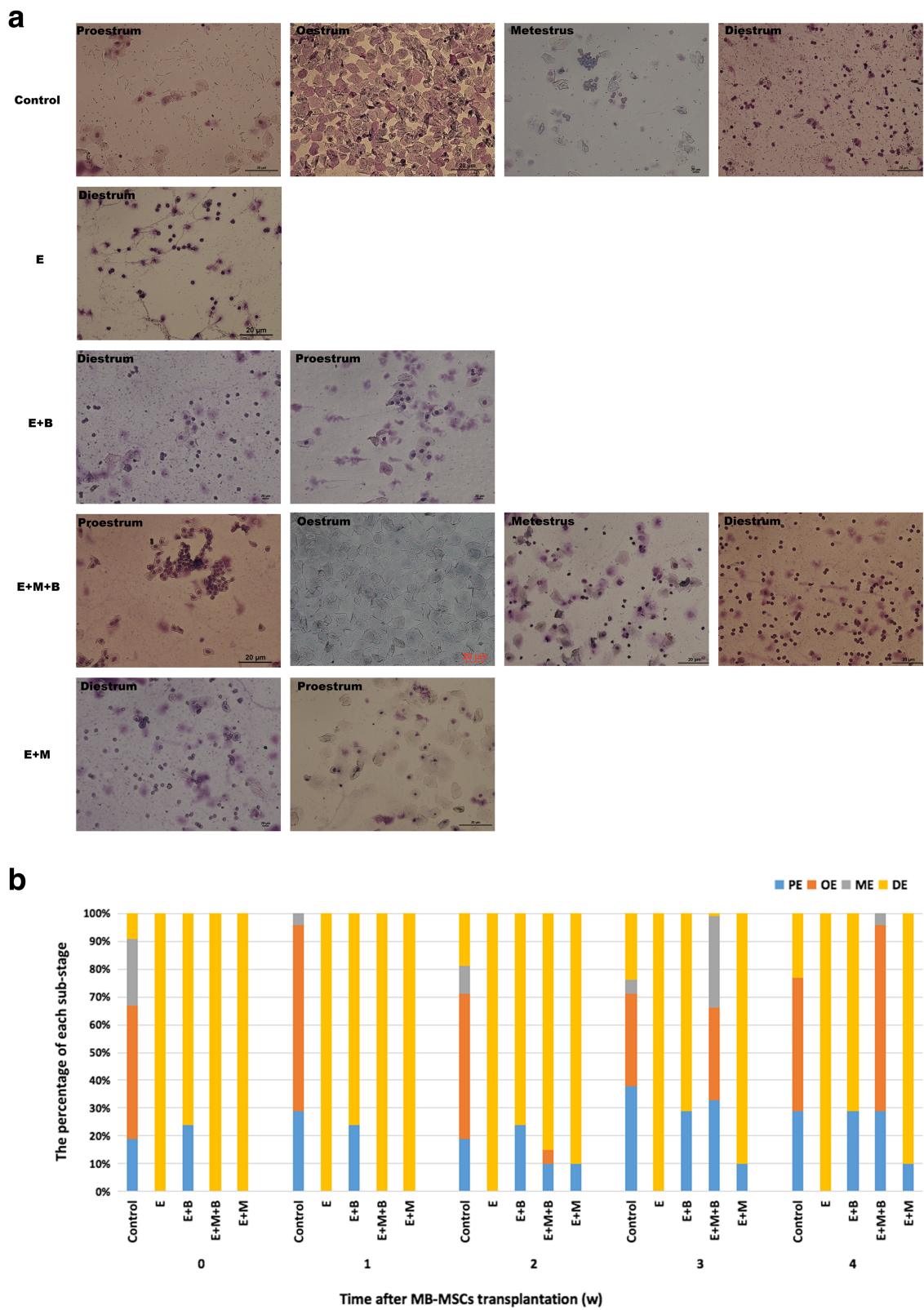
**The combined treatment improved ovarian morphology in POF mice**

In order to detect the effect of epirubicin administration on the total number of follicles in every stage, we randomly

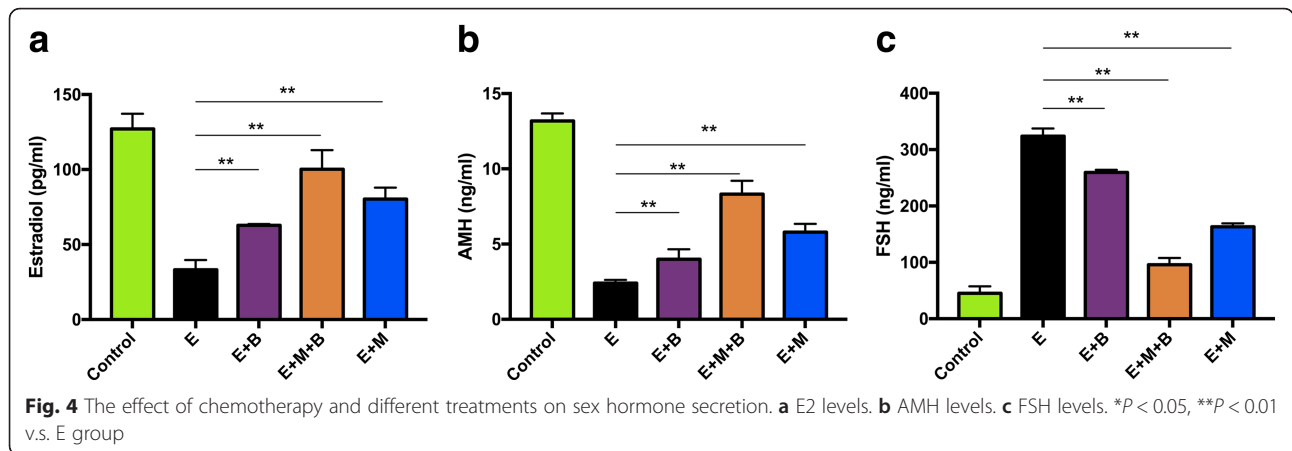
selected five slices of every ovary and classified and counted the follicles on the entire field of these five slices. Three ovaries from three mice were calculated in each group and the total follicles in each group were added and used for further analysis.

Epirubicin exposure resulted in a decrease in the number of primordial follicles, primary follicles, secondary





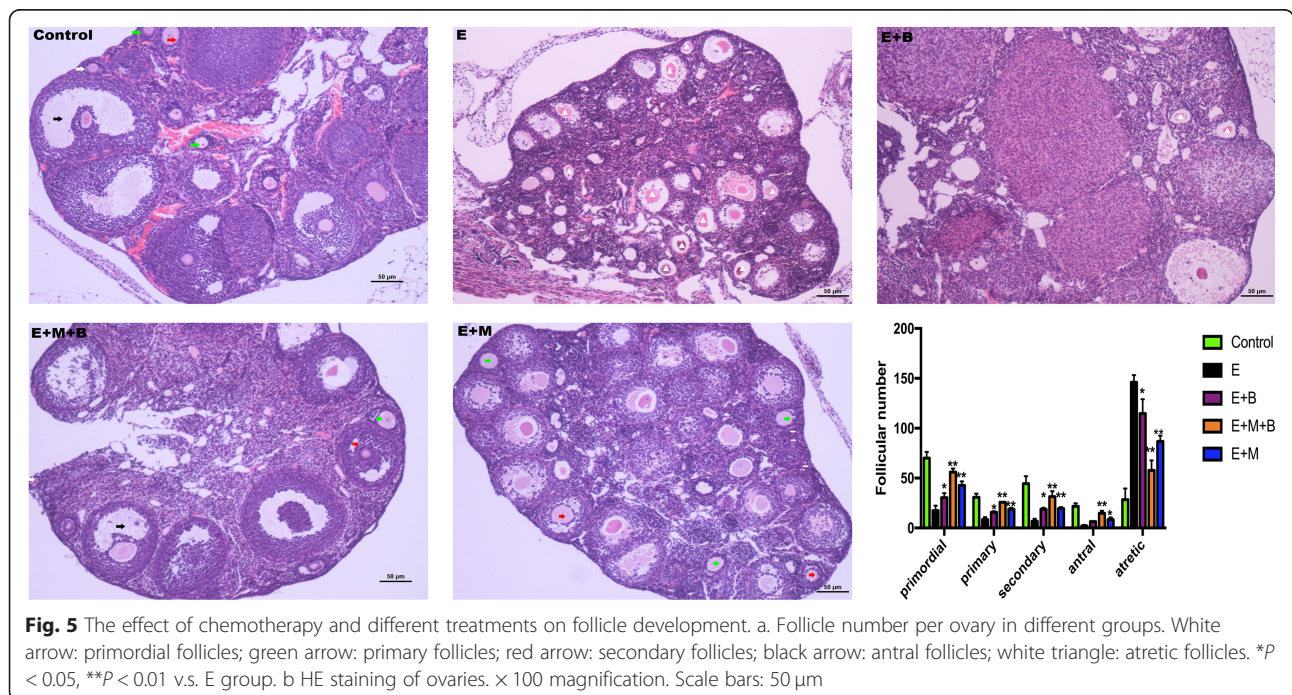
**Fig. 3** The effect of chemotherapy and different treatments on estrous cycles. **a** Vaginal smears in different groups.  $\times 200$  magnification. Scale bars: 20  $\mu\text{m}$ . **b** The percentage of sub-stages of proestrus (PE), oestrus (OE), metestrus (ME) and diestrus (DE) in different groups

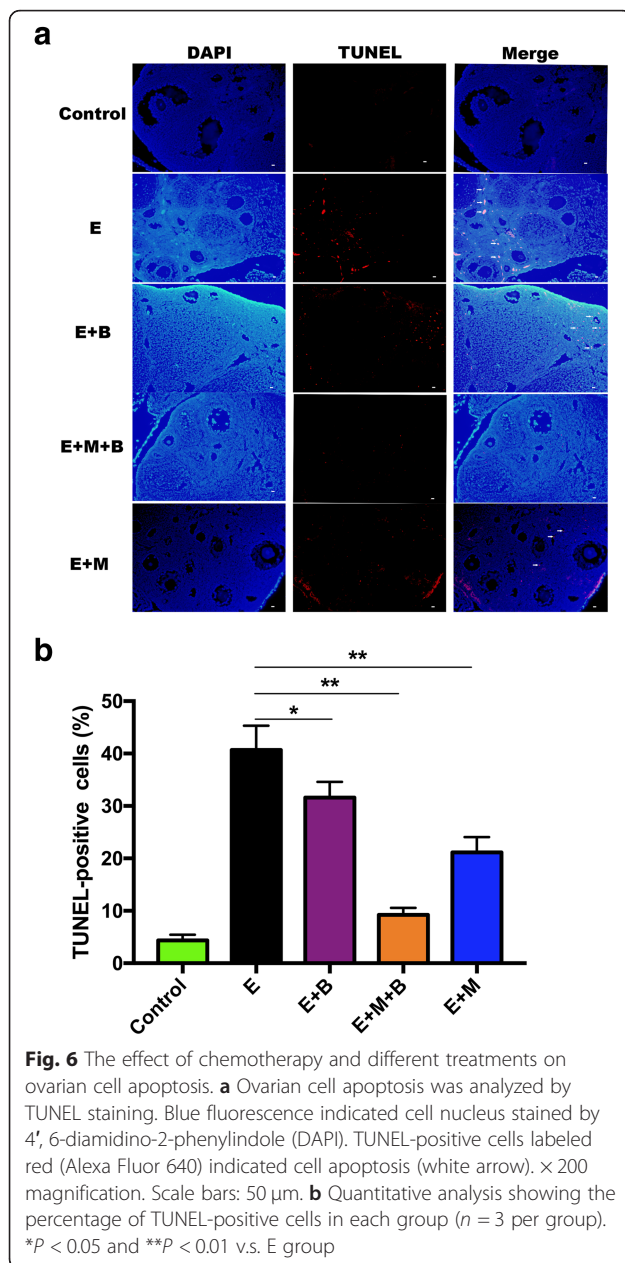


follicles and antral follicles ( $P < 0.01$ , Fig. 5), but an increase in the number of atretic follicles ( $P < 0.01$ , Fig. 5). However, the number of healthy follicles at all stages increased and the number of atretic follicles decreased in E + M + B group ( $P < 0.01$ , Fig. 5) and E + M group ( $P < 0.05$ , Fig. 5), respectively. A significant difference of primordial follicles, primary follicles, secondary follicles and atretic follicles was observed between E group and E + B group ( $P < 0.05$ , Fig. 5) except of antral follicles ( $P > 0.05$ , Fig. 5). Besides, E + M + B group indicated a more significant efficacy on follicular development than E + B group and E + M group, respectively ( $P < 0.05$ , Fig. 5).

**The combined treatment decreased ovarian cell apoptosis in POF mice**

To investigate if epirubicin induces ovarian cell apoptosis and the effect of MB-MSCs and/or BSTCR treatment, ovarian tissue slides of each group were stained with TUNEL kit. The rate of TUNEL-positive cells was significantly higher in mice exposed with epirubicin compared to control group ( $P < 0.01$ , Fig. 6b). The rate of TUNEL-positive cells was significantly decreased in POF mice after treatment of MB-MSCs and/or BSTCR. In addition, E + M + B group showed a stronger inhibition of cellular apoptosis compared to E + B group ( $P < 0.01$ , Fig. 6b) and E + M group ( $P < 0.01$ , Fig. 6b), respectively.





Besides, TUNEL-positive cells were only observed in granulosa cells and most of the apoptotic cells were observed in atretic follicles, which suggests that the increase of granulosa cell apoptosis may be one of the potential mechanisms of epirubicin-induced POF in mice.

#### Ovarian expression of GADD45b, CyclinB1 and CDC2

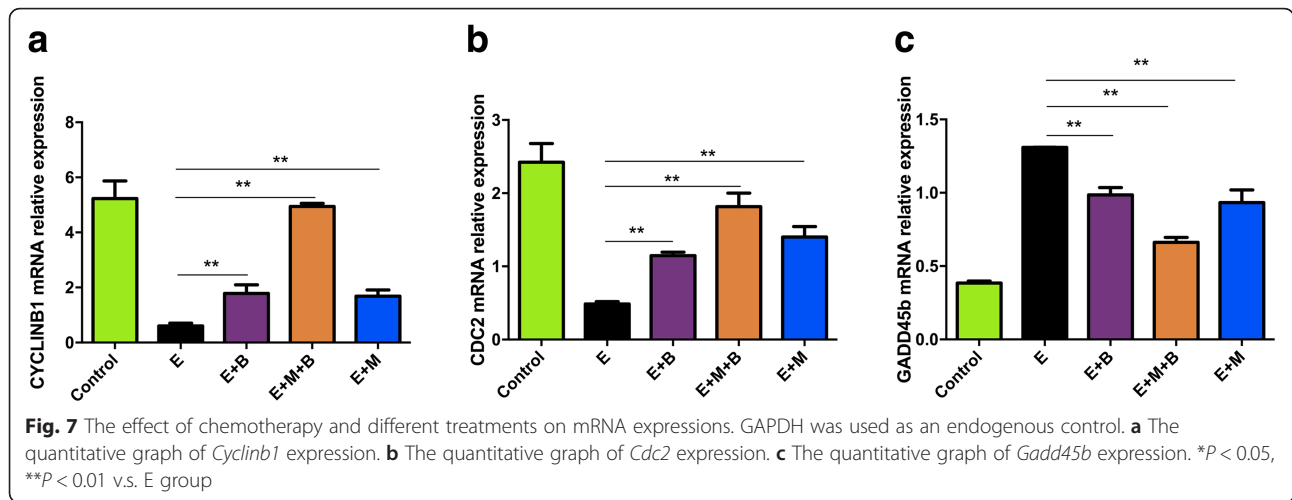
To detect whether the effect of MB-MSCs on GADD45b, CyclinB1 and CDC2 expressions in POF mice is consistent with the effect on damaged human GCs, their expressions were determined by RT-qPCR and western blot analysis. The results showed that GADD45b and pCDC2 mRNA and protein expressions were significantly higher in E

group compared with those in control group ( $P < 0.01$ , Figs. 7 and 8). GADD45b expressions were decreased in E + M + B, E + M and E + B groups compared with that in E Group ( $P < 0.01$ , Figs. 7 and 8), and a more significant decrease in protein expression was observed in E + M + B group compared with that in E + M group and E + B group ( $P < 0.05$ , Fig. 8). pCDC2 protein expressions were decreased in E + M + B and E + M groups compared with that in E Group ( $P < 0.01$ , Fig. 8). E + M + B group showed a more significant decrease than E + M and E + B groups ( $P < 0.01$ , Fig. 8). No significant difference in pCDC2 protein expression was observed between E + B and E groups ( $P > 0.05$ , Fig. 8). CyclinB1 and CDC2 expressions were down-regulated in E group compared with the control group ( $P < 0.01$ , Fig. 8). CyclinB1 and CDC2 expressions in E + M + B, E + M and E + B groups were significantly increased compared with E group ( $P < 0.01$ , Fig. 8). E + M + B group showed a more significant increase than E + M and E + B groups ( $P < 0.01$ , Fig. 8).

#### Discussion

Epirubicin is a first-line anti-tumor drug widely used in acute leukemia, lymphoma and breast cancer [3, 30, 31]. After treating human ovarian GCs with epirubicin in vitro, we have previously found that epirubicin inhibited the secretion of E2, progesterone, AMH, inhibin A and inhibin B and the proliferation of GCs, and promoted apoptosis of GCs. Studies have reported that cyclophosphamide may induce POF through PI3K/Akt/mTOR, Rictor/mTORC2/Akt/Foxo3a signalling axis [32, 33]. Cisplatin acts through PTEN/AKT/FOXO3a pathway [34]. Tripterygium glycosides act through serine/threonine kinase 11-p53-p21 signaling pathway [35]. In our previous study, a genome-wide transcriptional analysis using the Affymetrix GeneChip<sup>®</sup> identified 3599 significantly differentially expressed genes between granulosa cells treated/not treated with epirubicin groups, including 1962 upregulated genes and 1607 downregulated genes. The pathway analysis using the KEGG database showed that the cell cycle pathway was involved in 18 differentially expressed genes between granulosa cells treated/not treated with epirubicin groups. The microarray analysis results showed that of the three subtypes (GADD45a, GADD45b, and GADD45g) of GADD45, GADD45b was identified as a significantly differentially expressed gene. And we believe that epirubicin acts through activating GADD45b of the cell cycle pathway, which serves as one of the most important mechanisms of epirubicin-induced POF. In vitro experiments have also confirmed that epirubicin might inhibit the activity of CDC2/CyclinB1 complex by increasing the protein expression of GADD45b in the cell cycle pathway of ovarian GCs, resulting in inhibition of the proliferation of GCs and blocking in G2/M phase [22]. In this study, we

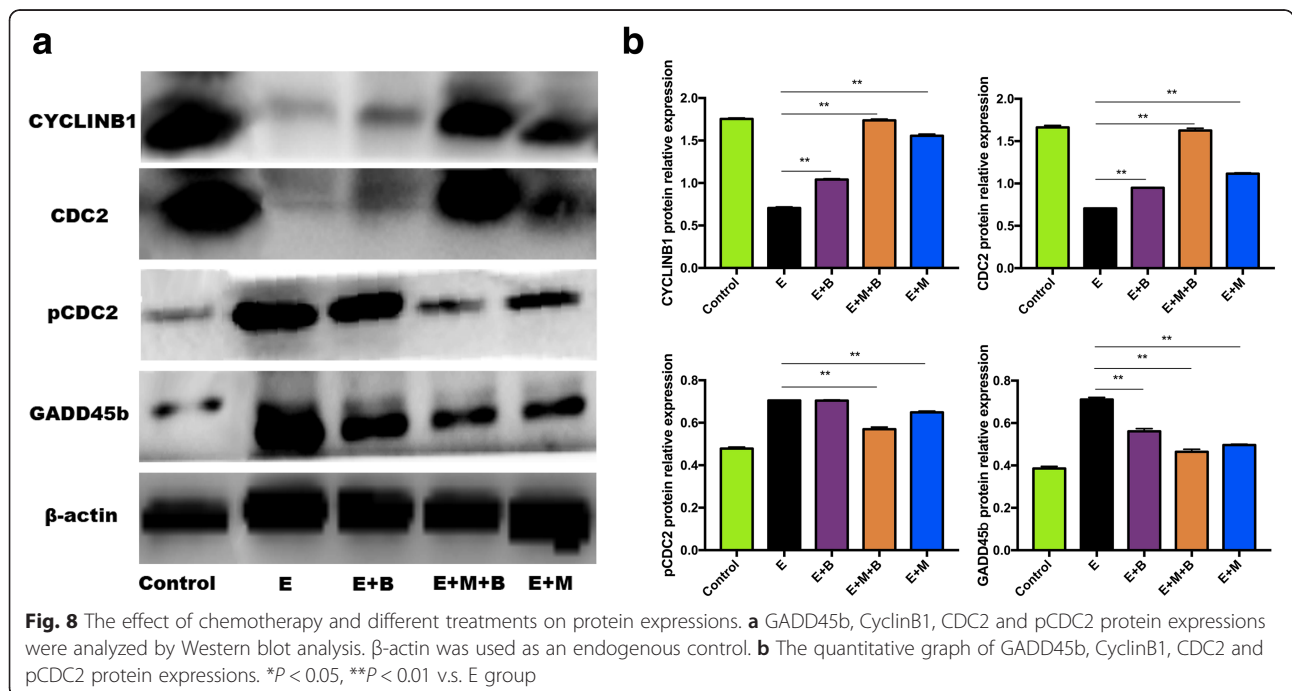




found that epirubicin treatment resulted in a decrease in the number of primordial, primary, secondary and antral follicles; an increase in the number of atretic follicles; an increase in ovarian in situ apoptosis; a decrease in serum E2 and AMH levels; an increase in serum FSH levels; and the stagnation in diestrus or proestrus. This is probably because epirubicin can promote the expressions of GADD45b and pCDC2 but inhibit the expressions of CyclinB1 and CDC2.

Hormone replacement therapy (HRT) is one of the therapeutic options for POF. However, although HRT is capable of alleviating clinical symptoms of POF, but it is incapable of restoring the reproductive ability. Long-term oral administration of estrogen and/or progesterone can also increase

the risk of venous thromboembolism (VTE), stroke, breast cancer, ovary cancer and other estrogen sensitive tumors [36–39]. Gonadotropin-releasing hormone antagonist (GnRHa) is also thought to be effective in preventing chemotherapy induced POF, which is attributed to the reduction in ovarian perfusion and inhibition of follicles into the growth stage [40]. However, there are also reports that GnRHa is not associated with the decrease in POF risk [41, 42]. Assisted reproductive technology (ART) such as cryopreservation of embryos, oocytes and ovarian tissues is not mature enough to be widely used in clinic [43]. The pluripotent stem cells hold a promise in the treatment of POF due to their exceptional self-replication ability. MB-MSCs have the advantages of self-renewal, high proliferative



potential, pluripotentiality and low immunogenicity [44, 45]. In early *in vitro* experiments, we found that MB-MSCs repaired epirubicin induced damage to human ovarian GCs by inhibiting GADD45b expression in the cell cycle pathway [22]. In epirubicin induced POF mice model, we found that MB-MSCs resulted in an increase in E2 and AMH levels and the number of preantral and antral follicles and a decrease in the number of atretic follicles, but the estrous cycle still showed no significant recovery on 28 d after MB-MSCs transplantation. Thus, we are expecting another way to improve damaged ovarian function with MB-MSCs. In Chinese traditional medicine, Shen governs reproduction, and thus invigorating Shen is helpful to improve the function of the reproductive system. BSTCR is a well-known traditional Chinese prescription capable of the expression of FSHR and IGF-1 mRNA, and thus it can promote the efficacy of gonadotropin to GCs and increase the reactivity of GCs to gonadotropin. We have previously found that BSTCR might recover the ovary reserve capacity via the BDNF pathway. BDNF is expressed in GCs and oocytes, and thus it plays a critical role in the development and maturation of oocytes. It is also involved in mitochondria assembly and motility, meiotic spindle configuration and cortical granule distribution during oocyte maturation, prohibiting JNK and P38 activation under stress conditions, and increasing cell viability [23, 24]. We expect that BSTCR can have an effect on the migration and survival of MB-MSCs act synergistically with MB-MSCs to improve damaged ovarian function.

We found that combined with BSTCR, MB-MSCs could result in the recovery of the estrous cycle, an increase in the number of promordial, primary, secondary and antral follicles, a decrease in the number of atretic follicles and ovarian cell apoptosis, an increase in serum E2 and AMH levels, and a reduction in serum FSH levels in epirubicin induced POF mice. However, although mice treated with MB-MSCs alone showed an increase in the number of follicles and sex hormone levels, the estrous cycle showed no obvious recovery. Thus, BSTCR was thought to act synergistically with MB-MSCs to improve damaged ovarian function.

As for the mechanism of MB-MSCs and BSTCR in improving ovarian function, according to the differentially expressed gene analysis previously, GADD45b might play a critical role in repairing epirubicin induced cell cycle arrest of GCs and promoting cell proliferation, and it could also have an effect on the CDC2/CyclinB1 complex and thus affect the cell cycle and proliferation of human ovarian GCs [22]. The RT-qPCR and western blot analysis showed that ovarian GADD45b and pCDC2 expressions were down-regulated, and CyclinB1 and CDC2 expressions were up-regulated in mice treated with MB-MSCs, BSTCR or MB-MSC combined with BSTCR. Importantly, MB-MSCs combined with BSTCR showed better therapeutic effects than BSTCR or MB-MSCs alone.

The GADD45 gene family encodes three related GADD45 proteins, including GADD45 $\alpha$ ,  $\beta$  and  $\gamma$ , with similar functions and sequences. Each gene is expressed in a number of mammal tissues such as heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis [46]. GADD45 proteins are involved in many cell functions, including DNA repair, cell cycle control, cell survival or apoptosis, cell senescence, maintenance of genomic stability and genotoxic stress [47–50]. The members of GADD45 gene family have similar but not identical functions in different apoptosis and growth inhibitory pathways. GADD45 $\alpha$ ,  $\beta$  and  $\gamma$  have been suggested to mediate different signal pathways via different stimuli and finally mediate cell apoptosis and senescence. Their expression levels are generally low under normal conditions but significantly increased under stress [48]. In this study, we found that epirubicin treatment induced high expression of GADD45b. The function of GADD45 as a stress sensor is mediated via a complex interplay of physical interactions with other cellular proteins implicated in cell cycle regulation and the response of cells to stress, notably PCNA, p21, cdc2/cyclinB1, and the p38 and JNK stress response kinases [51]. The GADD45 isoforms have also been implicated in the G2/M cell cycle checkpoint in human and mouse cells [52]. In this study, the RT-qPCR and western blot analysis revealed an increase in GADD45b and pCDC2 expressions and a decrease in CyclinB1 and CDC2 expressions in mice treated with epirubicin, indicating that epirubicin may regulate cell cycle arrest of ovary cells by up-regulating GADD45b protein expression and down-regulating CDC2 and CyclinB1 expressions. However, the combined therapy resulted in a decrease in GADD45b and pCDC2 expressions and an increase in CyclinB1 and CDC2 expressions, indicating that MB-MSCs combined with BSTCR may repair epirubicin induced ovarian function damage by down-regulating GADD45b expressions and up-regulating CDC2 and CyclinB1 expressions. These results were in good agreement with our previous *in vitro* studies.

## Conclusions

Although the treatment of POF with BSTCR or MB-MSCs alone results in an increase in the number of follicles and sex hormone levels, the estrous cycle shows no obvious recovery. MB-MSCs combined with BSTCR could result in the recovery of the estrous cycle, an increase in the number of promordial, primary, secondary and antral follicles, a decrease in the number of atretic follicles and ovarian cell apoptosis, an increase in serum E2 and

AMH levels, and a reduction in serum FSH levels in epirubicin induced POF mice. Importantly, it shows better therapeutic effects than BSTCR or MB-MSCs alone. This can be related to the inhibition of GADD45b expression and the promotion of CDC2 and CyclinB1 expressions. This study may be helpful for female patients of childbearing age with chemotherapy induced POF.

#### Abbreviations

AMH: Anti-Müllerian Hormone; ART: Assisted reproductive techniques; BCA: Bicinchoninic acid assay; BDNF: Brain derived neurotrophic factor; BSTCR: Bushen Tiaochong recipe; DAPI: 4', 6-diamidino-2-phenylindole; DOR: Diminished ovarian reserve; E2: Estradiol; ELISA: Enzyme-linked immunosorbent; FSH: Follicle-Stimulating Hormone; GADD45: The growth arrest and DNA damage inducible 45; GFP: Green fluorescent protein; GnRHa: Gonadotropin-releasing hormone agonist; H&E staining: Hematoxylin and eosin staining; HRT: Hormone replacement therapy; IGF-1: Insulin-like growth factors – 1; MB-MSCs: Menstrual blood derived mesenchymal stem cells; MSCs: Mesenchymal stem cells; PBS: Phosphate-buffered saline; POF: Premature ovarian failure; PVDF: Polyvinylidene difluoride; SDS: Sodium dodecyl sulfate; TUNEL: Terminal deoxynucleotidyltransferase-mediated deoxy-UTP nick end labeling; VTE: Venous thromboembolism

#### Acknowledgements

This work was supported by grants (81303108 to Xue Du) from the National Natural Science Foundation of China.

We appreciate the help of Dr. Yanjun Zhang from Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College for the gift adenovirus.

#### Authors' contributions

FG was responsible for the mice experiments on the effects of mesenchymal stem cells on POF mice and writing the manuscript. TX was responsible for the BSTCR preparation and the effects of BSTCR on POF mice. YZ was responsible for the Western blot analysis and ELISA analysis. XM was responsible for the TUNEL assay and HE staining. ZY was responsible for the POF model establishment. SH, YH, RM and YZ collected and analyzed the data and revised the manuscript. XD designed the experiment, revised the manuscript, supervised the study, and provided financial support. All authors read and approved the final manuscript.

#### Funding

This work was supported by grants (81303108 to Xue Du) from the National Natural Science Foundation of China.

#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

#### Ethics approval and consent to participate

Written informed consent was obtained from each donor, and this study was approved by the ethics committee of Tianjin Medical University (IRB2018-YX-089).

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

#### Author details

<sup>1</sup>Department of Obstetrics & Gynecology, General Hospital, Tianjin Medical University, NO.154, Anshan Road, Heping District, Tianjin 300052, People's Republic of China. <sup>2</sup>Reproductive Center, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, No. 88 Chang Ling Street, Xi Qing district, Tianjin 300112, China. <sup>3</sup>State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300020, China.

Received: 15 February 2019 Accepted: 6 July 2019

Published online: 16 July 2019

#### References

- Verdecchia A, Francisci S, Brenner H, et al. Recent cancer survival in Europe: a 2000-02 period analysis of EUROCARE-4 data. *Lancet Oncol.* 2007;8:784–96.
- Brenner H, Coebergh JW, Parkin DM, et al. Up-to-date monitoring of childhood cancer long-term survival in Europe: Leukaemias and lymphomas. *Ann Oncol.* 2007;18:1569–77.
- Moebus V, Jackisch C, Lueck HJ, du Bois A, Thomssen C, Kurbacher C, Kuhn W, Nitz U, Schneeweiss A, Huober J, Harbeck N, von Minckwitz G, Runnebaum IB, Hinke A, Kreienberg R, Konecny GE, Untch M. Intense dose-dense sequential chemotherapy with epirubicin, paclitaxel, and cyclophosphamide compared with conventionally scheduled chemotherapy in high-risk primary breast cancer: mature results of an AGO phase III study. *J Clin Oncol.* 2010;28(17):2874–80.
- Plosker GL, Faulds D. Epirubicin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in cancer chemotherapy. *Drugs.* 1993;45(5):788–856.
- Liu L, Mu LM, Yan Y, et al. The use of functional epirubicin liposomes to induce programmed death in refractory breast cancer. *Int J Nanomedicine.* 2017;12:4163–76.
- Tomasi-Cont N, Lambertini M, Hulsbosch S, et al. Strategies for fertility preservation in young early breast cancer patients. *Breast.* 2014;23(5):503–10.
- Ben-Aharon I, Bar-Joseph H, Tzarfaty G, Kuchinsky L, Rizel S, Stemmer SM, Shalgi R. Doxorubicin-induced ovarian toxicity. *Reprod Biol Endocrinol.* 2010;8:20.
- Morgan S, Lopes F, Gourley C, Anderson RA, Spears N. Cisplatin and doxorubicin induce distinct mechanisms of ovarian follicle loss; imatinib provides selective protection only against cisplatin. *PLoS One.* 2013;8(7):e70117.
- Levine MN, Bramwell VH, Pritchard KI, Norris BD, Shepherd LE, Abu-Zahra H, Findlay B, Warr D, Bowman D, Myles J, Arnold A, Vandenberg T, MacKenzie R, Robert J, Ottaway J, Burnell M, Williams CK, Tu D. Randomized trial of intensive cyclophosphamide, epirubicin, and fluorouracil chemotherapy compared with cyclophosphamide, methotrexate, and fluorouracil in premenopausal women with node-positive breast cancer. National Cancer Institute of Canada clinical trials group. *J Clin Oncol.* 1998;16:2651–8.
- Venturini M, Del Mastro L, Aitini E, Baldini E, Caroti C, Contu A, Testore F, Brema F, Pronzato P, Cavazzini G, Sertoli MR, Canavese G, Rosso R, Bruzzi P. Dose-dense adjuvant chemotherapy in early breast cancer patients: results from a randomized trial. *J Natl Cancer Inst.* 2005;97:1724–33.
- Zhou WB, Yin H, Liu XA, Zha XM, Chen L, Dai JC, Tao AD, Chen L, Ma JJ, Ling LJ, Wang S. Incidence of chemotherapy-induced amenorrhea associated with epirubicin, docetaxel and navelbine in younger breast cancer patients. *BMC Cancer.* 2010;10:281.
- Schover LR. Premature ovarian failure and its consequences: vasomotor symptoms, sexuality, and fertility. *J Clin Oncol.* 2008;26(5):753–8.
- Song D, Zhong Y, Qian C, Zou Q, Ou J, Shi Y, Gao L, Wang G, Liu Z, Li H, Ding H, Wu H, Wang F, Wang J, Li H. Human umbilical cord mesenchymal stem cells therapy in cyclophosphamide-induced premature ovarian failure rat model. *Biomed Res Int.* 2016;2016:2517514.
- Rones H, Kashi O, Meirou D. Prevention of chemotherapy-induced ovarian damage. *Fertil Steril.* 2016;105(1):20–9.
- Luo Q, Yin N, Zhang L, Yuan W, Zhao W, Luan X, Zhang H. Role of SDF-1/CXCR4 and cytokines in the development of ovary injury in chemotherapy drug induced premature ovarian failure mice. *Life Sci.* 2017;179:103–9.
- Lande Y, Fisch B, Tsur A, Farhi J, Prag-Rosenberg R, Ben-Haroush A, Kessler-Ickson G, Zahalka MA, Ludeman SM, Abir R. Short-term exposure of human ovarian follicles to cyclophosphamide metabolites seems to promote follicular activation in vitro. *Reprod BioMed Online.* 2017;34(1):104–14.
- Li D, Chen Y, Qi L, Ju X, Liu H, Wang G. Differentially expressed genes in cisplatin-induced premature ovarian failure in rats. *Anim Reprod Sci.* 2013; 137(3–4):205–13.
- Lai D, Wang F, Yao X, Zhang Q, Wu X, Xiang C. Human endometrial mesenchymal stem cells restore ovarian function through improving the renewal of germline stem cells in a mouse model of premature ovarian failure. *J Transl Med.* 2015;13:155.
- Xiao GY, Liu IH, Cheng CC, Chang CC, Lee YH, Cheng WT, Wu SC. Amniotic fluid stem cells prevent follicle atresia and rescue fertility of mice with premature ovarian failure induced by chemotherapy. *PLoS One.* 2014;9(9):e106538.

20. Wang Z, Wang Y, Yang T, Li J, Yang X. Study of the reparative effects of menstrual-derived stem cells on premature ovarian failure in mice. *Stem Cell Res Ther.* 2017;8(1):11.
21. Wang F, Wang L, Yao X, Lai D, Guo L. Human amniotic epithelial cells can differentiate into granulosa cells and restore folliculogenesis in a mouse model of chemotherapy-induced premature ovarian failure. *Stem Cell Res Ther.* 2013;4(5):124.
22. Yan Z, Guo F, Yuan Q, Shao Y, Zhang Y, Wang H, Hao S, Du X. Endometrial mesenchymal stem cells isolated from menstrual blood repaired epirubicin-induced damage to human ovarian granulosa cells by inhibiting the expression of Gadd45b in cell cycle pathway. *Stem Cell Res Ther.* 2019;10(1):4.
23. Xia T, Fu Y, Li S, Ma R, Zhao Z, Wang B, Chao C. Bu Shen Tiao Chong recipe restores diminished ovary reserve through the BDNF pathway. *J Assist Reprod Genet.* 2016;33(6):795–805.
24. Xia T, Luo SP, Fu Y, Han B. Effects of bushen tiaochong recipe containing serum on ovarian granulosa cell proliferation, steroidogenesis and associated gene expression in rats. *Chin J Integr Med.* 2007;13(3):200–5.
25. Chang EM, Lim E, Yoon S, Jeong K, Bae S, Lee DR, Yoon TK, Choi Y, Lee WS. Cisplatin induces Overactivation of the dormant primordial follicle through PTEN/AKT/FOXO3a pathway which leads to loss of ovarian Reserve in Mice. *PLoS One.* 2015;10(12):e0144245.
26. Xu X, Tan Y, Jiang G, Chen X, Lai R, Zhang L, Liang G. Effects of Bushen Tianjing recipe in a rat model of tripterygium glycoside-induced premature ovarian failure. *Chin Med.* 2017;12:10.
27. Tang CL, Li F, Sun L, et al. Therapeutic effect of Bushen Huoxue recipe on autoimmune premature ovarian failure mice established by immunization with recombinant porcine zona pellucid 4 antigen. *Chin J Integr Med.* 2013;19(6):439–45.
28. Song KK, Ma WW, Huang C, et al. Effect and mechanism of Bushen Huoxue recipe on ovarian reserve in mice with premature ovarian failure [J]. *J Huazhong Univ Sci Technolog Med Sci.* 2016;36(4):571–5.
29. Du X, Yuan Q, Qu Y, Zhou Y, Bei J. Endometrial mesenchymal stem cells isolated from menstrual blood by adherence. *Stem Cells Int.* 2016; 2016:3573846.
30. Menzel H, Müller A, Von Schilling C, Licht T, Peschel C, Keller U. Ifosfamide, epirubicin and etoposide rituximab in refractory or relapsed B-cell lymphoma: analysis of remission induction and stem cell mobilization. *Leuk Lymphoma.* 2008;49(7):1337–44.
31. Bhutani M, Kumar L, Vora A, Bhardwaj N, Pathak AK, Singh R, Kochupillai V. Randomized study comparing 4'-epi-doxorubicin (epirubicin) versus doxorubicin as a part of induction treatment in adult acute lymphoblastic leukemia. *Am J Hematol.* 2002;71(4):241–7.
32. Zhou L, Xie Y, Li S, Liang Y, Qiu Q, Lin H, Zhang Q. Rapamycin prevents cyclophosphamide-induced over-activation of primordial follicle pool through PI3K/Akt/mTOR signaling pathway in vivo. *J Ovarian Res.* 2017;10:56.
33. Zhang B-f HY, Liu X, Cheng Z, Lei Y, Liu Y, et al. The role of AKT and FOXO3 in preventing ovarian toxicity induced by cyclophosphamide. *PLoS One.* 2018;13(8):e0201136. <https://doi.org/10.1371/journal.pone.0201136>.
34. Chang EM, Lim E, Yoon S, Jeong K, Bae S, Lee DR, et al. Cisplatin induces overactivation of the dormant primordial follicle through PTEN/AKT/FOXO3a pathway which leads to loss of ovarian reserve in mice. *PLoS One.* 2015;10:e0144245.
35. Liu TE, Zhang L, Wang S, Chen C, Zheng J. Tripterygium glycosides induce premature ovarian failure in rats by promoting p53 phosphorylation and activating the serine/threonine kinase 11-p53-p21 signaling pathway. *Exp Ther Med.* 2015;10:12–8.
36. Gartlehner G, Patel SV, Feltner C, Weber RP, Long R, Mullican K, Boland E, Lux L, Viswanathan M. Hormone therapy for the primary prevention of chronic conditions in postmenopausal women: evidence report and systematic review for the US preventive services task force. *JAMA.* 2017;318(22):2234–49.
37. Canonico M, Plu-Bureau G, Scarabin PY. Progestogens and venous thromboembolism among postmenopausal women using hormone therapy. *Maturitas.* 2011;70(4):354–60.
38. Rantanen K, Tatlisumak T. Stroke in women - oral contraception, pregnancy, and hormone replacement therapy. *Curr Vasc Pharmacol.* 2013;11(1):58–73.
39. King J, Wynne CH, Assersohn L, Jones A. Hormone replacement therapy and women with premature menopause—a cancer survivorship issue. *Eur J Cancer.* 2011;47(11):1623–32.
40. Blumenfeld Z. How to preserve fertility in young women exposed to chemotherapy? The role of GnRH agonist cotreatment in addition to cryopreservation of embryos, oocytes, or ovaries. *Oncologist.* 2007;12(9):1044–54.
41. Demeestere I, Brice P, Peccatori FA, Kentos A, Gaillard I, Zachee P, Casanovas RO, Van Den Neste E, Dechene J, De Maertelaer V, Bron D, Englert Y. Gonadotropin-releasing hormone agonist for the prevention of chemotherapy-induced ovarian failure in patients with lymphoma: 1-year follow-up of a prospective randomized trial. *J Clin Oncol.* 2013;31(7):903–9.
42. Demeestere I, Brice P, Peccatori FA, Kentos A, Dupuis J, Zachee P, Casanovas O, Van Den Neste E, Dechene J, De Maertelaer V, Bron D, Englert Y. No Evidence for the Benefit of Gonadotropin-Releasing Hormone Agonist in Preserving Ovarian Function and Fertility in Lymphoma Survivors Treated With Chemotherapy: Final Long-Term Report of a Prospective Randomized Trial. *J Clin Oncol.* 2016;34(22):2568–74.
43. Harel S, Fermé C, Poirot C. Management of fertility in patients treated for Hodgkin's lymphoma. *Haematologica.* 2011;96(11):1692–9.
44. Meng X, Ichim TE, Zhong J, Rogers A, Yin Z, Jackson J, Wang H, Ge W, Bogin V, Chan KW, Thébaud B, Riordan NH. Endometrial regenerative cells: a novel stem cell population. *J Transl Med.* 2007;5:57.
45. Zhong Z, Patel AN, Ichim TE, Riordan NH, Wang H, Min WP, Woods EJ, Reid M, Mansilla E, Marin GH, Drago H, Murphy MP, Mineev B. Feasibility investigation of allogeneic endometrial regenerative cells. *J Transl Med.* 2009;7:15.
46. Zhang W, Bae I, Krishnaraju K, Azam N, Fan W, Smith K, Hoffman B, Liebermann DA. CR6: a third member in the MyD118 and Gadd45 gene family which functions in negative growth control. *Oncogene.* 1999;18(35):4899–907.
47. Tamura RE, de Vasconcellos JF, Sarkar D, Liebermann TA, Fisher PB, Zerbini LF. GADD45 proteins: central players in tumorigenesis. *Curr Mol Med.* 2012;12(5):634–51.
48. Moskalev AA, Smit-McBride Z, Shaposhnikov MV, Plyusnina EN, Zhavoronkov A, Budovsky A, Tacutu R, Fraifeld VE. Gadd45 proteins: relevance to aging, longevity and age-related pathologies. *Ageing Res Rev.* 2012;11(1):51–66.
49. Smith ML, Ford JM, Hollander MC, Bortnick RA, Amundson SA, Seo YR, Deng CX, Hanawalt PC, Fornace AJ Jr. p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. *Mol Cell Biol.* 2000;20(10):3705–14.
50. Gupta M, Gupta SK, Hoffman B, Liebermann DA. Gadd45a and Gadd45b protect hematopoietic cells from UV-induced apoptosis via distinct signaling pathways, including p38 activation and JNK inhibition. *J Biol Chem.* 2006;281(26):17552–8.
51. Liebermann DA, Tront JS, Sha X, Mukherjee K, Mohamed-Hadley A, Hoffman B. Gadd45 stress sensors in malignancy and leukemia. *Crit Rev Oncog.* 2011;16(1–2):129–40.
52. Zhu N, Shao Y, Xu L, Yu L, Sun L. Gadd45-alpha and Gadd45-gamma utilize p38 and JNK signaling pathways to induce cell cycle G2/M arrest in Hep-G2 hepatoma cells. *Mol Biol Rep.* 2009;36(8):2075–85.

## Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Ready to submit your research? Choose BMC and benefit from:**

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

**At BMC, research is always in progress.**

Learn more [biomedcentral.com/submissions](https://biomedcentral.com/submissions)

