Multiple treatments with human embryonic stem cell-derived mesenchymal progenitor cells preserved fertility and ovarian function of perimenopausal mice undergoing natural aging.

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Research Article

Keywords: Human embryonic stem cell-derived mesenchymal progenitor cells (hESC-MPCs), Ovarian function, Reproductive senescence, Fertility preservation of natural aging female, Myeloid-derived suppressor cells (MDSCs)

Posted Date: December 4th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3652097/v1

Version of Record: A version of this preprint was published at Stem Cell Research & Therapy on March 3rd, 2024. See the published version at https://doi.org/10.1186/s13287-024-03684-6.

Abstract

Objectives: There is no approved therapy to preserve ovarian health with aging. To solve this problem, we developed a long-term treatment of human embryonic stem cell-derived mesenchymal progenitor cells (hESC-MPCs) and investigated whether the cells retained the ability to resist ovarian aging, leading to delayed reproductive senescence.

Materials and Methods: In a middle-aged female model undergoing natural aging, we analyzed whether hESC-MPCs have a beneficial effect on the long-term maintenance of reproductive fecundity and the ovarian reservoir or how their transplantation regulates ovarian function.

Results: The number of primordial follicles and mice with regular estrous cycles were increased in perimenopausal mice underwent multiple introductions of hESC-MPCs compared to age-matched controls. The level of estradiol in the hESC-MPC group was similar to that of the young and adult groups. Embryonic development and live birth rate were increased in the hESC-MPC group compared with the control group, suggesting a delay in ovarian senescence by hESC-MPCs. In addition to the direct effects on the ovary, multiple-treatments with hESC-MPCs reduced ovarian fibrosis by downregulating inflammation and fibrosis-related genes via suppression of myeloid-derived suppressor cells (MDSCs) produced in bone marrow.

Conclusions: Multiple introduction of hESC-MPCs could be a useful approach to maintain ovarian function in female reproductive aging and that these cells are promising sources for cell therapy to postpone the ovarian aging and retain fecundity in perimenopausal women.

INTRODUCTION

The ovary has two main roles, as the female reproductive organ and a hormone secretion system. The ovaries function cyclically; in humans, their function declines with age from the mid-30s, and their menopause-disrupting endocrine function starts mid-50s as well (1). In fact, ovarian function declines early relative to that of other organs in the body and is also implicated in forecasting ovarian menopause (2). The mechanism underlying reduction in ovarian function with aging is a major question in reproductive medicine and has been investigated for several decades. In addition to the loss of ovarian reproductive function, menopause is an unavoidable part of aging and involves the deterioration of general health, such as by increasing the risks of cardiovascular disease, osteoporosis, vasomotor symptoms, depression, and cognitive impairment (3). Reproductive aging and menopause are increasingly significant health issues because more women plan to delay childbearing, and their life expectancy has increased (4). However, although many approaches have been applied to maintain the ovarian reservoir and to extend reproductive potential, effective clinically applicable protocols have not been identified.

A recent study focused on preventing pathological changes in ovarian cells by mTOR inhibition, which postponed follicular activation and development and then extended the follicle reserve and endocrine

function of the ovaries (1). Additionally, ovarian fibrosis in stromal interstitial tissues surrounding follicles is the key causal factor of obesity-induced ovarian dysfunction and the process is similar to that of reproductive aging (5). Indeed, a reduction in adverse factors, inducing a high level of inflammation, reactive oxygen species (ROS), DNA damage, and apoptosis, could improve follicle quality and ovarian lifespan (6–9).

Furthermore, several studies have focused on the biomolecular interactions taking place in human bone marrow during aging. In addition to remodeling of the mesenchymal stromal cell population in bone marrow (10), changes in macrophages and macrophage-like immature cells (myeloid-derived suppressor cells, MDSCs) also occur during aging in healthy or tumor-bearing hosts (11). MDSC consist of two large groups of cells: granulocytic MDSCs (G-MDSCs), which are phenotypically and morphologically similar to neutrophils, and monocytic MDSCs (M-MDSCs), which are similar to monocytes (12). These cells suppress innate and adaptive immunity and promote aging-related fibrosis and have been found in many other abnormal conditions, such as autoimmunity, infection, diabetes and cardiac aging (13, 14). Several studies have reported that increased MDSC numbers contribute to the development of these disorders, and the transplantation of mesenchymal progenitor cells (MPCs) obtained from various sources has beneficial effects on blocking or delaying the progression of diseases through suppression of inflammation and immune attack (15–17).

MPCs derived from adult and fetal tissues have been proposed as a new therapeutic cell source for the clinical treatment of various diseases (18). In fact, MPCs secrete multiple cytokines and growth factors, that can regulate immune reactions, apoptosis, cell survival and cell proliferation (19). However, the application of these tissue-specific MPCs still have some drawbacks; for example, the harvesting of MPCs demands invasive surgical procedures that may cause severe side effects and the manufacturing protocol for MPCs is difficult to standardize because of donor heterogeneity. Furthermore, MPCs have limited cell growth capacity in culture; therefore, it is difficult to obtain sufficient quantities for clinical use (20–22).

Recently, human embryonic stem cell-derived MPCs (hESC-MPCs) have been suggested as an alternative source to MPCs owing to their high proliferative capacity and ease of standardization. Our previous studies and many others have demonstrated that MPCs can restore function in models of premature ovarian insufficiency (23–25) and various other diseases (26–28). However, in a middle-aged female model undergoing natural aging, it was not determined whether hESC-MPCs have a beneficial effect on the long-term maintenance of reproductive fecundity and the ovarian reservoir or how their transplantation regulates ovarian function. In the present study, we found that multiple introductions of hESC-MPCs may contribute to preserving ovarian function in perimenopausal female mice by suppressing apoptosis and fibrosis and may maintain oocyte competence and delay reproductive senescence.

MATERIALS AND METHODS

Maintenance of hESC-MPCs

Differentiation and characterization of human ESC-MPCs were performed as described in our previous reports (23, 25, 29). Briefly, human CHA-hES15 ESCs (Korea Stem Cell Registry No. hES12010028) were detached by a mechanical method using a glass pipette (Corning, Corning, NY) and cultured in a Petri dish (Corning) for embryoid body (EB) formation. Fourteen days after EB formation, the cells were attached to culture dishes and outgrowth cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/low glucose (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, Gibco, Franklin Lakes, NJ), 0.1 mM beta-mercaptoethanol (Invitrogen, Carlsbad, CA), 1% nonessential amino acids (NEAAs, Gibco), and 1% penicillin-streptomycin (P/S, Gibco). Sixteen days after EB attachment, the outgrowth cells were subcultured and further maintained in DMEM/F12 medium (Gibco) supplemented with 10% FBS, 1% NEAA, 1% P/S, and 0.1 mM mercaptoethanol. These cells were defined as human ESC-MPCs and were used in this study. The characteristics of human ESC-MSCs were described in our previous reports (25, 29).

Animal experiments

Animals were housed in the Animal Care Facility of CHA University according to the institutional guidelines for laboratory animals under temperature- and light- controlled conditions with a 12-h daily cycle and were fed *ad libitum*. The experimental protocols for the use of animals were approved by the Institutional Animal Care and Use Committee of CHA University (IACUC 190050, 190176, and 200130).

To prepare the natural perimenopausal mouse model, 6-month-old mice were purchased from KOATECH (Pyeongtaek, Gyeonggi-do, Korea) and maintained until 10 months of age. Next, 10-month-old female mice with a regular estrous cycle were randomly assigned into two groups: a phosphate-buffered saline-injected control group (CON group) and an hESC-MPC-injected group (hESC-MPC group). hESC-MPCs were introduced by intravenous injection every month for 4 months to delay ovarian aging. To evaluate the reproductive performance of perimenopausal mice, 14-month-old female mice were individually caged with a young male for mating. The pregnancy rates and mean litter size were recorded on Day 18.5 postconception.

Estrous cycle analysis

Vaginal smears were collected in 100 μL of PBS at 9:00–10:00 every morning for 2 weeks. Smear samples were dropped on a glass slide and then air dried on a warm plate. Dried samples were stained with hematoxylin and eosin. The estrous cycle stages were determined as in a previous study (30). Consistent cycles of proestrus (Pro), estrus (Est), metestrus (Met), and diestrus (Di) repeated every 4–5 days were considered a "regular estrous cycle" (30). Irregular estrous cycles were defined as when the mice had at least one prolonged estrous cycle (more than 5 days) before the end of the observation period. The experiments were repeated four times (total n = 21 in the CON group; total n = 27 in the hESC-MPCs group), and the results are presented as the mean ± standard error of the mean (SEM).

Ovarian follicle counting

Ovarian follicle counting was performed as described in our previous reports (23). Briefly, the ovary was fixed in 4% formaldehyde and embedded in paraffin. Each ovary was serially sectioned to 5 μm thickness and stained with hematoxylin and eosin to evaluate follicle growth. Follicle counts were conducted on serially cut sections, counting every tenth section. Follicles (primordial, primary, secondary, antral follicles and zona pellucida remnants) were classified and counted. The follicles were classified as previously described (31). The percentage of follicles at each stage was calculated and compared between each group. The results are expressed as the mean ± SEM. (total n = 6 in the CON group; total n = 6 in the hESC-MPCs group).

Enzyme-linked immunosorbent assay (ELISA)

Plasma was harvested, and the levels of 17β-estradiol (E_2) and FSH were evaluated with ELISA kits (MyBioSource, San Diego, CA) according to the manufacturer's instructions. Briefly, 50 μl of blank, standard or plasma sample was added to each well. Then 50 μl of biotin-labeled antibody working solution was added to each well. The microplate was incubated for 45 min at 37°C. The plate was washed with wash buffer 3 times. Thereafter, 50 μl of HRP-streptavidin conjugate reagent was added to each well and incubated for 30 min at 37°C. The plate was washed with wash buffer 5 times. Then, 90 μl of TMB substrate was added to each well and incubated for 15 min at 37°C in the dark. Then, 50 μl of stop solution was added to each well and the absorbance (O.D. 450) was measured and recorded by a microplate reader (Varian Company, Australia). (total n = 21 in the CON group; total n = 20 in the hESC-MPCs group).

In vitro fertilization and embryo development

Epididymal sperm from 8 to 10-week-old males C57BL/6N mice were collected in 500 μL of HTF medium (Millipore) and allowed to capacitate for 1 hour before use. Cumulus-oocyte complexes (COCs) were inseminated for 4–5 hours in a 50 μL drop of HTF medium and then the fertilized embryos were quickly washed in drops of KSOM medium (Millipore) by using a pasture pipette to remove unbound sperm and cumulus cells. Embryos were cultured in KSOM medium in a humidified atmosphere with 5% CO_2. The number of blastocysts was counted on Day 5. Experiments were repeated at least 5 times.

Bone marrow cell harvest and flow cytometry

Bone marrow (BM) was harvested from femurs by flushing with RPMI medium. Cells were harvested by centrifugation at 800 x g for 2 min and incubated in 1X RBC lysis buffer for 3 min. The cells were washed and harvested again by centrifugation with the addition of PBS containing 1% FBS. The BM cells obtained were passed through a 70-μm cell strainer. The cells were incubated with fluorochrome-conjugated anti-mouse antibody for 1 hour at 4°C as follows. V450 mouse lineage antibody cocktail (BD), rat anti-mouse Ly-6A/E (Sca-1)-PE-Cy™7 (BD), and rat anti-mouse CD117 (c-Kit)-APC (BD) antibodies were stained together to quantify the frequency of $Lin^-Sca1^+c\text{-}Kit^+$ (LSK) cells. A rat anti-mouse CD34-FITC antibody (BD) was used to further distinguish the LSK population from the long-term (LT) and short-term (ST) hematopoietic stem cell (HSC) populations. The MDSC population was identified with a combination of antibodies against CD11b-PE-Cy™7 (Invitrogen), Ly6G-FITC (Invitrogen) and Ly6C-APC

(Invitrogen). Dead cells were excluded from the analysis by staining with 7-amino-actinomycin D (BD). The expression of the stained antibodies was measured using a FACSCantoTM II flow cytometer (BD Biosciences), and the acquired data were analyzed with FlowJo software (Tristar).

Sirius red staining

Paraffin sections of ovarian tissue were exposed to xylene to remove paraffin and rehydrated through sequential concentration changes starting with 100% ethanol, then 70% ethanol, and finally deionized water. Fibrotic areas in ovarian tissue sections were detected by sirius red staining (Abcam). The sections were incubated with a picro-sirius red solution targeting collagen for 60 minutes and then rinsed with acetic acid solution. The sections were dehydrated in absolute alcohol, and the slides were mounted and analyzed under a light microscope.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA isolation and cDNA synthesis were performed as previously described (32). SYBR Green PCR master mixes (Enzynomics, Daejeon, South Korea) were used, and all target genes were calculated using the comparative CT method. The expression levels of all genes were normalized to that of Gapdh. The primer sequences were *Gapdh*-F (TCAAGAAGGTGGTGAAGCAGG) *Gapdh*-R (CACATACCAGGAAATGAGCTT), *IL10*-F(TGGCCCAGAAATCAAGGAGC), *IL10*-R (CAGCAGA CTCAATACACACT), *IL6*-F (AGGATACCACTCCCAACAGACCT), *IL6*-R(CAAGTGCATCATCGTTGTTCATAC), *iNos*-F (ATTCAGATCCCGAAACGCTTCA), *iNos*-R (CCAGAACCTCCAGGCACACAGT), *Arg1*-F (AGCACTGAG GAAAGCTGGTC) and *Arg1*-R (TACGTCTCGCAAGCCAATGT).

Statistical analysis

All experiments were repeated at least three times. The results are shown as the means ± SEM. The statistical significance of differences was evaluated by one-way ANOVA with Duncan's post hoc test using SPSS ver. 18 software (SPSS Inc., Chicago, IL). Embryo development rates were analyzed by Student's *t* test in GraphPad Prism 7.0 software (GraphPad Software, Inc., CA, USA). *P* values < 0.05 were regarded as statistically significant.

RESULTS

hESC-MPCs contribute to maintaining estrus cycle regularity in perimenopausal mice

Reproductive cycles in middle-aged perimenopausal mice were analyzed after multiple-introductions of hESC-MPCs to evaluate the potential therapeutic function of these cells in reproductive aging. The cell introduction experiment was started in naturally aged mice at 10 months (44-weeks), which have ovarian function similar to that in human perimenopause (33). The evaluation of reproductive aging was tested in mice at 14 months (60-weeks), which have ovarian function similar to that in human menopause (Fig. 1A and Fig. 2A). Prior to the main experiments, we performed a preliminary experiment to determine the

appropriate number of cell introductions in the naturally aged mouse model (Fig. 1). The mice were maintained until 10 months of age (44 weeks of age), and then hESC-MPCs were introduced intravenously 1 time (1° I.V) and 4 times at monthly intervals (4° I.V). As shown in Fig. 1, the levels of sex hormones in the plasma showed a consistent pattern (plasma E2: 262.9 ± 15.6 pg/ml in 3-month-old, 271.1 ± 31.5 pg/ml in 10-month-old adult, 181.8 ± 8.2 pg/ml in 14-month-old control, 218.3 ± 8.3 pg/ml in 14-month-old 1° I.V, and 246.1 ± 12.5 pg/ml in 14-month-old 4° I.V; plasma FSH: 12.4 ± 0.8 ng/ml in 3-month-old, 16.9 ± 2.4 ng/ml in 10-month-old adult, 20.8 ± 1.4 ng/ml in 14-month-old control, 19.3 ± 1.5 ng/ml in 14-month-old 1° I.V, and 19.0 ± 1.3 ng/ml in 14-month-old 4° I.V). Therefore, we expected that multiple treatments of hESC-MPCs would have a better effect on fertility preservation in perimenopausal mice, and the 4° I.V group was chosen as the hESC-MPCs group (the mice were maintained until 10 months of age, and then hESC-MPCs were introduced intravenously at monthly intervals for 4 months) in the main study. Mice injected intravenously with saline as a mock control for the same duration were used as the control group (Fig. 2A). The estrous cycles, consisting of 4 stages, proestrus (Pro), estrus (Est), metestrus (Met), and diestrus (Di), were checked (Fig. 2B and 2C) and consistent cycles of Pro, Est, Met, and Di repeated every 4–5 days were referred to as a “regular estrous cycle” (Fig. 2D) (34).

Because reproductive aging can increase the length and irregularity of the estrous cycle, we evaluated the estrous cycles in 14-month-old mice with or without hESC-MPC introduction. In the main study, only 10-month-old mice showing regular estrous cycles were enrolled for the experiment. As cycle regularity was extremely low in 14-month-old mice in the control group, age-matched mice in the hESC-MPCs group showed relatively high estrous cycle regularity (hESC-MPCs: 24.7 ± 2.7% vs. CON: 3.1 ± 3.1%, $p < 0.05$) (Fig. 2E), suggesting restoration of ovarian function by hESC-MPCs.

hESC-MPCs maintained ovarian structure and function in perimenopausal mice

To further investigate whether hESC-MPC administration can delay the exhaustion of ovarian reserve, histological analysis of ovaries was performed (Fig. 3A). The number of primordial follicles, which represent ovarian reserve, was significantly higher in the hESC-MPCs group (31.3 ± 8.2) than in the age-matched control group (19.2 ± 5.0). On the other hand, the number of zona pellucida remnants (ZPRs), which are considered atretic follicles, was much lower in the hESC-MPCs group (hESC-MPCs: 19.5 ± 6.8%, CON: 111.4 ± 33.3%, $p < 0.05$; Fig. 3B). To rule out the possibility that the difference in contents occurred due to individual variation in ovarian volume, the ratio of each follicle count was normalized to the total number of follicles and compared (Fig. 3C). Interestingly, most follicles in the 14-month-old control group were ZPRs (51.7 ± 12.9); in contrast, the hESC-MPCs group had a significantly lower proportion of ZPRs (24.0 ± 4.6, $p < 0.01$). In addition, consistent with Fig. 3B, hESC-MPCs had a significantly higher proportion of primordial follicles than the 14-month-old control group (hESC-MPCs: 40.7 ± 5.6%, CON: 10.7 ± 3.2%, $p < 0.01$; Fig. 3C). These results suggest that hESC-MPC treatment can delay the exhaustion of ovarian reserve and may generate good-quality oocytes in even perimenopausal ovaries.

Recently, several studies reported that stromal fibrosis is an indicator of natural ovarian aging (5, 35, 36). We investigated whether hESC-MPC treatment may reduce of the reproductive age-associated fibrosis in the ovary. Picrosirius red (PSR), which stains that stained connective tissue, especially collagen I and III fibers, was applied to sampled ovaries. The fibrotic collagen fibers in ovaries were well stained intense red with PSR and increased in the 14-month-old control group (Control, Fig. 3D). However, multiple treatments of hESC-MPCs reduced this age-dependent ovarian fibrosis. These results suggest that hESC-MPCs can maintain the young ovarian environment by reducing ovarian fibrosis that might be associated with aging.

hESC-MPCs inhibit the increase in aging-related G-MDSCs and cellular inflammation in perimenopausal mice

Aging-related chronic inflammation contributes to fibrosis and a reduction in organ function (37). Thus, we examined the expression levels of inflammation- related genes in ovaries to determine whether aging-associated fibrosis in the ovary is related to an inflammation-mediated response. As shown in Fig. 4A, the expression of proinflammatory cytokines (IL6) and profibrotic cytokines (IL10 and Arg1) was significantly upregulated in the 14-month-old mouse group compared to young and 10-month-old mice groups. Importantly, hESC-MPC introduction effectively reduced the expression levels of these cytokines. We found that the total number of BM cells was significantly increased in the 14-month-old mouse group compared to the 10-month-old mouse group (Fig. 4B). In addition, the number and frequency of CD11b + cells, which may be monocyte, macrophages or granulocytes, were significantly decreased by transplantation of hESC-MPCs (Fig. 4C). These findings suggested that pro-inflammation and pro-fibrotic changes in aged mouse ovaries may be related to an altered hematopoietic composition in the BM.

The immunosuppressive and anti-inflammatory roles of MDSCs have been well demonstrated in fibrotic diseases in several tissues (38). Therefore, we next asked whether transplantation of hESC-MPCs into aged mouse affected the number and frequency of MDSCs in the BM. Mouse MDSCs are classified into two types based on the degree of expression of Ly6G and Ly6C and the expression of CD11b as follows: $CD11b^{+}Ly6G^{hi}Ly6C^{low}$, granulocytic MDSCs (G-MDSCs); $CD11b^{+}Ly6G^{low}Ly6C^{hi}$, monocytic MDSCs (M-MDSCs) (Fig. 4D). We found that the number and frequency of the G-MDSC subset in 14-month-old-mice were significantly reduced by transplantation of hESC-MPCs (Fig. 4E and 4F). These results suggest that hESC-MPCs may suppress the expansion of MDSCs with age and have beneficial anti-inflammatory and antifibrotic effects in the aging ovary.

hESC-MPCs improve the ovulation of mature eggs and embryo development in perimenopausal mice

We investigated whether hESC-MPCs can improve the ovulation of mature eggs and embryo development, which were decreased by aging. Although the percentage of ovulated mice did not differ among the three groups (Fig. 5A), the number of recovered (ovulated) eggs per mouse was significantly reduced in both groups of 14-month-old mice (14-month-old control, 3.6 ± 0.1 and hESC-MPCs, 4.6 ± 0.3 group) compared to 3-month-old mice (18.7 ± 2.6; Fig. 5B). Furthermore, the rate of blastocyst formation

was reduced with age (26.8 ± 6.4). In contrast, the hESC-MPCs group had significantly improved blastocyst formation (53.4 ± 7.4) compared with the 14-month-old control, to levels similar to that in the young control (48.7 ± 6.8; Fig. 5C and D). These results collectively suggest that hESC-MPCs successfully delayed reproductive senescence in perimenopausal mice.

hESC-MPCs rescue poor reproductive outcomes in perimenopausal mice

To further investigate the therapeutic function of hESC-MPCs on pregnancy outcomes in perimenopausal mice, we evaluated reproductive parameters, including mating rate, delivery rate, number of offspring and litter size, in both groups after hESC-MPC therapy. Female mice were mated with young adult male mice in both groups. The values for all parameters were significantly lower in perimenopausal (14-month-old) mice than in young control (3-month-old) mice; (all values shown in Fig. 5). On the other hand, hESC-MPC-treated mice had similar mating rates (Fig. 5E) and delivery rates (Fig. 5F) as young controls. Although litter size was not significantly different from that of 14-month-old control and hESC-MPC-treated mice (Fig. 5H), the hESC-MPCs group had a significantly increased average number of offspring per female (Fig. 5G). These results suggest that fertility can be partially maintained by hESC-MPCs even with age.

DISCUSSION

It is well known that menopause, or reproductive senescence, occurs with aging. In fact, hormone replacement therapy and assisted reproductive technologies are used for patients with advanced maternal age who hope to achieve pregnancy. However, these approaches have shown a low therapeutic effect in patients and do not contribute to maintaining fertility. Recently, to overcome these issues, stem cell therapy using mesenchymal progenitor cells/mesenchymal stem cells (MPCs/MSCs) from various adult or fetal tissues has been applied to achieve a therapeutic effect and inhibit reproductive senescence (39, 40). hESC-MPCs have been suggested as an alternative to tissue-derived MPCs due to their high proliferative capacity and ease of to standardization, but their therapeutic properties for maintaining ovarian function in perimenopausal females have not been determined. In the present study, we found that multiple introductions of hESC-MPCs may delay ovarian aging in perimenopausal female mice by reducing fibrosis and may contribute to preserving ovarian function.

Menopause is defined as cessation of the menstrual cycle for 12 months or more and is mainly caused by decreased secretion of 17β-estradiol (41). Menopause can be caused by surgery or cancer treatments (42). As women age, the menstrual cycle becomes less regular, and the amount of FSH increases in response to reduced levels of ovarian hormones. This process is called “menopausal transition”, “around menopause” or “perimenopause”. Perimenopause usually begins in women between 35 and 45 years of age, and menopause occurs at 47–51 years of age (43). Since human perimenopause occurs around the age of 38, according to the previous calculation method (6, 33), it can be expected that reproductive senescence will occur in mice from approximately 10 months of age. Additionally, the mean age of

menopause is approximately 51 years in women and is considered to be 15 months in mice. As shown in Fig. 2C-E, the rate of regular estrous cycles was significantly increased in the hESC-MPC-injected group compared with the control group, and the levels of E_2 and FSH in the hESC-MPC-injected group were more similar to those in the young adult group than the middle-aged control group (Fig. 1B and C). In addition, the percentage of primordial follicles was significantly higher in the treated group than in the age-matched control group. A significantly decreased number and proportion of ZPRs was found in the hESC-MPCs group compared to the control group (Fig. 3B and C). Consistent with these observations, not only reproductive performance but also the quality of blastocysts of perimenopausal mice were improved by hESC-MPC injections (Fig. 5). Based on these results, we have confirmed the therapeutic potential of hESC-MPCs for delaying ovarian aging and preserving fecundity in perimenopausal mice.

Recent studies have reported that various adverse factors that could induce inflammation, ROS, DNA damage, and apoptosis decrease follicle quality and ovarian lifespan (7–9). For example, the levels of the proinflammatory cytokine IL-1a are enriched in developing follicles, and their ovarian mRNA levels increase with age. In fact, IL-1a knockout significantly increased female fertility in 12-month-old mice (9). In addition, increased collagen deposition, indicative of tissue fibrosis, has been documented in the ovaries of postmenopausal women and animal models of reproductive aging (35, 44). Targeted removal of fibrotic collagen from the mouse ovary may extend the female reproductive lifespan (5). Therefore, the cellular events that cause ovarian fibrosis should be determined. In the present study, we also observed an age-dependent increase in ovarian fibrosis, which was reduced after multiple introductions of hESC-MPCs (Fig. 3D). To identify regulators of hESC-MPC-mediated ovarian fibrosis, we analyzed the status of bone marrow components among the experimental groups. Cell counts of BM and HSCs were similar in all groups. However, the number of MDSCs in the hESC-MPC group was decreased compared with that in the control group (Fig. 4B-F). Several studies have verified the expansion of MDSCs with age (11, 45) and MDSCs have a role in inducing aging-related cardiac fibrosis (13). In addition to increased numbers of MDSCs, there were a significant change in the levels of pro-inflammatory (IL6) and pro-fibrosis (IL10 and Arg1) cytokine molecules in the ovaries in the hESC-MPC group (Fig. 4A). In previous studies, intravenously delivered hESC-MPCs had a positive role in restoring of cisplatin-induced ovarian damage by reducing apoptotic signaling in stromal cells and increasing the proliferation of granulosa cells (25). Additionally, by local delivery of those cells in the same mouse model, we found that the secretome of hESC-MPCs may help prevent ovarian degeneration and retain female fecundity (23). Therefore, in the present study, we analyzed the effects of long-term exposure to hESC-MPCs on ovarian function in perimenopausal mice undergoing natural aging.

CONCLUSIONS

To the best of our knowledge, these data suggest firstly that multiple introductions of hESC-MPCs may delay ovarian aging by reducing inflammation and fibrosis via downregulation of MDSC production in BM and extend fertility and fecundity in perimenopausal female mice. Therefore, cell therapy using hESC-

MPCs would be a useful tool for fertility preservation in naturally aging females. However, the development of a safe introduction method into patients is still needed for clinical application.

Abbreviations

ESC
embryonic stem cell
MPC
mesenchymal progenitor cell
G-MDSC
granulocytic myeloid-derived suppressor cell
M-MDSC
monocytic myeloid-derived suppressor cell
mTOR
mammalian target of rapamycin
ROS
reactive oxygen species
EB
embryoid body
DMEM
Dulbecco’s modified Eagle’s medium
FBS
fetal bovine serum
IACUC
Institutional Animal Care and Use Committee
ELISA
Enzyme-Linked Immunosorbent Assay
HRP
horseradish peroxidase
TMB substrate
3,3',5,5'-Tetramethylbenzidinesubstrate
HTF medium
human tubal fluid medium
COCs
Cumulus-oocyte complexes
KSOM medium
potassium-supplemented simplex optimised medium
BM
Bone marrow
LSK

$Lin^{-}Sca1 + c\text{-}Kit^{+}$
I.V.
intravenous
WT
wild type
E2
estradiol
FSH
follicle stimulating hormone
ZPR
zona pellucida remnants
PSR
Picrosirius red

Declarations

ACKNOWLEDGMENTS

We thank all staff of ESC Research Team of CHA Advanced Research Institute for maintenance and differentiation of human PSCs.

AUTHOR CONTRIBUTIONS

E.Y.S and S.J contributed equally to this work. D.R.L. and S.H.H. conceived the project. E.Y.S, S.J, S.H.H., D.K.H and D.R.L. designed the experiments. E.Y.S., S.J., J.E.L., D.J. performed *in vitro* and *in vivo* experiments. E.Y.S, S.J., D.K.H, S.H.H. and D.R.L. wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

This research is partly supported by the Korean Fund for Regenerative Medicine (KFRM, 23A0206L1) of MSICT and MHW, and the Bio & Medical Technology Development Program (2022M3A9I2082319) and Basic Science Research Program (2019R1A6A1A03032888) of NRF of Republic of Korea.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that the dataset supporting the conclusions of this study is included within the article. The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experiments using hESCs were performed under authorization from the Institutional Review Board for Human Research at the CHA University Seongnam, Korea (Title of the approve project: Development of applying technology of homozygous somatic cell nuclear transferred-embryonic stem cells and its derivatives; Name of the institutional approval committee: CHA University Institutional Review Board; Approval number and date: 1044308-201712-LR-051-03 (Jan. 24, 2019) and 1044308-201712-LR-051-04 (Feb. 11, 2020)).

The experimental protocols for the use of animals were approved by the Institutional Animal Care and Use Committee of CHA University (Title of the approve project: A study for improvement of ovarian reproductive function using human embryonic stem cell-derived mesenchymal progenitor cells; Name of the institutional approval committee: CHA University IACUC; Approval number and date: IACUC 190050 (July 24, 2018), IACUC 190176 (July 31, 2019), and IACUC 200130 (Aug. 18, 2020)).

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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Figures

Figure 1

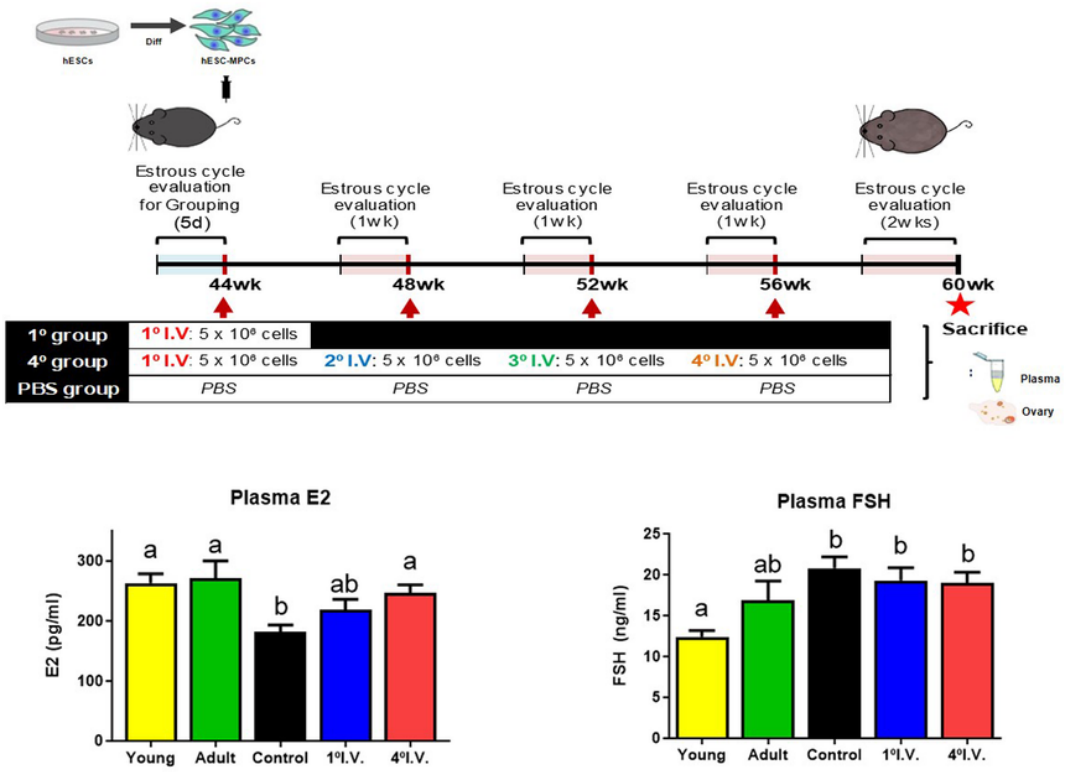

Figure 1

Effect of the number of human embryonic stem cell-derived mesenchymal progenitor cells (hESC-MPCs) on the plasma hormone levels in perimenopausal mice. (A) Experimental scheme of a preliminary experiment to determine the appropriate number of cell introductions. (B and C) The levels of sex hormones (serum 17β-estradiol (E2) and FSH) were measured by ELISA in plasma from various experimental groups (**young**, 3-month-old mice; **adult**, 10-month-old mice; **control**, 14-month-old mice; **1° I.V.**, 14-month-old mice treated once with hESC-MPCs intravenously at 10 months of age; **4° I.V.**, 14-month-old mice treated monthly for 4 months with hESC-MPCs. Different superscript letters indicate significant differences ($p < 0.05$).

Figure 2

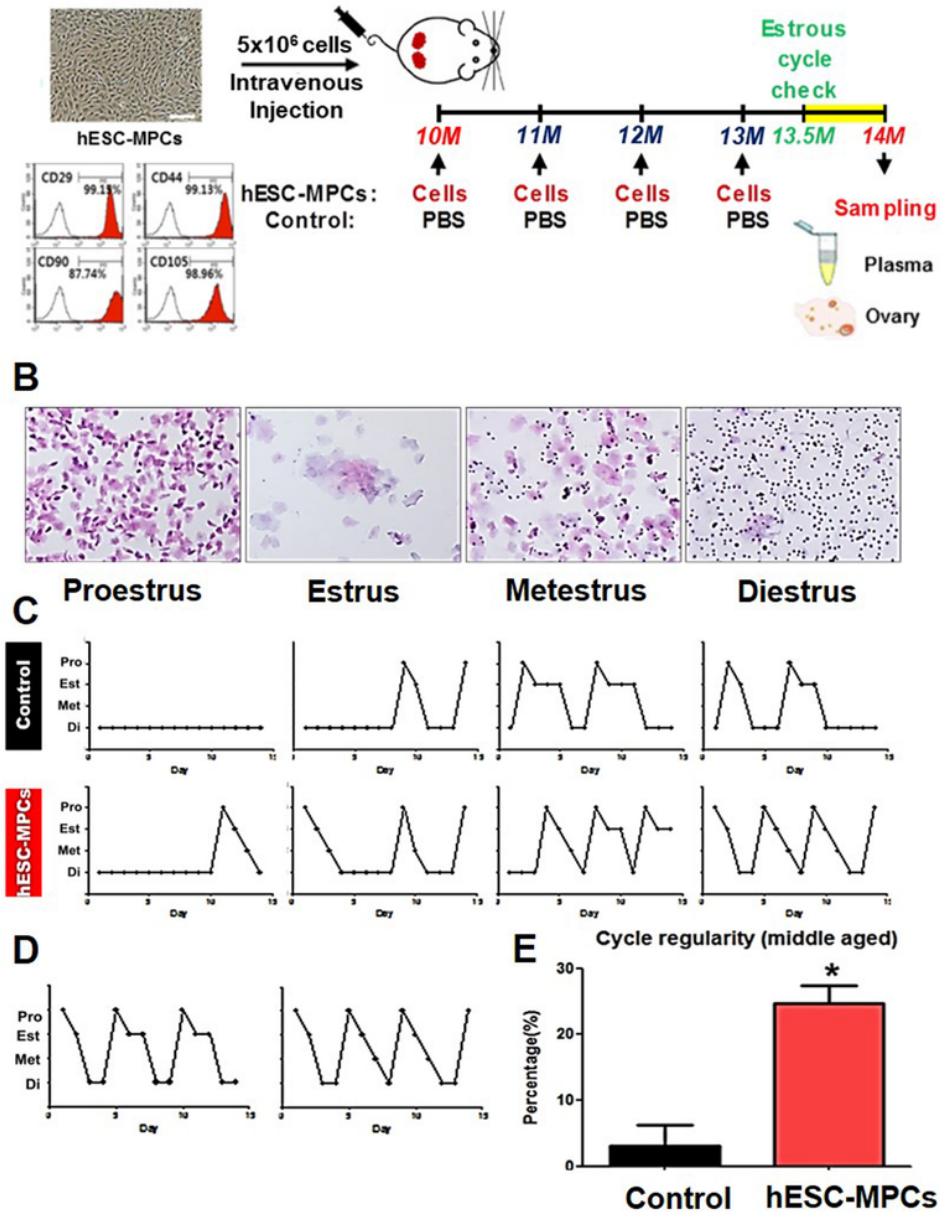

Figure 2

Effect of human embryonic stem cell-derived mesenchymal progenitor cells (hESC-MPCs) on the estrous cycles in perimenopausal mice. (A) Schematic illustrations of the experimental procedures used to evaluate the therapeutic effects of hESC-MPCs on reproductive aging in perimenopausal mice. (B) Representative images from hematoxylin-eosin (HE)-stained samples from vaginal lavage at each phase of the estrous cycle in C57BL/6 mice. (C) Staged estrus cycles of individual middle-aged control (14-month-old mice, upper) and hESC-MPC-multiple introduced mice (lower) (Pro, proestrus; Est, estrus; Met, metestrus; Di, diestrus). (D) Examples of regular estrous cycles within 2 weeks. (E) Estrus cycles were more regular in the hESC-MPCs group than in the control group as revealed by continuous vaginal smear continuous detection. *, significantly different ($p < 0.05$) (total n = 21 in the CON group; total n = 27 in the hESC-MPCs group). (total n = 20 in the CON group; total n = 21 in the hESC-MPCs group).

Figure 3

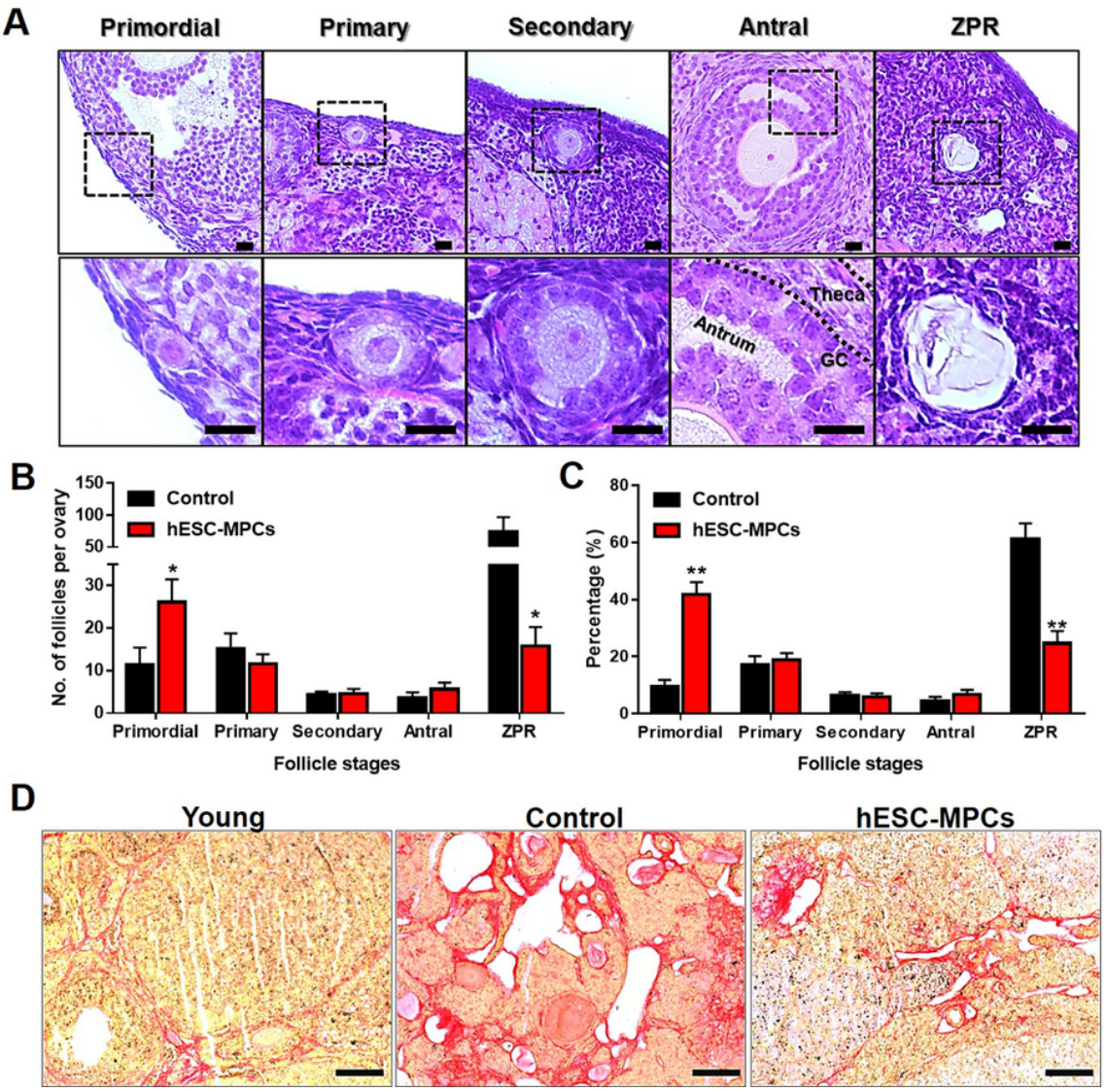

Figure 3

Maintenance of ovarian reserve by multiple introduction of human embryonic stem cell-derived mesenchymal progenitor cells (hESC-MPCs) in perimenopausal mice. (A) Follicles at different developmental stages in the ovaries. Scale bars = 20 μm. (B) Numbers of different types of follicles per ovary. (C) Quantification of the percentage of each follicle type per ovary. Significant differences are indicated (*$p < 0.05$, **$p < 0.005$). Theca, theca cell; GC, granulosa cell; Primordial, primordial follicle; Primary, primary follicle; Secondary, secondary follicle; Antral, antral follicle; ZPR, zona pellucida remnant. (total n = 6 in the CON group; total n = 6 in the hESC-MPCs group). (D) Representative images of picrosirius Red (PSR)-stained ovarian tissues from 3-month-old (young), 14-month-old (control), and 14-month-old mice treated monthly for 4 months with hESC-MPCs (hESC-MPCs). The intense red staining correlates with fibrotic regions.

Figure 4

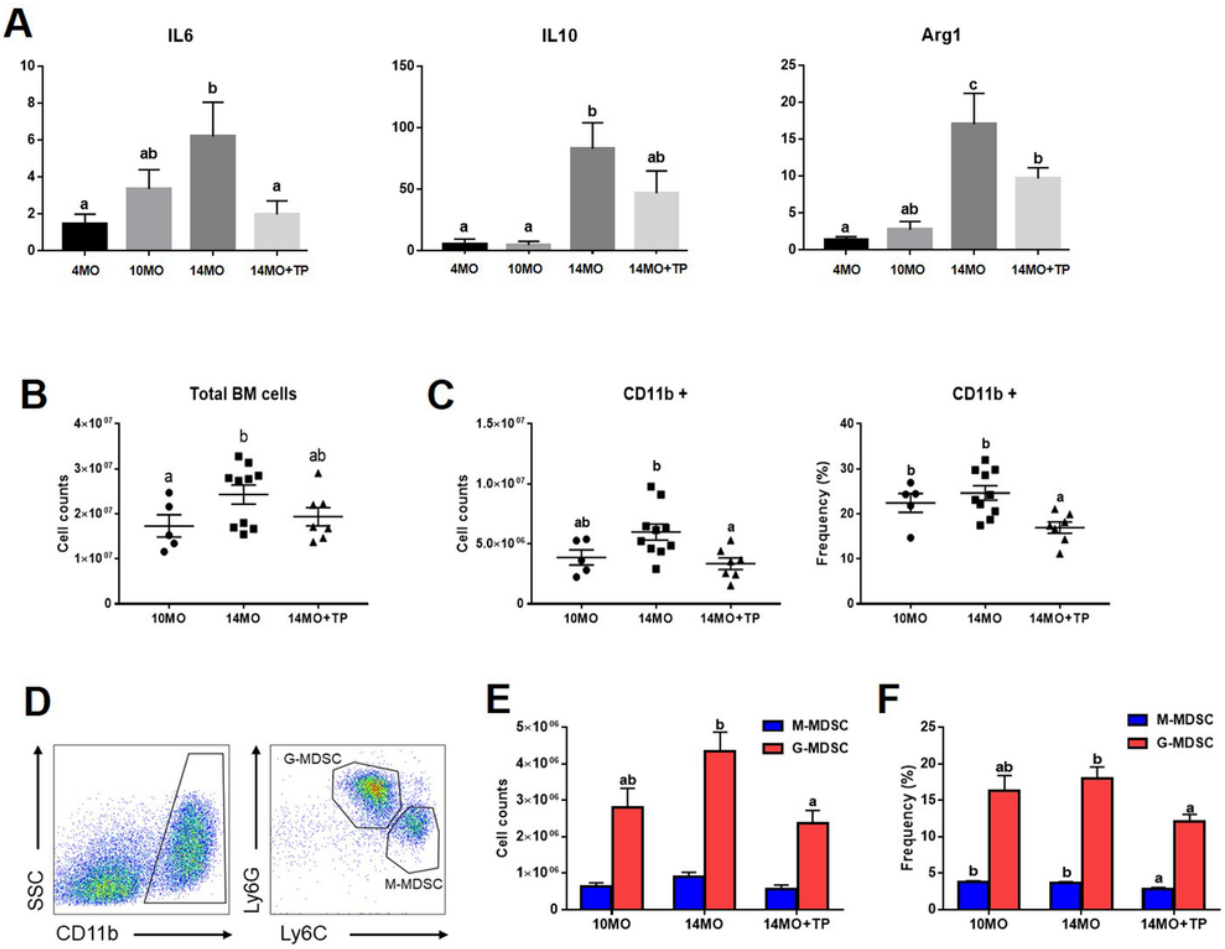

Figure 4

Effects of hESC-MPCs on the number of bone marrow (BM)-derived myeloid-derived suppressor cells (MDSCs) and inflammation-related genes in aged ovaries. (A) qRT-PCR analysis for of expression of IL6, IL10, and Arg1 in mouse ovaries from the 4-, 10- and 14-month-old groups treated/not treated with ESC-MPCs. (B) Total counts of BM cells from each group. (C) Cell counts and frequency of $CD11b^{+}$ cells in BM. (D) Representative FACS plots of the G-MDSC ($CD11b^{+}Ly6G^{hi}Ly6C^{low}$) and M-MDSC ($CD11b^{+}Ly6G^{low}Ly6C^{hi}$) populations in BM. (E-F) Cell counts and frequency of G-MDSCs and M-MDSCs. Different superscript letters indicate significant differences (p<0.05).

Figure 5

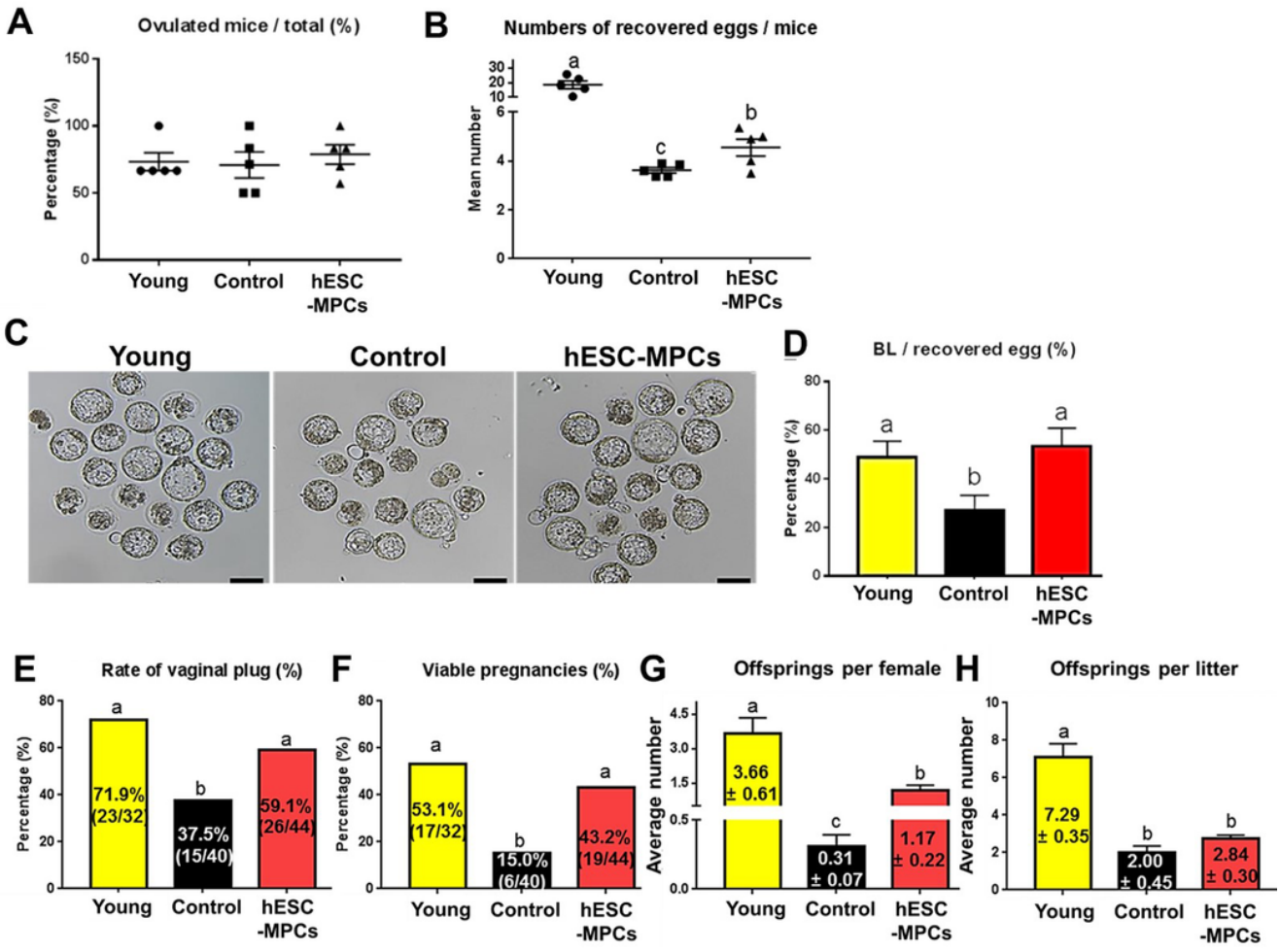

Figure 5

Effect of human embryonic stem cell-derived mesenchymal progenitor cells (hESC-MPCs) on ovulation, embryonic development, pregnancy and litter sizes in perimenopausal mice. (A) Ovulation rate, calculated as the number of ovulated mice per total mice. (B) The average number of eggs recovered per mouse. (C) Representative images of embryos after 120 hours of culture *in vitro*.Scale bars = 50 μm. (D) Percentage of blastocyst formation after in vitro fertilization. (E-H) Female mice from the young group and experimental group were individually caged with a young male for mating. (E) Percentage of plug-positive mice per total mice. (F) Percentage of mothers that delivered viable fetuses per mouse. (G) Average number of offspring per mouse. (H) Average number of offspring per litter. Three-month-old (young), 14-month-old (control), and 14-month-old mice treated monthly for 4 months with hESC-MPCs (hESC-MPCs). Different superscript letters indicate significant differences (p<0.05).

Supplementary Files

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