Research Paper: Assessment of In Vitro-Derived Germ Cells Contribution in Oogenesis in Female Mice Ovaries

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ABSTRACT

Introduction: Contrary to a common belief, most mammalian females lose the ability of Germ Cell (GC) renewal and oogenesis during fetal life. Although, it has been claimed that germ line stem cells preserve oogenesis in postnatal mouse ovaries, that postnatal oogenesis keeps producing functional and sufficient GCs in the case of infertility (caused by different reasons) is doubtful. On the other hand, there are many studies showing derivation of primordial GCs and late GCs from Embryonic Stem Cells (ESCs) in vitro. This study aimed to clarify the role of ESC-derived GCs in oogenesis.

Methods: Mouse ESCs via Embryoid Body (EB) formation were differentiated into GC lineage by adding Bone Morphogenetic Protein 4 (BMP4) and Retinoic Acid (RA) to the culture medium. Expression of GC markers was characterized by using Reverse Transcription Polymerase Chain Reaction (RT-PCR) and immunohistochemistry. Several 6- to 10-week-old female mice, sterilized using chemical agents, were injected with ESCs-derived GCs thorough their tail veins. To track the transplanted cells, their ovaries were immunohistochemically stained after two months.

Results: Expression of GC specific markers such as mouse vasa homologue (Mvh) and Deleted in Azoospermia-Like (DAZL) indicated that GCs were successfully developed from ESCs. Interestingly, there was no evidence of homing of GCs in the transplanted ovaries after transplantation of ESCs-derived GCs.

Conclusion: Our findings do not suggest any contribution of ESC-derived GCs within the sterilized mice ovaries.

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1. Introduction

Based on WHO report, up to 15% of reproductive-age couples suffer from infertility worldwide. In men, oligospermia, sperm immobility, varicocele, and so on were common causes of infertility and chlamydia, uterine fibroids, ovulation disorders, polycystic ovary syndrome, and so on were reported in women [1]. Women may definitely encounter pathologic or age-related infertility disorders, too. Females are born with a limited number of oocytes which would gradually disintegrate throughout the life [2]. In this regard, the idea of treating infertility by Stem Cell (SC) transplantation has been successfully tested using laboratory animals as a model. In a study, the investigators transplanted Bone Marrow (BM) and Peripheral Blood (PB) in adult sterilized female mice. Two months after transplantation, a vast range of oocytes in all developmental stages were detected within the ovaries [3]. Other researchers applied transplantation through different approaches and succeeded in detecting oocyte-like cells in the ovaries of female mice [4-7].

Another idea to overcome female infertility, regardless of age or the leading problems, is to produce gametes in vitro. Several scientists have studied derivation of Germ Cells (GCs) from SCs [8-12]. Induction of Embryonic Stem Cells (ESCs) into oocyte like cells was first reported by Hubner et al. [13]. Later, many scientists reported the production of female gonocytes by various strategies and different sources of SCs [8, 14-18]. However, there were no data available about the role of ESC-derived GCs in the sterilized ovaries. In this study, we attempted to determine the role of ESC-derived GCs (if any) in restoring oocyte generation, when the ESCs differentiated into GCs and transplanted into chemotherapy-treated female infertile mice.

2. Materials and Methods

Cell lineage and animal strain

The cell line used in this study was CGR8-GFP+ESC (Bonyakhte Company, Iran). The study strain comprised 6- to 10-week-old C57BL/6 mice (Razi Institute, Iran) that were kept in the Department of Anatomy of Tehran University of Medical Sciences, Tehran, Iran. Animal selection, all experiments, and subsequent care were all done according to the animal care instructions of Iran Veterinary Organization. The study was approved by the Ethics Committee. The animals were caged individually under 12:12 h light/dark cycle, at 22˚C, and humidity of 30%. They were fed by a balanced diet with free access to water. All experiments were done under aseptic conditions. The procedures, care, and sacrifices were the same for all animals.

Culture of embryonic stem cells

ESCs were cultured in knockout Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) containing 15% fetal bovine serum (FBS; Sigma, Germany), 1 mM glutamine (Invitrogen, USA), 100 U/mL penicillin (Sigma, Germany), 100 µg/mL streptomycin (Sigma, Germany), 1x non-essential amino acid (NEAA; Sigma, Germany), 0.1 mM 2-mercaptoethanol (2ME; Sigma, Germany), and 103 U/mL leukemic inhibitory factor (LIF; Sigma, Germany). Reaching an appropriate confluency, the cells were washed with phosphate buffer saline (PBS; Sigma, Germany). Then, using 0.25% trypsin (Sigma, Germany), the cells were detached from the bottom of the dish, centrifuged and thereafter, the supernatant was discarded. Finally, the cells were transferred into a new culture medium.

Embryoid body formation and differentiation

For Embryoid Body (EB) differentiation [19], ESCs were treated with 0.25% trypsin to form single cells. Then, they were transferred into an ESC medium containing 20 ng/mL Bone Morphogenic Protein 4 (BMP4) (GenWay Biotech, USA), without LIF. The cells were plated in hanging drops at a concentration of 800 or 1000 cells per 20 mL in Petri dishes (bacteriological, Sterilin). After 3 days, EBs were accumulated from the lids and then cultured in a medium enriched with 2 mM Retinoic Acid (RA) (Sigma, USA). Then, the dishes were transferred to the incubator (5% CO_2 at 37°C). After one week, EBs were collected from the dishes while 5 groups of EBs were prepared as (i) A group of ESCs without any treatment; (ii) A differentiated group treated with both BMP4 and RA; (iii) A differentiated group treated with BMP4 alone; (iv) A differentiated group treated with neither BMP4 nor RA, and (v) A differentiated group treated with RA alone.

Reverse transcription polymerase chain reaction

To evaluate the expression patterns of GC markers; DAZL (Deleted in azoospermia-like), MVH (mouse vasa homologue), c-kit, and Stra8 (Stimulated By Retinoic Acid 8) in the differentiated mouse ESCs, reverse transcription polymerase chain reaction (RT-PCR) was implemented. Expression of fragilis, a common marker between ESC and GC lineage; SCP3 (synaptonemal complex protein 3), a premeiotic marker; as well as Nanog and Oct4, as pluripotent markers were studied by

RT-PCR, too. As described in the method section, the expression patterns were investigated in 5 groups.

RNA was extracted using TRIzol® reagent (Invitrogen, Canada) according to the manufacturer's protocol. RT-PCR was performed using a cDNA synthesis kit (Bioneer, South Korea) adding 1 µg of total RNA according to the manufacturer's protocol. PCR was completed using Tag DNA polymerase (CinnaGene, Iran) in a 9600 GeneAmp® PCR system (PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA). After an initial denaturation at 94°C for 5 min, cDNA was subjected to 33 cycles of PCR. The primer sets are shown in Table 1. Testicular cells from adult testis were used as the positive control. We examined the expression of β-actin to normalize the RT-PCR reaction. PCR products were observed on 2% agarose gel.

Immunocytochemistry

After differentiation of ESCs, GC markers were detected by immunocytochemistry. To summarize, the process was done by fixation with 4% paraformaldehyde, permeabilization with 0.1% Triton X-100 (Sigma, Germany) and blocking with 10% rabbit serum (Sigma, Germany) [20]. In separate procedures, the cells were incubated for 2 hours at 37°C with rabbit polyclonal antibody to MVH, diluted 1:100 (Abcam, UK) and rabbit polyclonal antibody to DAZL, diluted 1:100. After washing with PBS, the secondary donkey anti-rabbit antibody labeled with phycoerythrin (PE); dilution, 1:100 (Abcam, UK) was applied for both samples for 3 hours. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen, USA) [21].

Immunohistochemistry/immunofluorescence

To evaluate the ability of ESC-derived GCs to home in the ovaries of treated mice, the ovaries were cryosectioned and then the expression of Green Fluorescent Protein (GFP) and VASA was investigated using fluorescence microscope to track GCs.

After cryosectioning and washing with PBS, the ovaries were fixed with Zamboni (American Mastertech, USA) for 20 minutes and incubated overnight. In brief, sections were washed and permeabilized in 0.1% Triton X-100 (Sigma, Germany) for 5 minutes, blocked with 3% BSA/TBST for 1 hour and then, incubated with primary antibody for 1 hour at room temperature. Primary antibody was anti-MVH rabbit polyclonal (1:100, Abcam). After washing in PBS, slides were incubated with secondary antibody for 45 minutes at room temperature. For immunofluorescence staining, secondary antibody was donkey anti-rabbit (1:100, Abcam), and coverslips were mounted with vectashield.

Transplantation

To sterilize recipients, 1 week earlier to cell injection while they were awake, 120 mg/kg cyclophosphamide (Sigma, Germany) and 12 mg/kg busulfan (Sigma, Germany) were administered using insulin syringe via peritoneum. SCs transplantation was completed 7 days later, and 1×106 cells were introduced intravenously via the tail vein using an insulin syringe, while the mice were awake [3]. Before injection, the differentiated cells were mixed with 50 µl culture medium and shaken to be distributed equally within the liquid, and then injected. In each group, 5 mice were included. In the control group, no cell transplantation was performed. Two months later, the ovaries were collected to be analyzed.

3. Results

Differentiation of embryonic stem cells into germ cells using bone morphogenetic protein 4 and retinoic acid

In the ESC group without any treatment measure; no expression of DAZL, a protein that in humans is encoded by the Dazl gene; MVH, a highly specific marker of germ cells; Stra8, a pre-meiotic marker; and SCP3, a primordial germ cell marker was detected. However; c-kit, a stem cell factor receptor; fragilis, a pluripotency marker; Oct4, a POU transcription factor that is expressed in embryonic stem (ES) cells; and Nanog, a pluripotency marker, were highly expressed (Figure 1). In the group enriched with BMP4 and RA, c-kit, Stra8, fragilis, DAZL, MVH, and SCP3 genes were expressed. However, the expression of Oct4 marker was noticed, too. No expression for Nanog was found in this group (Figure 1).

In the EBs treated with BMP4 alone, no expression of Stra8 and SCP3 was located; however, MVH, DAZL, c-kit, fragilis, Oct4, and Nanog genes were detectable (Figure 1). In the group without any differentiation factor, no cell was found to express MVH, DAZL, Stra8, and SCP3 genes, while, c-kit, fragilis, Oct4, and Nanog markers were expressed in this sample (Figure 1). In the last group, treated with RA alone; c-kit, fragilis, Oct4, MVH, DAZL, Stra8, and SCP3 markers were expressed, but no expression of Nanog was seen (Figure 1). It was shown that RA or its combination with BMP4 was appropriate inducers for differentiation of ESCs to GCs.

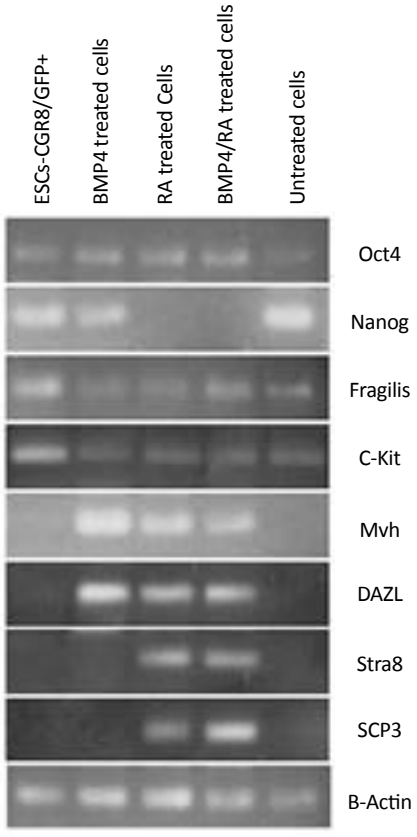

Figure 1. Molecular analysis of specific markers in differentiated and undifferentiated stem cells. RT-PCR analysis shows the expression of germ GC and ESC genes in differentiated and undifferentiated mouse ESCs. β-Actin was used as an internal control.

To study whether SCs gave rise to germ like cells, EBs were stained with anti-VASA and anti-DAZL antibodies. In VASA staining of the ESC group, no VASA marker was observed (Figure 2a), whereas, in two groups treated with BMP4-RA and BMP4 alone, some VASA positive cells were demonstrated (Figure 2b and 2c). In addition, in the group enriched with no differentiation factor, no positive cell was found by VASA marker (Figure 2d), but in the group treated with RA alone, there were some cells to be stained by VASA marker (Figure 2e).

For DAZL staining, no positive cell was detected in ESCs samples (Figure 3a); however, in EBs enriched with BMP4-RA, some cells were found to be positive for this marker (Figure 3b). Additionally, in the group using BMP4 alone, some DAZL-positive cells could be seen (Figure 3c). In the group with no differentiation factor, no cell was observed to be positive with regard to DAZL protein (Figure 3d); however, in the samples cultured with RA alone, a few numbers were shown to be positive to this marker (Figure 3e). So in the presence of RA, BMP4 or their combination, ESCs could develop into GCs.

Oocyte pool depletion by chemotherapy regimen

The sterilized ovaries in comparison with normal ones were considerably devoid of oocyte. However, there were still some follicular structures containing oocytes within the ovaries (14±16 versus 2±3) suggesting that chemotherapy drugs did not completely deplete the ovaries of recipients (Figure 4).

Homing of ESC-derived germ cells in sterilized female mice

No GFP positive cells or cells bearing VASA marker were observed in the ovaries, indicating that the ESC-derived GCs did not home in the chemotherapy-treated recipient adult mice ovaries (Figure 5). Therefore, no contribution of ESC-derived GCs in restoration of oogenesis in the unfertilized mice was noticed.

4. Discussion

In the present study, we showed that ESC-derived GCs were identical to those of E11.5 GCs in vivo. The differentiation protocol used in this study was a slightly modified method applied earlier [22, 23]. To distinguish GCs formed within EBs, MVH protein was employed as

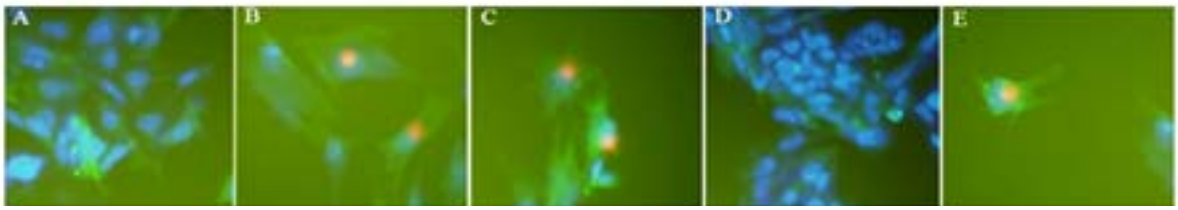

Figure 2. Immunostaining of VASA, germ cells specific marker. After 10 days of differentiation the nuclei were stained by DAPI (blue) and the cells were GFP positive (green). (A): The ESC group without any treatment; (B): The group treated with BMP4 and retinoic acid; (C): The group treated with BMP4 alone; (D): The group treated with no differentiation factor; and (E): The group treated with retinoic acid alone (×200).

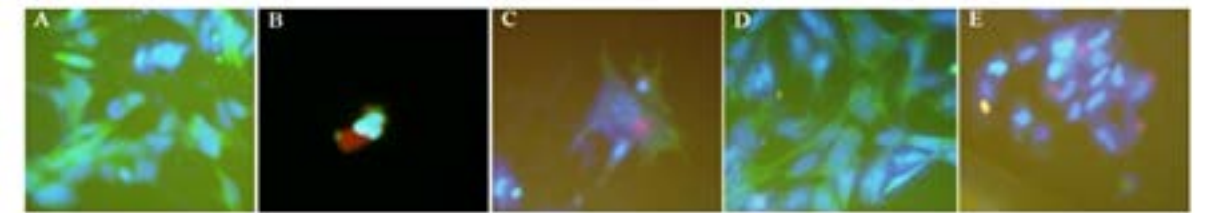

Figure 3. Immunostaining of DAZL, germ cells specific marker. After 10 days of differentiation, the nuclei were stained by DAPI (blue) and the differentiated cells were GFP positive (green). (A): The ESC group without any treatment; (B): The group treated with BMP4 and retinoic acid; (C): The group treated with BMP4 alone; (D): The group treated with no differentiation factor; and (E): The group treated with retinoic acid alone (×200).

a reliable discriminative marker, which was frequently used in previous studies [8, 24]. This marker was a germ-cell-specific ATP-dependent RNA helicase that was not expressed in pluripotent stem cells [25]. Furthermore, during the post-meiotic stage of both male and female gametogenesis, migrating GCs, in both mice and humans, first express VASA marker [26, 27]. Apart from VASA marker; DAZL, c-kit, Stra8, and fragilis were also candidate markers for detecting GCs, but they are expressed in ESCs as well. Therefore, they are not individually reliable for GC lineage to be detected. In this regard, we considered VASA as a key marker for detection of GCs, derived from mouse ESCs.

In the EBs cultured in the presence of BMP4 alone, some MVH-positive cells were observed within them; however, there were no MVH positive cells inside the EBs in the medium with neither BMP4 nor RA. It was demonstrated that BMP4 had a key role in the development of GC from pluripotent epiblast cells, and that BMP4, added to the EB culture, increased the formation of PGCs in vitro [10, 28-31]. Thus, our findings are consistent with previous studies demonstrating that BMP4 significantly boosted the derivation of GCs from ESCs [25, 32-34]. However, there was no spontaneous GC derivation from ESCs cultured in the medium without any differentiation factor. In addition, this finding was not in agreement with the studies reported about spontaneous development of ESCs into GCs [14].

On the other hand, in the group treated with RA alone, expression of VASA marker was noticed in some cells within the EBs. It was shown that RA developed ESCs into GC lineage without help of other differentiation factors such as BMP4. Therefore, our result agree with the statement that RA could stimulate PGC proliferation, and helped embryonic germ cells or ESCs to differentiate [35]. In the groups treated with BMP4-RA, BMP4 alone, and RA alone, the expression patterns in all groups were similar. So there was no difference be-

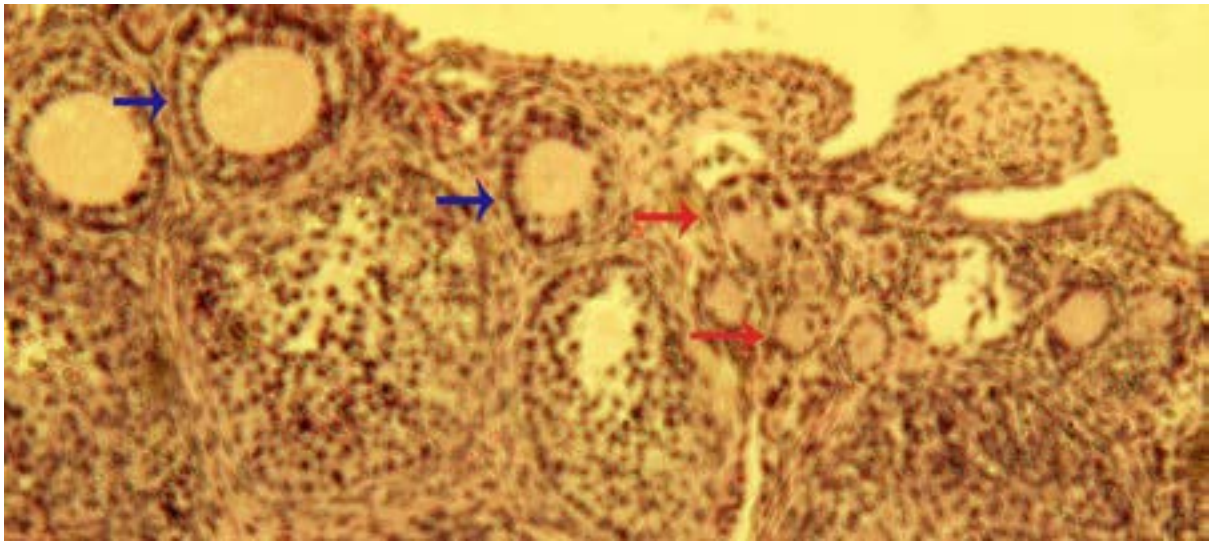

Figure 4. Histological section through chemotherapy-treated ovaries. Ovaries of recipient mice were treated with busulphan (12 mg/k) and cyclophosphamide (120 mg/kg). They did not completely eliminate follicles containing oocyte after a week. Blue arrows show the follicles with no oocyte, whereas the red ones show the follicles containing oocytes (×100).

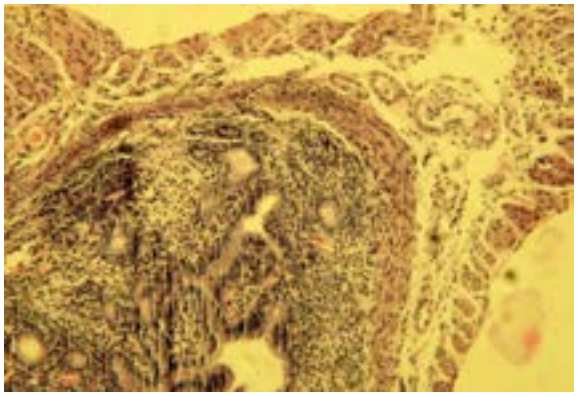

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Figure 5. Assessment the homing of transplanted germ cells. The ovaries of host mice were sterilized by busulphan (12 mg/k) and cyclophosphamide (120 mg/k). After a week, transplantation was performed by injecting about 106 cells into the mice tail vein. By passing the determined time, the ovaries were sectioned and analyzed. However, no donated differentiated cell was found to home in the recipient ovaries (×100).

tween the group treated with either BMP4 or RA, but in the BMP4 treated alone group, no sign of SCP3 could be found. It might indicate that for further development of GCs, the presence of RA is important. Moreover, it shows that the existence of RA in later stages rather than primary levels of GC differentiation is vital.

Furthermore, in our study, SCP3 was expressed in two differentiation groups. It indicates that in those groups, the cells could further develop into late GCs, and as a result, meiosis did likely happen in some cells within the EB structures. Apart from studies done to help infertile couples in vitro, several researchers have investigated on infertility in vivo as well. In our study, we transplanted ESC-derived GCs into the adult chemotherapy-treated recipient female mice via tail vein, but at the determined time, we could not detect any GFP positive differentiated cell in the recipient ovaries. However, Johnson et al. (2005) transplanted BM and PB, separately, via tail vein in the sterilized adult female mice [3]. Nonetheless, they could identify a number of donated oocytes within the recipient ovaries that was completely inconsistent with our finding.

On the other hand, Eggan et al. (2006), using a mouse model, evaluated the capacity of bone marrow cells within the peripheral blood for generation of any oocyte. They

Table 1. Primers used for the PCR analysis of ESC-derived germ cells.

Genes	Primer Sequences	Tm (°C)
Oct4	F: 5´-CACGAGTGGAAAGCAACTCA-3´ R: 5´-TTGGTTCCACCTTCTCCAAC-3´	58
Nanog	F: 5´-CTGCTCCGCTCCATAACTTC-3´ R: 5´-GCTTCCAAATTCACCTCCAA-3´	58
fragilis	F: 5´-AGCCTATGCCTACTCCGTGA-3´ R: 5´-GAGGACCAAGGTGCTGATGT-3´	58
VASA	F: 5´-ACCAAGATCAGGGGACACAG-3´ R: 5´-TAACCACCTCGACCACTTCC-3´	58
DAZL	F: 5´-AAGGCAAAATCATGCCAAAC-3´ R: 5´-TCCTGATTTCGGTTTCATCC-3´	57
Stra8	F: 5´-CTCCTCCTCCACTCTGTTGC-3´ R: 5´-GCGGCAGAGACAATAGGAAG-3´	57
SCP3	F: 5´-GGGGCCGGACTGTATTTACT-3´ R: 5´-TTCCCAGATTTCCCAGAATG-3´	57
c-kit	F: 5´-CTCACATAGCAGGGAGCACA-3´ R: 5´-ACAACTCACCCACACGCATA-3´	60
b-Actin	F: 5´- CGTCTTCCCCTCCATCG -3´ R: 5´-CTCGTTAATGTCACGCAC -3´	60

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showed that bone marrow cells existing in the peripheral blood or other blood cells played no role in oogenesis. However, those cells that migrated to the ovaries, resembled the leukocytes [36]. Begum et al. (2008) transplanted the radiated ovaries under the kidney capsules or inside the ovarian bursae of recipient mice. Eventually, they could not find any evidence to prove the theory of existence of extra ovarian GCs to support oogenesis after birth [37]. These results are in agreement with our findings and the dogma that a limited number of oocytes are formed during embryonic period and the number decreases with age.

From the clinical perspective, in the female gender infertility may occur due to different causes such as exposure to chemotherapy agents. These agents severely interfere with the existence of SCs in the specific organs of individuals like bone marrow. Thus, if there is an extra-ovarian source of GCs which deals with production of new oocytes despite use of chemical agents, the process cannot be further continued or at least suppressed. If we could find a source of SCs to take the place of those eliminated migrating SCs, we might succeed in having new oogenesis with the donated cells. We do not even have confirmed documents to prove the new theory of existence of extra ovarian source.

In conclusion, there are some reasons why these ESC-derived GCs cannot home in the recipient ovaries. First, the existence of an extra-ovarian source is yet to be proved. There is no vivid reason whether these presumptive extra-ovarian GCs exist. First, if it is not true, then transplantation of these cells will not result in oogenesis. Second, if there is such an extra ovarian pool, we do not know when, how, and in what circumstances, they become active and take the place of other GCs in the ovary. Since this theory has recently been proposed, modern techniques need to be developed to understand the genetic and epigenetic outcomes of this assumption.

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Conflict of Interest

All authors certify that this manuscript has neither been published in whole nor in part nor being considered for publication elsewhere. The authors have no conflicts of interest to declare.

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