Sertoli cell condition medium can induce germ like cells from bone marrow derived mesenchymal stem cells

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**ABSTRACT**

Objectives: Although many researchers have confirmed induction of germ cells from bone marrow mesenchymal stem cells (BMMSCs), there are no reports that confirm spontaneous differentiation of germ cells from BMMSCs. In this study, we have evaluated the effect of adult Sertoli cell condition medium (SCCM) as a mutative factor in the induction of germ cells from BMMSCs.

Materials and Methods: BMMSCs were collected from the bone marrow of 6-8-week old NMRI mice and their mesenchymal entities were proven using superficial markers (expression of CD44 and CD73 and non-expression of CD45 and CD11b) by flow cytometry. Their multi-potential entities were proved with differentiation to osteogenic and adipogenic cells for 21 days. Also isolated Sertoli cells were enriched using lectin coated plates and Sertoli cell condition medium (SCCM) was collected. Sertoli cells were identified by immunocytochemistry and Vimentin marker. The cells were then differentiated into germ cells with SCCM for 2 weeks. Finally induced cells were evaluated by RT-PCR and immunocytochemistry.

Results: Differentiation of mesenchymal stem cells to osteoblast and adipocyte showed their multi-potential property. Expression of CD44 and CD73 and non-expression of CD45 and CD11b confirmed mesenchemy cells. Immunocytochemistry and RT-PCR results showed expression of germ cells specific marker (Mvh).

Conclusion: This study confirmed the effect of SCCM as a motivational factor that can used for differentiation of germ cells from BMMSCs.

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**Introduction**

Germ cells are cells that undergo meiosis process in vivo. They are derived from Primordial germ cells (PGCs) in the two sexes when they migrate from dorsal mesentery in early gonads (1, 2). Infertility affects nearly 15% of couples and male factor is the main cause of infertility (3).

Obtaining germ cells from stem cells has emerged as an important strategy for treating infertility (1). Stem cells can be obtained from inner cell mass (ICM) of blastocyst (embryonic stem cells, ESCs), from extra-embryonic tissues such as placenta, Wharton’s jelly, umbilical cord blood and amniotic fluid (fetal stem cells), and from adult tissues as bone marrow, peripheral blood and others (adult stem cells) (4). In the past, investigators focused on ESCs (5-8) but in more recent years, there has been increasing focus on adult stem cells because of ethical reasons and the potential of ESCs to create tumor (9-11).

Differentiation of stem cells depends on their surrounding micro environments and motivational factors such as Sertoli cells (4). Sertoli cells are the only somatic cells that have a close connection to germ cells and play an important role in regulating cell cycle by secreting necessary factors for viability, proliferation and differentiation of the germ cell. These factors include activin, stem cell factor, insulin like growth factor and inhibiting factors such as ID protein (12, 13).

Many studies have shown that Sertoli cells induce postmeiotic development in vitro (14, 15). Some studies have shown that secretory products derived from Sertoli cell conditioned medium increases cell proliferation and enhanced dopaminergic neuronal differentiation of the 796RMB cell line (16). Sertoli cell conditioned medium can significantly push human embryonic stem cell (hESCs) differentiation of human embryonic mesenchym cells (hUMSCs) into germ cells (18). Conditioned medium collected from testicular cell cultures induced differentiation of embryonic stem cells into ovarian structures containing oocytes (19).

Recent studies have shown that mesenchymal stem cells can differentiate into germ cells, but there have been no studies that have confirmed spontaneous differentiation of germ cells from BMMSCs. This study is aimed at evaluating the role of adult Sertoli cell condition medium (SCCM) as a mutative factor that induces differentiation of germ cells from BMMSCs.

**Conclusion:** This study confirmed the effect of SCCM as a motivational factor that can used for differentiation of germ cells from BMMSCs.
Materials and Methods

Experimental animals

6-8 week-old NMRI male mice were maintained under standard conditions with free access to food and water. The ethics committee of Tehran University of Medical Sciences approved the animal experiments, in accordance with University guidelines.

BMMSCs isolation and culture

BMMSCs were collected from 6-8 week old NMRI mice by flushing method- aspiration. After centrifuging, suspended cells were plated in Dulbecco’s modified eagles medium (DMEM) (Gibco, Germany) enriched with 15% fetal bovine serum (FBS) (Gibco, Germany), 100 μ/l penicillin and 100 μg/ml streptomycin (Gibco, Germany). Then cells were incubated at 37 °C and 5% CO2 for two weeks. The medium was replaced every 3 days until sufficient confluence was observed. After 3 passages, their mesenchymal entity were proven using superficial markers (expression of CD44 and CD73 and non-expression of CD45 and CD11b) by Flow cytometry and their multi-potential entity were proven by their differentiation into osteogenic and adipogenic cells within 21days (20).

BMMSCs pluripotency

The cells obtained from third passage were cultured in osteogenic and adipogenic medium. The osteogenic medium consisted of DMEM enriched 10 μg/ml Ascorbic2-phosphate (Sigma, USA), 10 nM Dexamethasone (Sigma, USA), 10 mM B-Glycerol phosphate (Sigma, USA). Adipogenic medium consisted DMEM enriched 50 μg/ml ascorbic phosphate (Sigma, USA), 50 μg/ml indomethacin (Sigma, USA) 100 nM dexamethasone (Sigma, USA). The conditioned media were incubated in 95% humidified, 5% CO2 atmosphere at 37 °C. After 3 weeks, the cells were evaluated with alizarin red for osteogenic cells and oil red for adipogenic cells (9).

Alizarin red S staining

Osteoblast-differentiated cells were washed with PBS (Invitrogen, USA) and fixed in 10% formaldehyde (Sigma, USA) at room temperature for 15 min. Following two washes with PBS, cells were stained with 2% alizarin red S (Sigma, USA) (pH 4.2) for 20 min at room temperature. After removal of excess dye, the cells were rinsed 4 times with distilled water for 5 min and inspected under light microscopy and photographed.

Oil red O staining

Adipogenic-differentiated cells were washed with PBS, and 10% formaldehyde (Sigma, USA) was added along the sides of each well of the plate, after 10 min the formalin was removed from the wells. The working solution of oil red was added along the side of each well for 5 min, so that the cells were completely covered. They were then rinsed with tap water until the water ran clear. The hematoxylin counterstain was performed on each well so that the cells were completely covered and they were allowed to stand for 1 min and inspected under a phase contrast microscope.

Flow cytometry

In order to demonstrate the existence of mesenchymal stem cells obtained from bone marrow, superficial markers were analyzed using flow cytometry according to the chemicon protocol (21). The cells were cultured and after the third passage, they were harvested by trypsin (Invitrogen, USA). 1×10⁶ cells were used for analysis. Flow cytometric assay were performed and mesenchymal stem cells CD markers recognized, then Win MFI 2.9 software was used for analysis. CD44 (12-0441-81, ebioscience, USA) and CD73 (550257, BD Bioscience, USA) were used as mesenchymal stem cells markers and CD45 (341071, BD bioscience, USA) were used as hematopoietic and macrophage markers. The cells were incubated in 5 mg/ml FITC conjugated antibodies. FITC mouse IgG2A isotype control (11-4724, ebioscience, UK), Rat IgG1 Isotype control (ab18412, abcam, USA) were used as isotype control (21).

Sertoli cells isolation, culture and preparation condition medium

Sertoli cells were obtained from the testis of adult mice (6-8 weeks old) as previously described by Scarpino et al. Briefly, culture dishes were coated with 5 μg/ml of Datura Stramonium agglutinin (DSA; Sigma, USA) lectin in PBS at 37 °C for 1 hr. Then, the coated plastic dishes were washed three times with 0.5% BSA (Sigma). The mixed population of the testis cells obtained by enzymatic digestion was placed on lectin-coated dishes and incubated for 1 hr at 37 °C in a humidified atmosphere of 5% CO2 in air. After incubation, the non-adhering cells were collected after being washed twice with DMEM medium. Then DMEM and 10% FBS were added. After seven days the Sertoli cells formed a confluent layer (21). When the Sertoli cells formed a confluent layer, the condition medium was collected after 48 hr and centrifuged at 1000 g for 10 min. The supernatant was used as culture medium (16, 17).

Immunocytochemistry for characterization of adult Sertoli cells

Vimentin was detected in cultured Sertoli cells by immunocytochemistry. After fixation with 4% paraformaldehyde, permeabilization by 0.4% Triton X100 (Sigma, USA) and blocking with 10% goat serum (Sigma, USA), the cells were incubated for 2 hr at 37 °C with mouse monoclonal anti-vimentin antibody, diluted 1:100 (Sigma, USA). After washing with PBS, the secondary antibody, goat anti-mouse labeled with fluorescent isothiocyanate (FITC) diluted 1:100 (Sigma,
USA) was applied for 3 hr. Control cells were treated under similar conditions except for the removal of the first antibody. Nuclei were stained with 5μg/ml Dapi (Sigma, USA) (22).

**Induction of BMMSCs to germ cells**

SCCM prepared as described above, and BMMSCs that obtained from third passage, were cultured in this medium for 2 weeks. The control group consisted of cells that were cultured in DMEM, penicillin streptomycin (Gibco, Germany), FBS 10% (Gibco, Germany) for 2 weeks. The medium was replaced in the two groups every 3 days with fresh medium and the cells were incubated at 37 °C in an atmosphere of 95% air and 5% CO2. Finally the cells were evaluated with RT-PCR and immunocytochemistry to identify the differentiated cells.

**Immunofluorescence for identification of differentiated cells**

At first, the cells were washed with PBS twice and fixed with 4% paraformaldehyde. Triton X-100 was then added for penetrating the cells. Bovine serum albumin was used to prevent expression of non-specific proteins. Then primary antibody (anti-Mvh, ab13840, 1:100) was added and maintained at room temperature for 3 hr. Secondary antibody (goat anti-mouse labeled with fluorescent isothiocyanate (FITC) was added. After 2 hr of incubation, the antibody was thrown away and the cells were washed. Control cells were treated under similar conditions except for the removal of the first antibody. Nuclei were stained with DAPI (23).

**Reverse transcriptase polymerase chain reaction**

After two weeks of culture, the expression level of Mvh (Mouse vashemolog gene) was studied by reverse transcription polymerase chain reaction (RT-PCR). Primer sequences for Mvh were 5'-GAGGGGGAAGGAGCATTTCC and Rev 5'-TGGTATGTTGCACATTGCCCT and β-actin, 5' GGTTCG -ATGCCCTGAGGCTC and reverse 5'-ACTTCCGTTGCAGGAGGAGG for normalization in RT PCR. Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. RT-PCR was performed by cDNA synthesis kit (Bioneer, South Korea) using 1 μl of total RNA, Tag DNA polymerase (Gia gene, Tehran, Iran) in a Gene Amp PCR system 9600 (PerkinElmer Life and Analytical Sciences, Wellesley, MA). After initial denaturation at 94 °C for 5 min, cDNA was subjected to 33 cycles of PCR. Testicular cells from adult testis were used as positive control and H2O used as negative control. PCR products were discovered on 2% agarose gels, and the intensity of bands assayed by UVI doc gel documentation system (Avebury house 36 a union lane Cambridge CB4 1QB-UK).

**Statistical analysis**

The results were expressed as mean±SD. The statistical significance between the mean values was determined by one-way ANOVA analysis of variance, Tukey and Duncan post-test. P≤0.05 was considered significant.

**Results**

**BMMSCs isolation, identification and pluripotency**

Our results showed that spindle shaped BMMSCs replication reached as much as 80-90% of the original concentration within 5 and 8 days respectively. Our findings demonstrate that the cells differentiated to osteogenic, as shown with alizarin red and Adipogenic cells with oil red staining (Figure 1). BMMSCs also expressed mesenchymal superficial marker CD44 and CD73 significantly and did not express non-mesenchymal superficial marker CD45 and CD11b (Figure 2). Therefore these cells had the characteristics of mesenchymal and pluripotential cells.

**Sertoli cell isolation and characterization**

Sertoli cells cultured on lectin DSA-coated plates were initially round and adhered firmly to the bottom of the dish. After 1 day, the cells began to flatten; they gradually spread and took on an epithelioid appearance. The presence of Vimentin protein on Sertoli cells cytoplasm was confirmed by immunocytochemistry (Figure 3).

**BMMSCs differentiation**

BMMSCs were cultured in SCCM for 2 weeks. The cells showed expression levels of Mvh transcript by RT-PCR (Figure 4, 5). Also the presence of Mvh protein was confirmed by immunocytochemistry (Figure 6).
Inductive role of SCCM on mesenchymal stem cell

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Figure 2. Flow cytometric analysis for detection of superficial markers of stem cells (CD44 and CD73) (upper), Expression of superficial markers of hematopoietic and macrophage cells (CD45 and CD11b) (lower)

Figure 3. Microscopic image of the morphology of Sertoli cells derived from 6-8 week-old male mice. Sertoli cells were isolated by lectin DSA and characterized by immunocytochemistry. A-Monolayer Sertoli cells begin to flatten and spread following one day plating on lectin DSA (×200 magnification). B- Sertoli cells were positive for Vimentin in cytoplasm (green color) and nuclei were stained with DAPI (blue color) (×400 magnification). C- Negative control (scale bar =50 µm) (×400 magnification)

Discussion

Male fertility is related to spermatogenic cells. Many researchers have tried to generate male germ cells from adult stem cells, in order to develop therapies for male infertility (24). Mesenchymal stem cells derived from bone marrow are good alternatives to adult stem cells that can form a variety of cell types such as fat cells, cartilage, bone, etc (25).

We obtained mesenchymal stem cells from bone marrow and confirmed their multi-potentially properties by osteoblast and adipocyte condition medium. Alizarin red S and oil red staining. Then mesenchymal entity of the cells was confirmed by Flow cytometry. Superficial markers CD44 and CD73 were expressed significantly but CD45 and CD11b were not expressed. This results are similar to results of research performed by Domicini et al in 2006 (26).

Figure 4. Electrophoresis of PCR products on 2 percent agarose gels. After 14 days of cultivating mesenchymal stem cells in conditions medium, the expression of Mvh gene was studied by RT-PCR. 1-100bp DNA Ladder 2-negative control (H2O) 3-negative control (mesenchymal stem cells) 4-Sertoli cells condition medium 5-positive control (testis)

Figure 5. Ratio of Mvh gene has been shown by a histogram. (H2O= negative control, c-= negative control (mesenchymal stem cells), Sertoli cell condition medium (test group), c+=positive control (testicular cells))
Sertoli cells play a crucial role in the events of spermatogenesis. They secrete endocrine and exocrine factors (stem cell factor (SCF), glial cell line-derived neurotropic factor (GDNF), inhibin and FSH hormone) that are needed for maintaining viability, proliferation and differentiation of spermatogonial stem cells to spermatocytes and stimulating meiosis in vitro (30). Some researchers have co-cultured with Sertoli cells to induce germ cells from different stem cells (13, 31) but there are few studies to evaluate the effect adult Sertoli cell condition medium to induced germ cells. In this study, we have evaluated the effect of adult Sertoli cell condition medium (SCCM) to induced germ cells from BMMSCs.

The condition medium is the supernatant of cultured Sertoli cells that includes all of the secreted factors of Sertoli cells. It was prepared after 48 hr. Finally, BMMSCs were cultured in the SCCM medium for 2 weeks. Our findings confirmed the effect of SCCM in inducing germ cells. We also performed semi-quantities RT-PCR for expression Mvh and immunocytochemistry to evaluate induction of the Mvh protein and both experiments were positive. Mvh is a primary marker that express in germ cells and is a general marker for all germ cells but not in other cells. The expression Mvh is related to proliferating and differentiating germ cells and it is known as ATP-dependent RNA helicase (32).

Geens et al performed RT-PCR and immunocytochemistry for Vase and used SCCM as an inductive factor for germ cell differentiation from hESC. They extracted the condition medium from neonate mice Sertolicecell after 24 hr (17) and Fengming et al evaluated the effect of SCCM in induction of germ cell. They extracted condition medium from neonate rat Sertoli cell after 72 hr and their result were positive (33) but we used adult Sertoli cell condition medium after 48 hr.

Sertoli cells undergo several changes such as the division of Sertoli cells during the neonatal and peripubertal periods. During puberty, Sertoli cells undergo a maturational changes such as enlarged and tripartite nucleolus, lack of proliferative activity,
organization of blood-testis barrier (BTB), down-regulation or disappearance of former existed proteins (e.g., anti-müllерian Hormone (AMH), aromatase, and cytokeratin 18 (CK18)), and up-regulation or appearance of mature markers (e.g. androgen receptor (AR), GATA-1 and transferrin) (33). At the same time, the functional changes of Sertoli cells fall out from testis formation to spermatogenesis support. In the study, we evaluated the effect of adult Sertoli cells condition medium on the induction of germ cells from BMMSCS. Our findings showed that Sertoli cells condition medium induced formation of germ like cells from BMMSCS.

Conclusion
The study showed that the Sertoli cells condition medium is an appropriate culture system that can induce the expression of germ cell markers and is a good medium that can be used to differentiate germ cells from BMMSCS and can be useful for treating infertility. Future studies can be designed to evaluate the effect of SCCM in differentiation of other somatic stem cells.

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Conflict of interest
There is no conflict of interest.

References
1. Marques-Mari A, Lacham-Kaplan O, Medrano J, Pellicer A, Simón C. Differentiation of germ cells and gametes from stem cells. Hum Reprod Update. 2009; 15:379-390.
2. Shirazi R, Zarnani AH, Soleimani M, Abdolvahabi MA, Nayernia K, Kashani IR. BMP4 can generate primordial germ cells from bone-marrow-derived pluripotent stem cells. Cell Biol Int 2012; 36:1185-1193.
3. Valsangkar S, Bodhare T, Bele S, Sai S. An evaluation of the effect of infertility on marital, sexual satisfaction indices and health-related quality of life in women. J Hum Reprod Sci 2011; 4:80-85.
4. Alison MR, Poulson R, Forbes S, Wright NA. An introduction to stem cells. J Pathol 2002; 197:419-423.
5. Hübner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, et al. Derivation of oocytes from mouse embryonic stem cells. Science 2003; 300:1251-1256.
6. Toyooka Y, Tsuchikawa N, Akasu R, Noce T. Embryonic stem cells can form germ cells in vitro. Proc Natl Acad Sci U S A 2003;100:11457-11462.
7. Clark AT, Bodnar MS, Fox M, Rodriguez RT, Abeyta MJ, Firpo MT, et al. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. Hum Mol Genet 2004; 13:727-739.
8. Geijsen N, Horoschak M, Kim K, Gribnau J, Eggen K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 2004; 427:148-154.
9. Shireizely MH, Pasbakhsh P, Amidi F, Mehrannia K, Sobhani A. Comparison of differentiation potential of male mouse adipose tissue and bone marrow derived-mesenchymal stem cells into germ cells. Iran J Reprod Med 2013; 11:965.
10. Wakitani S, Takaoka K, Hattori T, Miyazawa N, Iwanaga T, Takeda S, et al. Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. Rheumatology 2003; 42:162-165.
11. Brickman JM, Burdon TG. Pluripotency and tumorigenicity. Nat Genet 2002; 32:557-558.
12. Mohammadi SM, Movahedin M, Koruji SM. A comparison between the colony formation of adult mouse spermatogonial stem cells in co cultures with sertoli and STO (mouse embryonic fibroblast cell line). Cell J 2010; 12:231-240.
13. Ahmed EA, Barten-van Rijbroek AD, Kal HB, Sadri-Ardekani H, Mizrak SC, van Pelt AM, et al. Proliferative activity in vitro and DNA repair indicate that adult mouse and human Sertoli cells are not terminally differentiated, quiescent cells. Biol Reprod 2009; 80:1084-1091.
14. Lee DR, Kim KS, Yang YH, Oh HS, Lee SH, Chung TG, et al. Isolation of male germ stem cell-like cells from testicular tissue of non-obstructive azoospermic patients and differentiation into haploid male germ cells in vitro. Hum Reprod 2010; 21:471-476.
15. Sousa M, Cremades N, Alves C, Silva J, Barros A. Developmental potential of human spermatogenic cells co-cultured with Sertoli cells. Hum Reprod 2002; 17:161-172.
16. Shemekh R, Saporta S, Cameron DF, Willing AE, Sanberg CD, Johe K, et al. Effects of Sertoli cell-conditioned medium on ventral midbrain neural stem cells: a preliminary report. Neurotox Res 2008; 13:241-246.
17. Geens M, Sermon KD, Van de Velde H, Tournaye H. Sertoli cell-conditioned medium induces germ cell differentiation in human embryonic stem cells. J Assist Reprod Genet 2011; 28:471-480.
18. Huang P, Lin LM, Wu XY, Tang QL, Feng XY, Lin GY, et al. Differentiation of human umbilical cord Wharton’s-jelly-derived mesenchymal stem cells into germ-like cells in vitro. J Cell Biochem 2010; 109:747-754.
19. Lacham-Kaplan O, Chy H, Trounson A. Testicular cell-conditioned medium supports differentiation of embryonic stem cells into ovarian structures containing oocytes. Stem Cells 2006; 24:266-273.
20. Rastegar T, Minae MB, Roudkani MH, Kashani IR, Amidi F, Abolhasani F, et al. Improvement of expression of α6 and β1 Integrins by the co-culture of adult mouse spermatogonial stem cells with SIM mouse embryonic fibroblast cells (STO) and growth factors. Iran J Basic Med Sci 2013; 16:134.
21. Scarpino S, Morena AR, Petersen C, Fröysa B, Söder O, Boitani C. A rapid method of Sertoli cell isolation by DSA lectin, allowing mitotic analyses. Mol Cell Endocrinol 1998; 146:121-127.

22. Anway MD, Folmer J, Wright WW, Zirkin BR. Isolation of Sertoli cells from adult rat testes: an approach to ex vivo studies of Sertoli cell function. Biol Reprod 2003; 68:996-1002.

23. Baazm M, Abolhassani F, Abbasi M, Habibi Roudkenar M, Amidi F, Beyer C. An improved protocol for isolation and culturing of mouse spermatogonial stem cells. Cell Reprogram 2013; 15:29-336.

24. Hou J, Yang S, Yang H, Liu Y, Liu Y, Hai Y, et al. Generation of male differentiated germ cells from various types of stem cells. Reproduction 2014; 147:R179-R188.

25. Hua J, Pan S, Yang C, Dong W, Dou Z, Sidhu KS. Derivation of male germ cell-like lineage from human fetal bone marrow stem cells. Reprod Biomed Online 2009; 19:99-105.

26. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8:315-317.

27. Nayernia K, Lee JH, Drusenheimer N, Nolte J, Wulf G, Dressel R, et al. Derivation of male germ cells from bone marrow stem cells. Lab Invest 2006; 86:654-663.

28. Ghasemzadeh-Hasankolai M, Batavani R, Esfamenadjad MB, Sedighi-Gilani M. Effect of zinc ions on differentiation of bone marrow-derived mesenchymal stem cells to male germ cells and some germ cell-specific gene expression in rams. Biol Trace Elem Res 2012; 150:137-146.

29. Drusenheimer N, Wulf G, Nolte J, Lee JH, Dev A, Dressel R, et al. Putative human male germ cells from bone marrow stem cells. Soc Reprod Fertil Suppl 2006; 63:69-76.

30. Oatley JM, Brinster RL. The germline stem cell niche unit in mammalian testes. Physiol Rev 2012; 92:577-595.

31. Zanganeh BM, Rastegar T, Roudkenar MH, Kashani IR, Amidi F, Barbarestani M. Co-culture of spermatogonial stem cells with Sertoli cells in the presence of testosterone and FSH improved differentiation via up-regulation of post meiotic genes. Acta Med Iran 2013; 51:1-11.

32. West FD, Mumaw JL, Gallegos-Cardenas A, Young A, Stice SL. Human haploid cells differentiated from meiotic competent clonal germ cell lines that originated from embryonic stem cells. Stem Cells Dev 2010; 20:1079-1088.

33. Yue F, Cui L, Johlura K, Ogawa N, Sasaki K. Induction of mid brain dopaminergic neurons from primate embryonic stem cells by coculture with Sertoli cells. Stem Cells 2006; 24:1695-1706.

34. Hai Y, Hou J, Liu Y, Liu Y, Yang H, Li Z, et al. editors. The roles and regulation of Sertoli cells in fate determinations of spermatogonial stem cells and spermatogenesis. Semin Cell Dev Biol 2014; 29:66-75.