Research Article

# Isolation, Culture and Characterization of Chicken Primordial Germ Cells

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## Abstract

Nowadays, production of recombinant proteins in eukaryotes is gaining good deal of attention. Transgenic chicken as a eukaryotic system has a high potential for producing recombinant proteins. Post-translational changes, especially glycosylation, are characteristic of the eukaryotic proteins. In practice we need to choose a proper expressing host when considering over-expression of a recombinant protein. Chickens are among the well-considered candidates for such application. Production of transgenic chickens could be achieved in different ways, including application of primordial germ cells. Primordial germ cells are progenitor of sperm and ovum. These cells are round, with a big nucleus and a cytoplasm with lipid and glycogen particles. The first step for having transgenic chickens is isolation and culture of the primordial gem cells. In the present study, these cells were isolated by centrifugation method in presence of ficoll and using magnetic cell sorting, and were cultured in optimal culture medium. These cells were finally characterized with defined methods, like Periodic acid-schiff staining, alkaline phosphates activity assessment, and antibody staining.

***Keywords*:** primordial germ cells, transgenic chicken, recombinant proteins

## Introduction

Scientific studies in the field of producing transgenic animals have progressed significantly as a model of various human diseases and for the purpose of producing recombinant drugs. Gordon produced the first transgenic mouse by introducing gene into pronuclear fertilized egg (Gordon et al., 1980). Producing transgenic chickens was also started from 1980, and first transgenic chicken was produced with the help of viral vectors and injecting them to the blastoderm cells (Petitte et al., 1990). Transgenic chickens have many priorities in comparison with other animals, and these capabilities have attracted the attention of many researchers around the world. Different methods have been used for producing transgenic chickens (Houdebine, 2002; Love et al., 1994; van de Lavoir et al., 2006). Nevertheless, primordial germ cells are known as the best option for producing transgenic chickens, because separating these cells is almost easy, they have a high germ line transmission, the ability to keep these cells in culture medium for genetic manipulation, and keeping these cells in culture medium does not reduce germ line transmission rate (Han, 2009).

Primordial germ cells (PGCs) are actually precursors of sperm and ovum. These cells were first described by Swift (Swift, 1915). These cells are round, have a big nucleus and a cytoplasm with lipid and glycogen granules. At first, around 50 cells were isolated from epiblast and move toward head fold to be located in germinal crescent. After forming embryo cardiovascular system, these cells migrated toward genital ridge through blood and are finally located in primitive gonad (Kunwar et al., 2006). This type of PGCs migration is specific to the poultry, and is different from mammals. Specific route of chicken primordial germ cells has made their isolation available in different points including from germinal crescent, blood, and

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primitive gonad (Fujimoto et al., 1976). In the present study, primordial germ cells were isolated from blood by centrifuge in the presence of ficoll at stage 14 HH (Hamburger and Hamilton) , and were isolated from primitive gonad using magnetic cell sorting (MACS) at stage 28 HH, then by optimizing growth conditions in culture medium which was done by presence of feeder cells and using growth factors, these cells were cultured in laboratory. After being sorted, these cells were characterized; using specific methods; like Periodic acid-Schiff staining (PAS) staining, alkaline phosphates (AP) staining, and specific antibodies. To the best of my knowledge, this is the first report of isolation and characterization of primordial germ cells in Iran.

## Materials and Methods

### Chicken embryos

Fertilized chicken (*Gallus gallus domesticus*, Ross breed) eggs were obtained from a commercial hatchery and incubated in a rotary egg incubator (DORNA System, Iran) at 38°C at 60% humidity with rocking at an angle of 90 degree every 90 minutes.

## Isolation of primordial germ cells

### Isolation with Ficoll

Under a laminar hood, the blunt end of the eggs was cut horizontally with a diamond cutter. After checking that embryos had developed to the Hamburger–Hamilton stage 14 HH (Hamburger and Hamilton, 1992), blood was collected from 30 embryos and placed in Dulbecco's minimal essential medium (DMEM; Biosera, UK) containing 10% fetal bovine serum (FBS, Invitrogen, USA). After centrifugation at 800xg for 10 minutes, pellet of blood cells was resuspended in 16% Ficoll-400 (Sigma-Aldrich Corp., Germany), was covered with 6.3% Ficoll-400 solution, and was centrifuged at 800xg for 30 minutes (Yasuda et al., 1992). Primordial germ cells were collected at the interface between the 16% and 6.3% Ficoll phases. Ficoll was removed by centrifuging the samples twice at 200xg for 8 minutes.

### Isolation with magnetic cell sorting (MACS)

From eggs incubated for 5.5 days (stage 28) (Hamburger and Hamilton, 1992), embryos were removed and rinsed three times with calcium-free phosphate buffered saline (PBS). Using fine glass needle under a stereomicroscope, the genital ridge (with its mesonephrus attached) was dissected out. Then gonadal tissue was dissociated into cells in a solution of 0.25% trypsin/0.02% EDTA (Biosera, UK) at 37°C for 10 minutes. Dissociated cells were placed in DMEM containing 10% FBS. The dispersed cells were filtered through a 100-µm mesh, and then washed with PBS and centrifugated at 300xg for 10 minutes. To isolate primordial germ cells, gonadal cells were incubated with anti-stage specific embryo antigen (anti-SSEA)-1 microBeads (Miltenyi Biotech, Germany) in 20 µl mixed with 80 µl of Buffer (PBS supplemented with 0.5% BSA and 2mM EDTA) for 15 minutes at 4°C. Cells were washed with 1 ml of buffer, and were resuspended in 200 µl of buffer and subsequently loaded onto MACS columns (Kim et al., 2004). Using the magnetic field of a MACS separator, PGC cells bound to anti-SSEA-1 microbeads were isolated and were cultured in Knockout DMEM (KO-DMEM; Invitrogen, USA) medium.

### Short-term culture of primordial germ cells

PGC cells are sensitive cells and need a specific culture medium for growth. This appropriate medium was optimized after a while, as these cells have the ability of growth in this medium. Primordial germ cells were seeded in 24 well cell culture plate with or without the presence of mitotically inactivated STO cells ($3 \times 10^4$ cells/cm$^2$) in KO-DMEM, which was supplemented with 7.5-10% (vol/vol) FBS, 2.5-5% (vol/vol) chicken serum (Sigma-Aldrich, USA), 1× Penicillin (100 U/ml)/Streptomycin(100 µg/ml) (Biosera, UK) , 2mM glutamine (Sigma-Aldrich, USA), 1mM pyruvate (Sigma-Aldrich, USA), 10 mM nonessential amino acids (Sigma-Aldrich, USA), 10 mM HEPES (Sigma-Aldrich), 0.15 mM β-mercaptoethanol (Sigma-Aldrich, USA), 2 ng/ml mouse leukemia inhibitory factor (LIF) (Sigma-Aldrich, USA), 3-12 ng/ml human basic fibroblast growth factor (Sigma-Aldrich, USA), and 6-10 ng/ml stem cell factor (Sigma-Aldrich, USA). The seeded cells were then cultured in a $CO_2$ incubator at 37.5°C in an atmosphere of 5% CO2 in air with 90% relative humidity. Half of the culture medium was replaced with new culture medium every two days, and the old culture medium was replaced totally after one week.

### Periodic acid-Schiff (PAS) staining

Isolated PGCs and PGC colonies were fixed in 4% paraformaldehyde (PFA; Electron microscopy sciences, USA) in PBS (vol/vol) for 10 minutes. After rinsing in PBS, the cells were then immersed in periodic acid solution (Sigma-Aldrich, USA) for 5 minutes, and subsequently were treated with

Schiff's reagent (Sigma-Aldrich, USA) for 15 minutes. All procedures were performed at room temperature, and the stained PGCs were observed under a white-light microscope (Olympus BX51, Japan).

**Alkaline phosphates staining**

After fixation with 4% PFA for 10 minutes, cells and colonies were washed three times with alkaline phosphatase (AP) buffer, and then covered with AP solution (1ml of for each coverslip). After 15 minutes, cover slips were washed with AP buffer and were observed under a microscope (Olympus BX51, Japan). The AP buffer contained 100mM Tris-HCl (Merck, USA), 100mM NaCl (Merck, USA), 5mM $MgCl_2$ (Merck USA), 0.05% Tween 20 (Merck, USA), with a pH of 9.5. AP solution was prepared by mixing 120µl of 1% BCIP (5-bromo-4-chloro-3-indolyl phosphate; Fermentas, USA) in 100% DMF (dimethylformamide; Merck, USA), 120µl of 1.5% NBT (nitro blue tetrazolium; Fermentas, USA) in 70% DMF, and 5 ml of AP buffer.

**Immunocytochemistry**

Isolated PGCs and PGC colonies were fixed with 4% PFA for 10 minutes. After washing with PBS (three times, five minutes each), cells were permeabilized with 0.5% Triton X-100 (Merck, USA) for 5 minutes. After washing with PBS, to minimize nonspecific binding of antibodies, the fixed cells were blocked for 45 minutes with a blocking buffer consisting PBS with 5% (v/v) BSA. Then, cells were washed three times with PBS, and were incubated with each of the primary antibodies including mouse anti-SSEA-1 (1:100; Santa Cruz Biotechnology Inc., USA), mouse anti-SSEA-4 (1:150; Santa Cruz Biotechnology Inc., USA), rabbit anti-VASA (1:200; Santa Cruz Biotechnology Inc., USA), After incubation for 2 hours in the primary antibody solution in a humid chamber at room temperature, the cells were washed three times with PBS. Then, cells were incubated with either of the secondary antibodies, donkey anti-rabbit IgG conjugated to Cy3 (Jackson ImmunoResearch, USA), donkey anti-rabbit IgG conjugated to HRP (Jackson ImmunoResearch, USA), donkey anti-mouse IgM conjugated to HRP (Jackson ImmunoResearch, USA), , and goat anti-mouse IgG conjugated to HRP (Dianova, Germany), in a dark humid chamber for 1 hour at room temperature. After washing with PBS, coverslips were mounted on the slide with the application of 40 µl antifade plus DAPI (DENAzist Asia, Iran) or DAB staining (Fermentas, USA) and analyzed under a fluorescence or light microscope (Olympus BX-UCB, Japan). Negative controls, without the use of primary antibody, were only stained with the secondary antibody.

## Results

**Characterization of primordial germ cells**

The isolated PGCs (from HH stages 18 and 28) contained a large nucleus and many prominent vacuoles in the cytoplasm (Fig. 1A). PAS (periodic acid-Schiff) staining of PGCs produced a diffuse staining pattern throughout the cytoplasm indicating a cytoplasm rich in glycogen particles (Fig. 1B). Staining with alkaline phosphatase activity, a pluripotency cell marker, also was positive for primordial germ cells (Fig. 1C). Many antibodies are used for showing the cell epitopes of primordial germ cells. For example, in human we can refer to SSEA-3 ،SSEA-4 and TRA-1 (Henderson et al., 2002), in mouse, SSEA-1 is recognized as the pluripotency marker (Resnick et al., 1992). In chicken, different markers like SSEA-1 ،SSEA-3 ،SSEA-4 and EMA-1 has been reported (Jung et al., 2005). In the present study, Immunostaining with the pluripotency markers, SSEA-1 (stage-specific embryonic antigen 1) and SSEA-4 (stage-specific embryonic antigen 4), demonstrated that PGCs were strongly stained for these markers (Fig. 1D, 1E). In addition, to determine whether isolated PGCs also express the germ cell-specific protein, we used immunofluorescence to detect VASA (CVH), a RNA processing protein important for germ cell survival and specification (Kuramochi-Miyagawa et al., 2010). Immunostaining with an antibody to VASA illustrated that in Primodial germ cells it was localized throughout the cytoplasm (Fig. 1F). This is consistent with the reported cytoplasmic localization of CVH in avian germ cells (Tsunekawa et al., 2000).

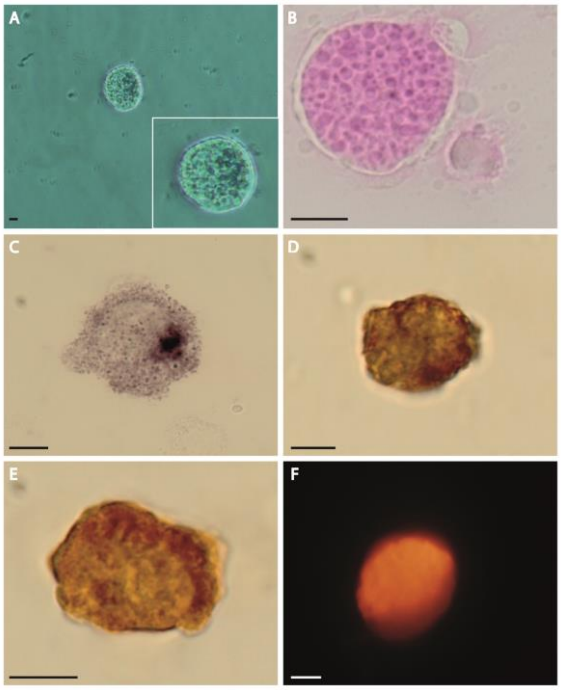


**Figure 1.** Characterization of PGCs. A) A single PGC under white light microscope, B) A single PGC Stained with PAS, C) A single PGC stained for AP activity, D) A single PGC immunostained for SSEA-1, E) A single PGC immunostained for SSEA-4, F) A single PGC immunostained for VASA.
Scale bar represents 5µm.

## Short-term culture of primordial germ cells

A number of experiments were carried out to identify the most appropriate concentration of sera and growth factors for in vitro culture of PGCs. The best results obtained with STO as feeder cells, 10

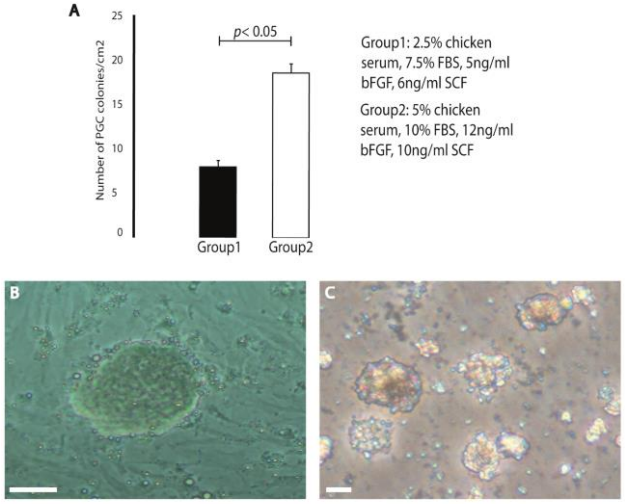


**Figure 2.** Culture of primordial germ cells. A) Optimization culture medium for PGCs . B) A single colony under white light microscope after 2 weeks. C) Apoptosis of the colonies occurred in the absence of the growth factors (Scale bar represents 50µm).

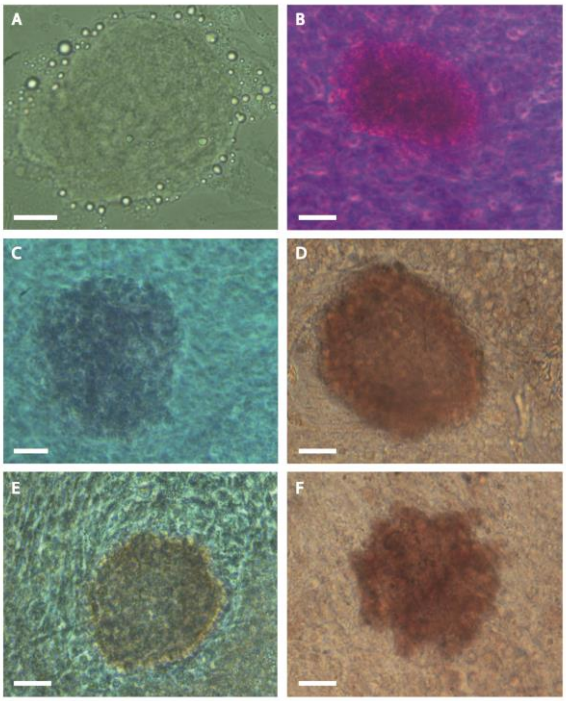


**Figure 3.** Characterization of colonies of PGCs. A) A single colony under white light microscope, B) A single colony stained with PAS, C) A single colony stained for AP activity, D) A single colony immunostained for SSEA-1, E) A single colony immunostained for SSEA-4, F) A single colony immunostained for VASA (Scale bar represents 25µm).

Percent FBS, 5 percent chicken serum, 12ng/ml of human basic fibroblast growth factor, and 10ng/ml of stem cell factor (Fig 2A). Typical colonies of PGCs were observed two weeks after culture (Fig. 2B). Apoptosis of the colonies occurred even in the absence of the growths factor (Fig 2C). Cell colonies resembling ES colonies were formed, with the appearance of discs with smooth ridges and densely packed cells or multilayer congregates. Primordial germ cells form colony after 7 to 10 days. These colonies were adhered to the surface of STO cells. In the present study, they had a circular appearance with a specific margin (Fig 2B).

## Characterization of colonies

Colonies of primordial germ cells (Fig 3A) also were characterized with specific methods. These colonies were positive with periodic acid Schiff (Fig 3B) and alkaline phosphatase (Fig 3C). Staining with pluripotency markers, SSEA-1 (Fig 3D) and SSEA-4 (Fig 3E) were positive. To confirm the germ-cell identity of the colonies, we detected the vasa which has been shown to be a specific germ-cell marker (Fig 3F).

## Discussion

Primordial germ cells and the colonies obtained from PGCs were characterized by staining with PAS, AP, and specific antibodies. Primordial germ cells were cultured under different conditions; in the presence and absence of feeder cells, growth factors and using simple or enrichment culture medium. But as primordial germ cells are sensitive to environmental conditions, they need enrichment culture, growth factors, and feeder cells for growth. Three factors are more important than others e.g. SCF, bFGF, and LIF. In zebrafish, epidermal growth factor, bFGF, kit ligand-a, and stromal cell derived factor-1b were used (Fan et al., 2008). Also, in mouse and human bFG, SCF, and LIF have been used mainly for growing PGCs (Matsui et al., 1992; Shamblott et al., 1998).

In the present study, these factors were added to the culture medium for growing primordial germ cells. The best growth conditions for PGCs are provided in the presence of three factors. Fibroblastic growth factor is activating some signal pathways like MEK/ERK signal, or changing the expression of some genes like IL17RD, also LIF and SCF factors are stopping apoptosis pathways through controlling the expression of tTGase and inhibiting the fragmentation of DNA so these factors play an important role in survival and proliferation of primordial germ cells (Choi et al., 2010; Pesce et al., 1993). Presence of STO feeder cells is important in the culture medium from two aspects; first, these cells provide factors and nutrition for growth of PGCs, and second, a physical connection is made between PGCs and STO cells which help the growth and maintenance of primordial germ cells *in vitro*. It has been shown that physical connection between PGCs and surrounding cells are important in aggregation of these cells in genital ridge (Gomperts et al., 1994) and it's similar for colony formation in culture medium.

Providing an optimized medium to grow PGCs in culture is very important, because a significant amount of primordial germ cells is needed for producing transgenic chickens, and as the number of isolated cells is low in each separation, significant amount can be achieved by culturing and proliferate them in optimized culture medium. On the other hand, by the presence of primordial germ cells in culture medium, it is easier to create changes in genome of primordial germ cells. After confirming the gene transfer into the genome of these cells, injecting these transformed cells into the embryo is done to get the transgenic chicken with the desire capability.

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## References

1. Choi J. W., Kim S., Kim T. M., Kim Y. M., Seo H. W., Park T. S., Jeong J. W., Song G. and Han J. Y. (2010) Basic fibroblast growth factor activates MEK/ERK cell signaling pathway and stimulates the proliferation of chicken primordial germ cells. PloS one 5:e12968.

2. Fan L., Moon J., Wong T., Crodian J. and Collodi P. (2008) Zebrafish primordial germ cell cultures derived from vasa::RFP transgenic embryos. Stem Cells Dev 17:585-597.

3. Fujimoto T., Ukeshima A. and Kiyofuji R. (1976) The origin, migration and morphology of the primordial germ cells in the chick embryo. The Anatomical record 185:139-145.

4. Gomperts M., Garcia-Castro M., Wylie C. and Heasman J. (1994) Interactions between primordial germ cells play a role in their migration in mouse embryos. Development (Cambridge, England) 120:135-141.

5. Gordon J. W., Scangos G. A., Plotkin D. J., Barbosa J. A. and Ruddle F. H. (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proceedings of the National Academy of Sciences of the United States of America 77:7380-7384.

6. Hamburger V. and Hamilton H. L. (1992) A series of normal stages in the development of the chick embryo. Developmental Dynamics 195:231-272.

7. Han J. Y. (2009) Germ cells and transgenesis in chickens. Comparative Immunology, Microbiology and Infectious Diseases 32:61-80.

8. Henderson J. K., Draper J. S., Baillie H. S., Fishel S., Thomson J. A., Moore H. and Andrews P. W. (2002) Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem cells (Dayton, Ohio) 20:329-337.

9. Houdebine L.-M. (2002) The methods to generate transgenic animals and to control transgene expression. Journal of Biotechnology 98:145-160.

10. Jung J. G., Kim D. K., Park T. S., Lee S. D., Lim J. M. and Han J. Y. (2005) Development of novel markers for the characterization of chicken primordial germ cells. Stem Cells 23:689-698.

11. Kim J. N., Kim M. A., Park T. S., Kim D. K., Park H. J., Ono T., Lim J. M. and Han J. Y. (2004) Enriched gonadal migration of donor-derived gonadal primordial germ cells by immunomagnetic cell sorting in birds. Mol Reprod Dev 68:81-87.
12. Kunwar P. S., Siekhaus D. E. and Lehmann R. (2006) In vivo migration: a germ cell perspective. Annual review of cell and developmental biology 22:237-265.
13. Kuramochi-Miyagawa S., Watanabe T., Gotoh K., Takamatsu K., Chuma S., Kojima-Kita K., Shiromoto Y., Asada N., Toyoda A., Fujiyama A., Totoki Y., Shibata T., Kimura T., Nakatsuji N., Noce T., Sasaki H. and Nakano T. (2010) MVH in piRNA processing and gene silencing of retrotransposons. Genes & development 24:887-892.
14. Love J., Gribbin C., Mather C. and Sang H. (1994) Transgenic birds by DNA microinjection. Bio/technology (Nature Publishing Company) 12:60-63.
15. Matsui Y., Zsebo K. and Hogan B. L. (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70:841-847.
16. Pesce M., Farrace M. G., Piacentini M., Dolci S. and De Felici M. (1993) Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). Development (Cambridge, England) 118:1089-1094.
17. Petitte J. N., Clark M. E., Liu G., Verrinder Gibbins A. M. and Etches R. J. (1990) Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. Development (Cambridge, England) 108:185-189.
18. Resnick J. L., Bixler L. S., Cheng L. and Donovan P. J. (1992) Long-term proliferation of mouse primordial germ cells in culture. Nature 359:550-551.
19. Shamblott M. J., Axelman J., Wang S., Bugg E. M., Littlefield J. W., Donovan P. J., Blumenthal P. D., Huggins G. R. and Gearhart J. D. (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells. Proceedings of the National Academy of Sciences 95:13726-13731.
20. Swift C. H. (1915) Origin of the definitive sex-cells in the female chick and their relation to the primordial germ-cells. American Journal of Anatomy 18:441-470.
21. Tsunekawa N., Naito M., Sakai Y., Nishida T. and Noce T. (2000) Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. Development (Cambridge, England) 127:2741-2750.
22. van de Lavoir M., Mather-Love C., Leighton P., Diamond J. H., Heyer B. S., Roberts R., Zhu L., Winters-Digiacinto P., Kerchner A., Gessaro T., Swanberg S., Delany M. E. and Etches R. J. (2006) High-grade transgenic somatic chimeras from chicken embryonic stem cells. Mech Dev 123:31-41.
23. Yasuda Y., Tajima A., Fujimoto T. and Kuwana T. (1992) A method to obtain avian germ-line chimaeras using isolated primordial germ cells. Journal of reproduction and fertility 96:521-528.