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Cultivation and Biological Characterization of Chicken Primordial Germ Cells

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ABSTRACT

The purpose of this work was to investigate the isolation, culture process of chicken gonadal primordial germ cells (PGCs) and study their biological characterization. PGCs were harvested from 5.5-day-old chicken embryonic genital ridges and explanted onto chicken embryonic fibroblasts (CEFs). The results showed that the primary cultivation of chicken PGCs on their own gonadal stroma cells were better than CEFs at first two days for reproduction. The conditioned media supported the growth and colony formation of PGCs for a prolonged time in vitro *and maintained a normal diploid karyotype, which were positively stained by alkaline phosphatase (AKP), periodic acid Schiff (PAS) and reacted with anti-SSEA-1, SSEA-3, Oct4, Blimp1 and Sox2. Real-time PCR showed that they expressed the stage specific genes CVH, Blimp1 and Dazl, the stem cell specific genes Sox2, Pouv and Nanog. They also formed the embryoid bodies (EBs). These results suggested that the chicken PGCs cultured* in vitro *not only had strong self-renewal ability, but also had the potential capability of multi-lineage differentiation.*

Key words: Chicken, Primordial germ cells, Biological characteristics, *in vitro*

INTRODUCTION

Primordial germ cells (PGCs) arise early in embryogenesis and migrate to the germinal crescent region at stage 4, and then enter the developing blood vascular system (Naito et al. 2015). It is precursors of the male and female gametes and develops into sperm, or egg cells (Nieuwkoop and Sutasurya 1979), which carry genetic information through sexual reproduction to future generations (Johnson et al. 2011, Naito et al. 2015). It is a pluripotent stem cell resource that has been discovered after embryonic carcinoma cells (ECCs), teratocarcinoma stem cells (TSCs) and embryonic stem cells (ESCs) (Matin et al. 2004, Nayernia et al. 2004). Under the combined effect of growth factors and feeder cell layer, PGCs proliferate and develop for long-term and maintain undifferentiation for embryonic Germ Cells (EGs), just like the state of embryonic stem cells with totipotency of developmental potential. Establishment of the EG for the study of germ cell development and looking for new effective carrier for genetically modified animal operations have great value (Matsui et al. 1992, Resnick et al. 1992). The first bona fide germ cells, the PGCs, leave the epiblast with the extraembryonic mesoderm at the posterior primitive streak, and a group of around 40 PGCs locates at the base of the allantois (Mintz and Russell 1957). These cells successively pass around provisionally into the bloodstream during the stages 13–15 and then migrate to the gonads and differentiate into female or male gametes (Kuwana and Rogulska 1999, Naito et al. 2015). However, getting the PGCs are restricted. Chicken as an

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acceptance model organism (Cogburn et al. 2007) have advantages of population distribution and samples can be obtained easily. PGCs have been obtained mainly from germinal crescent (Petitte et al. 1990, Park and Han 2012) and embryonic blood (van de Lavoir et al. 2006, Macdonald et al. 2010, Miyahara et al. 2014, Naito et al. 2015). However, their numbers have been quite limited (Park et al. 2003) and couldn't provide sufficient materials for the transgenic manipulation (Shiue et al. 2009). Therefore, chicken PGCs from embryonic gonad and long-term *in vitro* culture could be adequate resource for genetic manipulation.

Several studies have been made on chicken PGCs since Dubois reported it in 1969 (Dubois 1969). Allioli et al. (1994) reported that chicken PGCs isolated from the gonads could proliferate *in vitro* for several days with three-culture system, respectively. Park and Han (2002) established a long-term *in vitro* culture system for chicken gonadal PGCs. They isolated PGCs from the undifferentiated gonads of stage 28 chicken embryos and maintained their specific characteristics for up to two months *in vitro*.

The purpose of this work was to investigate the isolation, culture process of chicken gonadal primordial germ cells (PGCs) and study their biological characterization.

MATERIAL AND METHODS

Experimental animals

All the animal procedures were approved by the Institutional Animal Care and Use Committee of Chinese Academy of Agricultural Sciences. Chicken embryos were provided by the Experimental Animal Base Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing. The use of animals in research and all experimental procedures involving chicken embryos were conducted in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School.

Preparation of CEFs

Fibroblasts were isolated from chicken embryos in incubated eggs for eight days, and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with high glucose containing 10% fetal bovine serum (FBS, HyClone), then treated with 10 μg/mL Mitomycin-C (Roche) for 2 h after-third generation. CEFs were used as the feeder cells for PGCs and were rinsed with Ca^{2+} and Mg^{2+}free PBS five times (Hong, Moon et al. 1998).

Isolation and *in vitro* culture of chicken PGCs

Fertile eggs were incubated at 38°C and 50% humidity for 5.5 days. Gonads of the chicken embryos developed at stage 28 (Hamburger and Hamilton 1951), were isolated using stereoscopic microscope, then embryonic gonadal tissues were collected and dissociated with 0.125% trypsin-0.02% EDTA. After neutralized with DMEM containing 10 % FBS, the gonadal cells were collected by centrifugation.

The precipitated cells were re-suspended in PGCs cell culture medium, which consisted of DMEM medium replenished with 10% FBS (Gibco, New York, USA), 2% chicken serum and supplemented with 10 ng/mL of human stem cell factor (hSCF; Pepro Tech, London, UK), 10 units/mL of leukemia inhibitory factor (LIF; Pepro Tech, London, UK), 20 ng/mL of human basic fibroblast growth factor (bFGF; Pepro Tech, London, UK), 1 × penicillin/streptomycin (Life Technologies). Cell suspensions were seeded into 6-well culture plates with CEFs as feeding layer (Choi et al. 2010) at a density of 1×10^4/well, and cultured at 37.5℃ in 5% CO_2. The PGCs colonies were dissociated and moved into fresh plates with CEFs using trypsin-EDTA treatment for subculture (Liu et al. 2014).

PAS staining and alkaline phosphatase activity assay

For PAS staining, the cell colonies of the cultured chicken PGCs were fixed with 4% paraformaldehyde for 20 min and washed 3×5 min with PBS. The cell colonies were then submerged in periodic acid solution (Sigma-Aldrich) for 5 min at room temperature. After washing twice with PBS, the cell colonies were immersed in Schiff's solution (Sigma-Aldrich) for 15 min at room temperature. Then PAS-stained PGC colonies were observed under an inverted microscope after washing twice with PBS. AKP activity was detected by AKP substrate kit (Sigma-Aldrich) according to the manufacture's instruction. Images were captured with a computer-assisted video camera (IX-71 inverted research microscope, Olympus, Japan).

Karyotyping

The chicken PGCs were incubated in 0.5 µg/mL colcemid (Karyomax, Invitrogen) at 37.5°C in 5% CO_2 for 5 h, then the chicken PGCs were dissociated with 0.125% trypsin-0.02 % EDTA. The cells were then centrifuged and re-suspended in 0.075 M KCl solution at 37℃. After 30 min, the cells were centrifuged at 200 g for 8 min and the pellet was fixed in 3:1 methanol: glacial acetic acid. For metaphase analysis, the cell suspension was dropped on the frozen glass slides, stained with Giemsa (Amresco, Solon, OH, USA) and analyzed for the six pairs of macro-chromosomes and the sex chromosomes. At least 20 metaphase spreads were counted for every chicken PGC passage.

Immunocytochemistry staining

For immunocytochemical analysis, the chicken PGCs were fixed in 4% paraformaldehyde (PFA) in PBS and permeabilized with 0.4% Triton/X-100. Blocking with 10% goat serum was performed for 1 h prior to the incubation with primary antibodies. The cells were incubated with primary antibodies ant-SSEA-1, SSEA-3, BLIMP1, Oct4 and Sox2 at 4℃ overnight, followed by with goat anti-rabbit Alexa Fluor 594 (red) -labeled secondary antibody (A11072, Invitrogen) for 1 h. DAPI was used to label the cell nuclei, and images were captured with a fluorescence microscope (Nikon TE-2000-E inverted microscope, Japan).

RT-PCR analysis

Total RNA was extracted from the PGC colonies at third passage using TRIzol (Invitrogen). After DNase I treatment to remove the potential contamination of genomic DNA, 2.0 µg of total RNA were reverse transcribed into cDNA using an RNA PCR Kit (AMV) Ver 3.0 (Takara, China). The gene expression analysis was detected by ABI StepOnePlus real-time

Table 1 - The primer information for PGC identification.

PCR thermal cycling instrument (USA). The stage specific genes CVH, BLIMP1 and Dazl, the stem cell specific genes Sox2, Pouv and Nanog were detected (the primers information shown in Table 1), the PCRs reaction were performed by the PCR Master Mix Kit (Promega, USA), and the PCR products were visualized by electrophoresis on 2.5 % agarose gels.

Gene name	Primer sequences	Circles	Product length (bp)	Tm (℃)
CVH	F: 5'- ATGATGAATGTGCTGTTGAG-3'	35	149	50
	R: 5'- AGGAATGCTGTGCTTCTG-3'			
BLIMP1	F: 5'- ACCTCACATTCCAGAAGAC-3'	35	108	50
	R: 5'- GAAGGACTTGGTGTAGTAGAT-3'			
DAZL	F: 5'- CAATATGGTACTGTGAAGGAG-3'	35	126	50
	R: 5'- GACACTGATCTGTGATTCTAC-3'			
SOX2	F: 5'- GTCACCTCCTCGTCTCAT-3'	35	140	51
	R: 5'- GCACTCTGGTAGTGTTGG-3'			
POUV	F: 5'- CAAGGACCTCAAGCACAA-3'	35	89	50
	R: 5'- TGAACATCTTCCCATAGAGC-3'			
NANOG	F: 5'- GCCTGTATCCGTTCATGG-3'	35	140	51
	R: 5'- GTCATAATCCACATTGGTAGAG-3'			
GAPDH	F: 5'-GAGGGTAGTGAAGGCTGCTG-3'	35	113	51
	R: 5'-CATCAAAGGTGGAGGAATGG-3'			

Embryoid body formation

The three passages of PGCs were used for EBs formation. PGC cell colonies were digested with 0.125% trypsin-0.02% EDTA for 1min at 37℃, gently broken into single cell and cultured on a gelatin-coated dish in suspension for two weeks in DMEM supplemented with 15 % FBS. The medium was changed every two days. The cultures were maintained at 37.5℃ in a humidified 5% CO_2 incubator.

Statistical analysis

Statistical analyses of the data were performed with a one-way ANOVA, followed by the Tukey-Kramer honestly significant difference (HSD) test for the three sets of results. AP-value of less than 0.05 was considered significant. Statistical analyses were done with a JMP○,R Statistical Discovery Software (SAS Institute, Cary, NC).

RESULTS

Culture and characterization of chicken PGCs

In this study, the primary cultivation of the chicken PGCs were plated together with their own gonadal stroma cells. The PGCs formed cell colonies after two days (Fig. 1A). On the contrary, directly cultured chicken PGCs with CEFs formed PGCs colonies less efficiently (Fig. 2). After subculture of the single PGCs were attached to the surface of CEFs and grown up into small cell colonies after seeding for 24 h (Fig. 1B), the small cell colonies turned to bigger cell clusters after three days (Fig. 1C). The PGCs were cultured for five weeks and treated for subculture on an average of three days. The PGCs cell morphology and growth characteristics are the basis identification of PGCs (Meyer 1964). The size of PGCs, large amounts of glycogen granules in the cytoplasm and large nuclei are bigger than somatic cells. Therefore, PAS stained of PGCs can be set apart from somatic cells (Jung et al. 2005). The presence of alkaline phosphatase activity is an important indicator for the cells in the undifferentiated state, like ES cells (O'Connor et al. 2008). Undifferentiated PGCs were with high AKP activity, hence the differentiation of PGCs cultured *in vitro* by AKP staining could be examined. This study showed that PGCs were stained mauve with AKP staining and red by PAS staining (Fig. 1E-F).

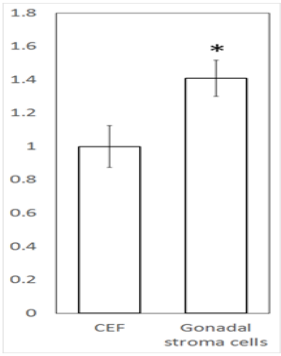

Figure 2 - Colony forming efficiency of PGCs under two different conditions. Under the gonadal stroma cells condition PGCs could form cell colonies more efficiency than the condition of CEF. ($*P < 0.05$).

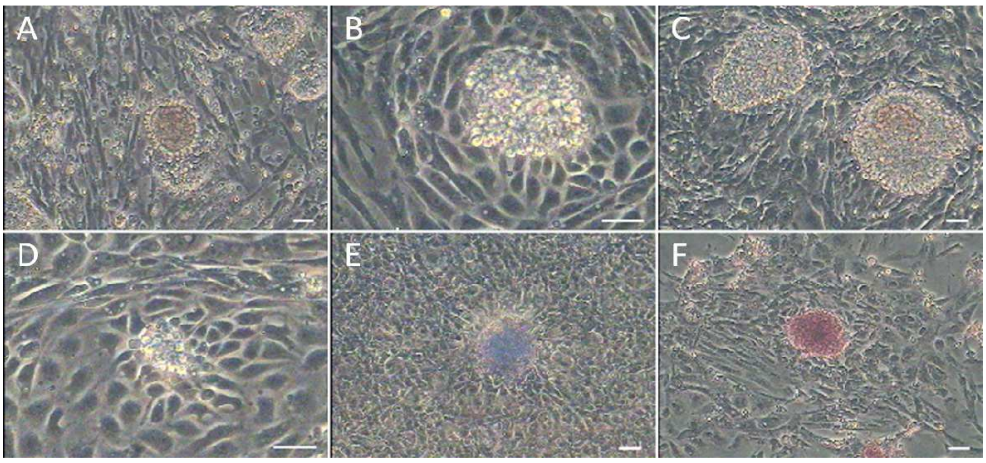

Figure 1 - Morphology of PGCs. (A) On day 2 of primary culture, PGCs had a distinct ‘‘colony’’ shape. (B) On 24h of subculture, PGCs grown up into small cell colonies. (C) On day 3 of subculture, the small cell colonies turned to bigger cell clusters. (D) On day 3 of primary culture, the gonadal stroma cells apoptosis cause PGCs differentiation. (E-F) PGCs were stained mauve with AKP staining and red by PAS staining (scale bar: 100μm).

Karyotype and chromosome number of chicken PGCs

The chromosome karyotype was checked in fifth passage. Karyotype analysis revealed that the PGCs were diploid (2n=78) with nine pairs of macrochromosomes and 30 pairs of microchromosomes (Fig. 3). The sex chromosome type was ZZ or ZW. PGCs karyotype was no variation after subculture.

Immunocytochemistry staining of chicken PGCs

The key PGC genes in humans include NANOS3, BLIMP1, TFAP2C, STELLA, TNAP, KIT, OCT4, and NANOG (Irie et al. 2015). Human ES express cell-surface antigens SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, while mouse ES cells express SSEA-1 (Henderson et al. 2002). The three passages of PGCs expressed stage-specific surface

makers SSEA-1, SSEA-3, BLIMP1 and stem cell makers Oct4, Sox2 (Fig. 4).

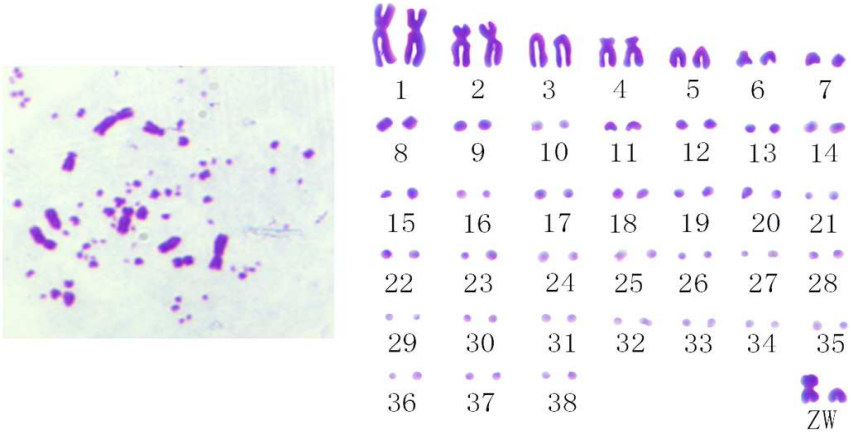

Figure 3 - Chromosome at metaphase (left) and karyotype (right) of chicken PGCs (♀) ZW type. The diploid chromosome number in chicken PGCs was 2n=78, consisting of nine pairs of macrochromosomes and 30 pairs of microchromosomes.

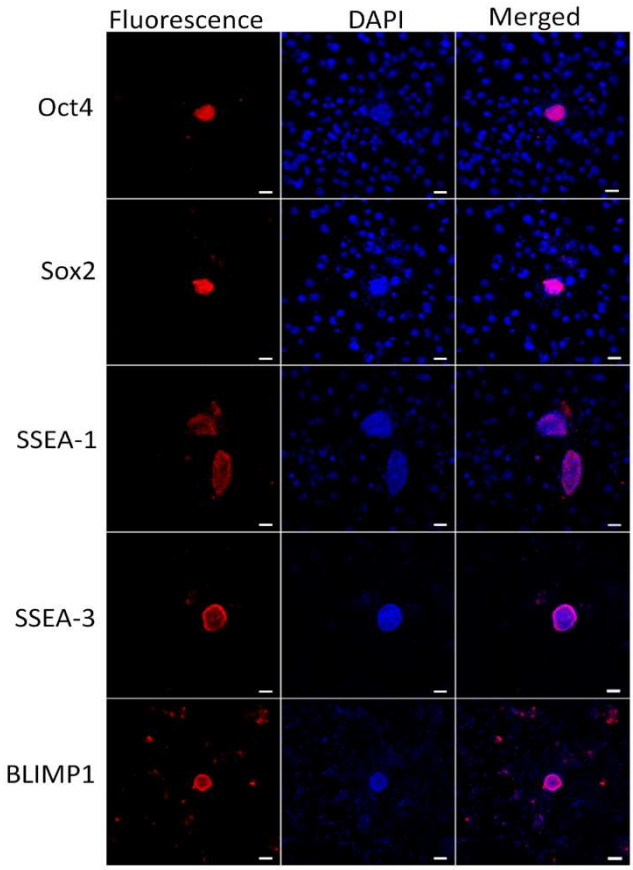

Figure 4 - Surface marker detection of chicken PGCs by immunofluorescence staining. The results show that Oct4, Sox2, SSEA-1, SSEA-3 and BLIMP1 are positively expressed (scale bar: 100μm).

RT-PCR for detection of specific gene expression results

The analysis of CVH, Blimp1, Dazl, PGCs marker gene, and Sox2, POUV, Nanog, stem cell gene, by

RT-PCR (Fig. 5) showed that the cell colonies in three passages were PGCs, which were detected for stem cell maintenance.

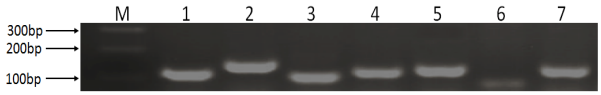

Figure 5 - Detection of PGCs markers by RT-PCR. M: Marker; 1: GAPDH (113 bp); 2: CVH (149 bp); 3: BLIMP1 (108 bp); 4: DAZL (126 bp); 5: SOX2 (140 bp); 6: POUV (89 bp); 7: NANOG (140 bp).

EBs formation

To induce EB formation from the PGCs, the undifferentiated PGCs at passage three were removed from the feeder layers and cultured in EB formation flasks with a non-factors medium and a suspension condition. Initially, PGCs were shown in single round cell colonies with a bright refraction (Fig. 6A). After three days, PGCs aggregated gradually and formed simple EBs (Fig. 6B), and two days later, the number of EBs increased (Fig. 6C). After culturing a week, the size of EBs increased (Fig. 6D). On day 10, a single-layer columnar cells were observed at the edge of the suspending EB clusters; the single-layer cell boundary were clear; the cells in the internal parts of the EBs had a trend from open growth to dense growth, and some EBs formed cavities in the center area without any columnar cells (Fig. 6E). At the second week, EBs had a further growth in size, most of them were in round shape as before, but some of them were in irregular shape combined multiple round cell clusters (Fig.6F).

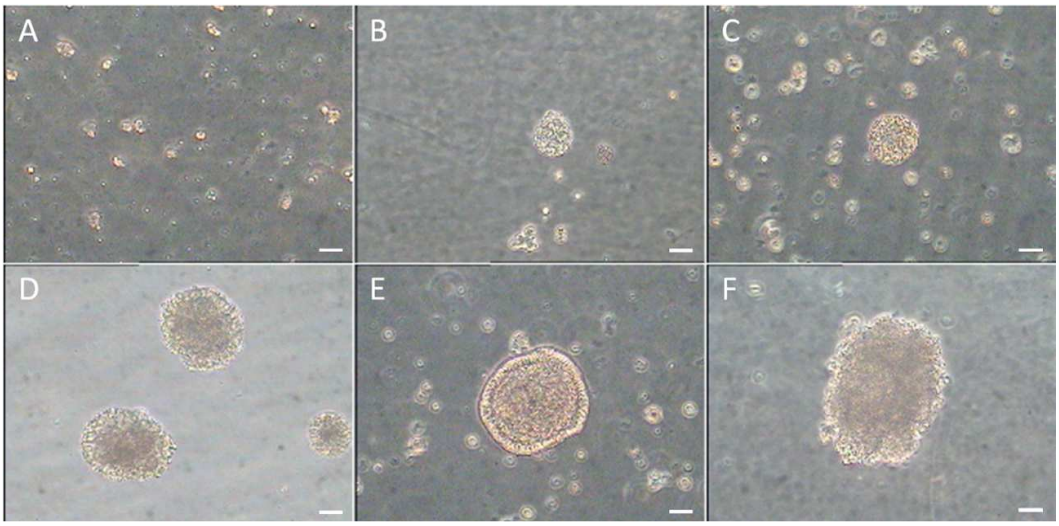

Figure 6 - EBs formation from PGCs. (A) On primary culture, PGCs were in single round cell. (B) After three days PGCs aggregated gradually and formed simple EBs. (C) On day 5 of culture, the number of EBs increased. (D) On a week of culture the size of EBs increased. (E) On day 10, the edge of the EB clusters appeared a single-layer columnar cells. (F) On two weeks of culture, EBs had a further growth in size (scale bar: 100μm).

DISCUSSION

The primary culture of the chicken PGCs were plated together with their own gonadal stroma cells; cultured PGCs had a distinctive cellular morphology consisting of a large nucleus and a cluster of large vacuoles present in the cytoplasm, ground round PGCs cluster with less differentiated cells from the PGCs. There were gonadal stroma cells, which provided adequate nutrition to the PGCs at the first two days just like *in vivo*, but their supply was not up with the PGCs for a long time. After three days, the gonadal stroma cells apoptosis led to the rupture of feeder layer and caused PGCs differentiation.

Previous studies (Yang and Petitte 1994, Choi et al. 2010) have shown that CEFs provide growth environment and signals for the PGCs attachment of adherent growth and secreted growth factor inhibited their differentiation, as feeder layer was a

good choice. The primary cultured and 1st passage of CEFs act as feeder without highly pure fibroblast, but higher than four passages fibroblast, easy chromosome anomalies, may decline the functions as feeder layer. On the other hand, the quality of feeder layer cells have a great relationship with the time of Mitomycin-c treat, rinsing Mitomycin-c cleanly, or not and proper seeding density. High-density feeder layer cells not only produce contact inhibition of the normal cells to reduce the energy, but also make excessive culture fluid drain. Low-density fibroblasts cannot contiguous to form feeder single-layer. Hence, three passage CEFs was used at a density of 1×10^5/well, washing with PBS eight times after Mitomycin-c treatment as feeder layer. The chicken PGCs could be maintained for over 10 passages in an undifferentiated state on a feeder layer made with well-grown CEFs.

Twice diploid karyotype is the prerequisite for the cell growth and functioning. Chickens have nine pairs of macrochromosomes and 30 pairs of microchromosomes with sex chromosomes Z and W (Pollock and Fechheimer 1976, Fechheimer 1990). In this study, the chromosome karyotype of fifth passage was examined, which showed that chicken embryos PGCs were able to maintain a normal chromosome after subculture of five passages.

In this study, the chicken PGCs could propagate *in vitro* for five weeks in the culture medium replenished with hSCF, LIF and bFGF. The chicken PGCs expressed Cvh, Blimp1 and Dazl (van de Lavoir et al. 2006a), and the transcription factors POUV, Nanog and Sox2 acted cooperatively to maintain the pluripotency in both humans and mouse ESC (van de Lavoir et al. 2006b). Finally, the formation of EBs *in vitro* shoed that the chicken PGCs were potentially capable of multi-lineage differentiation.

CONCLUSION

The results of this study confirmed that the cells derived from stage 28 chickens genital ridge exhibited a morphology and epitope profiles that were similar to those of duck ES and EG cells after extended periods in culture. The primary cultivation of chicken PGCs on their own gonadal stroma cells were better than CEFs at first two days. The chicken PGCs cultured on the CEFs were successfully grown. CEFs could be used to support the growth of PGCs *in vitro* for five weeks. The chicken PGCs could form EBs *in vitro*.

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