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—Original Article—

Isolation, Culture and Biological Characteristics of Primordial Germ Cells from Beijing Fatty Chicken

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Abstract. The aim of this study was to explore the isolation and culture process of Beijing fatty chicken primordial germ cells (PGCs) and investigate their biological characteristics. The PGCs isolated from the genital ridges of Beijing fatty chicken (*Gallus domesticus*) embryos after 5.5 days of incubation were co-cultured with mice embryonic fibroblasts (MEF). The results showed that the PGCs of the Beijing fatty chicken were positive for periodic acid Schiff (PAS) and alkaline phosphatase (AKP) staining. These cells could proliferate for a prolonged time *in vitro* and maintain diploid karyotype. Immunocytochemical staining showed that they expressed SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81, and real-time PCR showed that they expressed Cvh, CDH and Dazl. They could form simple embryoid bodies and differentiate into osteoblasts in vitro. In addition, after transfected with pEGFP-N3, pEYFP-N1 and pDsRed-N1 vectors by liposomal transfection, enhanced green, yellow and red fluorescent protein-positive cells could be visualized using a laser confocal microscope. The above results suggested that PGCs from the Beijing fatty chicken not only had strong self-renewal ability, but also had the potential to differentiate towards mesoblast cells. These cells are suitable for genetic manipulation as nuclear donors.

Key words: Beijing fatty chicken, Biological characteristics, Differentiation, Primordial germ cells

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As progenitors of spermatogonia and oogonia, PGCs are characterized by their pluripotency, and therefore represent a good model for the study of embryo development *in vitro*. Owing to their physiological and developmental characteristics, the avian, especially the chicken, has great value in transgenic research. An efficient transfer system of using chicken gonadal PGCs for producing germline chimeras has been established [1]. Production of transgenic chickens will supply tremendous bioactive materials for human pharmaceutics and functional food development. In chicken germ cell research, it is occasionally considered that SSEA-1 and PAS-positive cells are presumptive PGCs, and the pluripotency of candidate PGCs has been confirmed by induction of germline transmission via the transfer of cells into recipient embryos [2]. Apparently, the former method is somewhat insufficient to fully characterize chicken PGCs, while the latter is too complicated and time-consuming. Therefore, additional effort to identify the characterization of chicken PGCs comprehensively is urgently required for further development of avian transgenic systems.

In this report, PGCs were isolated from the genital ridges of Beijing fatty chicken embryos. After proliferating *in vitro* for a prolonged period, they can maintain diploid karyotype, express a set of typical markers of ES cells and differentiate into osteoblasts *in vitro*. Furthermore, the results demonstrated that the chicken PGCs had a relatively high cloning efficiency and could be used as nuclear donors to accept transgenes readily.

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Materials and Methods

Preparation of mouse embryonic fibroblasts (MEF)

Fibroblasts were isolated from mouse embryos on day 13.5, and cultured in Dulbecco's modified Eagle's (DMEM) medium (Gibco) containing 5% fetal bovine serum (FBS, HyClone), and then treated with 10 μg/ml mitomycin C (Roche) for about 30 min after a 3–5 generation subculture. Cells to be used as feeder cells for PGCs were rinsed with Ca^{2+} and Mg^{2+}-free PBS three times and subcultured again with DMEM medium.

Isolation of Beijing fatty chicken embryonic PGCs

The gonadal cells, which included the PGCs, were retrieved from the gonads of Beijing fatty chicken embryos incubated at 38 C and 60% humidity for 5.5 days. After rinsing three times with PBS to remove residual yolk, gonadal tissues were collected carefully with sharp tweezers under a microsurgery scope, and then dissociated in 0.25% trypsin-0.02% EDTA at room temperature (RT) for 5 min. After inactivation of the trypsin-EDTA with DMEM containing 15% FBS, the cells were harvested by centrifugation [2].

PGCs of the duck and mouse were collected from the day 7 chicken embryos and day 13 mouse embryos, respectively, and the culture conditions used for these PGCs were the same as those for

Table 1. Primer sequences for RT-PCR analysis of target genes

Gene	Accession No.	Forward primer	Reverse primer	Size/bp
Cvh	AB004836	CCTTGCAGCCTTTCTTTGTC	GCCTCTTGATGCTACCGAAG	145
CDH	XM_423051	CAACCGGACCAATAAGATGG	TGAGCGTGTCCTCATACAGG	182
Dazl	NM_204218	CCCTTTAAGTCCCGGTTCTC	CCACTGAGCAGCCAATGTAA	139
cPouV	DQ867024	AAATGTGTGAAGCCCAGTCC	GCTCTGGAGCTGAAGCTGTT	123
cNanog	DQ867025	CAGCAGACCTCTCCTTGACC	TTCCTTGTCCCACTCTCACC	187
Sox2	D50603	AGGCTATGGGATGATGCAAG	GTAGGTAGGCGATCCGTTCA	163
β-actin	NM_205518	CCAGCCATCTTTCTTGGGTA	ATGCCAGGGTACATTGTGGT	141

the chicken PGCs.

Culture of Beijing fatty chicken PGCs

The gonadal cells were cultured in 24-well plates (Corning) at 37.5 C in a water-saturated atmosphere of 95% air and 5% CO_2 with PGCs cell culture media consisting of DMEM, 15% FBS, 1% non-essential amino acid, 0.03 mM purine and pyrimidine, 1 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 5.5×10^{-5} M β-mercaptoethanol, 100 μg/ml of streptomycin, 100 IU/ml of penicillin, 5 ng/ml of human stem cell factor (hSCF; Pepro Tech, London, UK), 10 IU/ml of murine leukemia inhibitory factor (mLIF; Pepro Tech) and 10 ng/ml of human basic fibroblast growth factor (bFGF; Pepro Tech) [3].

For subculture, the colonies of chicken PGCs were rinsed three times with PBS to remove dead cells and digested with 0.25% trypsin-0.02% EDTA for 3 min; the reaction was terminated by 15% FBS in DMEM, and the PGCs were then reseeded into microwell plates with feeder cells.

Periodic acid-Schiff and alkaline phosphatase staining

Subcultured cells were fixed in 4% neutral paraformaldehyde for 10 min and rinsed twice in PBS. For PAS staining, fixed cells were immersed in periodic acid solution for 5–10 min and sequentially immersed in Schiff's Solution for 15 min at RT. After washing with 1 × PBS twice, PAS-stained PGCs were observed under an inverted microscope. Cells were stained for AKP using a BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium) alkaline phosphatase substrate kit (Wuhan Boster, China), according to the manufacturer's instructions. Images were captured with a computer-assisted video camera (IX-71 inverted research microscope, Olympus, Japan).

SYBR Green real-time PCR analysis of gene expression

Total RNA was extracted from PGC colonies at the third passage and putative EG colonies at the sixth passage with an RNAprep pure Micro Kit (TIANGEN, China). The cDNA was synthesized using an RNA PCR Kit (AMV) Ver 3.0 (Takara, China). The gene expression analysis was detected by ABI StepOnePlus real-time PCR thermal cycling instrument (USA). The gene expression level was quantified relative to the expression of ACTB (β-actin) gene, and the specificity of PCR products was confirmed by melting curve analysis. Real-time PCR was performed in triplicate in a 20 μl mixture containing 10 μl SYBR Premix Ex Taq buffer (TaKaRa, China), 0.4 μl ROX Reference Dye, 0.8 μM forward and reverse primer (Table 1), 1 μl template cDNA and 7 μl ddH_2O. The cycling conditions consisted of an initial 10 sec at 95 C followed by 40 cycles of two-temperature cycling, 5 sec at 95 C (for denaturation) and 34 sec at 60 C (for annealing and polymerization). The expression level was calculated by the 2-ΔΔCt method to compare the relative expression.

Immunocytochemistry

To analyze marker expression of Beijing fatty chicken PGCs, the cell colonies were fixed in 4% paraformaldehyde for 15 min at RT, washed twice (5–10 min each) in Tris buffered saline with Tween-20 (TBST; 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20), treated with 0.1% Triton X-100/ PBS for 10 min, washed twice (5–10 min each) in TBST buffer and incubated in 3% bovine serum albumin (BSA)/PBS (blocking solution) for 30 min. The primary antibodies were anti-SSEA-1, anti-SSEA-4, anti-TRA-1-60 and anti-TRA-1-81 from an ES Cell Characterization Kit (Chemicon, Temecula CA, USA). All antibodies were diluted in PBS (SSEA-1, SSEA-4, 1:40; TRA-1-60, TRA-1-81, 1:100) and incubated with samples overnight at 4 C. The next day, the colonies were washed three times with TBST buffer and incubated with secondary antibodies (diluted in PBS 1:100) at RT for 1 h. The secondary antibodies were FITC (fluorescein isothiocyanate)-conjugated goat anti-mouse IgG+IgM for SSEA-1, TRA-1-60 and TRA-1-81 and FITC-conjugated goat anti-mouse IgG for SSEA-4 (Wuhan Boster, China). Fluorescent signals were visualized with a confocal microscope (Nikon TE-2000-E inverted microscope, Japan).

Karyotyping

Beijing fatty chicken PGCs were incubated with colcemid (final concentration 0.1 μg/ml) for 4 h at 37 C in 5% CO_2, harvested, centrifuged and resuspended in 0.075 M KCl prewarmed to 37 C. After incubation for 30 min at 37 C, the cells were pelleted again, fixed with 3:1 methanol:acetic acid at 4 C and washed three times with the fixative. Finally, the cell suspension was dropped onto chilled glass slides and stained with Giemsa (Amresco, Solon, OH, USA). At least 20 metaphase spreads were counted for every chicken PGC passage [4].

Embryoid body formation

Beijing fatty chicken PGCs were digested with 0.25% trypsin-0.02% EDTA for 1 min at 37 C, gently broken into small clumps and cultured on a gelatin-coated dish in suspension for 4–8 days in

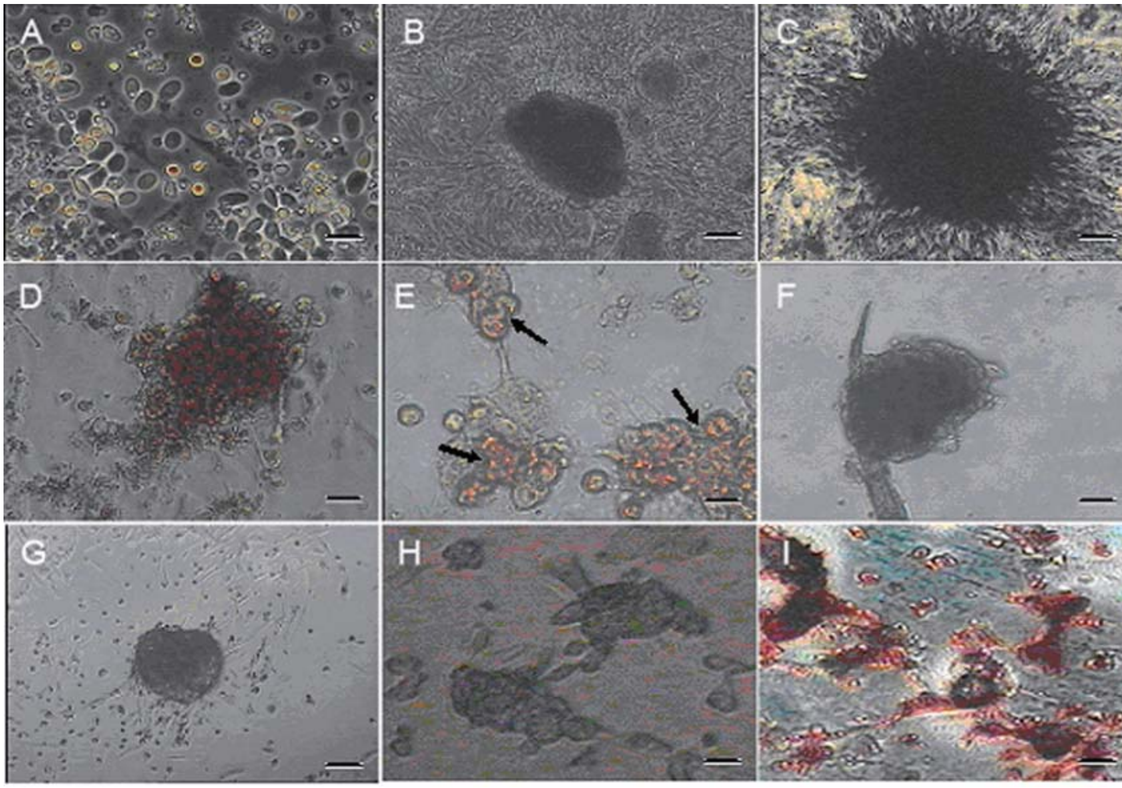

Fig. 1. Cellular morphology and identification of chicken primordial germ cells (PGCs). A: Beijing fatty chicken PGCs with gonadal somatic cells. Scale bar: 35 μm. B: Chicken PGC colony at the second passage on MEF; the colony is uniformly round, multilayered and well-delineated. Scale bar: 80 μm. C: AKP reactivity in a colony formed at the third passage. Scale bar: 80 μm. D: PAS reactivity in a colony formed at the third passage. Scale bar: 80 μm. E: Gonadal PGCs are indicated by arrows after PAS straining. Scale bar: 35 μm. The PGCs tended to gather and form colonies. It is difficult to obtain the picture of a single PGC. F: Embryoid bodies formed in suspension culture after eight days. Scale bar: 100 μm. G: Differentiated cells expanded when EBs were plated onto a gelatin-coated culture dish. Scale bar:100 μm. H: Morphology of osteoblasts induced by chicken PGCs, which consisted of aggregated cells. Scale bar: 35 μm. I: Histochemical staining for Alizarin Red identified discrete mineralized nodules. Scale bar: 35 μm.

a medium consisting of 75% DMEM, 15% FBS, 2 mM L-glutamine, 1% nonessential amino acid, 0.1 mM β-mercaptoethanol and 1 μM all-trans-retinoic acid. The medium was changed every two days [5].

Induction of PGC differentiation to osteoblasts

Beijing fatty chicken PGCs were resuspended in DMEM containing 15% FBS, 100 μg/ml of streptomycin, 100 IU/ml of penicillin, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, 0.1 μM dexamethasone and 1 μM all-trans-retinoic acid for 9–15 days. The medium was replaced every 3 days.

Cultured cells were fixed in 4% paraformaldehyde for 15 min, washed in PBS twice and stained for 5 min with 1% solution of Alizarin Red. Plates were washed in PBS twice, and bone nodule colonies were counted [6].

Expression of three fluorescent protein genes in Beijing fatty chicken PGCs

To lay the foundations for further research on gene transfer, the influences of transfection with foreign genes of three fluorescent proteins (pEGFPN3, pEYFP-N1 and pDsRed1-N1) were assessed. PGCs were seeded in a 24-well plate one day before transfection. A mixture of the plasmid DNA (μg, Clontech, CA, USA) and Lipofectamine 2000 (μl, Invitrogen; 1:2 to 1:3) was added to the wells containing the cells and serum-free medium [4]. The medium was changed after 6 h, and cells were examined at 24, 48 and 72 h and 1 and 2 weeks after transfection using a Nikon TE-2000-E inverted microscope at excitation wavelengths of 433-588 nm to determine the transfection efficiency and morphology of the positive transfected cells. For each experiment group, images were captured from 10 visual fields, and confocal fluorescence microscopy was used to measure the total and positive cell counts in each field to determine the transfection efficiency. The effect of exogenous genes on the cells was measured by cell motility using trypan blue [7].

Results

Culture and characterization of Beijing fatty chicken PGCs

In primary culture, Beijing fatty chicken PGCs formed nest col-

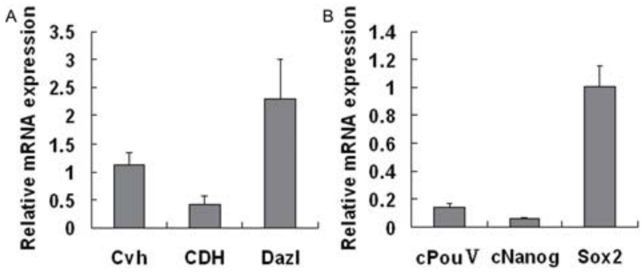

Fig. 2. Analysis of mRNA expression by real-time RT-PCR. A: Expression of Cvh, CDH and Dazl at the third passage. B: Expression of cPouV, cNanog and Sox2 in chicken EG cells at the sixth passage.

onies after three days. The PGCs colonies were maintained without any observable morphological differentiation until the third passage (Fig. 1A and B). PGCs were stained dark blue with AKP staining and stained red by PAS histochemistry (Fig. 1C, D and E).

Chicken vasa homologue (Cvh), chicken dead end homologue (CDH) and deleted in azoospermia-like (Dazl) genes, as the PGCs marker genes, were analyzed by real-time RT-PCR (Fig. 2A) [8]. The results revealed that most of the cells in colonies within three passages were PGCs. Additionally, the expressions of cPouV, cNanog and Sox2, which were proven to be critical for stem cell self-renewal and pluripotency, were also detected in putative EG colonies at the sixth passage (Fig. 2B).

Differentiation in vitro

Beijing fatty chicken PGCs showed a specicial morphology and formed simple embryoid bodies (EB) readily (Fig. 1F) in the presence of serum in a suspension culture, which suggested that the PGCs were capable of differentiation into a variety of cell types *in vitro*. When plated onto a gelatin-coated culture dish, EBs grew with differentiated cells (Fig. 1G).

PGCs cultured in DMEM containing 15% FBS, 100 μg/ml of streptomycin, 100 IU/ml of penicillin, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, 0.1 μM dexamethasone and 1 μM all-trans-retinoic acid formed distinct nodular colonies. Histochemical staining for Alizarin Red identified discrete mineralized nodules (Fig. 1H and I).

Marker expression by immunocytochemistry

A set of antibodies recognizing specific cell surface antigens has been used to characterize PGCs [9, 10]. Human ES cells are characterized by the expression of cell-surface antigens, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, and the lack of SSEA-1, while mouse ES cells express SSEA-1, but not the others [11]. Compared with the mouse and duck PGCs in the same figure, chicken PGCs expressed SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81 (Fig. 3).

Karyogram and chromosome number of chicken PGCs

After counting at the first and third passage, this study showed that 97% of the Beijing fatty chicken PGCs were diploid (2n=78) with 9 pairs of macrochromosomes and about 30 pairs of microchromosomes (Fig. 4). The sex chromosome type was ZZ (♂)/ZW (♀).

Expression of three fluorescent protein genes in Beijing fatty chicken PGCs

The three fluorescent protein genes, pEGFP-N3, pEYFP-N1 and pDsRed1-N1, were all highly expressed (Fig. 5). The transfer efficiencies of the yellow (pEYFP-N1) and red (pDsRed1-N1) fluorescent proteins were significantly lower than those of the green fluorescent proteins (pEGFP-N3). Positive cells were observed at 12 h after transfection, and the numbers and intensity increased markedly and reached a maximum at 48 h to 72 h. The numbers of positive cells decreased at 1 week, but a few dispersed positive cells remained after 2 weeks.

Discussion

Isolation and culture of chicken PGCs

Recent reports indicated that PGCs remain restricted to the germ line [13, 14]. Chicken EG and ES cells contribute substantially to somatic tissues but not to the germ line [15]. The production of germline chimeras is very important in understanding germ cell development and specification, but it is difficult to fractionate sufficient numbers of PGCs for manipulation and implantation into developing embryos. We have attempted several times to transfer PGCs into recipient embryos to produce germline chimeras, but all our attempts have failed. Cvh, CDH and Dazl have been found to be expressed exclusively in chicken PGCs [14], while cPouV, cNanog and Sox2 are known as three critical transcription factors that act cooperatively to maintain pluripotency in both mouse and human embryonic stem cells [16]. In this study, we have obtained Beijing fatty chicken PGCs from embryos after 5.5 days of incubation. The PGCs could propagate *in vitro* for a month in culture media supplemented with SCF, LIF and bFGF, which are the most important growth factors for the survival and proliferation of mouse PGCs [12]. These cells satisfied the criteria used for defining chicken PGCs by using the expression of markers. They formed simple embryoid bodies in the suspension culture *in vitro*, which suggested that the Beijing fatty chicken PGCs were capable of differentiation into a variety of cell types *in vitro*.

Induction of PGCs differentiation to osteoblasts

Dexamethasone combined with β-glycerophosphate and ascorbic acid was the most potent inducer of osteogenic differentiation. The induction process also depended on the time of application; the most effective induction time is at 14 days after induction culture. In the presence of β-glycerophosphate and ascorbic acid, serum-fed osteoblast cultures will spontaneously differentiate along a well-characterized and ordered developmental pathway to form mineralized bone nodules. These nodules demonstrate the morphological and biochemical characteristics of embryonic/woven bone and are a useful quantitative assay of osteoblast commitment and bone formation *in vitro* [17, 18].

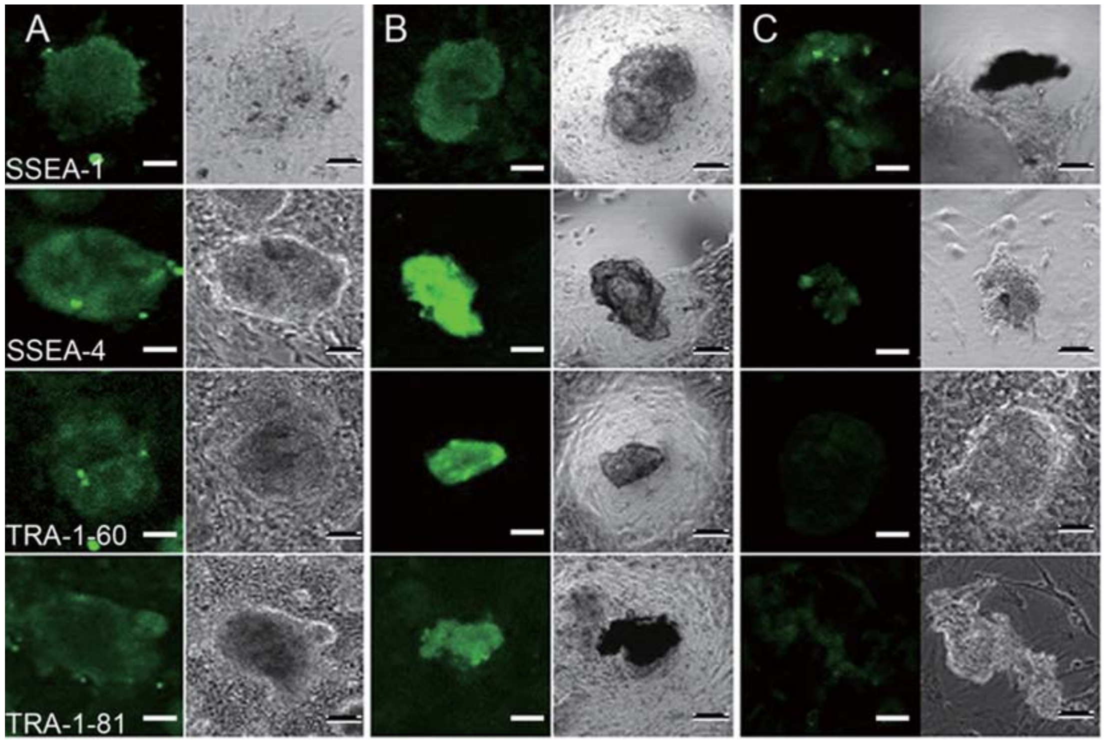

Fig. 3. Characterization of colony-forming in duck, chicken and mouse PGCs by a set of antibodies recognizing specific cell surface antigens at the third passage. Duck PGCs expressed SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81(A). Chicken PGCs expressed SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81(B). Mouse PGCs expressed SSEA-1 slightly, but not SSEA-4, TRA-1-60 and TRA-1-81(C). In contrast, no reactivity was detected in the MEF feeder. Scale bar: 35 μm.

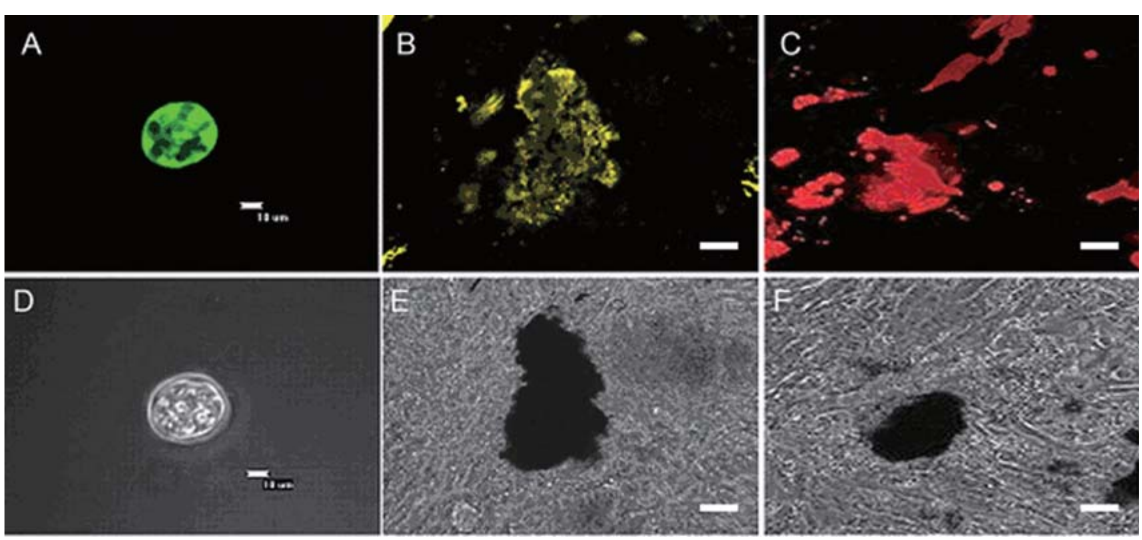

Fig. 5. Comparative figures of fluorescent protein expression at 48 h. A, D: Fluorescent protein expression of pEGFP-N3 at 48 h. Scale bar: 10 μm. B, E: Fluorescent protein expression of pEYFP-N1 at 48 h. Scale bar: 80 μm. C, F: Fluorescent protein expression of pDsRed1-N1 at 48 h. Scale bar: 80 μm.

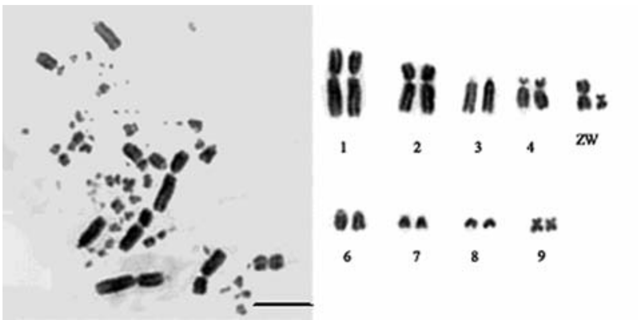

Fig. 4. Chromosome at metaphase (A) and karyotype (B) of the Beijing fatty chicken PGCs (♀). There are 9 pairs of macrochromosomes and about 30 pairs of microchromosomes. Scale bar: 10 μm.

Chromosome analysis

Cells possess a characteristic chromosome number, shape and structure, which remain very stable in normal cells. Therefore, karyotype analysis is a major method for distinguishing normal cells from variants. The International Poultry karyotype criterion defines poultry chromatin as comprising 8 pairs of macrochromosomes and about 30 pairs of microchromosomes with sex chromosomes Z and W [19]. Chromosomal diploid numbers range widely in poultry; most species have 78 and 82, and the number of macrochromosomes for *Gallus domestica* is 6–9, while the number of microchromosomes is 24–35.

Expression of exogenous genes

Expression levels and species-independent efficiency of fluorescence genes have been used as markers to observe the expression, contribution and function of target proteins in live cells and organisms [20]. The DNA concentration, lipofectine concentration, DNA incubation time and presence of serum all affected transfection efficiency, which was identical to the results obtained in research on Vero cells, HeLa cells and some other cell lines [21–23]. In this study, the transfected cells were not significantly less viable than the control cells.

In summary, this report comprehensively demonstrated the characterization of pluripotent PGCs from the Beijing fatty chicken, and the data obtained will be useful for investigating the differentiation of germ cells. Furthermore, these cells are suitable for genetic manipulation and can be used as nuclear donors using the nuclear transfer technique. The technical platform now established will support conservation of endangered species and their genetic materials at the cellular level.

Acknowledgments

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