

Derivation and characteristics of pluripotent embryonic germ cells in duck¹

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ABSTRACT Embryonic germ (EG) cells derived from primordial germ cells are undifferentiated and pluripotent stem cells. In our study, primordial germ cells isolated from the genital ridges of Beijing duck (*Anas domestica*) embryo at stage 28 were co-cultured with mice embryonic fibroblasts. Duck EG cells grew with a high nuclear:cytoplasm ratio and were positive for periodic acid-Schiff and alkaline phosphatase staining. The duck EG cells could proliferate for more than a month in repeated subculture and maintain a diploid karyotype. They expressed the pluripotent markers such as stage-specific embryonic antigen-1, stage-specific embryonic

antigen-4, tumor rejection antigen-1-60, and tumor rejection antigen-1-81. Furthermore, the EG cells could form embryoid bodies and differentiate to osteoblast in vitro. This study explored the isolation and culture process of duck EG cells using the culture methods of chicken EG cells and investigated biological characteristics of Beijing duck EG cells. Our results showed that methods published for the culture of chicken EG cells could be applied to the culture of duck EG cells. Duck EG cells will be useful for the differentiation of EG cells in avian species.

Key words: Beijing duck, embryonic germ cell, biological characteristic, derivation

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INTRODUCTION

Pluripotent stem cells have been derived from 2 embryonic sources. Embryonic stem (ES) cells are derived from the inner cell mass of preimplantation embryos, and embryonic germ (EG) cells are derived from primordial germ cells (PGC). Such cells have been characterized in mammals, especially in humans (Shamblott et al., 1998). Primordial germ cells are the progenitors of the sperm or eggs. In avian species, PGC first arise from the epiblast and migrate to the germinal crescent at stage 4, after approximately 18 h of incubation. After 50 to 55 h of development, PGC migrate to the gonad and subsequently produce functional sperm and oocytes (Swift, 1914; Hamburger and Hamilton, 1951; Fujimoto et al., 1976). Recent reports indicate that PGC maintain their restriction to the germ line (Naito

et al., 2004; Van de Lavoie et al., 2006a). Chicken EG and ES cells contribute substantially to somatic tissues but not to the germ line (Van de Lavoie et al., 2006b). Duck is similar to the chicken in physiological and developmental characteristics. However, little research has been done on duck EG cells. Therefore, the establishment of an isolation and culture system of duck EG cells is critical for the study of avian stem cells. In this study, the duck EG cells were derived from duck gonadal PGC. The duck EG cells could proliferate for more than a month by using the culture media of chicken EG cells, maintain normal karyotype, express a set of typical markers of ES cells, and be induced to differentiate into osteoblast in vitro.

MATERIALS AND METHODS

Preparation of Mouse Embryonic Fibroblasts

Mouse fibroblasts were isolated from 13.5 d post-coitum embryos and cultured (10^4 cells·cm⁻²) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, San Diego, CA) containing 5% fetal bovine serum (FBS; HyClone, Tauranga, New Zealand). After a 3- to 5-generation subculture, mouse embryonic fibroblasts

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were treated with 10 $\mu\text{g}/\text{mL}$ of mitomycin C (Sigma, St. Louis, MO) for about 30 min, then rinsed with PBS and subcultured again with DMEM medium.

Isolation of Duck Gonadal Cells

The Beijing duck embryonic gonad tissue at stage 28 (Hamburger and Hamilton, 1951; 7 d of incubation), including the PGC, was dissociated in 0.25% trypsin–0.02% EDTA at room temperature (RT) for 5 min. After the inactivation of trypsin-EDTA with DMEM containing 15% FBS, the cells were harvested by centrifugation (Park and Han, 2000).

Culture of Duck EG Cells

For primary culture, cell suspension containing both PGC and somatic cells at a density of $1 \times 10^5/\text{well}$ was cultured in 24-well plates (Corning Inc., Corning, NY) in a 37°C incubator with 5% CO₂ with complete medium consisting of DMEM, 15% FBS, 1% nonessential 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 $\mu\text{g}/\text{mL}$ of streptomycin, 100 IU/mL of penicillin, 5 ng/mL of human stem cell factor (SCF; Peprotech, London, UK), 10 IU/mL of murine leukemia inhibitory factor (LIF; Peprotech), and 10 ng/mL of human basic fibroblast growth factor (bFGF; Peprotech). For about 3 to 7 d, the cultured PGC formed colonies (Han et al., 2002).

For subculture, the colonies of duck EG were rinsed with PBS to remove dead cells and digested with 0.25% trypsin-0.02% EDTA for 3 min, then terminated by 15% FBS in DMEM and reseeded into microwell plates

with feeder cells. The EG cell colonies were passaged at an interval of 3 to 7 d on average.

Periodic Acid-Schiff and Alkaline Phosphatase Staining

Subcultured cells were fixed in 4% neutral paraformaldehyde for 10 min and rinsed twice in PBS. For periodic acid-Schiff (PAS) staining, fixed cells were immersed in periodic acid solution for 5 to 10 min at RT and then immersed in Schiff's solution for 15 min at RT. After being rinsed with PBS twice, PAS-stained EG cells were observed under an inverted microscope.

For alkaline phosphatase staining, we used a 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium alkaline phosphatase substrate kit (Boster, Wuhan, China; Park and Han, 2000) according to the instructions of the manufacturer. Images were captured with a computer-assisted video camera (IX-71 inverted research microscope, Olympus, Tokyo, Japan).

Immunocytochemistry

To analyze marker expression of duck EG colonies, the cell colonies at the third passage were fixed in 4% paraformaldehyde for 15 min at RT and washed twice (5 to 10 min each) in Tris buffer saline with Tween-20 (TBST; 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20) and treated with 0.1% Triton X-100 for 10 min and then washed twice (5 to 10 min each) in TBST buffer and incubated in 3% BSA (blocking solution) for 30 min. The primary antibodies were anti-stage-specific embryonic antigen-1 (SSEA-1), anti-stage-specific embryonic antigen-4 (SSEA-4), anti-tumor rejection an-

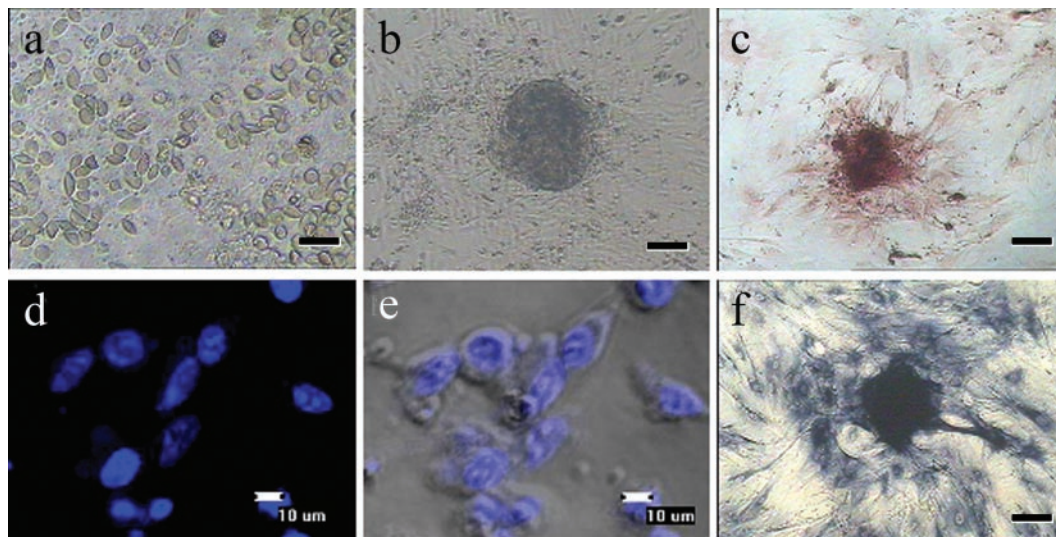


Figure 1. Cellular morphology and identification of duck embryonic germ (EG) cells. a) Duck primordial germ cells with gonadal somatic cells; scale bar: 35 μm . b) Duck EG colony on mice embryonic fibroblast at the second passage. The colony is uniformly round, multilayered, and well delineated; scale bar: 80 μm . c) Periodic acid-Schiff reactivity formed in a colony at the third passage; scale bar: 100 μm . d, e) Duck EG cells stained with Hoechst 33258, which shows that duck EG cells were composed of a large nucleus and a relatively small amount of cytoplasm. f) Alkaline phosphatase reactivity formed in a colony at the third passage; scale bar: 100 μm . Color version available in the online PDF.

tigen-1-60 (**TRA-1-60**), and anti-tumor rejection antigen-1-81 (**TRA-1-81**) from ES Cell Characterization Kit (Chemicon, Temecula, CA; Fang et al., 2006). All antibodies were diluted in PBS (TRA-1-60 and TRA-1-81, 1:100; SSEA-1 and SSEA-4, 1:40) and incubated with samples overnight at 4°C. The next day, colonies were washed with TBST buffer 3 times and incubated with secondary antibodies (diluted in PBS 1:100) at RT for 1 h. The secondary antibodies were fluorescein isothiocyanate (**FITC**)-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 (Booster). Fluorescent signals were visualized with a

confocal microscope (Nikon TE-2000-E inverted microscope, Tokyo, Japan).

Karyotyping

Duck EG cells were incubated with colcemid (final concentration 0.1 $\mu\text{g}/\text{mL}$) for 4 h at 37°C in 5% CO_2 and then harvested, centrifuged, and resuspended in prewarmed 0.075 M KCl. 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 with the fixative 3 times. Finally, the cell suspension was dropped on precooled glass slides and stained with Giemsa (Am-

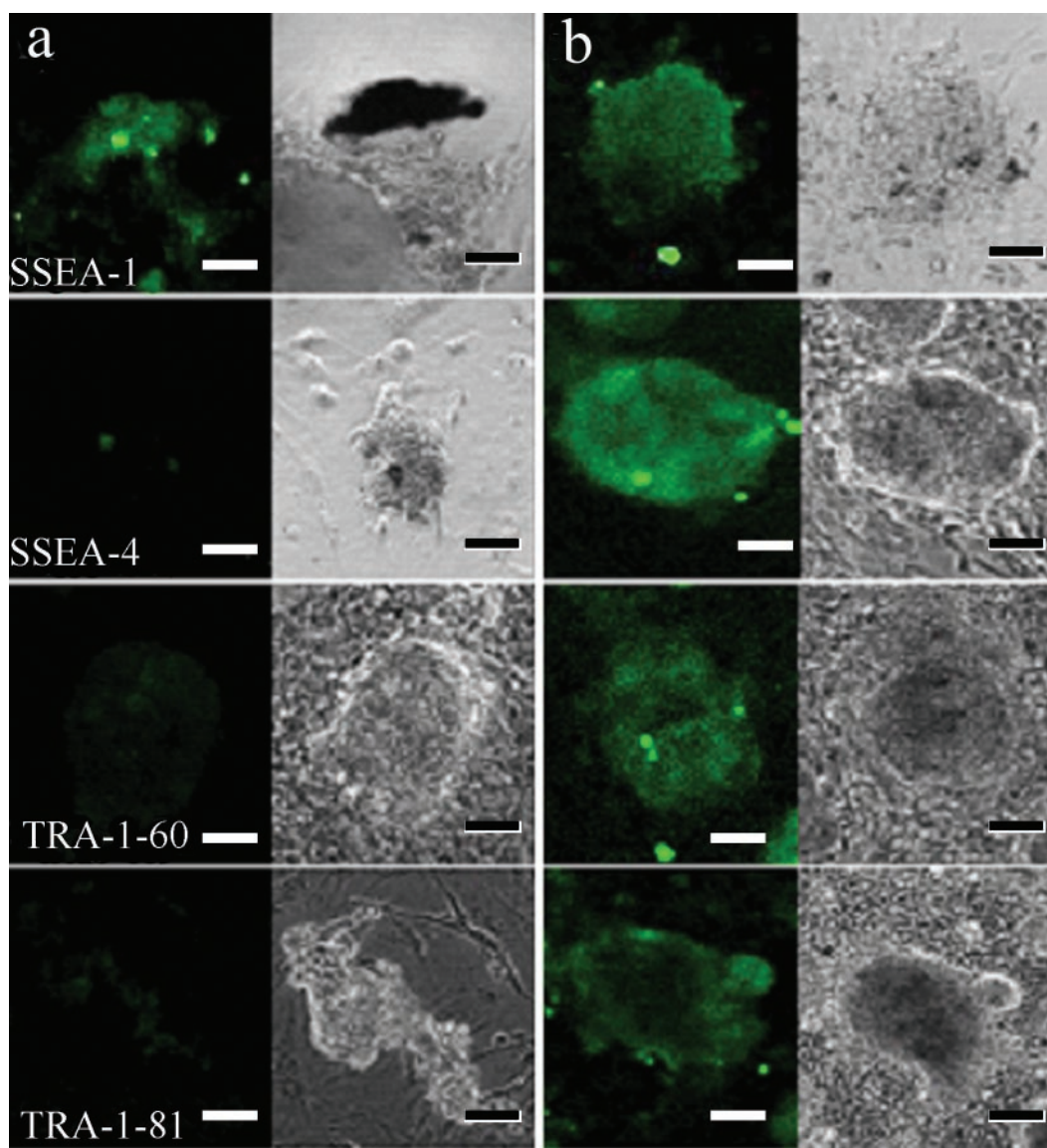


Figure 2. Characterization of duck embryonic germ (EG) colonies by a set of antibodies recognizing specific cell surface antigens at the third passage. Mouse EG colonies were used as the control group for characterization of the markers. Mouse EG colonies expressed anti-stage-specific embryonic antigen-1 (SSEA-1) but not anti-stage-specific embryonic antigen-4 (SSEA-4), tumor rejection antigen-1-60 (TRA-1-60), and tumor rejection antigen-1-81 (TRA-1-81) (a). Duck EG colonies expressed SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 (b). In contrast, no reactivity was detected in the mice embryonic fibroblast feeder, and antibody reactivity is not uniform within a colony. Scale bar: 35 μm . Color version available in the online PDF.

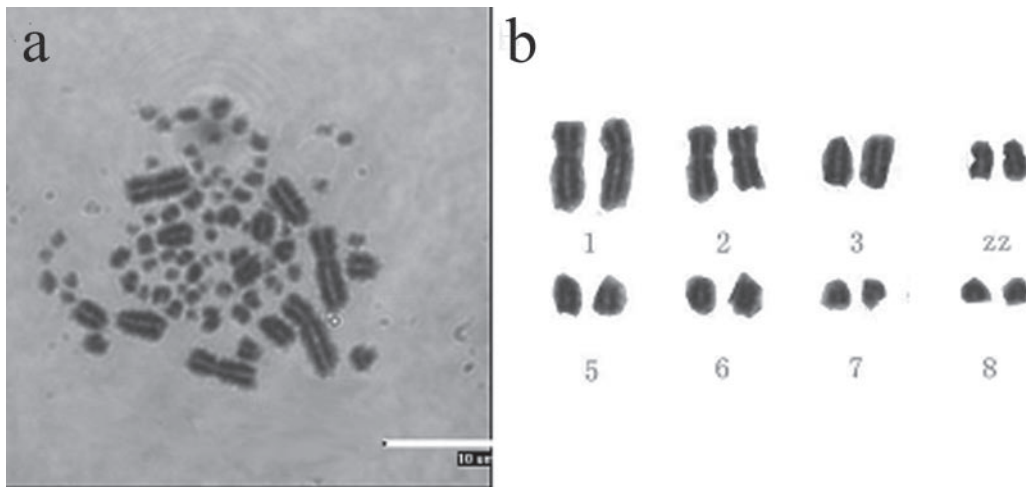


Figure 3. Chromosomes at metaphase (a) and karyotype (b) of the Beijing duck embryonic germ (EG) cells (male). There are 8 pairs of macrochromosomes and 31 pairs of microchromosomes. Because of the large number of microchromosomes, it was difficult to determine if the karyotype of duck EG cells was normal; however, we were able to ensure that duck EG maintained a diploid karyotype.

resco, Solon, OH). At least 20 metaphase spreads were counted for every passage of duck EG cells (Liu et al., 2008; Wu et al., 2008).

Embryoid Body Formation

Duck EG colonies at the third passage were digested with 0.25% trypsin –0.02% EDTA for 1 min at 37°C and then gently broken into small clumps and cultured in suspension on dishes in medium consisting of 75% DMEM, 15% FBS, 2 mM L-glutamine, 1% nonessential amino acid, 0.1 mM β -mercaptoethanol, and 1 μ M all-*trans*-retinoic acid for 4 to 8 d. The medium was changed every 2 d. During this period, the cells aggregated to form embryoid bodies (EB), and then the EB was plated onto gelatin-coated dishes and cultured to allow the expansion of different types of cells (Amit et al., 2004).

Stimulation of EG Cells Differentiation to Osteoblasts

Duck EG cells at the third passage were resuspended in DMEM containing 15% FBS, 100 μ g/mL of strep-

tomycin, 100 U/mL of penicillin, 50 μ g/mL of ascorbic acid, 10 mM β -glycerophosphate, 0.1 μ M dexamethasone, and 1 μ M all-*trans*-retinoic acid for 9 to 15 d. The medium was replaced every 3 d (Buttery et al., 2001).

Differentiated cells were fixed in 4% paraformaldehyde for 15 min, washed in PBS twice, and stained with 1% solution of Alizarin Red for 5 min. Plates were washed in PBS twice, and bone nodule colonies were observed under the microscope (Buttery et al., 2001).

RESULTS AND DISCUSSION

Culture and Characterization of Duck EG Cells

Duck EG cells were derived from Beijing duck gonadal PGC collected at stage 28. After about 3 d of culture, the cell colonies with the appearance of grapes were formed. These cells subsequently formed nest colonies at about 7 d. The colonies of EG cells were passaged at an interval of 5 to 7 d. These colonies were maintained for up to 7 passages and proliferated for more than a month in repeated subculture. The morphology of the duck EG colonies was uniformly round,

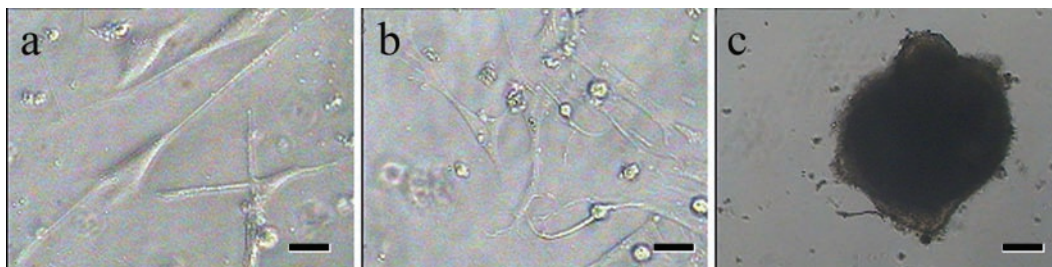


Figure 4. Morphology of differentiated cells and embryoid bodies (EB) induced by duck embryonic germ (EG) cells. a) The outgrowth of the EB contained cells with a typical neuronal morphology; scale bar: 35 μ m. b) The outgrowth of the EB contained cells with special morphology; scale bar: 35 μ m. c) Embryoid bodies formed in suspension after 8 d of culture; scale bar: 100 μ m. Color version available in the online PDF.

multilayered, and well delineated. The duck EG cells had a large nucleus and a relatively small amount of cytoplasm. The duck EG cells can be easily identified by PAS reaction, which stains for glycogen in cytoplasm (Meyer, 1964). Alkaline phosphatase activity is one of the characteristics of pluripotent stem cells (Park and Han, 2000). Duck EG cells were stained dark blue by AKP staining (Figure 1).

In our study, the duck culture media of EG cells supplemented with SCF, LIF, and bFGF is similar to that of chicken EG cells (Park and Han, 2000); SCF, LIF, and bFGF were the most important growth factors for the survival and proliferation of PGC in the mouse. For murine PGC culture, SCF was required for cell survival and proliferation, LIF was necessary as a mitogen in combination with SCF, and bFGF was absolutely required for the long-term culture of mouse PGC and led to the derivation of large colonies of EG cells (Resnick et al., 1992). Recent study proved that chicken PGC maintain their restriction to the germ line (Van de Lavoie et al., 2006a), whereas chicken EG and ES cells contribute substantially to somatic tissues but not to the germ line (Van de Lavoie et al., 2006a,b). Therefore, more research is needed to get the chimera in duck PGC and EG cells.

Marker Expression of Duck EG Colonies

Cell surface antigens provide invaluable tools for the identification of cell differentiation. A set of antibodies (SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81) recognizing specific cell surface antigens was used to characterize the EG colonies (Jung et al., 2005; Fang et al., 2006). Compared with mouse EG colonies, the duck EG colonies expressed SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 (Figure 2).

Stage-specific embryonic antigens that are developmentally regulated during early embryogenesis are widely used as markers to monitor the differentiation of ES cells. As the keratan sulfate-associated antigens, TRA-1-60 and TRA-1-81 are also characteristic of hu-

man embryonic carcinoma cells. Human ES cells are characterized by the expression of the cell surface antigens, SSEA-4, TRA-1-60, and TRA-1-81, and by the lack of SSEA-1 (Henderson et al., 2002). Mouse ES colonies expressed SSEA-1 but not the others. Rabbit EG cells did not express surface antigens recognized by the 4 antibodies (Fang et al., 2006). The surface antigens of duck EG colonies in our study are different from that of mouse, human, and rabbit EG cells.

Karyogram and Chromosome Number of Duck EG

Cells possess characteristic chromosome numbers, shape, and structure, which remain very stable in the 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 30 pairs of microchromosomes with sex chromosomes Z and W (Ladjali-Mohammed et al., 1999). The chromosomes in 100 duck EG cells were counted at the first and third passage. The normal duck EG cells were diploid ($2n = 78$), containing 8 pairs of macrochromosomes and 31 pairs of microchromosomes. The sex chromosome type is ZZ (male)/ZW (female). The results showed that 97% of the cells were diploid (Figure 3).

Differentiation In Vitro

The ability to differentiate in vitro into simple or complex EB in suspension culture is one of the characteristics of pluripotent ES cells (Evans and Kaufman, 1981) and EG cells (Resnick et al., 1992). Duck EG cells after 3 passages could form EB with a heterogeneous morphology in the presence of serum in a suspension culture for about 8 d (Figure 4). Therefore, the duck EG cells were capable of differentiation in vitro.

Embryonic germ cells have the potential to differentiate to all fetal and adult cell types and might represent

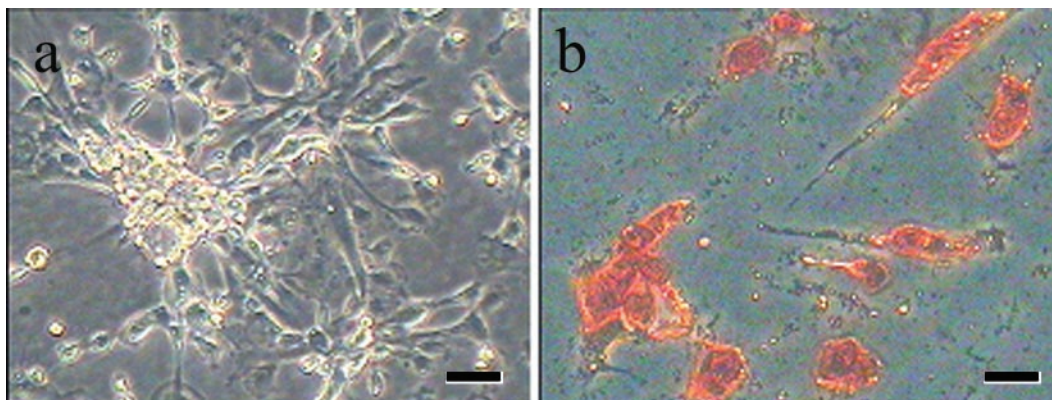


Figure 5. Morphology and identification of osteoblasts induced by duck embryonic germ (EG) cells. a) Morphology of osteoblasts induced by duck EG cells that consisted of aggregated cells; scale bar: 35 μ m. b) Histochemical staining for Alizarin Red identifies discrete mineralized nodules; scale bar: 35 μ m. Color version available in the online PDF.

a useful cell source for tissue engineering and repair. Duck EG cells can be differentiated toward the osteoblast by supplementing serum-containing media with ascorbic acid, β -glycerophosphate, dexamethasone, and all-*trans*-retinoic acid. Embryonic stem cell differentiation into osteoblasts was characterized by the formation of discrete mineralized bone nodules. Histochemical staining for Alizarin Red indicated discrete mineralized nodules (Figure 5). This could be applied to obtain purified osteoblasts to analyze mechanisms of osteogenesis and for use of EG cells in skeletal tissue repair.

In this study, we have obtained the duck EG cells from PGC of stage 28 using the similar culture media of chicken EG cells. The duck EG cells could propagate for more than a month by repeated subculture and expressed pluripotent markers that are enriched in undifferentiated ES cells. They could form simple EB in the suspension culture in vitro and differentiate to osteoblasts, which all suggested that the duck EG cells were capable of differentiation in vitro.

This report is the first to successfully demonstrate the characterization of pluripotent EG cells from Beijing duck. Our results will be useful for investigating the differentiation of EG cells in avian species.

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