Conservation of Red Junglefowl Biodiversity by Primordial Germ Cell Cryopreservation

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ABSTRACT

Primordial germ cell (PGC) can be cryopreserved and used as a potential tool for the conservation of avian biodiversity. By transplantation of donor PGCs to recipient embryos to generate germline chimeras, the PGCs can migrate to the developing gonads where germ cells are produced thereby enabling a reproduction of the offsprings derived from the donor PGCs through mating of these germline chimeras. From the cryopreserved PGCs, *in vitro* propagation is required to gain sufficient active PGCs for further transplantation therefore a culture condition for Red junglefowl PGCs was studied and herewith reported. A co-culture system was employed, using feeder cells derived from a quail embryo and Kav-1 media containing 5% each of fetal bovine and chicken serum. The cultured PGCs grew in colonies of two distinct forms either composed of aggregated round cells or spreading flattened cells. These cultured PGCs were positive to PAS staining and SSEA-1 indirect immunofluorescent detection, suggesting that PGC characteristics are retained. A biological analysis of both distinctive colonies remains to be explored. The culture condition used in this study can generally support Red junglefowl PGC survival and growth which could be employed in the protocol for Red junglefowl conservation by cryopreserved PGCs.

Key words: primordial germ cell, culture, red junglefowl, cryopreservation

INTRODUCTION

Red Junglefowl has been suggested for being the ancestor of world domestic chickens (Fumihito et al., 1996) of which a rich biodiversity can offer a vast resource for the development of subspecies, breeds and lines of descendants including ornamental chickens and commercial poultry. Our previous studies suggested that domestication process partly involving man-made genetic selection resulted in the reduction of anti-oxidant capacity. This could be a risk factor for survivability of domestic chickens living under stressful conditions such as high stocking density in industrialized poultry farming. The healthy population of Red Junglefowl could therefore serve as a gene bank to ensure the continuation of chicken species.

Although the Red Junglefowl biodiversity seems critically essential, it is at risk of decline. Effective and timely implementation of conservation program is therefore crucial for ecological homeostasis. The maintenance of living stocks as a traditional conservation approach could be hampered by various threats i.e. limited food sources and habitats, poaching and illegal trade, climate changes, and emerging diseases. In avoidance of the trouble of maintaining the living stocks, focused technology for preservation of avian genetics has been on cryopreserved semen (Blesbois, 2007) by which homozygous recessive female characters could hardly be recovered. In addition, the problem of low fertilizing ability of frozen/thawed avian semen remains to be corrected (Long, 1996; Makhafola et al., 2009). Some promising tools for conservation of mammalian species such as embryo and oocyte cryopreservation (Prentice and Anzar, 2011) and cloning (Marshall, 2000; Shimozawa et al, 2002) are not yet possible in avian. Effective means to conserve and restore the avifauna biodiversity has therefore been in search of science.

Biotechnology as a promising tool to create newborn from genetic materials could be potentially applied in part of the restoration protocol; however, current available technology such as cloning has been unsuccessfully tried in avian species. The later development of germline stem cell technology has been introduced as an effective alternative. From cryopreserved primordial germ cells (PGCs), chimeric birds can be produced by transplantation of cultured PGCs to recipients of either the same or different species (Naito et al., 1994). Donor-derived offspring are then produced by breeding of these germline chimeras (Wernery et al., 2010).

Germline chimera technology has been successfully developed with varied efficiency among reports (Macdonald et al., 2010; Naito et al., 2010; Park, 2003; van de Lavoir et al., 2006; Tajima et al., 1993; Wernery et al., 2010). PGC propagation *in vitro* appears to be indispensable to obtain sufficient number of PGCs for transplantation and so to increase the success rate of PGC homing to the developing gonads. The reported culture protocols are mostly based on chicken PGC experiment, which might need adjustments for culturing PGCs derived from other species.

This study therefore aimed to establish an in vitro culture protocol for the propagation of PGCs collected from Red Junglefowl and to cryopreserve the cultured PGCs for future creation of germline chimera.

MATERIALS AND METHODS

Embryos and PGC isolation

Ten fertilized eggs of white ear-lobed Red Junglefowl (*Gallus gallus gallus*) were used for PGC collection. The PGCs were collected from blood circulation of the embryos at 13-15 developmental stage, stage identification was according to Hamburger & Hamilton (1951). To obtain the embryos of 13-15 staged development, the eggs were subjected for 50-55 hour incubation provided the conditions of 38.5 °C, 55% relative humidity and 1-hour interval rotation. Under a stereoscope (Olympus, SZ51), a fine glass micropipette was inserted into the vitelline vein through a small window on the shell priorly made. The aspirated blood was mixed with Kav-1 media containing 5% each of fetal bovine serum and chicken serum before transferring onto a double layer of 5.5% and 11% nycodenz in a centrifuge tube, which was subsequently centrifuged at 4 °C with 400 g force for 30 min. After centrifugation, the fluid between the two layers of nycodenz suspended the PGCs was aspirated and transferred to another tube for further pelleting. The pellet was washed thrice, and then re-suspended in 200 µL of Kav-1 media containing 5% each of fetal bovine serum and chicken serum.

The cell suspension, consisting of a mixed population of PGC and blood cells was examined under a phase-contrast inverted microscope (Olympus, CK40) for further purification of PGCs by manual aspiration using a glass micropipette. The PGCs were differentiated from blood cells by morphology (Zhao and Kuwana, 2003).

Feeder cells

Feeder cells were employed in this study to support the culture of wild chicken PGC using a protocol reported by Kuwana et al. (1996). The feeder cells were priorly screened for the capacity to support PGC survival and growth, using commercial broiler chicken PGCs for screening. Each feeder tested was derived from a quail embryo of 13-15 developmental stage. A hind gut sample from the last somite to the distal end of the embryo was excised under a stereoscope (Olympus, SZ51) and transferred to a culture dish containing Kav-1 plus 5% each of fetal bovine serum and chicken serum. The sample was cut into tiny pieces before being transferred into a 12.5 cm^2 tissue culture flask (BD Falcon, USA) that was later incubated at 38 °C. Propagation of the tissue sample derived cells was continued with the media changed at every 3 days until reaching confluency. Subpassage was performed at a splitting ratio of 1:3, using 0.1% Trypsin-EDTA to detach the cells from the flask. The 10^{th} and higher passages of these embryonic fibroblast-like cells were used for PGC culture.

Preparation of mitotically inactivated quail embryonic fibroblast-like cells

The 10^{th} and higher passages of quail embryonic fibroblast-like cells were grown in 25 cm^2 tissue culture flask (Corning, USA) to confluency. Cellular mitotic activity was inactivated by applying 10 µg/mL Mitomycin C (Sigma, USA) treatment at 38 °C. The duration of treatment was varied from 1, 2, 3 and 4 hours to find the optimum condition justified by a completed inactivation of mitotic activity with the least cell death. The mitotically inactivated cells were subsequently treated with 0.1% Trypsin-EDTA until the cells detached from the flask. After washing the cells thrice with and re-suspending in Kav-1 plus 5% each of fetal bovine serum and chicken serum, the cell suspension was counted and approximately 4×10^4 cells were loaded in each well of a 96-well plate pre-coated with rat tail type-I collagen, and then were incubated at 38 °C for 2 hours for them to attach to the surface. The cells were washed with Kav-1 plus 5% each of fetal bovine serum and chicken serum and observed under a phase-contrast inverted microscope. A mono-layer completely covering the well surface was anticipated.

PGC culture

The PGCs were loaded on the feeder cell layer in Kav-1 media containing 5% each of fetal bovine and chicken serum at a density of 100-200 cells/well before being incubated at 38 °C, half of the media was changed every other day. Subculture was performed when the proliferative PGCs aggregated in large colony of more than 30 cells per colony. The PGC colonies were detached from the surface and disaggregated by gently blowing and pipetting, respectively. Trypsinization with 0.1% trypsin-EDTA was applied to detach the tightly adherent colony if present.

Characterization of cultured PGC

The cultured cells were examined for PGC characteristics, including positive stage specific embryonic antigen 1, SSEA-1 expression (D'Costa and Petitte, 1999) and Periodic Acid-Schiff, PAS staining (Meyer, 1964). Briefly, the cultured cells were fixed by applying freshly prepared 4% paraformaldehyde. After 10 min of incubation at room temperature, the cells were washed thrice with PBS, pH 7.4 for subsequent examination of SSEA-1 expression or PAS staining.

For SSEA-1 detection, indirect immunofluorescent assay was used. Briefly, the cells were incubated with 1:50 dilution of monoclonal anti-mouse SSEA1 (Santa Cruz Biotechnology, USA) for 2 hr at room temperature then washed twice with PBS, pH 7.4. Following washing, 1:200 dilution of secondary antibody, goat anti-mouse IgG-FITC (SantaCruz Biotechnology, USA) was applied onto the cells and incubated for 1 hr at room temperature, and then repeated washing. The cells were subjected for nuclear counterstaining using 5µg/mL Hoechst 33342 (Sigma, USA) before being observed under a phase-contrast inverted fluorescent microscope (Olympus IX71).

PAS staining was performed by incubating the cell with periodic acid for 10 min then thoroughly washing with PBS, pH 7.4. The cells were subsequently incubated with freshly prepared shift reagent for 30 min then were washed with PBS, pH 7.4. The PAS stained cells were observed under an inverted microscope (Olympus, CK40).

Cryopreservation

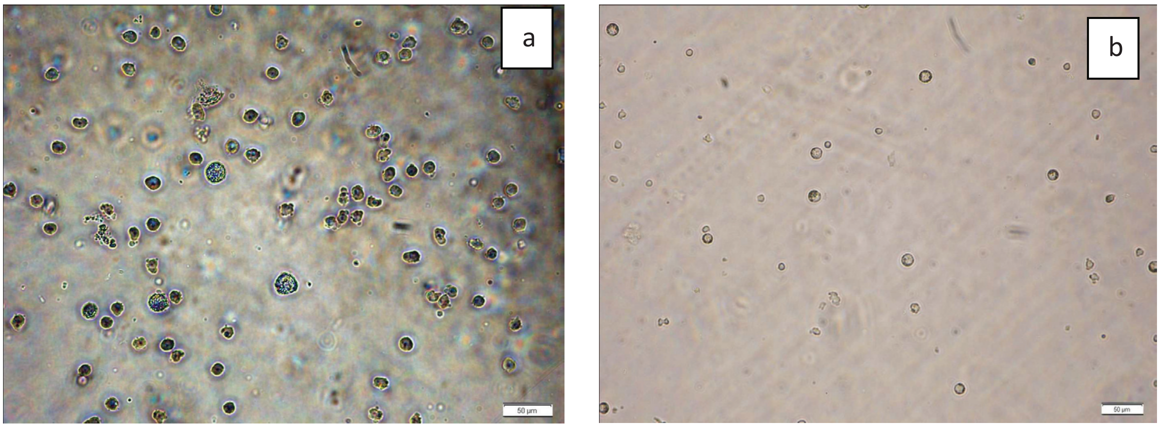
The cultured PGCs were cryopreserved in liquid nitrogen using fetal bovine serum containing 10% DMSO as a freezing media. Briefly, the cultured PGCs were harvested, washed in PBS, pH 7.4, resuspended in the freezing media before being transferred into a cryo-tube and then cooled down to -80 °C at the rate of approximately 1 °C/min using a Bicell bio-freezing vessel (Nihon Freezer Co., Ltd, Japan). The frozen sample was then stored in liquid nitrogen.

RESULTS AND DISCUSSION

PGC isolation and purification

In this study, the embryos at developmental stages of 13-15 based on Hamburger and Hamilton scores (1951) were used for collection of the blood circulating PGCs. Avian PGCs transmigrate from germinal crescent into the blood circulation at stage 11 and remain in the circulation until the embryo reaches stage 17 when the PGCs again transmigrate into the developing gonads (Fujimoto *et al*., 1976; Kuwana, 1998). PGC collection from the embryo younger than stage 13 was fairly difficult due to very small blood vessel. Furthermore, less numbers of PGCs were present in the circulation. For the embryo older than stage 15, a decrease in the numbers of isolated PGCs as compared to those collected from 13-15 staged embryos was experienced. This is in line with the report by Tajima et al. (1999) in which the number of circulating PGCs was found at maximum at stage 14-15 then declining due to the migration to the germinal ridges at stage 15-16. We also noticed that more blood was channeled to supply the developing organs causing a difficulty in aspirating the total blood completely from the vitelline vein.

In the whole blood sample collected from the embryo, PGC can be distinguished from blood cells by different morphology under a phase contrast inverted microscope (fig 1a). The PGC is round in shape and larger in size with large nucleus that reflects the oblique illumination observing under a dark-field inverted microscope. The population of PGCs in the whole blood sample was found relatively small. After the purification with nycodenz density gradient, a significant number of blood cells were removed giving much higher proportion of the PGCs (fig 1b) in the semi-purified sample. The final purification by manual aspiration of each microscopically identified PGC yielded absolute purified PGCs (fig 1c) for further used in *in vitro* cultivation.



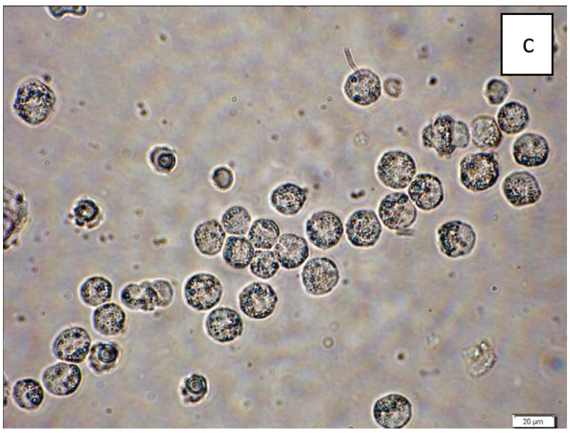


Figure 1. PGC collection and purification. Small number of PGCs were observed in the blood sample collected from a red junglefowl embryo (a). After a primary purification by nycodenz density gradient, most of the blood cells were removed resulting in a significant increase of PGC concentration (b). A single population of PGCs (c) was gained following a final purification by manual aspiration under a phase contrast inverted microscope.

The number of PGCs purified from the whole blood sample of each embryo was in a range of 4-20 cells, which is relatively small compared to a range of 100-200 PGCs isolated from each commercial broiler chicken embryo.

Feeder cell preparation

The embryonic hindgut sample attached to the surface of the tissue culture flask after 24 hours of incubation in Kav-1 media containing 5% each of fetal bovine serum and chicken serum at 38 °C. A propagation of fibroblast-like cells was initially observed after day 2 of culture. Starting from the edge of the tissue, the fibroblast-like cells continued on growing to form a dense patch of mono-layer surrounding the tissue sample (fig 2) which took approximately 10 days.

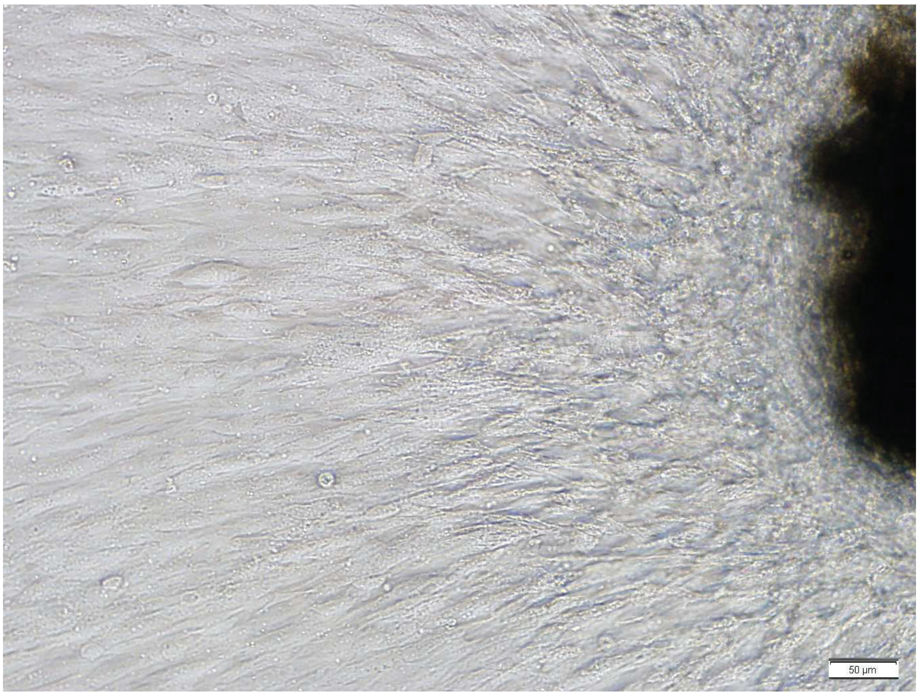


Figure 2. Fibroblast-like cells continued on growing to form a dense patch of mono-layer surrounding the tissue sample

Subculture using 0.1% Trypsin-EDTA yielded a nice single cell suspension. The early passages appeared morphologically non-homogeneous with different average doubling times between the passages. This might suggest that the culture contained a mixed population. By passaging, the uniformity of the cultured cells was gradually increased. The passage containing a morphologically homogeneous cell population with a constant doubling time for 3 consecutive passages was used for PGC culture. In this study, the 34^{th}- 40^{th} passages were used as feeder for the culture of Red Junglefowl PGCs.

The optimum duration of Mytomycin C treatment for inactivating the mitotic activity of quail embryonic fibroblast-like cells found in this study was 4 hours. Incomplete inactivation was presented in the trials using the shorter treatment durations while a significant increase of cell death was observed in the trial with the longer duration.

Culture of PGCs

The PGCs loosely attached to the feeder layer after 24 hours of incubation in Kav-1 media containing 5% each of fetal bovine serum and chicken serum at 38 °C. After a few days, the PGCs aggregated in small islets consisting of 4-7 cells each (fig 3).

Slow proliferation was observed in the first weeks of culture, subsequently the proliferation rate was significantly increased. By the end of week 2, some large colonies consisting of more than 30 cells each were grown. Interestingly, the propagated colonies appeared in 2 distinct forms, one comprised tightly aggregated round cells (fig 4a) and the other consisted of spreading flattened cells (fig 4b). Biological differences between the two forms remain to be explored.

Figure 3. PGCs aggregated in small islets consisting of 4-7 cells/colony

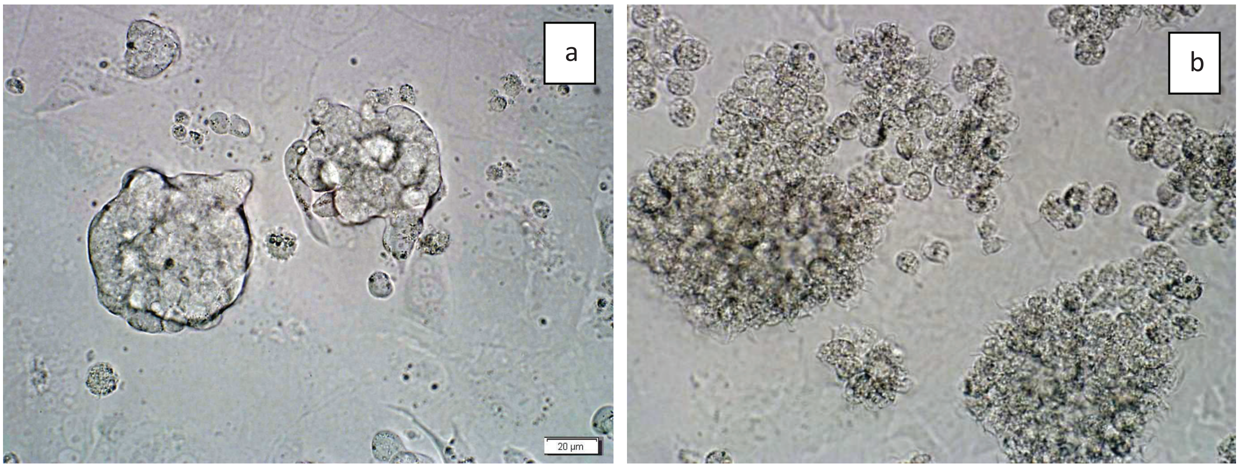


Figure 4. PGC co-culture with feeder cells: Two distinct forms of colonies composed of aggregated round cells (a) or spreading flattened cells (b) were observed.

The tightly aggregated round cell composing colonies attached loosely to the feeder layer; therefore, subculture was achieved by simply blowing and pipetting. Trypsin-EDTA was used to detach the other form of colony which bound tighter to the feeder layer making it difficult to be dispersed by blowing and pipetting. In this study, the culture and subpassage of Red Junglefowl PGCs can be maintained to the 8th passage which accounted for a total culture period of 3 months.

Characterization of cultured PGCs

The cultured PGCs were stained pink while the feeder cells were negative to PAS staining (fig 5). PGC has been characterized as a PAS positive cell due to large accumulation of glycogen in the cytosol (Macdonald *et al.*, 2010). The PAS positive staining therefore suggested that the general PGC character was retained in the newly proliferated cells under the culture conditions used in this study.

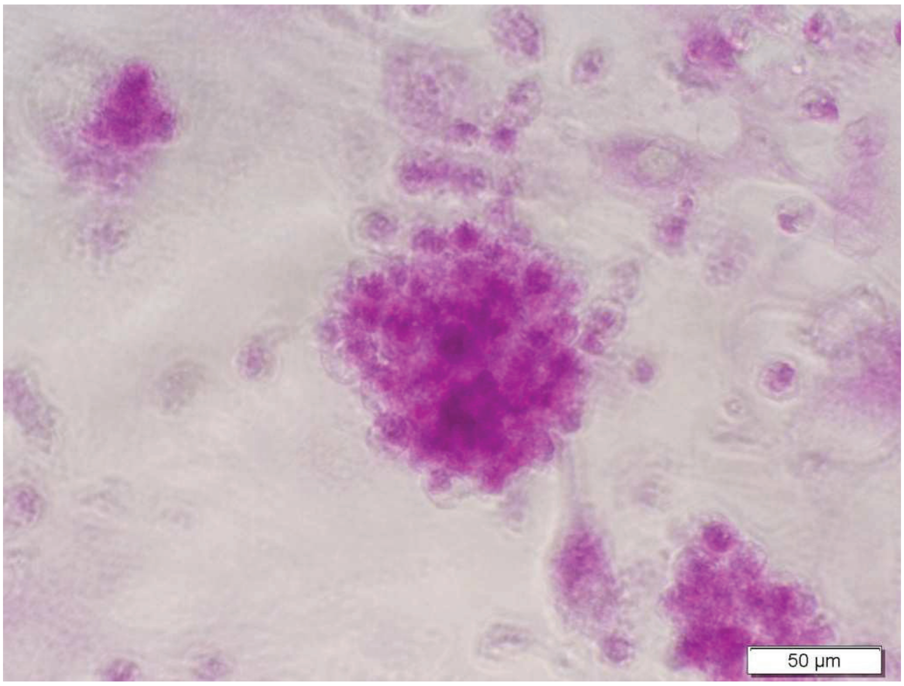


Figure 5. Positive PAS staining of cultured PGC colonies.

The culture cells were confirmed for their stem cell marker, SSEA-1 by immunofluorescent assay (fig 6) using anti-mouse SSEA1 (mouse monoclonal) and FITC conjugated goat anti-mouse IgG as the primary and secondary antibody, respectively.

A positive result as presented by green fluorescent on the cultured cells together with the PAS positive staining suggested that these grown cells are PGCs. It can be derived from this study that Red junglefow PGCs can be propagated *in vitro* by using Kav-1 medium containing 5% each of fetal bovine and chicken serum and embryonic Japanese quail derived feeder cells.

Although the feeder cells can support avian PGC survival and proliferation in this study as well as in other reports (Choi et al., 2010; Naito et al., 2010; Tang et al., 2007), the main obstacle found is the consistency of the feeder prepared for each culture. As being primary embryonic fibroblast-like cells, feeder characteristics including morphology, growth behavior and response to mytomycin C treatment as well as the capacity in supporting PGC culture were subjected to differences even among passages within line of the cells prepared from each embryo. Establishment of a novel cell line having consistent characters and being capable of promoting avian PGC growth is therefore worthwhile.

A feeder-free culture system is another approach to avoid the above mentioned problem. More importantly, this could avoid a possible contamination of the feeder cells in the PGC harvest to be used for transplantation in the production of chimeric birds.

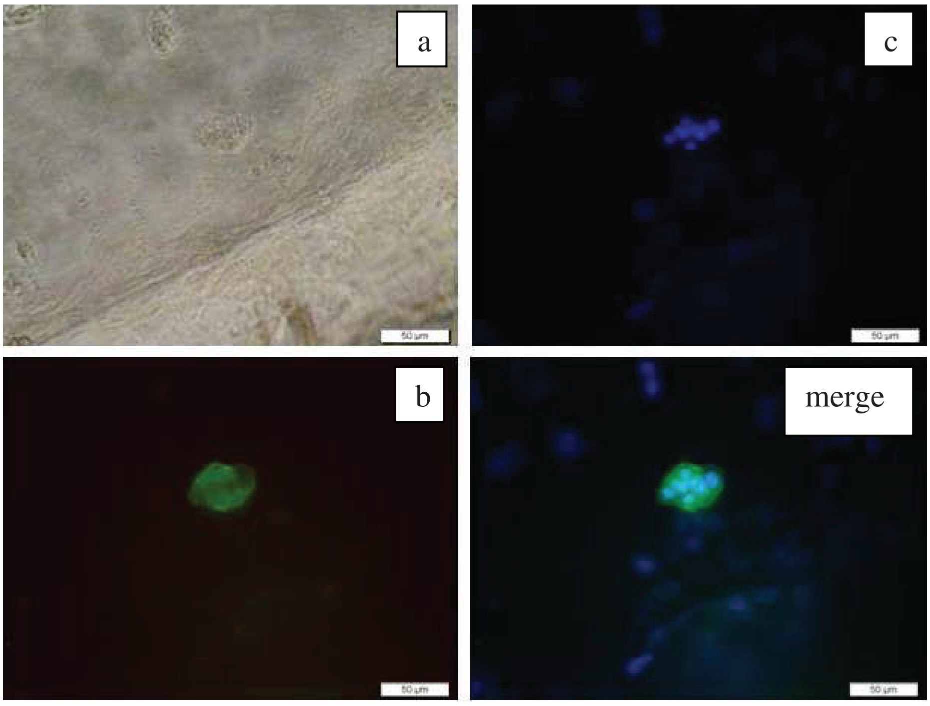


Figure 6. SSEA-1 expression on the cultured PGCs. The PGC colony identified by phase-contrast microscopy (a) was positive to SSEA-1 immunofluorescent staining (b). Nuclear counter staining with Hoechst 33342 confirmed the SSEA-1 localization on the cell surface (c, merge).

Conditioning medium and several survival and growth factors i.e. LIF, bFGF have been experimented (Shiue et al., 2009; Choi et al., 2010); however, the PGC culture performance was apparently inferior to the co-culture system using an embryo derived feeder cell.

The cultured PGCs were cryopreserved in liquid nitrogen using serum containing 10% DMSO as a freezing media. The cryopreserved cells were highly viable (>90 % viability) suggesting that this cryopreservation protocol is acceptable for preservation of Red junglefowl PGCs. Biodiversity of the collected samples will be explored and used for conservation and sustainable utilization planning.

ACKNOWLEDGEMENT

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