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Establishment of a pheasant (*Phasianus colchicus*) spermatogonial stem cell line for the production of interspecies germ line chimeras

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ABSTRACT

Background: Spermatogonial stem cells (SSCs) are important for the production of interspecies germ line chimeras. The interspecies germ cell transfer technique has been suggested as a way to conserve endangered birds. Our objective was to develop a technique for restoring endangered birds by developing interspecies germ line chimeras between pheasant (*Phasianus colchicus*) and chicken (*Gallus gallus*) with SSCs.
Results: SSCs were isolated from the surgically removed testis of a pheasant. Growth conditions for pheasant SSCs were established by co-culturing STO (SIM mouse embryo-derived thioguanine and ouabain resistant) cells and pheasant SSCs. The colony-forming cells divided and proliferated stably to yield an established SSC line. Pheasant SSCs showed strong reactivity for GDNF family receptor alpha1 (*GFRα1*) marker. Finally, production of germ line chimeras was attempted by transferring pheasant SSCs into recipient embryos. Although final embryo survival was 5.6% (20/354), the initial survival rate was 88% (312/354). To measure the percent transfer of donor SSC to gonads, the pheasant SSCs were labeled with PKH 26 fluorescent dye. We observed 30% donor cells and 9.48% c-kit/CD117-positive cells in the gonads of recipient chickens. Donor SSCs were thus stably engrafted in the recipient gonads.
Conclusions: This study showed that SSCs can be used as a tool for the conservation of endangered birds and the production of germ line chimeras. Our findings yield insights into how we may use the pheasant spermatogonial stem cell line for efficient production of interspecies germ line chimeras and ultimately, to the restoration of endangered birds.

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1. Introduction

Germ cells are unique, with an important role in the transmission of genetic information from one generation to the next through meiosis and mitosis. Primordial germ cells (PGCs) are the origin of the germ cell lineage [1] and a source of pluripotent germ cells after sexual maturation. In the male germ cells, gonocytes migrate through the seminiferous tubule to the basement membrane and differentiate into spermatogonial stem cells (SSCs) [2]. SSCs are important germ line stem cells for spermatogenesis in the testis. These male germ line stem cells share characteristics with other adult stem cells and both are capable of self-renewal and differentiation into spermatozoa [3].

SSC transplantation was first reported in mice; since then, this characteristic of SSCs has been suggested as a potential tool for producing interspecies germ line chimeras [4], which are of particular importance in efforts to restore endangered species.

Although sperm cryopreservation and artificial insemination can be used to support endangered species preservation, their utility is limited in non-domesticated species [5]. Germ cell techniques have been used for recent restoration efforts [6]. Studies of restoration in mammals are underway, but progress is slowed by long generation intervals. Avian species, unlike mammals, have the advantages of a relatively brief generation interval and high productivity so they can grow more quickly than mammals [7]. Avian species are good models for experimental studies in basic research and biotechnology [8], but SSCs have not been fully characterized in birds. Most recent studies of avian germ cells have focused on restoration of endangered bird species. Approximately 12.5% of the 9920 bird species in the word are endangered, and conservation efforts through natural breeding continue to encounter difficulties [5].

Kang et al. [9,10] produced interspecies germ line chimeras of pheasant and chicken by using PGCs. The pheasant is physiologically

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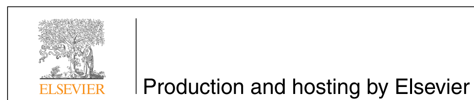

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and developmentally similar to the chicken. Interspecies transplantation of PGCs and SSCs can allow the generation of donor-derived offspring [11]. In fact, restoration of endangered avian species using SSCs enables the application of chicken germ line transmission technologies to the pheasant, making it possible to produce interspecies germ line chimeras in a chicken-pheasant system. Transfer of foreign genes into chicken embryos, establishment of in vitro culture of germ cells, and production of chimeric chickens and interspecies germ line chimeras [12,13,14,15,16] can be used for the restoration of endangered birds. We attempted to develop interspecies germ cell transfer techniques and to produce interspecies germ line chimeras for the restoration of endangered birds by using SSCs. In this study, we established a pheasant SSC line for transplantation to generate interspecies germ line chimeras of pheasant and chicken.

2. Materials and methods

2.1. Experimental animals

Five adult male (45–50 weeks old) Korean ring-necked pheasants (*Phasianus colchicus*) in non-breeding season and five female chickens (*Gallus gallus*) were used as the donors and recipients, respectively, of SSCs. The birds were maintained at the university poultry farm of Jeju National University, South Korea. Birds were kept in cages and the poultry shed was maintained at an ambient temperature of 25 ± 1°C. Procedures for bird management, reproduction, and embryo manipulation followed the standard operating protocol of our laboratory. We followed appropriate quality standards for all experimental protocols and animal handling. Birds were offered feed and water ad-libitum.

2.2. Retrieval of donor SSCs

Experimental procedures for SSC retrieval were performed at the affiliated laboratories of the Jeju National University, Jejusi, South Korea. The testes from adult male (45–50 weeks old) pheasants were surgically removed and manually decapsulated by removing the tunica albuginea. The exposed parenchyma was cut into pieces and washed with PBS (Sigma, St Louis, MO, USA). Testicular single cell suspensions were selected by two-step enzymatic digestion and then used for individual cell separation. Testes were transferred to digestion medium containing Dulbecco PBS (DPBS; Gibco BRL, Bethesda, MD) consisting of 1 mg/mL collagenase type IV (Sigma) and 1 mg/mL hyaluronidase (Sigma). Digestion was performed for 10 min by shaking (150 rpm) at 37°C to dissociate tubules. Testicular tissues were dissociated by gentle pipetting in 0.25% trypsin-EDTA (Gibco BRL) at 37°C for 5 min. Testicular cells containing SSCs were retrieved by passing through nylon mesh and then through 40 μm filters (BD Falcon, Franklin Lakes, NJ, USA). After centrifugation, the supernatant was removed and dissociated cells were washed twice in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Invitrogen, Carlsbad, CA, USA).

2.3. In vitro culture of testicular cells

Dissociated cells were cultured in the 6-well plates with modified DMEM containing 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA), nonessential amino acids (NEAA; Gibco Invitrogen), 10 ng/mL human basic fibroblast growth factor (bFGF; Sigma), and 100 ng/mL human insulin-like growth factor-1 (IGF-1; Sigma).

2.4. Co-culturing and growth factors of SSCs

Testicular germ cells were cultured in 24- and 6-well plates. Pheasant somatic cells were co-cultured with mitomycin C-treated STO cells and incubated for 1 d in a 5% CO_2 incubator. After incubation, the cells were washed twice with PBS. Pheasant SSCs were added in DMEM with 20% FBS, 100 U/mL penicillin (Gibco Invitrogen), and 100 μg/mL streptomycin (Gibco Invitrogen) in a humidified 5% CO_2 incubator; the medium was exchanged twice a week. We studied the effect of these various factors on SSC growth and optimized the medium (Table 1). Four different media were used to evaluate growth factors and cells growth.

2.5. Immunofluorescence assay

Cell surface antigen expression of cultured cells was analyzed by immunofluorescence techniques. Several antibodies, including stage-specific embryonic antigen-1 (*SSEA-1*), *SSEA-3*, *SSEA-4* (all Millipore, Billerica, MA, USA), *Oct4* (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and *GFRα1* (R&D Systems, Minneapolis, MN, USA) were used to trace the proliferative properties of pheasant SSCs. The following primary monoclonal antibodies were used to detect surface-antigen expression: anti-*SSEA-1*; anti-*SSEA-3*; anti-*SSEA-4*; anti-*oct4*; and anti-*GFRα1*. Fluorescein isothiocyanate (FITC)-labeled secondary antibodies, appropriate to the species and isotype of the primary antibody, were used to detect primary antibody binding. The cultured cells were fixed for 15–20 min at room temperature. After washing twice with 1 × rinsing buffer, cells were incubated in blocking solution for 30 min at room temperature. Primary antibodies were diluted to working concentrations of 1:10–1:50 in blocking solution and incubated at room temperature for 1 h, followed by incubation with secondary antibodies (final concentration 1:200) for 30 min at room temperature. The stained cells were observed under an inverted microscope (IX70; Olympus, Tokyo, Japan).

2.6. Paul Karl Horan (PKH) 26 labeling

To access donor and recipient embryos, gonadal cells containing pheasant SSCs were labeled with PKH 26 fluorescent dye (Sigma) for 5 min before transfer. The cells were suspended in 1 mL Diluent C from the PKH 26 mini kit. The egg window of the recipient embryos was sealed twice with parafilm (Bemis, Neenah, WI, USA) and positioned with the pointed end down until the next treatment. The labeled SSCs were monitored under the IX70 fluorescence microscope.

2.7. Transfer of SCCs into recipient embryos

Donor SSCs were transferred to the recipient embryos through a small window made at the pointed end of the eggs. Approximately 2 μL cell suspension from donor pheasant SSCs was transferred into the dorsal aorta of 2.5 d old chicken embryos with a 50-μL microcapillary tube (Sigma). The egg window was sealed with parafilm and then laid down with the pointed end at the bottom.

2.8. Fluorescence-activated cell sorting (FACS)

CD117 is known as the c-kit receptor, which is expressed in germ cells. CD117 was used as a specific marker of donor-derived SSCs in

Table 1
Media composition.

Components	Medium-1 (M1)	Medium-2 (M2)	Medium-3 (M3)	Medium-4 (M4)
Type of media	DMEM	DMEM	DMEM/F12	DMEM/F12
FBS (%)	10	10	10	5
Penicillin (U/mL)	100	100	100	100
Streptomycin (μg/mL)	100	100	100	100
NEAA (%)	–	1	1	1
LIF (U/mL)	–	10^3	–	–
bFGF (ng/mL)	–	–	10	10
IGF-1 (ng/mL)	–	–	100	100

the gonads of recipient chickens. Propidium iodide (PI, 1 mg/mL; Gibco Invitrogen) was used to discriminate live cells. The FACS Calibur (Becton Dickinson, San Jose, CA, USA) was used to identify c-kit/CD117-positive cells.

2.9. Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) using SPSS v 12.0 software (SPSS, Chicago, IL, USA). Significant differences between the means of different groups at P values < 0.05 were analyzed by Tukey's b-test. Values are expressed as means ± standard deviations (SD).

3. Results

3.1. Proliferation efficiency of pheasant SSCs through co-culture and effect of growth factors

In this study, our aim was to co-culture pheasant SSCs, which is an established technique in chickens, but the major constraint we faced was poor growth rate. Therefore, we decided to use basal cells and growth conditions were established through co-culture with the STO cells and somatic cells of pheasant. Pheasant SSCs were proliferated through co-culture. When SSCs were cultured with STO cells, proliferation efficiency increased rapidly ($P < 0.05$). However, proliferation in the absence of basal cells was very slow; after 2 weeks of culture, the cells started to die. The chicken somatic cells exhibited substantial proliferation rates. Pheasant somatic cells showed a reduced STO cell effect, but relatively high levels of cell proliferation (Fig. 1).

We used this study to reveal the effect of growth factors such as leukemia inhibitory factor (LIF), bFGF, IGF-1, and FBS on cell growth to optimize the medium for in vitro culture of pheasant SSCs. The pheasant SSCs proliferated efficiently in various culture conditions with strong proliferation in M3 and M4; M1 and M2 produced lower rates of SSC growth (Fig. 2). M4 supplemented with 5% FBS, bFGF, and IGF-1 yielded significantly higher growth ($P < 0.05$) than M3 supplemented with 10% FBS. This improvement in proliferation at low-level FBS (5%) suggested these cells require lower concentrations of FBS in the growth medium.

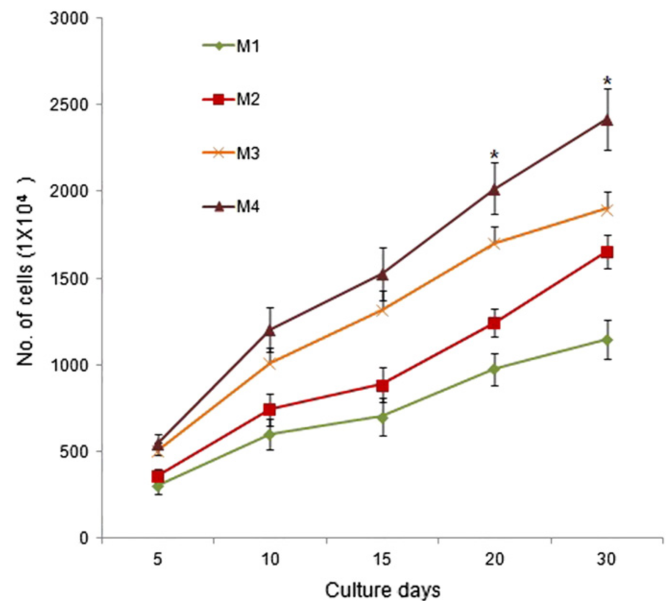

Fig. 2. Growth curves of pheasant SSCs in different media over 30 d. Medium-1 (M1) contained DMEM supplemented with 10% FBS; Medium-2 (M2) contained DMEM supplemented with 10% FBS and LIF; Medium-3 (M3) contained DMEM/F12 supplemented with 10% FBS, bFGF, and IGF-1; and Medium-4 (M4) contained DMEM/F12 supplemented with 5% FBS, bFGF, and IGF-1. M1 and M2 produced lower growth rates than did M3 and M4. M4 yielded the most rapid growth of pheasant SSCs. Data are reported as mean ± SD. *Significance at $P < 0.05$.

3.2. Establishment of pheasant SSC line and reactivity of SSCs to antibodies

The pheasant SSC line was established and it is continuing to undergo stabilization. Cells were not passaged many times, but the SSC line was established. SSCs are adherent cells that proliferate by adhering to the surface of the culture vessel. A high rate of proliferation was observed in the colony-forming cells (Fig. 3).

To analyze the proliferative properties of pheasant SSCs, the cells were examined with 5 antibodies *SSEA-1*, *SSEA-3*, *SSEA-4*, *Oct4*, and *GFRα1*. Reactivity was too weak to clearly detect SSCs in a mixed somatic cell population. Pheasant SSCs exhibited strong reactivity to *GFRα1*, a SSC-specific marker (Fig. 4), but did not react with antibodies

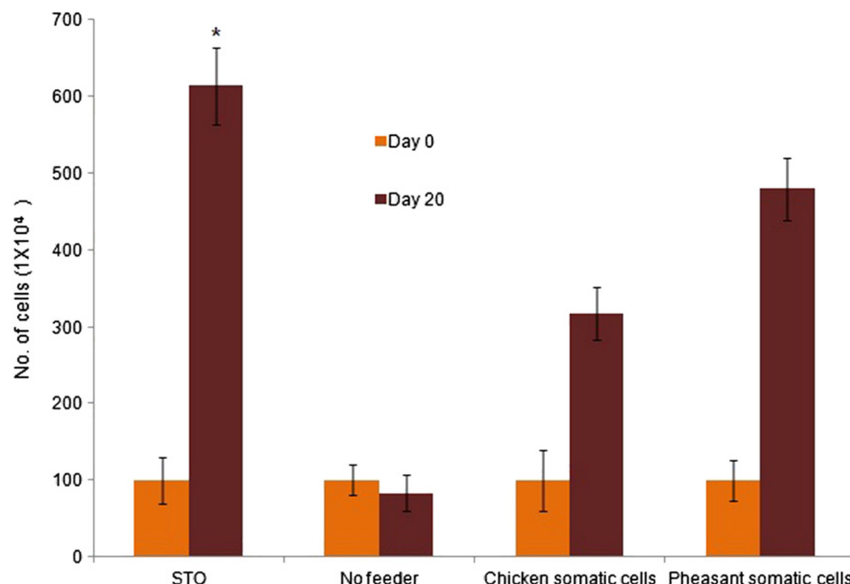

Fig. 1. STO cells and proliferation of pheasant SSCs in a co-culture system. Data are reported as mean ± SD. *Significance at $P < 0.05$.

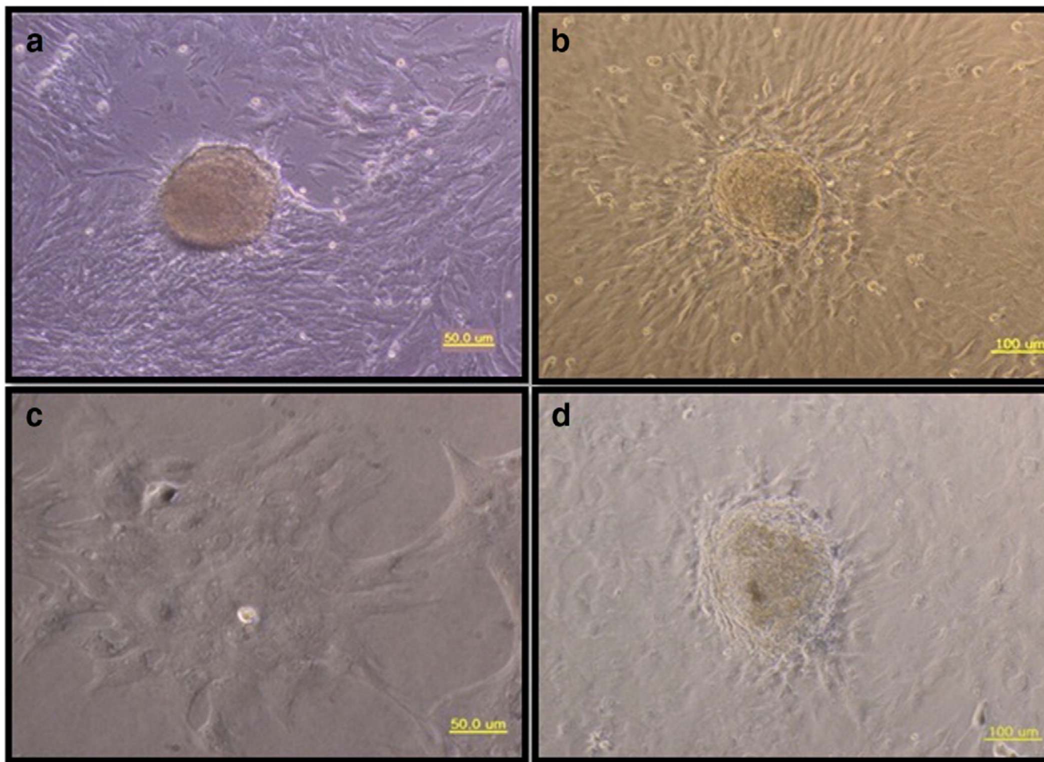

Fig. 3. Morphological characteristics of the pheasant spermatogonial stem cell line after subculture. (a) Chicken and (b) pheasant SSCs exhibited similar morphologies at 8 d; (c) pheasant SSCs maintained typical cell aggregation characteristics at 13 d (passage 3); and (d) maintained the characteristics and potential for aggregation and sphere formation at 25 d of culture (passage 6). Scale bar = 50 μm in (a) and (c); 100 μm in (b) and (d).

to *SSEA-1*, *SSEA-3*, *SSEA-4*, and *Oct4*. Thus, we continued the study with *GFRα1*.

3.3. Production of germ line chimeras by transferring donor SSCs into recipient

Germ line chimeras were produced by transplanting pheasant SSCs into recipient chicken embryos. The pheasant SSCs were transferred into 354 recipients; after 19 d, only 24 embryos were viable. The low rate of embryo development was preceded by an initially high survival rate (312/354); only 20 chicks hatched (5.6%). Donor cells were labeled with PKH 26 fluorescent dye to examine the percentage of donor SSCs in the recipient embryos. We transferred 10,000 and 20,000 labeled donor-derived SSCs to recipient embryos and found more cells in the gonads of chicken embryos with higher injected doses of SSCs (Fig. 5). Of 20 hatched chicks, only six had PKH 26-positive donor SSCs. As a result, 30% donor-derived SSCs were observed in the gonads of recipient chickens. Thus, we have established stable transfer techniques, but the percentage of PKH 26-positive cells was very low (5.3%). The distribution of stem cells in the transplanted SSCs was confirmed by FACS analysis. The c-kit/CD117-positive cells were found at a rate of about 9.48%, showing stable engraftment in the recipient gonads (Fig. 6).

4. Discussion

Each animal species possesses unique genetic and physiological characteristics. These features can be used to differentiate germ and somatic cells, analyze the expression of sex-specific genes, and to conserve genetic resources by using interspecies germ line chimeras. Furuta et al. [17] reported that germ cells can be used for conservation of native Ehime chickens (an endangered Japanese chicken). In this

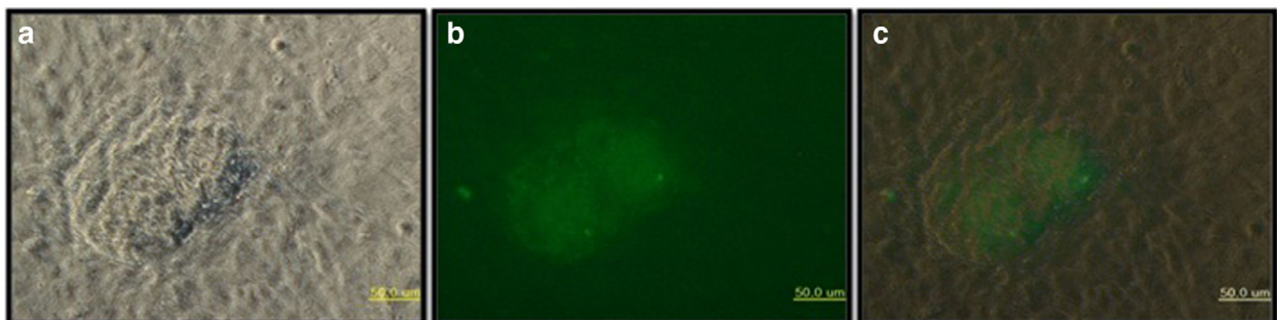

Fig. 4. Immunofluorescence detection of *GFRα1* marker on pheasant SSCs. (a) Phase-contrast microphotograph of an SSC colony; (b) green fluorescence of *GFRα1* antibody; and (c) merged figures (a) and (b). Pheasant SSCs were incubated with *GFRα1*. Scale bar; (a), (b), and (c) = 50 μm.

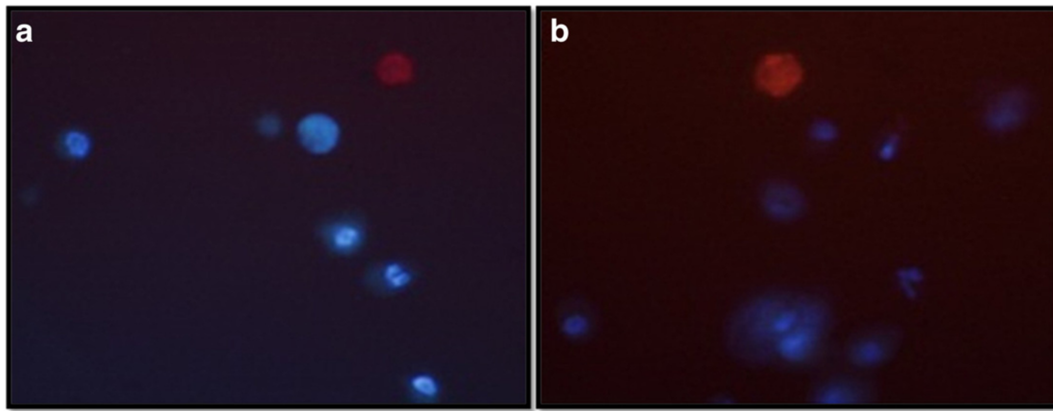

Fig. 5. PKH 26-labeled SSCs in the gonads of recipient embryos. Injection of 10,000 (a) and 20,000 (b) donor-derived SSCs. Red cells are labeled with PKH 26 cells and blue cells are PI-stained.

study, we established a SSC line to develop an interspecies germ line chimeric system that may facilitate restoration of endangered species.

Recent advances in chicken embryo manipulation methods have allowed researchers to produce germ line chimeras derived from the transfer of PGCs and methods for gene targeting in avian pluripotent cells have been considered. Kang et al. [9] produced interspecies germ line chimeras of pheasant and chicken. In this study, we used SSCs to produce interspecies germ line chimeras and transfer these interspecies SSCs into recipient embryos, a method that can be used to restore endangered birds. Moreover, we demonstrated that SSCs can be used to produce chimeric birds, thus advancing the study of germ cells. Pheasant SSCs transferred into chicken embryos migrated to the gonads and entered heterologous spermatogenesis in recipient embryos. Interspecies chimeras have thus become a powerful tool to preserve endangered birds [9].

It is essential to purify SSCs to increase the productivity of germ line chimeras and their transmission. For in vitro culture of SSCs, it is important to retrieve highly pure SSCs from the testes. To ensure purity, several isolation methods have been developed, such as using surface markers for specific proteins [18,19,20] and extracellular matrix [21,22]. In this study, we treated the pheasant testes with collagenase, hyaluronidase and trypsin to retrieve purified SSCs. Methods for in vitro culture and isolation of SSCs have recently been developed [23,24,25,26,27,28,29,30,31]. These cells were transferred into recipient testes and transgenic animals have been produced continuously [32,33,34].

In general, an established cell line proliferates and is stably differentiated through 100 passages without apoptosis or necrosis.

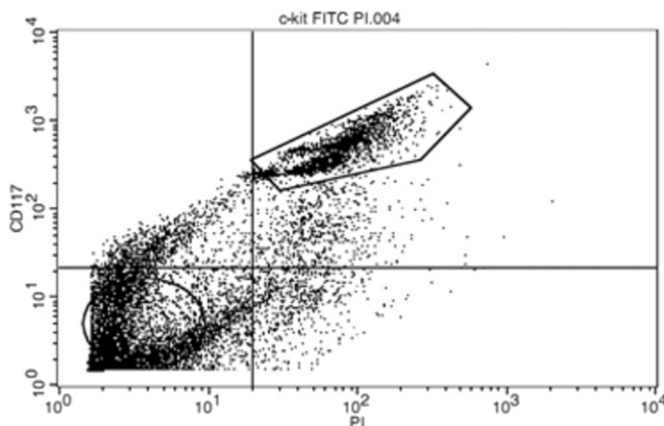

Fig. 6. The proportion of stem cells distributed in the donor PKH-26 cells.

As shown in Fig. 3, we sub-cultured the cells continuously, thus demonstrating establishment of the SSC line for the restoration of endangered birds. Colony-forming cells divided and proliferated stably in the positive direction. However, it is generally accepted that in vitro isolation and proliferation methods have not been clearly established for SSCs, perhaps because of a lack of information regarding appropriate in vitro culture conditions and specific markers of chicken germ line stem cells. In particular, the concentration of SSCs in the testis is extremely low; as few as 1 in 3333 cells have been reported in adult mouse testis [35], and 1 in 500 cells in adult rat testis [21]. Therefore, it is necessary to establish a highly pure SSC line from numerous types of cells and in vitro culture systems.

It is important to optimize the culture conditions (culture medium, co-cultured cells, and growth factors) for successful in vitro culture. Nagano et al. [23] confirmed the influence of in vitro culture conditions on SSCs. They also demonstrated the influence of culture medium, co-cultured cells, and growth factors on the proliferation and differentiation of SSCs. The success of long-term proliferation in culture is dependent on culture conditions [36]. For effective differentiation of SSCs, our cells were co-cultured with sertoli cells. In this study, SSCs were co-cultured with STO cells and proliferated by co-culturing both SSCs and STO cells (Fig. 1). Furthermore, Ryu et al. [19] found that SSCs proliferated in the presence of growth factors including glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and *GFRα1*. *GFRα1* is known to promote proliferation of SSCs. Interestingly, we detected significant expression of *GFRα1* in pheasant SSCs and showed strong reactivity for *GFRα1* (Fig. 4). In this study, we cultured SSCs in growth media with and without growth factors to identify the optimal culture conditions for pheasant SSCs. We obtained the best results with DMEM supplemented with 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, nonessential amino acids, 10 ng/mL bFGF, and 100 ng/mL IGF-1 (Fig. 2). These findings clearly indicated that growth factors such as LIF, bFGF, IGF-1, and FBS are important for the growth and proliferation of SSCs.

Lee et al. [37] established a germ line chimeric system for transferring testicular cells into heterologous testes. The testicular cells from juvenile and adult Korean Ogol chickens (KOC) were transplanted into White Leghorn (WL) testes. In this study, we transferred SSCs into 354 recipient embryos. Although embryo survival incidence was low (20/354), initial survival rates were sufficient. Subsequently, 30% donor-derived SSCs were observed in the gonads of recipient chickens. We confirmed the stability of our transfer techniques, but the percentage of PKH 26-positive cells was very low (5.3%). We believe that our findings support the development of an interspecies germ cell transfer system for the restoration of endangered birds.

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Author contribution

Proposed the theoretical frame: JHK, SJO, DKJ; Conceived and designed experiments: JHK, SJO, DKJ; Contributed reagents/materials/analysis tools: SWK, NEK; Wrote paper: JHK, NS, SSS; Performed the experiments the experiments: JHK, NS, SSS, RKM; Analyzed the data: JHK, NEK, MG.

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