An improved protocol for stable and efficient culturing of chicken primordial germ cells using small-molecule inhibitors

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Abstract

At present, the most reliable method for creating genetically modified chickens is the modification of the DNA sequence of primordial germ cells (PGCs). However, during embryogenesis, only a small number of chicken PGCs can be obtained. Therefore, *in vitro* PGC culturing is necessary to obtain sufficient cells for further genetic engineering. Previously reported PGC culturing methods lack versatility. We report here a new protocol for stable and efficient culturing of chicken PGCs using small-molecule inhibitors. The growth rate of PGCs was investigated following the addition of three small-molecule inhibitors, including blebbistatin, into the culture medium. Chicken PGC survival and proliferation rates increased after the addition of small-molecule inhibitors, compared with the untreated control. Blebbistatin was shown to be the most effective inducer of PGC growth. Long-term culturing of PGCs with blebbistatin maintained the morphology of typical PGCs, and these cells expressed marker proteins such as chicken vasa homolog (CVH) and NANOG. Additionally, PGCs transfected with a fluorescent protein gene were shown to migrate into the gonads of the recipient embryo, and progeny derived from PGCs cultured by this method were efficiently obtained. These results demonstrate that small-molecule inhibitors represent a useful tool for stable and efficient chicken PGC culturing.

Keywords: chicken, primordial germ cells, small-molecule inhibitor, chicken vasa homolog, NANOG

Introduction

Recently, remarkable advances in genome editing technologies, such as the transcription activator-like effector nuclease (TALEN) (Miller et al. 2011) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) (Cong et al. 2013; Mali et al. 2013) systems, have been achieved, allowing successful genetic modifications in various animal species (Nemudryi et al. 2014). Although DNA, RNA, and protein microinjections are mainly performed at the one-cell stage using fertilized eggs, these procedures and *in vitro* fertilization are extremely difficult to perform in chickens (Nishijima and Iijima 2013). Therefore, research on genetically modified (GM) chickens has been focused on the genetic modification of germ cells. *In vitro* and *in vivo* methods of gene transfer using primordial germ cells (PGCs) have been especially studied (Nishijima and Iijima 2013). Initially, the generation of GM chickens using PGCs was achieved using retroviral vectors (Vick et al. 1993). Additionally, the generation of GM chickens using cultured PGCs was achieved with non-viral genetic modification, such as gene transfer using transposons (Leighton et al. 2008; Macdonald et al. 2012; Park and Han 2012), as well as gene knockout by homologous recombination (Schusser et al. 2013). Recently, ovalbumin knockout chickens (Park et al. 2014), and green fluorescent protein knock-in chickens containing a large deletion in the vasa homolog (CVH) locus (Taylor et al. 2017) have been created using TALEN in cultured PGCs. Additionally, ovalbumin and ovomucoid knockout chickens (Oishi et al. 2016), with loxP site knock-in targeting the immunoglobulin heavy chain locus (Dimitrov et al. 2016) have been created using the CRISPR/Cas9 system in cultured PGCs. In these chickens, recombination of the loxP site by Cre recombinase caused a large deletion exceeding 20 kb in loxP knock-in PGCs *in vitro*. These examples demonstrate that genome editing of cultured chicken PGCs will likely become widely utilized in basic studies and applied research in the future. However, a stable and efficient method for the culturing of chicken PGCs is required, and the establishment of a PGC culture system should facilitate GM chicken generation.

PGCs are first observed in the extra-embryonic ectoderm during mouse embryogenesis, and they migrate along the intestinal wall to the genital ridge that will become gonads. Once the PGCs reach the genital ridge, they proliferate while maintaining their characteristics by interacting with the stromal cells of the gonads. In the chicken, PGCs are first observed at the center of the blastoderm as CVH-positive cells during early embryogenesis, and they migrate to the extra-embryonic tissue (germinal crescent) according to primitive streak formation (Tsunekawa et al. 2000). Then, the PGCs enter the dorsal aorta from the germinal crescent and circulate in the blood stream in order to reach the genital

ridge. Although there are differences in the migration paths, the migration of germ cells is similar in mice and chickens. When PGCs cannot interact with the stromal cells of the gonads, due to a failure to migrate, they immediately undergo apoptosis (Tres et al. 2004), and mouse PGCs are known to undergo rapid apoptosis *in vitro* (Pesce et al. 1993).

In chickens, PGCs were shown to successfully proliferate *in vitro* (Lavoir et al. 2006), but their maintenance in the undifferentiated state and the establishment of PGC lines using leukemia inhibitory factor (LIF), fibroblast growth factor 2 (FGF2), and stem cell factor (SCF) are difficult (Naito et al. 2010). Furthermore, the activation of apoptosis-related proteins, such as caspase 3 and caspase 9, was detected in cultured chicken PGCs, and epidermal growth factor (EGF) was shown to only partially inhibit the activation of these proteins (Ge et al. 2009). Recently, culturing methods using FGF2, insulin, and activin have been developed and have shown that chicken PGCs can be grown very efficiently (Whyte et al. 2015).

In this study, we hypothesized that inhibiting the relevant factors of apoptotic signals in stem cells using a small-molecules inhibitor in the PGC culture system would stabilize as well as enhance the proliferation of PGCs. Therefore, we investigated whether addition of small-molecule inhibitors contributes to the stabilization of chicken PGCs in vitro.

Materials and methods

Experimental animals and animal care

Freshly laid, fertilized, unincubated Barred Plymouth Rock (BPR) and White Leghorn (WL) eggs were purchased from Haraigawa Egg Farm (Fukuoka, Japan) and Akita Co. (Fukuyama, Japan). They were maintained in an isolated facility at the University Animal Farm, Hiroshima University, Japan. The experimental methods were approved by the animal use and care committee of Hiroshima University.

Isolation and culture of chicken PGCs

Chicken whole blood samples containing PGCs were collected from a BPR embryo at Hamburger Hamilton (HH) stage 13-15 (Hamburger & Hamilton 1951). The blood was dispersed in 500 µl PGC culture medium. Each culture experiment utilized PGCs derived from one embryo. The PGC culture medium was described in a previous study (Whyte et al. 2015), with some modifications. Briefly, KnockOut DMEM (Thermo Fisher Scientific, Waltham, MA, USA) was supplemented with 1× B-27 Supplement Minus Vitamin A (Thermo Fisher Scientific), 1× EmbryoMAX nucleosides (Merck, Darmstadt, Germany), 1× MEM non-essential amino acids (Thermo Fisher Scientific), 0.5 mM monothioglycerol (Wako Pure Chemical Industries, Osaka, Japan), 1× Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque, Kyoto, Japan), 10 ng/ml human FGF2 (PeproTech, Rocky Hill, NJ, USA), 1 unit/ml heparin (Merck), and 25 ng/ml human/mouse/rat activin A (PeproTech). The whole blood samples containing PGCs were cultured in a 24-well plate without feeder cells at 38°C, 5% CO_2, and 3% O_2, and subcultured every 2–4 days. The passaging of PGCs depended on their growth. PGCs were frozen in STEM-CELLBANKER (Nippon Zenyaku Kogyo, Fukushima, Japan) and stored at −80°C.

Inhibitor treatment

PGCs were cultured using the protocol described above. PGCs were cultured in a medium supplemented with different concentrations (0.25–2 µM) of the following inhibitors: blebbistatin, H-1152, and Z-VAD (Wako Pure Chemical Industries). The initial number of PGCs was adjusted to approximately 250 cells/well, and cells were seeded in 96-well culture plates (n = 3). The number of PGCs was evaluated using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) using a subset (approximately half) of the

cultured cells every other day of incubation, and the luminescent signal was measured by a 2030 Multilabel Reader ARVO X4 (PerkinElmer, Waltham, MA, USA). This assay represents a method for determining the number of viable cells based on the quantitation of ATP levels, which signal the presence of metabolically active cells. Doubling times were calculated by using an algorithm available online (www.doubling-time.com/).

Generation of lentiviral particles for inducible expression of ZsGreen1 genes

The internal ribosome entry site (IRES) was removed from the pLVSIN-EF1alpha-IRES-ZsGreen1 vector (Takara Bio, Shiga, Japan) using an In-Fusion HD cloning system (Takara Bio) following the manufacturer's instructions. The pLVSIN-EF1alpha-ZsGreen1 vectors were transformed into competent DH5-alpha cells (Toyobo, Osaka, Japan) and purified using an EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). The purified pLVSIN-EF1alpha-ZsGreen1 vectors were used with the Lenti-X Packaging Single Shots system (Takara Bio) to generate lentivirus particles in Lenti-X 293T cells (Takara Bio). Briefly, Lenti-X 293T cells were cultured to about 80% confluence in cell culture dishes (100 mm diameter) with 8 ml of FreeStyle 293 Expression Medium (Thermo Fisher Scientific). For each transfection, 30.0 µg of pLVSIN-EF1alpha-ZsGreen1 vector in 600 µl of distilled water was added to a Lenti-X Packaging Single Shots system, the components were mixed, and the mixture was incubated at room temperature (20–25°C) for 10 min. The entire mixture was added dropwise to one Petri dish containing a Lenti-X 293T cell culture and mixed by gentle rocking. The cells were incubated at 37°C with 5% CO_2 for 6 h, after which the medium was changed. Next, the cells were incubated for a further 72 h, and the culture medium was collected. The presence of lentivirus particles in the medium was determined using the Lenti-X GoStix reagent (Takara Bio) following the manufacturer's protocol. The lentiviral particles in the medium were concentrated 100 times using Lenti-X concentrator reagent (Takara Bio) following the manufacturer's instructions. The concentrated viral titer was estimated using a Lenti-X p24 Rapid Titer Kit (Takara Bio) following the manufacturer's instructions. The viral particles were aliquoted and stored at −80°C until use.

Transduction of PGCs with lentiviral particles

Lentiviral particles expressing the ZsGreen1 transgenes were used to transduce PGCs. Briefly, PGCs (1×10^6 cells) were cultured in culture dishes (100-mm diameter) containing PGC culture medium supplemented with 0.25 µM blebbistatin. Polybrene solution was added to the cell culture to a final concentration of 4 µg/ml and mixed by gentle rocking. About

200 µl of a thawed lentiviral aliquot (at a titer of about 100 multiplicity of infection) was added to the cells dropwise, and the cells and virus were mixed by gentle rocking. Transduced cells were cultured overnight, then the medium was replaced with a fresh culture medium, and the cells were cultured for 1 week. The ZsGreen1 positive cells were picked up and cloned by limiting dilution. Then, the cloned PGCs were analyzed by immunofluorescence staining to identify the clones that maintained the expression of marker proteins CVH and NANOG.

Immunofluorescent staining

The cultured PGCs were collected, pelleted, and washed three times with Dulbecco's phosphate-buffered saline (PBS; Nissui-Pharmaceutical, Tokyo, Japan) containing 1% bovine serum albumin (BSA; Thermo Fisher Scientific), with centrifugation for 5 min at 100 × *g* after every washing. The cells were fixed in 4% paraformaldehyde/PBS for 30 min at room temperature (20–25°C). Then, the cells were washed with 1 mM glycine (Wako Pure Chemical Industries)/1% BSA-PBS and permeabilized with 0.1% Triton-X 100 (Nacalai Tesque)/PBS for 5 min at room temperature (20–25°C). Following permeabilization, cells were washed again three times with 1% BSA/PBS and treated with PBS containing anti-CVH mouse monoclonal antibody (hybridoma supernatant; Nakano et al. 2011) and anti-chicken NANOG rabbit polyclonal antibody (1:50; Nakano et al. 2011) in the presence of 1% BSA for 1 h at 37°C. After this treatment, they were washed with 1% BSA/PBS and incubated with Alexa594-conjugated anti-rabbit IgG (H+L) goat antibody (1:100; Invitrogen, Carlsbad, CA, USA) and Alexa488-conjugated anti-mouse IgG (H+L) goat antibody (Invitrogen) for 1 h at 37°C, followed by washing three times with 1% BSA/PBS. The stained cells were dispersed in VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and observed under an inverted fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Creation of chimeric chickens

PGCs (approximately 5,000 cells) were prepared at 2,500 cells/µl using KnockOut DMEM containing 2% chicken serum. The suspended cells (2 µl) were injected into the subgerminal cavity of a WL embryo recipient that was irradiated with 3 Gy gamma rays at Stage X (Eyal-Giladi and Kochav, 1976). Stage X recipient embryos were moved into a substitute shell, which was filled with egg white and sealed with clear wrap. The transplanted embryo was cultured at 38°C for three days at 60% relative humidity (System II). The embryos were

again transferred to large host eggshells, sealed with clear wrap, and cultured at 38°C and 60% relative humidity for an additional 4 days or until hatching (System III, Perry 1988). After the 4 days, embryos were isolated from the yolk, washed with PBS, and the gonads were removed. The presence of fluorescent cells in the gonads was observed using a fluorescence stereomicroscope (SZX12, Olympus).

Progeny test

Chimera roosters were crossed with wild-type BPR hens by artificial insemination. The offspring were screened for the color (black or yellow) of their feathers to identify donor PGC-derived offspring.

Results

Small-molecule inhibitor treatment of chicken PGCs

The proliferative activity of male chicken PGCs was examined after the addition of three small-molecule inhibitors (blebbistatin, H-1152, and Z-VAD) into the culture medium. Among the four concentrations used, the most efficient concentration to increase PGC number for each inhibitor was 0.25 μM for blebbistatin and H-1152 and 0.5 μM for Z-VAD (Fig. 1). The number of PGCs that was induced by each small-molecule inhibitor reached up to $4.21 \times 10^5 \pm 0.23$ cells with blebbistatin (Fig. 1a), $2.68 \times 10^5 \pm 0.12$ cells with H-1152 (Fig. 1b), and $2.50 \times 10^5 \pm 0.12$ cells with Z-VAD (Fig. 1c) from 250 cells in 10 days. The most efficient concentration to increase PGC number with each inhibitor resulted in a 3.84-fold increase for blebbistatin (Fig. 1a), 2.45-fold increase for H-1152 (Fig. 1b), and 2.28-fold increase for Z-VAD (Fig. 1c) compared with that in control cells. In terms of doubling time, PGCs treated with blebbistatin doubled in 0.81 days, those treated with H-1152 and Z-VAD doubled in 0.9 days, and control cells doubled in 1.16 days. These findings regarding survival and proliferation of PGCs suggest that using small-molecule inhibitors efficiently promotes the proliferative capacity of PGCs.

Characterization of chicken PGCs *in vitro*

Chicken PGCs obtained from HH stage 13-15 (Hamburger & Hamilton 1951) male embryonic blood were cultured using culture medium supplemented with blebbistatin. The long-term (28 days) cultured PGCs maintained the morphology of typical chicken PGCs, such as a spherical shape and glycogen granules in their cytoplasm (Fig. 2a and 2b). PGCs were subcultured every 2–4 days to maintain the appropriate proliferation rates. After cryopreservation and subsequent reculturing, these PGCs were shown to maintain the same morphology. The success rate of PGC culture was about 50–60%, and the sex-chromosome type of cultured PGCs was ZZ.

Chicken PGCs were transduced with the lentivirus carrying an expression cassette for ZsGreen1 fluorescent protein, and the expression of CVH and NANOG was analyzed using immunofluorescent staining in these cells. Fluorescence microscopy detected the expression of ZsGreen1 (Fig. 2d and 2h); CVH was detected in the cytoplasm (Fig. 2e and 2f) and NANOG in the nucleus of most PGCs (Fig. 2i and 2j).

In order to demonstrate that the cultured chicken PGCs can migrate to the gonads in chimeric chickens, we transplanted PGCs expressing ZsGreen1 into a recipient embryo from

a fertilized egg derived from WL chickens. After seven days of culture, many ZsGreen1-positive cells were observed in the gonads (Fig. 2k and 2l).

Germline transmission of cultured PGCs after transplantation

In order to demonstrate that the cultured chicken PGCs can differentiate into functional gametes in recipient gonads, cultured male BPR PGCs (5,000 cells) were transplanted into the subgerminal cavity of WL embryo recipients at Stage X. The recipient embryos were hatched, survived to sexual maturity, and were then mated to wild-type chickens. BPR offspring were obtained from surrogate recipient chickens (Fig. 3a), and the efficiency was high (93%) even when using long-term (177 days) cultured PGCs (Fig. 3b). This result indicates that PGCs maintained in medium containing blebbistatin were germline competent.

Discussion

The cultivation of cells that can contribute to the germ line is important for the generation of GM chickens, as well as the long-term safeguarding and management of poultry genetic resources. Chicken PGCs collected from the germinal crescent or embryonic blood were transfected with a viral vector carrying a gene and were then transplanted into recipient embryos in the first reported case of successful genetic modification in chickens (Vick et al. 1993). Since then, chicken PGCs, together with embryonic stem (ES) cells, have been the target cells for the generation of GM chickens. To date, PGC culture systems have been focused on the reconstruction of the PGC niche *in ovo,* maintained by humoral factors and cell-to-cell contacts. In contrast to this, our approach is focused on the inhibition of apoptotic signaling that occurs when PGCs are not in their niche.

Culture methods based on similar principles were previously developed for the maintenance of primate ES cells and induced pluripotent stem (iPS) cells. For example, the apoptosis of human ES and iPS cells is induced by the loss of cell-cell adhesion; however, these cells can be cultured even without this adhesion if apoptotic signaling inhibitors are used (Ohgushi et al. 2010). Moreover, the use of apoptotic signaling inhibitor enables the cloning of chicken stem cells (Nakano et al. 2011). The apoptosis of these cells likely serves to eliminate cells that do not incorporate into the epiblast layer, similar to the process affecting ectopic PGCs (Tres et al. 2004).

Here, we showed that all investigated small-molecule inhibitors (blebbistatin, H-1152, and Z-VAD) increased the proliferative activity of PGCs. During apoptotic signaling, crosstalk between Akt and mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling occurs (Stankiewicz and Linseman 2014). Rho-associated protein kinase (ROCK), in the apoptotic signaling pathway, suppresses Akt and ERK through phosphatase and tensin homolog (PTEN). Akt and ERK, on the other hand, promote the expression of B-cell lymphoma 2 (Bcl-2), which prevents the release of cytochrome c from mitochondria, inhibiting apoptosis.

Blebbistatin, an inhibitor with a high affinity toward myosin (Kovács et al. 2004), was demonstrated to induce the proliferation of PGCs most effectively among all investigated small-molecule inhibitors. Blebbistatin inhibits myosin ATPase activity and inhibits acto-myosin-based motility. It acts to detach the actin in the acto-myosin conformation by binding between the nucleotide binding site and the actin-binding cleft of myosin, and the relaxation of the acto-myosin myofilament leads to several biological effects, one of which is the suppression of blebbing. Blebbing is one of the defined features of apoptosis, in which the cytoskeleton is broken up and causes the membrane to bulge outward during programmed

cell death. These bulges the segment cytoplasm (include fragmented nucleus and/or organelles) and separate them from the cell as apoptotic bodies (Wickman et al. 2013). Interestingly, similar to that observed in dissociated human ES cells (Ohgushi et al. 2010), inhibiting myosin hyperactivation was more effective than inhibiting caspases in chicken PGC cultures, but the mechanism could not be elucidated.

The inhibition of ROCK, which is upstream of myosin hyperactivation, was also effective for the maintenance of PGC characteristics. Additionally, we demonstrated that chicken PGCs grown using our culturing method maintained the ability to migrate to the gonads and that their germline transmission capacity was retained even after long-term culturing. The high germline transmission was attributed not only to the maintenance of PGC properties but also to the effective irradiation of host embryos by gamma ray.

In addition, PGC culture systems using small-molecule inhibitors are also available for other chicken species such as WL and Rhode Island Red (data not shown), other than BPR. There is a possibility that these methods can be applied not only for chickens but also for widely used birds, such as quail and zebra finch, in basic biology for important birds in the long-term safeguarding and management of genetic resources.

In conclusion, we developed a new, stable, and efficient protocol for the culturing of chicken PGCs by using small-molecule inhibitors. PGCs were shown to express CVH and NANOG, and they could be maintained in long-term subcultures. Additionally, we demonstrated that they could migrate into the gonadal ridge and could be transplanted into recipient embryos, allowing the development of chimeric chickens and germline transmission. These results indicate that small-molecule inhibitors are useful for the maintenance of stable chicken PGC cultures and may help accelerate genome editing of chickens to be used for future basic and applied research.

Compliance with Ethical Standards

Ethical approval: The experimental methods were approved by the animal use and care committee at the institution of Hiroshima University

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Figure legends

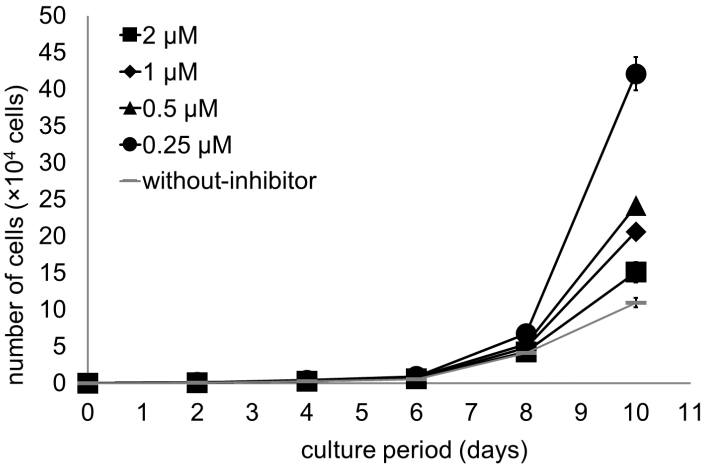
Fig. 1 Proliferation of PGCs in medium supplemented with small-molecule inhibitors
PGCs were treated with different concentrations of small-molecule inhibitors, including blebbistatin (a), H-1152 (b), and Z-VAD (c) (n = 3).

Fig. 2 Characterization of PGCs cultured with blebbistatin
Chicken whole blood samples containing PGCs were collected from a BPR embryo at HH stage 13-15, and the blood was dispersed in PGC culture medium containing blebbistatin (a). PGCs showed a spherical shape and glycogen granules in the cytoplasm (arrow in a). PGCs cultured with blebbistatin for 28 days (b). CVH and NANOG immunofluorescence staining. PGCs cultured with 0.25 μM blebbistatin for 75 days. Typical PGC morphology (c and g). ZsGreen 1 was transduced into PGCs by a lentiviral vector (d and h). PGCs were stained using anti-CVH monoclonal antibody (e) and NANOG polyclonal antibody (i), while the cell nuclei were visualized using DAPI (f and j). Scale bar, 100 μm. ZsGreen1-expressing PGCs, cultured with 2.5 μM blebbistatin for 80 days, were transplanted into stage X recipient embryos, and ZsGreen1-positive cells in gonads were observed in day 7.5 embryos. Day 7.5 chicken gonads (k). ZsGreen1-positive cells (l).

Fig. 3 Chicken PGCs propagated in blebbistatin are germline competent.
PGCs cultured with blebbistatin are germline competent. Male PGCs were injected into host embryos, hatched, and raised to sexual maturity. The photograph shows several offspring of a surrogate host rooster (a). The frequency of germline transmission from donor PGCs in surrogate host chickens is shown (b).

Figure 1. Ezaki et al.

a



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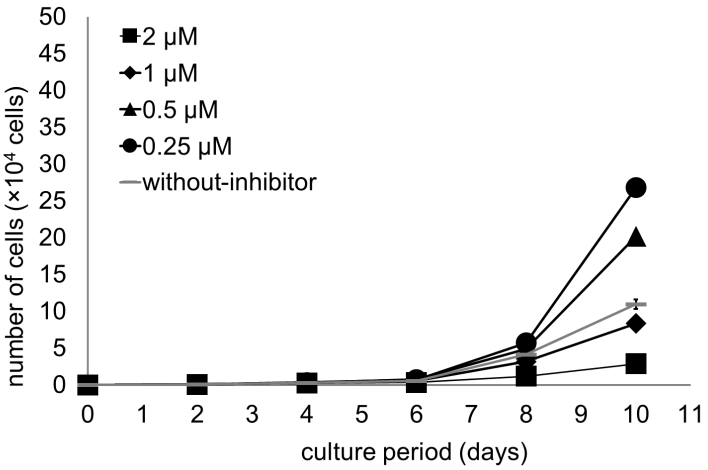

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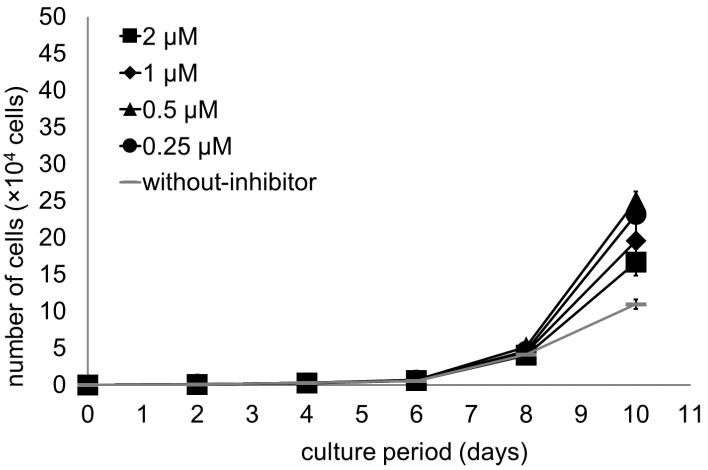

Figure 2. Ezaki et al.

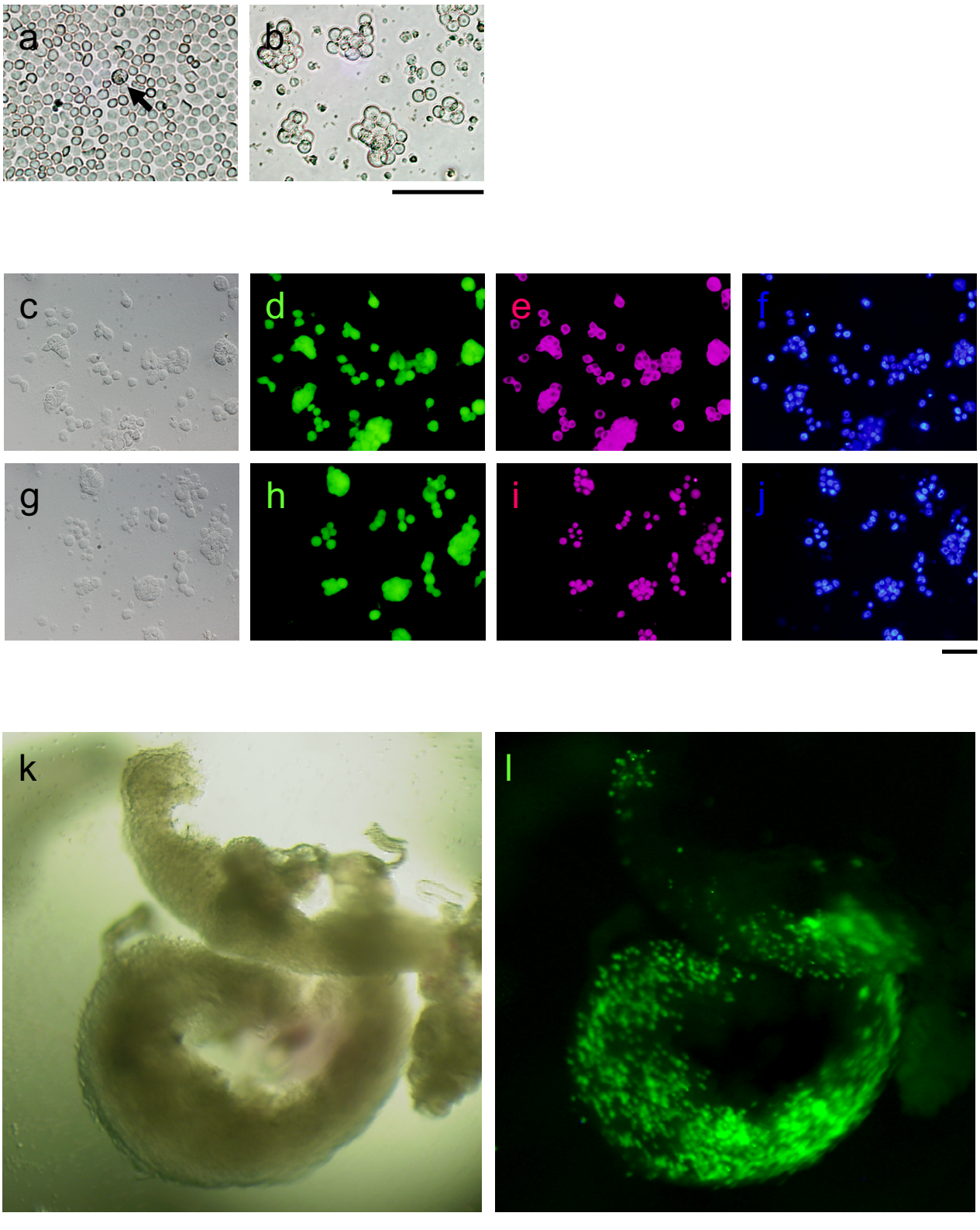

Figure 3. Ezaki et al.

a

b

Founder birds	Days in culture	Eggs set	Chicks hatched	PGC-derived offspring
#362	196	217	58 (27%)	13 (22%)
#7186	177	99	14 (14%)	13 (93%)
#7721	50	360	121 (34%)	92 (76%)