In vitro culture and characterization of duck primordial germ cells

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ABSTRACT This study aimed to isolate, culture, and characterize duck primordial germ cells (PGCs) and to compare these cells with chicken PGCs. We first cultured Muscovy duck (*Cairina moschata*) circulating PGCs and gonadal PGCs (gPGCs) in the modified serum-containing medium used to amplify chicken PGCs. gPGCs were found to proliferate better in serum-free chemically defined medium than in serum-containing medium. Thereafter, gPGCs were similarly isolated from 2 other duck breeds, the Pekin duck (*Anas platyrhynchos*) and the hybrid mule duck (*C. moschata* × *A. platyrhynchos*), and amplified for a limited period of time in the chemically defined culture condition, but sufficiently to be characterized and transplanted. Cultured gPGCs of all 3 duck breeds were characterized by Periodic acid-Schiff staining, immunocytochemical staining, and expression analysis of germline-specific and pluripotency genes. Cultured duck gPGCs colonized the gonads after being genetically labeled and injected into recipient embryos. Taken together, these results demonstrate that duck PGCs retain their germline characteristics after being isolated, expanded in vitro, and genetically modified. Further studies are required to establish the optimal conditions for long-term culture of duck PGCs, which may involve supplementing the culture medium with other growth factors or compounds.

Key words: primordial germ cell (PGC), Muscovy duck (*Cairina moschata*), Pekin duck (*Anas platyrhynchos*), mule duck (*C. moschata* × *A. platyrhynchos*)

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INTRODUCTION

Primordial germ cells (PGCs), the progenitors of gametes, are a unique cell type with germline competence in avian species (van de Lavoir et al., 2006) and can thus be used for numerous applications. Semen is widely cryopreserved to maintain the germplasm of many poultry species and endangered birds (Saint Jalme et al., 2003; Blesbois et al., 2005). However, it is impossible to cryopreserve avian ova. PGCs are an ideal alternative to preserve the germplasm of both sexes (Nakamura, 2016). However, the isolation and in vitro culture of waterfowl PGCs have been rarely described, in contrast with the many studies of chicken PGCs, and there are only a few reports concerning germ cells of waterfowls.

Chicken PGCs can be isolated from the blood of early embryos (stage HH 15 to 16; Hamburger and Hamilton 1951) before they enter the developing gonads and from gonads of embryos incubated for 7 d (stage HH 28 to 30). The proliferation rates of chicken circulating and gonadal PGCs (**gPGCs**) remain high upon long-term culture. When cultured chicken PGCs were injected into the blood circulation of chicken embryos (stage HH 15 to 16), PGCs further colonize and proliferate in the gonads (van de Lavoir et al., 2006; Macdonald et al., 2010; Whyte et al., 2015). The culture conditions of chicken PGCs have been standardized following progressive improvements made based on knowledge of the signaling pathways that control proliferation and self-renewal of these cells. Consequently, PGC derivation is currently reproducible in several strains of chicken.

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Genetic engineering of PGCs has also been performed to investigate gene functions in basic research (Taylor et al., 2017). Transgenic chicken cell lines have been generated by genetically modifying PGCs using genome editing technologies (Macdonald et al., 2012; Park and Han, 2012). Some of these cell lines were established to produce recombinant proteins, such as cytokines and growth factors, while others were generated using knockout technologies to study the functions of various genes, such as those encoding immunoglobulin and allergenic proteins including ovalbumin (Leighton et al., 2008; Schusser et al., 2013; Park et al., 2015; Oishi et al., 2016; Taylor et al., 2017). Recently developed DDX4 (also known as VASA and chicken vasa homolog [**CVH**]) knockout chickens may be a universal recipient for germline transplantation and genotype transmission (Taylor et al., 2017).

Ducks, which diverged from chickens more than 60 million years ago, are a major type of poultry used to provide meat worldwide and eggs in some Asian countries. The Muscovy duck (*Cairina moschata*) is usually raised for meat production due to its large body size and high growth efficiency. The mallard (*Anas platyrhynchos*) is also reared for meat production. However, some strains, such as Tsaiya ducks (*Anas platyrhynchos*), which are a Taiwanese mallard duck breed, can lay more than 320 eggs annually and are thus also used for egg production. The mule duck, another major duck breed produced for meat in China, Taiwan, and some south-eastern Asian countries, is a hybrid produced by crossing a Muscovy drake and a Pekin duck. More than 90% of the French food delicacy foie gras (fatty liver) produced in France is obtained from male mule ducks (Marie-Etancelin et al., 2008). These various uses of numerous breeds reflect the importance of ducks in the poultry industry worldwide.

Only a few duck cell types have been cultured and established as cell lines (Guan et al., 2010; Olivier et al., 2010). To develop a system for in vitro culture of duck PGCs similar to that established for chicken PGCs, we aimed to isolate, culture, and amplify PGCs from Muscovy, Pekin, and mule ducks. First, we attempted to isolate PGCs from the circulating embryonic blood (stage HH 15 to 16, embryonic day (E) 5) and gonads (stage HH 28–30, E9) of Muscovy duck embryos. Second, we optimized the culture conditions for duck PGCs by replacing chicken serum with ovotransferrin in the culture medium and then attempted to obtain PGCs from Pekin and mule ducks. These cultured PGCs were characterized by analyzing germ cell markers and compared with cultured chicken PGCs. Finally, we assessed the migration of cultured duck PGCs toward the genital ridges and their colonization of the gonads following xenogeneic and allogeneic transplantation into chicken and duck embryos, respectively. In summary, we isolated, cultured, and characterized PGCs from 3 duck breeds, 2 of which belonged to 2 major genera of duck, namely, *Cairina* and *Anas*.

MATERIALS AND METHODS

Ducks and Egg Incubation

All ducks, including Wujie black Muscovy ducks (*C. moschata*), Pekin ducks (*A. platyrhynchos*), and mule ducks, which were generated by crossing a male *C. moschata* Muscovy duck with a female *A. platyrhynchos* Kaiya duck, were hatched from eggs produced at the Ilan Branch of the Taiwan Livestock Research Institute (Council of Agriculture, Executive Yuan, Taipei, Taiwan). Leghorn chicken embryos (*Gallus gallus*) were purchased from the Animal Drugs Inspection Branch of the Animal Health Research Institute (Council of Agriculture, Executive Yuan, Taipei, Taiwan). Eggs were incubated in an automatically turning humidified incubator at 37°C. All animal experiments were conducted with the ethical approval of the Ilan Branch of the Taiwan Livestock Research Institute (No. 105–11).

Isolation and in Vitro Culture of PGCs

Circulating PGCs (**cPGCs**) were obtained by seeding approximately 2 μL of blood isolated from embryos at stage HH 15 to 16 (Hamburger and Hamilton, 1951), corresponding to E3 chicken embryos and E5 Muscovy duck embryos, into 300 μL of medium in a well of a 48-well plate. gPGCs were obtained by plating dispersed gonadal tissues isolated from stage HH 28 to 30 embryos, corresponding to E7 chicken embryos, E8 Pekin and mule duck embryos, and E9 Muscovy duck embryos, together with 500 μL of medium in a well of a 24-well plate. gPGCs were easily separated from somatic cells by collecting nonadherent cells at 1 d after plating of gonadal tissues. To isolate cPGCs and gPGCs, one-third of the total volume of culture medium was replaced by fresh culture medium every 3 d. When cells became confluent, all the medium was replaced and cells were sub-cultured into larger wells. All PGCs were maintained at 37°C in 5% CO_2. FAcs (fibroblast growth factor 2 (**FGF2**), Activin A and chicken serum) and FAot (FGF2, Activin A and ovotransferrin) media were prepared as described by Chen et al. (2018). Briefly, FAcs medium was diluted Dulbecco's modified eagle medium (**DMEM**) (1:3 ratio of sterile deionized water and calcium-free high-glucose DMEM) containing 1× B-27 supplement, 2 mM GlutaMAX, 1× non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.2% chicken serum (all from Gibco®, Grand Island, NY, USA), 1× nucleosides (EMD Millipore, Billerica, MA, USA), 2 mg/mL ovalbumin (Sigma-Aldrich, St. Louis, MO, Germany), 0.1 mg/mL sodium heparin (Sigma-Aldrich), 25 ng/mL human Activin A, and 4 ng/mL human FGF2 (both from R&D Biosystems, Minneapolis, MN, USA). The composition of FAot medium was the same as that of FAcs medium, except that chicken serum was replaced by 10 μg/mL ovotransferrin (Sigma-Aldrich).

Isolation and Primary Culture of Embryonic Fibroblasts

To prepare somatic cells as a control, primary embryonic fibroblasts (**EFs**) were isolated from embryos at stage HH 37, corresponding to E11 chicken embryos, E15 Pekin duck embryos, E16 mule duck embryos, and E18 Muscovy duck embryos. Following removal of the head, limbs, and viscera, the embryos were minced in 0.25% trypsin-EDTA and incubated for 15 min at 37°C. After filtration through a 100-μm nylon mesh (Falcon, Waltham, MA, USA), EFs were cultured in DMEM containing 10% FBS (Hyclone, Logan, UT, USA), 2 mM GlutaMAX, 1× non-essential amino acids, 1 mM sodium pyruvate, and 1× antibiotics (Gibco®). EFs were sub-cultured when 80% confluent and cells at passage 5 were characterized as a negative control.

Periodic Acid-Schiff Staining

gPGCs were washed twice and centrifuged at 1,200 rpm for 5 min, and the pellet was resuspended in cold Dulbecco's phosphate-buffered saline (**DPBS**) without Ca^{2+}/Mg^{2+} (Gibco®). The cell suspension was placed onto a Superfrost™ Plus slide (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 20 min at ambient temperature to allow cell attachment. Adherent cells were fixed in DPBS containing 4% paraformaldehyde and stained with Periodic acid-Schiff (**PAS**) using a PAS Kit (Sigma-Aldrich) according to the manufacturer's protocol.

Immunocytochemistry and Flow Cytometry

Cells attached to a Superfrost™ Plus slide (Thermo Fisher Scientific) and those in suspension (5×10^5 cells) were fixed in paraformaldehyde (Sigma-Aldrich) and permeabilized in 0.1% Tween-20 (Sigma-Aldrich) for 10 min. Thereafter, cells were incubated with 0.125 μg of an Alexa Fluor® 488-conjugated anti-stage-specific embryonic antigen-1 (SSEA-1) antibody, a mouse IgM isotype control FITC-conjugated antibody (eBioscience, San Diego, CA, USA), or a rabbit anti-CVH antibody (as previously described by Raucci et al. 2015) overnight at 4°C in 500 μL of blocking buffer, which comprised DPBS containing 1% bovine serum albumin (Sigma-Aldrich). Cells labeled with the anti-CVH antibody were subsequently stained with 1 μg of donkey anti-rabbit IgG conjugated with Alexa Fluor® 594 (for microscopy) or Alexa Fluor® 488 (for flow cytometry) (Invitrogen, Carlsbad, CA, USA) in 500 μL of blocking buffer for 1 h at room temperature. Slides were washed with DPBS and mounted using ProLong™ Gold Antifade Mountant with DAPI (Life Technologies, Carlsbad, CA, USA). Images were acquired using a Leica TCS SP5 II confocal microscope (Leica Microsystems, Bensheim, Germany). Flow cytometry was conducted on a Cytomics FC500 cytometer (Beckman Coulter, Brea, CA, USA). Data were analyzed using CXP Analysis Software (Beckman Coulter).

RNA Isolation and Reverse Transcription PCR

RNA was extracted from cultured cells using TRIzol® reagent (Invitrogen), according to the manufacturer's instructions. After purification, samples were resuspended in DNase/RNase-free distilled water and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). To confirm the RNA purity and integrity, the ratios of A260/280 were found to be in a range of 1.80 to 2.10 for each RNA sample and those also display the RNA integrity number higher than 9.00 by the detection using an Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip (Agilent Technologies, Santa Clara, CA, USA) (Supplementary Figure 1). In total, 500 ng of total RNA was treated with DNase (Invitrogen) and then reverse-transcribed using a High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™, Foster City, CA, USA). The transcription of various genes was determined by performing PCR using species-conserved primer sets (Table 1; Supplementary Table 1). The housekeeping genes *GAPDH* and *RPS17* were used as the internal control. The reaction mixture contained 1 U of Ultra-Pure Taq PCR Master Mix (Geneaid Biotech, New Taipei City, Taiwan), 10 μM of each primer, 25 ng of cDNA, and 20 μL of ultra-pure water. The following conditions were used: 94°C for 5 min, followed by 30 cycles at 94°C for 20 s, 59°C for 30 s, and 72°C for 20 s. PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide.

Cell Proliferation Assay

Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) (Tominaga et al., 1999). In total, 5×10^4 gPGCs were seeded into each well of a 24-well plate in 500 μL of FAcs or FAot medium. Thereafter, 50 μL of CCK-8 reagent was added to each well in order to attain the 1:10 ratio recommended by the manufacturer. Cells were incubated in a CO_2 incubator at 37°C for an additional 4 h. Absorbance at 450 nm was measured using a microplate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA). The relative total cell number was calculated by comparing absorbance of the samples with a standard curve generated using known numbers of chicken PGCs. The fold increase in the total cell number was calculated using the following formula: relative total cell number at day N/relative total cell number at day 1.

Table 1. Primer sets used for RT-PCR analysis of PGCs.

Gene	Full name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product length (bp)	Accession number
DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX4)	CAG ACC GCA TGC TTG ATA TG	CAG CCA GCC TCT GAA CTT CT	135	XM_013099917
DAZL	Deleted in azoospermia-like (DAZL)	TCA CTG ACA GGA CTG GTG TTT C	ATT GCT GGT CCC AGT TTC AG	127	XM_013108529
OCT4	POU domain class 5 transcription factor 3 (POU5F3)	GTT GTC CGG GTC TGG TTC T	GTG GAA AGG TGG CAT GTA GAC	189	NM_001309372
SOX2	SRY (sex determining region Y)-box 2 (SOX2)	CAC AAC TCC GAG ATC AGC AA	TAT AAT CCG GGT GCT CCT TC	127	XM_005024706
NANOG	Homeobox protein NANOG (NANOG)	GGT TTC AGA ACC AAC GGA TG	GTG GGG GTC ATA TCC AGG TA	121	XM_013092900
SALL4	Spalt-like transcription factor 4 (SALL4)	CTA TTT TGC TCC CGA TGC TG	CGT CTT GTG GAC TCC CAC TT	112	XM_005010435
PIWIL1	Piwi-like RNA-mediated gene silencing 1 (PIWIL1)	CCG AAA TGG AGA AGA TGT GAG GA	TGT GAT TAG GGA TGC TGA CTG G	179	NM_001098852
PIWIL2	Piwi-like RNA-mediated gene silencing 2 (PIWIL2)	GCT CCG TCA TCG GCT TCG T	TTC TTG GGC AGG CAG TGG TT	163	XM_015273687
CXCR4	C-X-C motif chemokine receptor 4 (CXCR4)	AAG AGG AGG TCA GCC ACA GA	TTT CAA CCG GAT CTT CTT GC	156	NM_204617
PRDM14	PR/SET domain 14 (PRDM14)	AAG GCA AAG TGG TCA ACA CC	AGT TCA CCA GGG ACA TCC AG	138	XM_013101163
PRDM1	PR/SET domain 1 (PRDM1)	CCC ACG AGT GTC AGG TTT GT	AGG TGC ACA AAC TGG GTG AA	133	XM_015284539
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	GAG GGT AGT GAA GGC TGC TG	CAT CAA AGG TGG AGG AAT GG	113	XM_005016745
RSP17	Ribosomal protein S17 (RPS17)	ACA CCC GTC TGG GCA ACG ACT	CCC GCT GGA TGC GCT TCA TCA	129	NM_204217

Lentiviral Transduction and Establishment of a Transgenic Chicken PGC Line

Chicken and duck PGCs were infected with recombinant lentiviral particles at a multiplicity of infection of 3 to induce expression of EGFP under the control of the PGK promoter. Viral particles were produced by co-transfecting 293T cells with the pCMVΔR8.91, pMD.G, and pAS7w.EGFP.puro plasmids, which encode viral capsid elements and EGFP. The procedure and materials used to prepare viral particles were provided by the National RNAi Core Facility at Academia Sinica in Taiwan. Clonal EGFP-expressing chicken PGC (EGF::PGC) lines were established by fluorescence-activated cell sorting (FACSAria III, BD Biosciences, San Jose, CA, USA) and amplified for further use. To detect EGFP gene insertion in the genome of chicken EGF::PGC lines, PCR was performed using a mixture containing 1 U of Ultra-Pure Taq PCR Master Mix (Geneaid Biotech), 10 μM of each primer (EGFP forward: 5′- CCT ACG GCG TGC AGT GCT TCA GC-3′; EGFP reverse: 5′- CGG CGA GCT GCA CGC TGC GTC CTC-3′), and 100 ng of genomic DNA (gDNA) from each cell line, or ultra-pure water as the negative control in a total volume of 20 μL. The cycling conditions were as follows: 94°C for 5 min, followed by 35 cycles at 94°C for 20 s, 60°C for 45 s, and 72°C for 45 s. To determine the gender of each chicken EGF::PGC line, the same PCR mixture mentioned above instead of primers was used. A pair of primers routinely used for sexing in chicken (2550F: 5′- GTT ACT GAT TCG TCT ACG AGA-3′; 2718R: 5′- ATT GAA ATG ATC CAG TGC TTG-3′ (Fridolfsson and Ellegren, 1999)) was provided to this reaction, and the cycling conditions were as follows: 94°C for 5 min, followed by 35 cycles at 94°C for 20 s, 50°C for 30 s, and 72°C for 30 s.

Gonadal Migration Assay

EGFP-positive chicken and duck PGCs (1×10^6 cells) were centrifuged and resuspended in 100 μL of FAot medium containing 1 μL of 2.5% Patent Blue V solution (Sigma-Aldrich). After making a small hole in each recipient egg using a mini-electric driller, 1 μL of the cell suspension (approximately 10^4 cells) was transferred into the dorsal aorta of each recipient embryo at stage HH 15 to 16 via microinjection with a sharp glass capillary (inner diameter: 30 μm). The hole was sealed with Tegaderm^{TM} Film (3M Medical, Maplewood, MN, USA). To observe the colonization of embryonic gonads by donor cells, embryos were isolated and dissected to reveal the entire gonads at 1 wk after injection (E10 for chicken embryos and E17 for duck embryos). The gonads of chicken recipients were also collected for further molecular analysis. Images were acquired using an optical microscope (Leica Z16 APO, Leica Microsystems) equipped with a Canon EOS 7D camera (Canon, Tokyo, Japan).

Identification of Species-specific Genomic Sequences Following Xenotransplantation

The gonads of injected E10 chicken embryos (stage HH 36) were isolated using tweezers. gDNA was

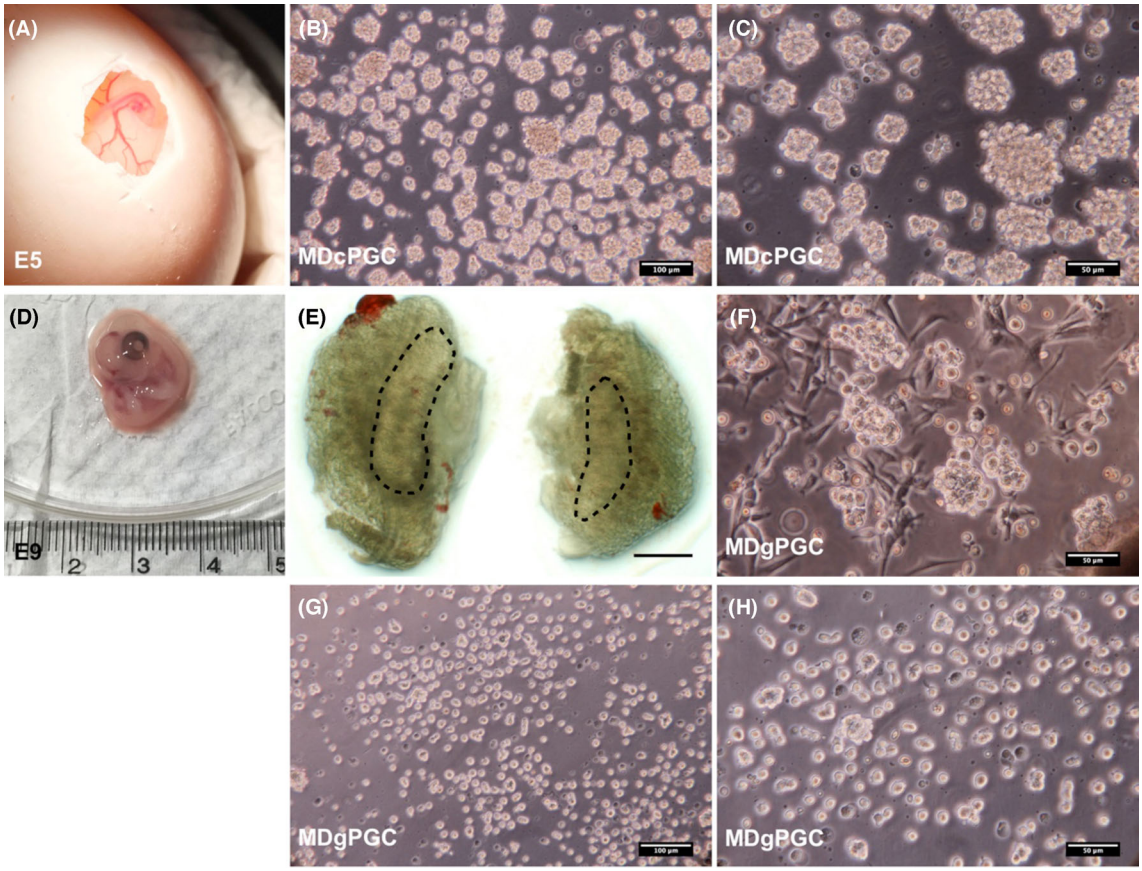

Figure 1. Generation of Muscovy duck PGCs. (A) Blood was collected from the dorsal aorta of E5 Muscovy duck embryos at stage HH 16. (B) MDcPGCs were obtained after 35 d of culture in FAcs medium. Scale bar: 100 μm. (C) MDcPGCs formed clusters and were highly confluent after 35 d of culture. Scale bar: 50 μm. (D) An E9 Muscovy duck embryo (stage HH 28). (E) Embryonic gonads, indicated by dotted lines, were collected and dispersed to obtain PGCs. Scale bar: 0.5 mm. (F) MDgPGCs were cultured from dispersed gonads and easily isolated from adherent stromal cells after 1 d of culture. Scale bar: 50 μm. (G and H) MDgPGCs remained proliferative in FAcs medium after 5 d of culture. Scale bars: 100 and 50 μm, respectively.

immediately extracted using an EasyPure Genomic DNA Spin Kit (Bioman, New Taipei City, Taiwan) according to the manufacturer's instructions, dissolved in DNase/RNase-free distilled water, and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). To detect duck PGCs in chicken gonads, PCR was performed using a mixture containing 1 U of Ultra-Pure Taq PCR Master Mix (Geneaid Biotech), 10 μM of each species-specific primer (chicken-specific forward: 5′-CCT CCC AGT CCC AGT AAG AAG TAG-3′; chicken-specific reverse: 5′-CAA CAT GAT GGG CGA GTG CT-3′; duck-specific forward: 5′-GGA TTT CTA GGC CCT TGG-3′; and duck-specific reverse: 5′-CAG TTG GTT AAG GCC GTA-3′), the primer sequences were derived as previously described by Liu et al. (2012), and 100 ng of gDNA from each transplanted recipient, 100 ng of gDNA from non-injected control gonads, or ultra-pure water as the negative control in a total volume of 20 μL. The cycling conditions were as follows: 94°C for 5 min, followed by 35 cycles at 94°C for 20 s, 59°C for 30 s, and 72°C for 20 s. PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Quantitative data are presented as mean ± SEM. The level of significance was calculated using the Student's *t*-test or a one-way ANOVA and Tukey's post hoc test. $P < 0.05$ was considered significant. For each test with more than 3 independent samples, the P value and statistical significance of comparisons are indicated.

RESULTS

In Vitro Culture of Muscovy Duck PGCs

Muscovy duck PGCs obtained from embryonic blood or gonads were initially expanded using the same conditions as those used to culture chicken PGCs. Muscovy duck circulating PGCs (**MDcPGCs**) were obtained by seeding embryonic blood collected from the dorsal aorta of an E5 embryo into FAcs medium (Figure 1A). We seeded PGCs isolated from each embryo in a separate well. The cells were sub-cultured when they reached approximately 80% confluency (Figure 1B and C).

Table 2. Cell expansion and culture duration of chicken and duck PGCs.

Culture medium	Type of PGCs	Number of wells with cell expansion[1]/total number of wells[2] (%)	Culture duration (days)[3]
FAcs	CcPGCs	6/10 (60.0)	>250[3]
	CgPGCs	7/10 (70.0)	>250[4]
	MDcPGCs	2/32 (6.3)	52.0 ± 6.0
	MDgPGCs	26/32 (81.3)	5.6 ± 0.2
FAot	CgPGCs	7/10 (70.0)	>250[4]
	MDgPGCs	22/24 (91.7)	8.9 ± 0.4
	PDgPGCs	13/24 (54.2)	4.8 ± 0.2
	MUDgPGCs	21/24 (87.5)	8.3 ± 0.4

[1]A well with cell expansion was defined as a culture well containing more than 1×10^5 expanded cells.
[2]PGCs isolated from each avian embryo were seeded in one separate well.
[3]The day on which cells stopped proliferating. Data are presented as mean ± SEM.
[4]CcPGCs and CgPGCs proliferated indefinitely in both types of media.

MDcPGCs proliferated in small clusters (Figure 1C). More than 1×10^5 cells were obtained after 1 mo of culture. However, the percentage of wells with cell expansion was lower for MDcPGCs (6.3%; 2/32) than for chicken circulating PGCs (CcPGCs; 60.0%; 6/10) (Table 2). Proliferation was assessed by seeding 1×10^4 cells into 1 well of 24-well plate. Cells were sub-cultured into a larger well every 3 d. Each well from a 24-well plate are sub-cultured into a well in 12-well plate after 3 d of culture, and into a well of 6-well plate. With each sub-culture, after transfer cells and the old medium to the larger well, equal volume of fresh medium was added. After 8 d of culture, there were 51.9×10^4 CcPGCs, but only 8.8×10^4 MDcPGCs (Figure 2A). In addition, the doubling time of CcPGCs was approximately half that of MDcPGCs (Figure 2B). CcPGCs continued to proliferate for more than 250 d in FAcs medium. By contrast, MDcPGCs were sub-cultured after approximately 50 d and stopped proliferating (Table 2).

Muscovy duck gonadal PGCs (**MDgPGCs**) were isolated from the gonads of E9 Muscovy duck embryos and plated in FAcs medium (Figure 1D and E). After 1 d of culture, MDgPGCs were easily isolated by gently detaching them from adherent stromal cells (Figure 1F). Following the isolation from stromal cells, MDgPGCs rapidly propagated upon sub-culture (Figure 1G and H). Robust cell expansion was observed in 81.3% (26/32) of wells after 3 d of culture, and MDgPGCs continued to proliferate for an average of approximately 6 d (Table 2). However, MDgPGCs stopped proliferating after 1 wk of culture. By contrast, chicken gonadal PGCs (CgPGCs) continued to proliferate for a long duration after forming clones, similar to CcPGCs (Table 2).

Comparison of MDgPGCs Cultured in Serum-containing and Serum-free Media

Based on the previous results, the proliferation of MDgPGCs, which had the highest percentage of cultures with cell expansion, was assessed further. When cultured in FAcs medium, which contained FGF2, Activin A, and chicken serum, MDgPGCs proliferated as aggregates and the number and size of these aggregates increased from day 2 to 7. By contrast, upon culture in FAot medium, which contained ovotransferrin instead of chicken serum, MDgPGCs still proliferated but formed fewer aggregates (Figure 3A). The relative total cell number and the fold increase in the total cell number were determined by performing the CCK-8 colorimetric assay. This demonstrated that MDgPGCs expanded during the first 2 d of culture in both types of media (Figure 3B) and that the total cell number was 3.5-fold higher on the third day than on the first day of culture (Figure 3C). MDgPGCs did not become fully confluent in either type of media, and the total cell number started to decrease from day 4. This decrease was more prominent in FAcs medium than in FAot medium. Consequently, the relative total number

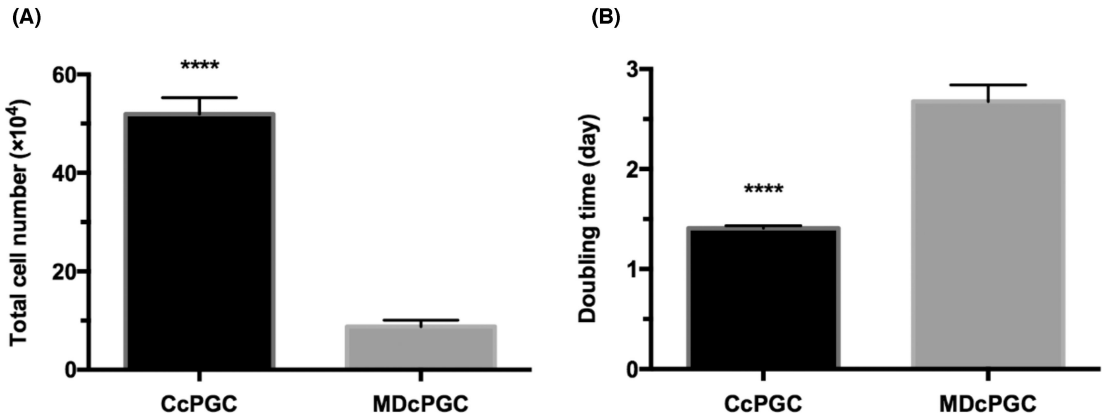

Figure 2. Growth assay of CcPGCs and MDcPGCs. (A) The total number of CcPGCs and MDcPGCs after 8 d of culture in FAcs medium. (B) Doubling time of CcPGCs and MDcPGCs. A total of 1×10^4 cells were seeded, and the total cell number was counted after 8 d of culture. The doubling time was calculated (Roth V. 2006 Doubling Time Computing, available from http://www.doubling-time.com/compute.php). Data are expressed as mean ± SEM from at least 3 independent experiments. $^{****}P < 0.0001$.

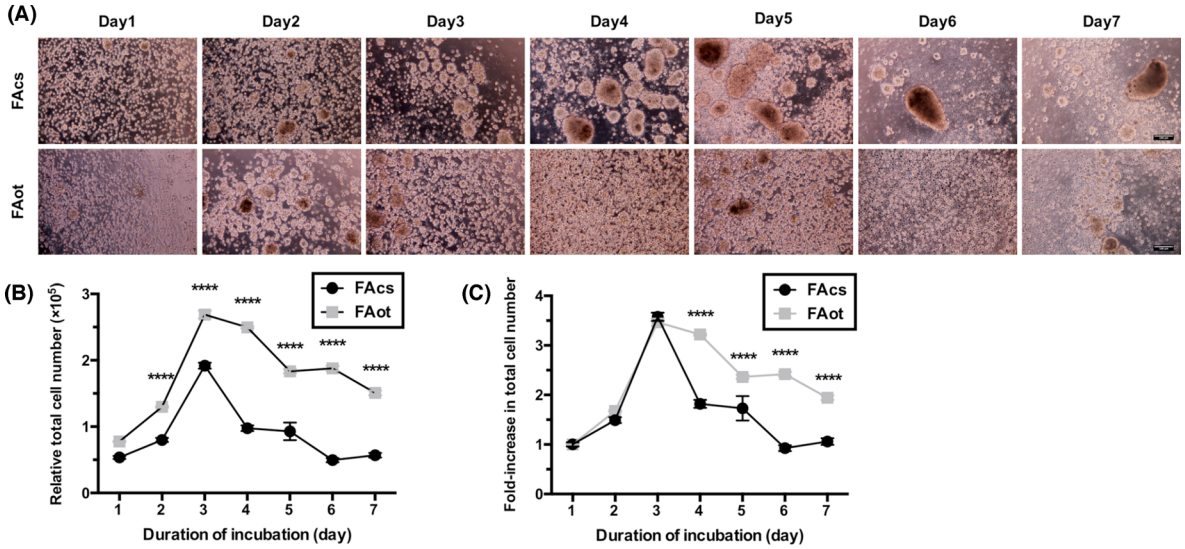

Figure 3. Comparison of MDgPGCs cultured in FAcs and FAot media. (A) Proliferation of MDgPGCs in FAcs and FAot media over 1 wk. Scale bar: 100 μm. (B) Proliferation efficiency of MDgPGCs in FAcs and FAot media over 1 wk. The relative cell number was determined by comparing absorbance of the samples in the CCK-8 assay with a standard curve generated using known numbers of chicken PGCs. Data are presented as mean ± SEM. $^{****}P < 0.0001$. (C) Fold change in the relative total cell number compared with the relative number of MDgPGCs seeded. Data are presented as mean ± SEM. $^{****}P < 0.0001$.

of MDgPGCs was higher in FAot medium than in FAcs medium from day 4 to 7 (Figure 3C).

In Vitro Culture of Chicken and Duck gPGCs

MDgPGCs proliferated better in FAot medium than in FAcs medium; therefore, MDgPGCs, Pekin duck gonadal PGCs (**PDgPGCs**), and mule duck gonadal PGCs (MUDgPGCs) obtained from individual embryos were cultured in the former medium. CgPGCs were also cultured as a control. Chicken and duck gPGCs remained large and round upon suspension culture in FAot medium (Figure 4). The percentage of cultures with cell expansion for CgPGCs was 70% (7/10), and 7 cell lines were established (Table 2). Robust cell expansion was found in 22 of 24 wells seeded with MDgPGCs,

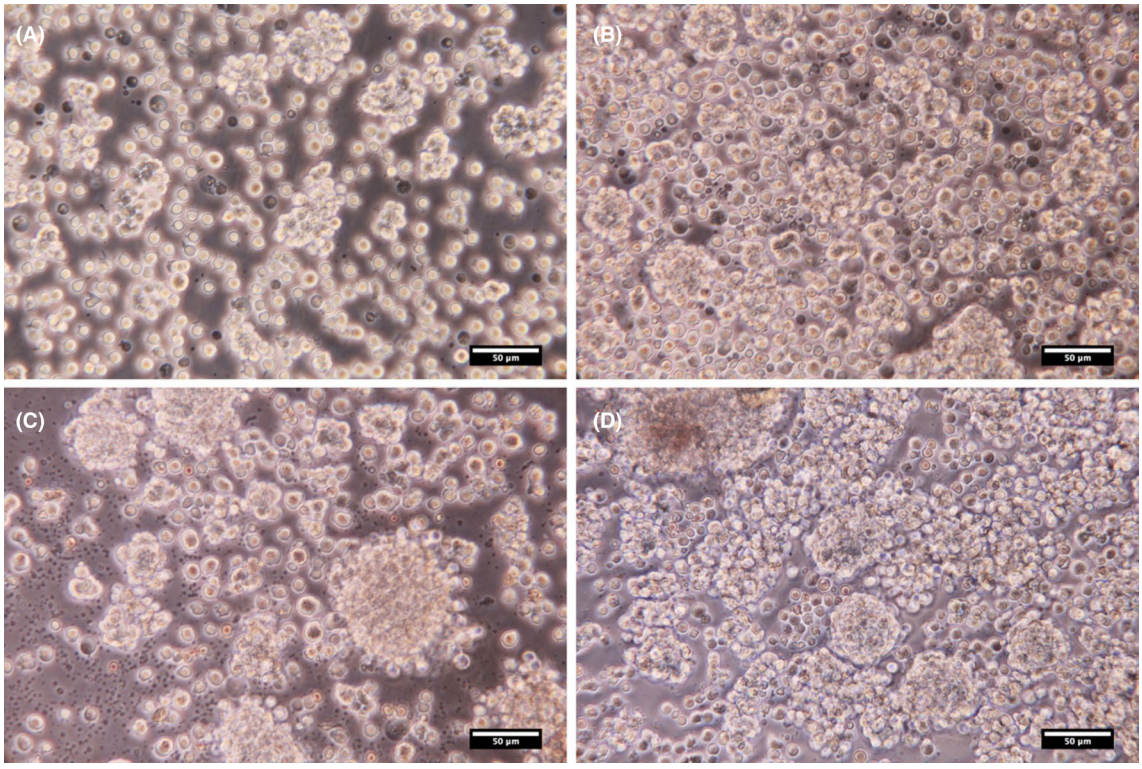

Figure 4. Culture of chicken and duck gPGCs in FAot medium. (A) Image of CgPGCs cultured for 105 d (35 passages). Scale bar: 50 μm. (B) Images of MDgPGCs, (C) PDgPGCs, and (D) MUDgPGCs cultured for 1 wk. Scale bar: 50 μm.

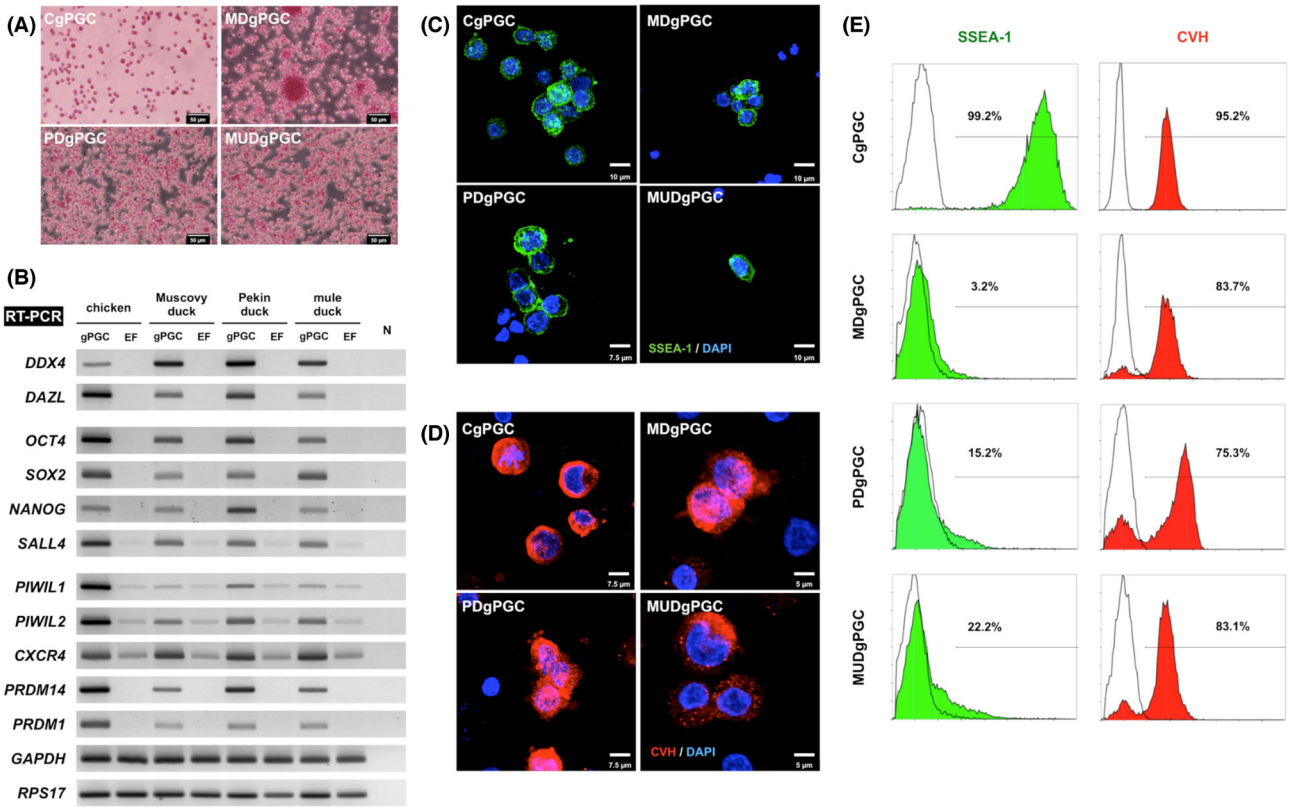

Figure 5. Characterization of chicken and duck gPGCs. (A) Staining of CgPGCs, MDgPGCs, PDgPGCs, and MUDgPGCs with PAS. Scale bar: 50 μm. (B) RT-PCR analysis of CgPGCs, MDgPGCs, PDgPGCs, and MUDgPGCs. Expression of germline-specific markers (*DDX4* and *DAZL*), pluripotency markers (*OCT4*, *SOX2*, *NANOG*, and *SALL4*), and other PGC-specific markers (*PIWIL1*, *PIWIL2*, *CXCR4*, *PRDM14*, and *PRDM1*) was evaluated. The housekeeping gene *GAPDH* and *RPS17* was used as the internal control. EFs from each breed were used as somatic cells for comparison. Water was used as a negative control in the reverse transcription reaction. (C) Immunofluorescence staining of the pluripotency marker SSEA-1 in CgPGCs (scale bar: 10 μm), MDgPGCs (scale bar: 10 μm), PDgPGCs (scale bar: 7.5 μm), and MUDgPGCs (scale bar: 10 μm). SSEA-1-positive cells: green; DAPI nuclear staining: blue. (D) Immunofluorescence staining of the germ cell marker DDX4 in CgPGCs (scale bar: 7.5 μm), MDgPGCs (scale bar: 5 μm), PDgPGCs (scale bar: 7.5 μm), and MUDgPGCs (scale bar: 5 μm). DDX4-positive cells: red; DAPI nuclear staining: blue. (E) Flow cytometric analysis of immunocytochemical staining of SSEA-1 and DDX4 in CgPGCs, MDgPGCs, PDgPGCs, and MUDgPGCs. The percentage of SSEA-1 or DDX4-positive cells is shown in each panel. gPGCs were stained with mouse IgM isotype antibodies or rabbit IgG secondary antibodies as a control. Ten thousand cells were assessed per sample.

corresponding to a percentage of 91.7% (Table 2). The percentage of wells with cell expansion for PDgPGCs and for MUDgPGCs was 54.2% (13/24) and 87.5% (21/24), respectively. CgPGCs proliferated for more than 250 d in FAot medium, corresponding to at least 50 passages. By contrast, MDgPGCs/MUDgPGCs and PDgPGCs proliferated for an average of approximately 8 and 5 d, respectively (Table 2). Thereafter, proliferation slowed and the relative total cell number did not increase. We hypothesize that the culture medium must be supplemented with an additional factor(s) for long-term culture of duck PGC lines, regardless of their embryonic and/or genetic origin.

Immunocytochemical Characterization of Cultured Chicken and Duck gPGCs

We sought to characterize and compare CgPGCs, MDgPGCs, PDgPGCs, and MUDgPGCs cultured in vitro. All cells were stained with PAS (Figure 5A), indicating their germ cell nature. Consistently, cells were labeled with an anti-SSEA-1 antibody (Figure 5C). However, staining of duck gPGCs with this antibody was heterogenous, with the percentage of SSEA-1-positive cells ranging from 3.2% to 22.2%. By contrast, up to 99.2% of CgPGCs were SSEA-1-positive, reflecting their homogeneity (Figure 5E). These results indicate that cell marker expression differs between chicken and duck gPGCs. The germline-specific marker DDX4 was detected in cultured chicken and duck gPGCs by immunostaining with a specific antibody (Figure 5D). Flow cytometric analysis revealed that 95.2%, 83.7%, 75.3%, and 83.1% of CgPGCs, MDgPGCs, PDgPGCs, and MUDgPGCs were DDX4-positive, respectively (Figure 5E). Some duck gPGCs were DDX4-negative, indicating the presence of non-germinal-derived cells.

Reverse Transcription PCR Analysis of Chicken and Duck gPGCs

Gene expression in MDgPGCs, PDgPGCs, and MUDgPGCs was analyzed by semi-quantitative reverse transcription PCR (**RT-PCR**). CgPGCs were used as

a positive control, while EFs derived from each breed were used as a negative somatic cell control. All gPGCs expressed the germline-specific markers *DDX4* and *DAZL* (Figure 5B). With regard to pluripotency genes, *SOX2* and *NANOG* were expressed in CgPGCs but were barely detected in duck gPGCs; however, *OCT4* (also known as *POUV* and *POU5F3*) and *SALL4* were expressed in all cells. *OCT4* and *NANOG* were lowly expressed in MDcPGCs (Supplementary Figure 2). *PIWIL1* and *PIWIL2*, which belong to the PIWI-like family and play critical roles in chicken germ cells, were expressed in all 3 types of duck gPGCs (Figure 5B). Expression of *PIWIL1* was lower in duck gPGCs than in CgPGCs. *CXCR4*, which is involved in migration and gonadal homing of PGCs, as well as *PRDM14* and *PRDM1*, which are key factors in establishment of the germline lineage in mammals, were expressed in all gPGCs (Figure 5B).

We analyzed expression of these genes in CgPGCs and MDgPGCs cultured for various durations. MDgPGCs cultured for more than 1 wk still expressed several markers, including *DDX4*, *DAZL*, *SALL4*, and *CXCR4* (Figure 6). With regard to pluripotency genes, expression of *OCT4*, *SOX2*, and *NANOG*, but not of *SALL4,* in MDgPGCs decreased as the culture duration increased. The same was true for expression of *PIWIL1*, *PIWIL2*, *PRDM14*, and *PRDM1*. mRNA expression of these genes was almost undetectable in MDgPGCs cultured for 14 d. However, all genes were stably expressed in CgPGCs regardless of the culture duration (Figure 6). CgPGCs highly expressed these genes upon culture for up to 250 d, which reflected the stability of these cells in vitro.

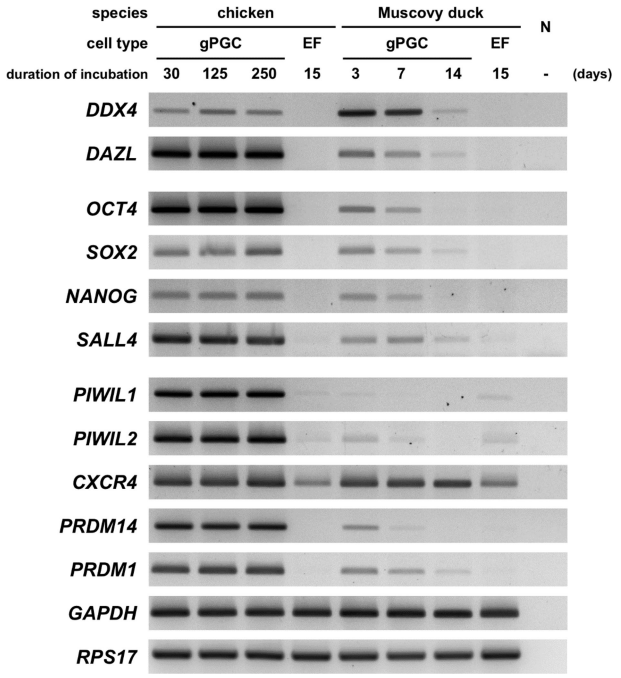

Figure 6. Expression of PGC markers in MDgPGCs during culture in vitro. mRNA expression of germline-specific markers (*DDX4* and *DAZL*), pluripotency genes (*OCT4*, *SOX2*, *NANOG*, and *SALL4*), and other PGC markers (*PIWIL1*, *PIWIL2*, *CXCR4*, *PRDM14*, and *PRDM1*) was examined by RT-PCR. *GAPDH* and *RPS17*, both ubiquitously expressed genes, were used as the internal control. All germline-specific markers were expressed in CgPGCs cultured for 30, 125, and 250 d. Expression was analyzed in MDgPGCs cultured for 3, 7, and 14 d. EFs from each breed served as a somatic cell control. Water was used as a negative control in the reverse transcription reaction.

Gonadal Migration of gPGCs After Allogeneic and Xenogeneic Transplantation

To assess the developmental potential of duck gPGCs cultured in vitro, these cells were genetically labeled with EGFP and then transferred into the dorsal aorta of an E5 Muscovy duck embryo or an E3 chicken embryo (Figure 7A). Upon delivery of the EGFP-containing vector (Figure 7B), CgPGCs (Figure 7C) and MDgPGCs (Figure 7D) highly expressed EGFP. The clonal chicken EGF::PGC lines, which were used as the positive controls, migrated toward the gonadal ridge (Figure 7A and C); PGC migration was observed at E10 in 3 of 5 chicken embryos transplanted with these male cells. Similarly, EGFP-positive MDgPGCs injected into the circulation of an E5 Muscovy duck embryo migrated toward the gonadal ridge and colonized the gonads (Figure 7A and D); PGC migration was observed at E17 in 3 of 7 Muscovy duck embryos transplanted with these cells. The presence of duck genetic material in gonadal tissues of chicken embryos injected with duck gPGCs was investigated by genetic analysis using chicken- and duck-specific primers (Liu et al., 2012). Duck genetic material was detected in the gonads of recipient chicken embryos via this approach (Figure 7E), suggesting that cultured duck gPGCs colonized the gonads.

DISCUSSION

The present study is to isolate, amplify, characterize, and compare PGCs from different duck breeds. A protocol for long-term culture of chicken PGCs is well-established. It is of interest to determine whether PGCs of other avian species can be cultured using similar or modified conditions, which attracts several research teams to establish the PGC culture system for interested non-chicken avian species. We therefore initially cultured duck PGCs in the medium containing FGF2, Activin A, and insulin, which is used to culture chicken PGCs and human embryonic stem cells (Vallier et al., 2005; Whyte et al., 2015). PGCs were successfully isolated from the circulating blood and gonads of Muscovy duck embryos using this approach. However, it took a considerable amount of time to obtain a large number of MDcPGCs, and the percentage of cultures with apparent cell expansion for these cells was very low even though they initially proliferated for a few days. MDgPGCs aggregated in FAcs medium, which may have prevented their long-term proliferation. Cell aggregation was greatly reduced when cells were cultured in FAot medium instead of FAcs medium. Moreover, culture in FAot medium slightly increased

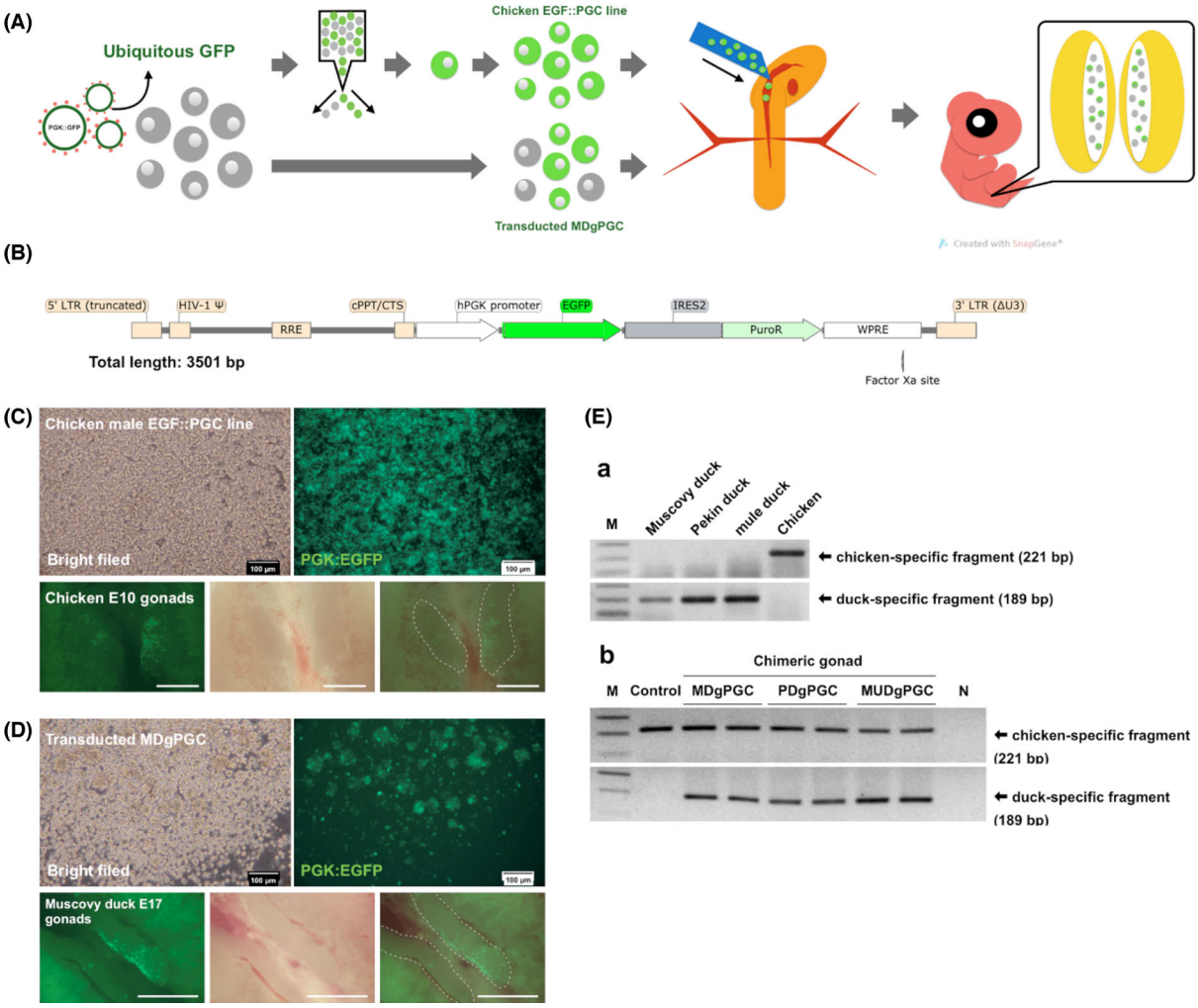

Figure 7. Homing of transplanted gPGCs to the gonads of recipients. (A) Graphical summary of the procedures used to functionally characterize the germ cell properties of PGCs expanded in vitro. The ability of transplanted chicken and duck PGCs to home to the gonads of recipients was assessed. (B) Structure of pAS7w.EGFP.puro, which had a total length of 3,501 bp. Features were visualized using SnapGene Viewer 4.0 (GSL Biotech LLC, USA). (C) Transplantation of cultured chicken EGF::PGCs into chicken embryos. Chicken male EGF::PGCs (upper panel) had a round shape when grown in suspension and ubiquitously exhibited green fluorescence, demonstrating expression of the transgene. Scale bar: 100 μm. After transplantation of EGF::PGCs into the blood circulation of an E3 chicken embryo and incubation for 7 d, donor cells were detected in the gonadal region (dotted lines). Scale bar: 500 μm. (D) Allogeneic transplantation of virally transduced MDgPGCs into E5 Muscovy duck embryos. Some MDgPGCs were EGFP-positive at 2 d after viral transduction (upper panel). Scale bar: 100 μm. EGFP-expressing MDgPGCs were detected in the gonads of E11 duck embryos (lower panel, dotted lines). Scale bar: 500 μm. (E) The presence of duck genetic material in the gonads of recipient chickens after transplantation of duck gPGCs. (a) Species-specific primer sets were used to amplify a chicken-specific PCR product (221 bp, upper panel) and duck-specific amplicons (189 bp; lower panel) from the 3 duck breeds. (b) Confirmation of the presence of duck gPGCs in the gonads of recipient chicken embryos at 7 d after transplantation. Duck-specific amplicons were observed in the gonads of chicken embryos transplanted with MDgPGCs, PDgPGCs, and MUDgPGCs (lower panel). Chicken-specific amplicons were observed in all samples as a positive control (upper panel).

the proliferation of MDgPGCs. Although the percentage of cultures with cell expansion for PDgPGCs and for MUDgPGCs was relatively high upon culture in FAot medium, these cells stopped proliferating after a relatively short duration. However, proliferating duck gPGCs exhibited typical features of germ cells similar to chicken gPGCs, which were used as controls and cultured under similar conditions. gPGCs were typically stained with both PAS staining, SSEA-1 and CVH (DDX4) antibodies. SSEA-1 is one of the most specific markers of stem and germ cell in mammals and aves (Shamblott et al., 1998; Park and Han, 2000; Lavial et al., 2009; Hayashi et al., 2011). However, the heterogenous results obtained in duck gPGCs indicate a species-specific difference on the expression of SSEA-1 in PGC among species. Numerous genes are specifically expressed in chicken PGCs and were similarly expressed in cultured duck PGCs. *DDX4* and *DAZL* are specifically expressed in germ cells of chicken embryos from the early cleavage stage (Tsunekawa et al., 2000; Nakamura et al., 2013). Lavial et al. (2009) reported that ectopic expression of *DDX4* results in reprogramming of chicken embryonic stem cells to a germ lineage fate. A *DDX4*-null chicken model was recently reported to exhibit female sterility due to loss of germ cells during meiosis (Taylor et al., 2017). This

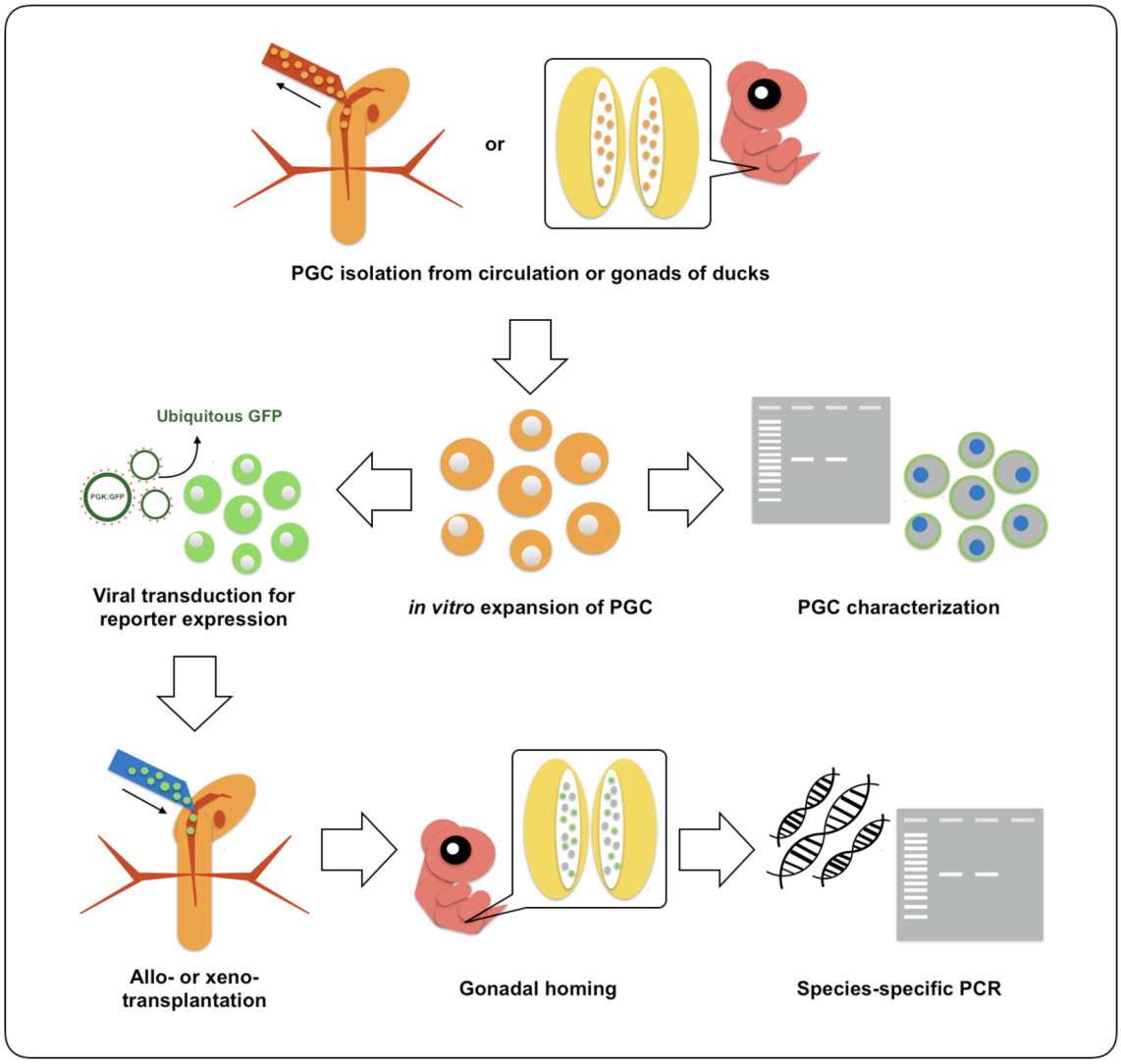

Figure 8. Graphical summary of the isolation, in vitro expansion, genetic modification, and functional characterization of duck cPGCs and gPGCs.

suggests that *DDX4* plays a key role in germ cell specification and development in avian species. In the present study, expression of *DDX4* was similar in duck and chicken gPGCs. *DAZL* is expressed in chicken germ cells at early development stages, similar to *DDX4*. Knockdown of *DAZL* results in apoptosis of chicken PGCs (Lee et al., 2016), suggesting this gene is important for germline development.

The pluripotency genes *OCT4*, *SOX2*, *NANOG*, and *SALL4* are expressed in chicken blastoderm cells, embryonic stem cells, and PGCs (Jean et al., 2015). In the present study, these genes were expressed in cultured duck PGCs, suggesting that their role in the maintenance of germ cell identity and their involvement in the maintenance of pluripotency in embryos are conserved. The PIWI protein family plays a major role in the germline lineage in Drosophila and mammals (Cox et al., 2000). Knockdown of *PIWIL1/CIWI* or *PIWIL2/CILI* in chicken PGCs induces the activation of retrotransposons and results in DNA double-strand breakage (Kim et al., 2012; Rengaraj et al., 2014), indicating these genes have a protective function in chicken PGCs. Similarly, *PRDM1/BLIMP1* and *PRDM14* are crucial for establishment of the mammalian germline lineage (Ohinata et al., 2005; Yamaji et al., 2008). Expression of both genes was initially observed in duck and chicken gPGCs, but was undetected in MDgPGCs after 2 wk of culture.

Expression of all the germline-specific and pluripotency genes decreased over time and was not detected in duck gPGCs after 2 wk of culture. Consequently, these cells stopped proliferating, regardless of which duck breeds they were they derived from. This indicates that these culture conditions are unsuitable for long-term culture of duck PGCs in vitro. However, duck gPGCs cultured for a short duration migrated to and colonized the genital ridges when injected into embryos. These obtained results might facilitate germline transmission of germline chimeras after development of the gonads (Macdonald et al., 2010; Nakamura et al., 2013). SDF1, which is the ligand of CXCR4 and is required for transmigration of cells through blood vessels

in chickens (Stebler et al., 2004), was also expressed in duck gPGCs. This may facilitate the migration of cultured duck gPGCs. Injected duck gPGCs colonized the gonads of both chicken and duck embryos. This may reflect phylogenetic conservation of the molecular and cellular mechanisms governing germ cell homing in chickens and ducks and indicates that putative interspecies chimeras can be generated using PGCs (Liu et al., 2012; van de Lavoir et al., 2012). Therefore, this approach could be used to develop a platform for the reproduction of endangered avian species. In addition, the sex of recipient is critical for donor germ cell development after transplantation. Only few donor PGCs could undergo a complete process of gametogenesis and formed functional gametes in opposite-sex recipient (Naito et al., 1999; Liu et al., 2012). In the present study, the mix-sex pooled MDgPGCs were used for transplantation, a part of donor cells thus could find the environment with proper sex for further development in recipients.

In conclusion, we successfully developed a method to isolate PGCs from 3 major duck breeds and to expand these cells in vitro (Figure 8). The characteristics of cultured duck PGCs were similar to those of chicken PGCs. Following xenogeneic and allogenic transplantation into embryos, cultured duck gPGCs homed to and colonized the gonads. Together, these results suggest that PGCs isolated from the 3 duck breeds remained pluripotent and maintained their germline characteristics. However, the culture conditions must be improved, perhaps by supplementing the culture medium with an additional factor(s), for long-term culture of duck PGCs in vitro.

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