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Identification and characterization of primordial germ cells in a vocal learning Neoaves species, the zebra finch

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ABSTRACT: The zebra finch has been used as a valuable vocal learning animal model for human spoken language. It is representative of vocal learning songbirds specifically, which comprise half of all bird species, and of Neoaves broadly, which comprise 95% of all bird species. Although transgenesis in the zebra finch has been accomplished, it is with a very low efficiency of germ-line transmission and far from the efficiency with a more genetically tractable but vocal nonlearning species, the chicken (a Galloanseriformes). To improve germ-line transmission in the zebra finch, we identified and characterized its primordial germ cells (PGCs) and compared them with chicken. We found striking differences between the 2 species, including that zebra finch PGCs were more numerous, more widely distributed in early embryos before colonization into the gonads, had slower timing of colonization, and had a different developmental gene-expression program. We improved conditions for isolating and culturing zebra finch PGCs *in vitro* and were able to transfect them with gene-expression vectors and incorporate them into the gonads of host embryos. Our findings demonstrate important differences in the PGCs of the zebra finch and advance the first stage of creating PGC-mediated germ-line transgenics of a vocal learning species.—Jung, K. M., Kim, Y. M., Keyte, A. L., Biegler, M. T., Rengaraj, D., Lee, H. J., Mello, C. V., Velho, T. A. F., Fedrigo, O., Haase, B., Jarvis, E. D., Han, J. Y. Identification and characterization of primordial germ cells in a vocal learning Neoaves species, the zebra finch. FASEB J. 33, 13825–13836 (2019). www.fasebj.org

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Primordial germ cells (PGCs) are the precursor cells of sperm and oocytes that contain the genetic information for transfer to the next generation. Therefore, besides being important for understanding basic aspects of development and evolution, PGCs have been utilized for the generation of germ-line chimeras (1). Among birds, PGCs have been best characterized in chicken (*Gallus gallus*), where they have been found in the central region of the early chicken embryo until Eyal-Giladi and Kochav X (EGK.X) stage (2–4). Thereafter, at Hamburger Hamilton 4 (HH4) stage, they assemble in the germinal crescent (4–6). From around HH11, the PGCs begin migrating to the extraembryonic region, then circulate in the bloodstream, and finally colonize the gonads, where they differentiate into embryonic germ cells (7, 8). Because of these unique characteristics, chicken PGCs have been isolated, manipulated, and used for basic and applied research, including generation of transgenic animals (1).

Although approaches developed in chicken have been useful for the characterization and manipulation of PGCs in other Galloanserae species (*e.g.*, quail and ducks) (9, 10), among species representing the more distantly related and breadth of the 10,000+ Neoaves species, production of a transgenic chimera using gonadal cells has only been

ABBREVIATIONS: cDAZL, chicken DAZL; DAZL, deleted in azoospermia-like; DDX4, DEAD-box helicase 4; DEG, differentially expressed gene; DIG, digoxigenin; EGK, Eyal-Giladi and Kochav; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GO, Gene Ontology; GSC, gonadal stromal cell; HH, Hamburger Hamilton; NANOG, Nanog homeobox; PGC, primordial germ cell; PIWIL1, piwi-like RNA-mediated gene silencing 1; POUV, POU domain class 5 transcription factor 3; RNA-Seq, RNA sequencing; SSEA-1, stage-specific embryonic antigen-1; zDAZL, zebra finch DAZL

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reported for the houbara bustard (*Chlamydotis undulata*) (11–13). One Neoaves species, the zebra finch (*Taeniopygia guttata*), is a representative songbird model organism used to investigate the neurobiological basis of vocal learning and human speech-related disorders (14, 15). Like many Neoaves, the zebra finch differs in development, namely being more altricial and requiring more parental care at hatching (16, 17). Zebra finch eggs are laid at an earlier stage than chicken (EGK.VI *vs.* EGK.X) (16). The total incubation time of zebra finch eggs is shorter than that of chickens (14 d *vs.* 21 d); however, the zebra finch takes more time to reach early developmental stages, indicating faster development in chicken in early stages (17). Additionally, at oviposition, germ cell–specific genes are expressed at higher levels in zebra finch embryos than in chicken embryos (16). These features indicate that characteristics of PGCs in zebra finch embryos may differ from those in chicken. However, until now, there have been no comprehensive studies of any Neoaves PGCs.

Here, we report for the first time the identification and characterization of zebra finch PGCs. We found important differences in zebra finch PGCs compared with those of chicken, including in embryonic location, developmental timing, gonadal gene expression, and ability to culture. We developed conditions to transgenically modify and incorporate donor PGCs into zebra finch host embryonic gonads. We believe that this study lays essential groundwork for improving transgenic technologies in Neoaves.

MATERIALS AND METHODS

Experimental animals

Zebra finches were maintained under a 12:12-h light/dark cycle at 22–23°C and provided with a seed blend, vitamin-enriched fruit pellets, and mineral grit. A hanging nest box and *ad libitum* hay for nesting material were placed in each cage (59 × 42 × 40 cm). Eggs were collected every 2 h from the onset of the light cycle and stored at 17–20°C for up to 7 d. White Leghorn chickens were managed according to our standard operation protocols. The care and experimental use of finches and chickens was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-161117-4-1 and SNU-190401-1, respectively) and The Rockefeller University Institutional Animal Care and Use Committee.

Embryo and gonad sample preparation

Zebra finch EGK.VI embryos were collected from oviposited eggs, and EGK.X embryos were collected from eggs incubated for 6 h postoviposition (16). The egg-laying time of chickens was recorded and intrauterine eggs of EGK.VI were harvested using an abdominal massage technique (18). Chicken EGK.X embryos were collected from oviposited eggs. To collect HH4, 6, 26, 28, 30, 32, and 38 embryos, zebra finch and chicken eggs were incubated with intermittent rocking at 37–38°C under 60–70% relative humidity until the appropriate time as previously described (3, 5, 17).

Gonads or gonads with mesonephri were collected from the abdomen of zebra finch embryos from eggs incubated for 5–8.5 d (120–200 h). The correct embryonic stages were identified according to incubation time and developmental features (17). The size of embryonic gonads at each stage was measured using imaging software, NIS-Elements (Nikon, Tokyo, Japan), which can measure area or size within a microscope image, before sampling. In addition, sexing of the embryos was performed by chromodomain-helicase-DNA-binding protein (*CHD*)*1* gene PCR amplification of the genomic DNA extracted from extraembryonic tissues using previously described primers (19). All experiments stated hereafter were repeated at least 3 times with 3 individual samples unless otherwise noted.

Whole-mount *in situ* hybridization

Hybridization probes for zebra finch deleted in azoospermia-like (z*DAZL*) were made from total RNA of zebra finch EGK.VI embryos that was reverse transcribed using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was amplified using z*DAZL*-specific primers (forward, 5′-TGTGGAAACCCAGCACTCAA-3′ and reverse, 5′-TGGAGGCATTACGTAATTTC-3′). The PCR product was cloned into a pGEM-T Easy Vector System (Promega, Madison, WI, USA). After sequence verification by Sanger single-molecule sequencing, recombinant plasmids containing the target genes were amplified using T7- and SP6-specific primers (20) to prepare the template for labeling the hybridization probes. Digoxigenin (DIG)-labeled sense and antisense z*DAZL* hybridization probes were transcribed *in vitro* using a DIG RNA Labeling Kit (Roche, Basel, Switzerland). DIG-labeled probes for zebra finch DEAD-box helicase 4 (*DDX4*) (ID: CK304812; GenBank; *https://www.ncbi.nlm.nih.gov/genbank/*) were generated using zebra finch *DDX4*-specific primers (forward, 5′-ATTGTGTTGGGGCAGGTCTG-3′ and reverse, 5′-CTTGCTGTTCTTTAAATGCACTG-3′) (21), and the chicken DAZL (c*DAZL*) hybridization probes were prepared as previously reported in Lee *et al*. 4.

Whole-mount *in situ* hybridization of chicken and zebra finch embryos at EGK.VI, EGK.X, HH4, and HH6 and gonads with mesonephri at HH28 was performed using a standard protocol that uses an anti-DIG alkaline phosphatase–conjugated antibody and visualization through a 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride colorimetric reaction (22). Afterwards, *in situ*–hybridized zebra finch embryos were paraffin-embedded and sectioned (thickness, 8–10 μm) and mounted with ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific), and evaluated under a fluorescence microscope (Nikon). *In situ* hybridization of all stages was performed on 5 embryos/stage.

Immunohistochemistry on tissue sections

Embryonic gonads with mesonephric tissues of chicken and zebra finch at HH28 were paraffin-embedded and sectioned (thickness, 8–10 μm). After deparaffinization, sections were washed 3 times with 1-time PBS and blocked with a blocking buffer (5% goat serum and 1% bovine serum albumin in PBS) for 1 h at room temperature. Sections were then incubated at 4°C overnight with primary antibodies, mouse anti–stage-specific embryonic antigen-1 (SSEA-1), rat anti–SSEA-3, mouse anti–SSEA-4 (MilliporeSigma, Burlington, MA, USA), mouse anti-integrin β1, mouse anti-integrin α6 (MilliporeSigma), and rabbit anti-DAZL (4) (1:200 dilution in blocking buffer). After washing 3 times with PBS, sections were incubated with fluorescence-conjugated secondary antibodies (Alexa Fluor 594 or 488; Thermo Fisher Scientific) for 1 h at room temperature. After washing 3 times with PBS, sections were mounted with ProLong Gold antifade reagent with DAPI and visualized on a confocal fluorescence microscope (Carl Zeiss GmbH, Oberkocken, Germany).

Flow cytometry

Chicken and zebra finch embryonic gonads at HH28 and zebra finch whole embryos at EGK.VI and X (n = 3, respectively) were dissociated with 0.25% EDTA. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were then incubated with anti-DAZL antibodies for 1 h on ice. After being washed with PBS, cells were incubated with FITC-conjugated secondary antibodies (Alexa Fluor 488) for 30 min on ice. The cells were analyzed with FACSCalibur (BD Biosciences, San Jose, CA, USA), and subsequent analyses were performed using FlowJo software (Treestar, Ashland, OR, USA).

In vitro culture of zebra finch PGCs

For each culture, about 3–5 pairs of gonads were retrieved from zebra finch embryos at HH28 under a dissecting microscope. The gonads were collected in a 1.5-ml microcentrifuge tube with 500 μl of 0.25% trypsin-EDTA and incubated at 37°C for 10 min. After adding the same volume of PGC culture medium, gonadal cells were centrifuged at 280 g for 5 min and the cell pellet was resuspended in 500 μl of PGC culture medium composed of knockout DMEM (Thermo Fisher Scientific), 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 2% chicken serum (MilliporeSigma), 1× nucleosides (MilliporeSigma), 2 mM L-glutamine (Thermo Fisher Scientific), 1× nonessential amino acids, β-mercaptoethanol, 10 mM sodium pyruvate, 1× antibiotic-antimycotic (Thermo Fisher Scientific), and 10 ng/ml human basic fibroblast growth factor (MilliporeSigma). The cells were cultured in a CO_2 incubator maintained at 37°C with 5% CO_2 and 60–70% relative humidity. Most of the somatic cells were attached to the bottom of the plate, whereas PGCs were in suspension. When being subcultured, the PGC-enriched suspension was transferred into a 1.5-ml microcentrifuge tube, centrifuged at 280 g for 5 min, and the cell pellet was harvested. The attached cells were harvested by 0.25% trypsin-EDTA dissociation for 5 min followed by centrifugation at 280 g for 5 min. A fraction of the adherent cells was then used as a feeder layer and all of the cells in suspension were subcultured at 4- to 5-d intervals for up to 30 d.

Western blot analysis

To confirm the fact that cDAZL antibodies work in zebra finch PGCs, Western blot analysis was performed. The zebra finch protein was isolated from whole embryonic gonadal cells by dissociation in RIPA lysis buffer (Thermo Fisher Scientific) with 100× protease-phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA). The chicken protein was isolated from cultured PGCs and used as a positive control. Approximately 5 μg of protein was loaded for separation on 15% SDS-PAGE gels. Resolved proteins were transferred onto a Hybond 0.45 PVDF membrane (GE Healthcare, Waukesha, WI, USA) and blocked with 3% skim milk for 1 h at room temperature (MilliporeSigma). The blocked membrane was incubated overnight at 4°C in the presence of 1:500-diluted anti-cDAZL antibody or 1:200-diluted anti–β-actin antibody (sc-47778; Santa Cruz Biotechnology, Dallas, TX, USA) and then incubated with horseradish peroxidase–conjugated (sc-2004) or alkaline phosphatase–conjugated secondary antibody (S3721; Santa Cruz Biotechnology) at a dilution of 1:500 and 1:7500 at room temperature for 1 h. Immunoreactive proteins were visualized using the ECL Western blotting detection system (GE Healthcare) or nitro-blue tetrazolium chloride–5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt solution (Roche).

Immunocytochemistry on cultured cells

Zebra finch PGCs cultured for 20–30 d were dried on glass slides and fixed in 4% paraformaldehyde for 10 min, washed 3 times with PBS, and permeabilized with 0.1% Triton X-100 for 10 min. After washing with PBS, the cells were blocked with a blocking buffer (5% goat serum and 1% bovine serum albumin in PBS) for 1 h and then incubated with anti-DAZL antibodies diluted 1:200 in blocking buffer at 4°C overnight. Following 3 washes with PBS, cells were incubated with FITC-conjugated secondary antibodies (Alexa Fluor 488) for 1 h at room temperature. Cells were finally mounted with ProLong Gold antifade reagent with DAPI and analyzed under a confocal fluorescence microscope (Carl Zeiss GmbH). For use as a negative control, adherent cells [gonadal stromal cells (GSCs)] cultured separately until passage 4 were harvested by 0.25% trypsin-EDTA dissociation. After inactivation of trypsin-EDTA, the cell pellet was resuspended with PBS, dried on glass slides, and stained as previously described.

Scanning electron microscopy

Zebra finch PGCs cultured for 20–30 d were fixed in 2% glutaraldehyde and postfixed in 1% osmium tetroxide at 4°C for 2 h. After dehydration in a graded series of increasing concentrations of ethanol, the samples were immersed in hexamethyldisilazane and then dried. The samples were coated with gold palladium using a low-vacuum coater Leica EM ACE200 (Leica Microsystems, Buffalo Grove, IL, USA) and observed using a Sigma field emission scanning electron microscope (Carl Zeiss GmbH).

RT-PCR and quantitative RT-PCR

Total RNA samples of PGCs or GSCs cultured for 20–30 d were prepared using Trizol reagent (Thermo Fisher Scientific). Total RNA samples were then reverse transcribed into cDNAs using the SuperScript III Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The cDNAs were amplified by PCR using specific primer sets of predicted zebra finch POU domain class 5 transcription factor 3 (*POUV*), Nanog homeobox (*NANOG*), *DAZL*, and *DDX4* along with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Supplemental Table S1). PCR reactions comprised 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min.

Total RNA samples of chicken or zebra finch embryonic gonads at HH28 were prepared using Trizol reagent and cDNAs were synthesized using the SuperScript III Reverse Transcription Kit. Gene-expression levels were measured using EvaGreen dye (Biotium, Hayward, CA, USA) and a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Quantification of relative gene expression was calculated using the following formula: $DC_t = C_t$ of the target gene – C_t of *GAPDH*. Primer set information is listed in Supplemental Table S2.

RNA sequencing expression analysis

Chicken and zebra finch embryos were incubated at 37–38°C for 6 d, removed from the egg and placed in PBS EGTA (ethylene glycol tetraacetic acid) (2 mM). Gonad pairs were dissected from individual embryos and 1 limb saved for PCR sexing as described above. RNA was extracted from single gonad pairs using the Qiagen (Germantown, MD, USA) RNeasy Plus Micro Kit. RNA from 3 individual males and 3 individual females from both chicken and zebra finch (12 samples total) were used to construct libraries. RNA samples were quantified using the Advanced Analytical Technologies (Ankeny, IA, USA) Fragment Analyzer High Sensitivity Total RNA Assay (DNF-472). RNA sequencing (RNA-Seq) libraries were

prepared with 40 ng total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490S; New England Biolabs, Ipswich, MA, USA) followed by NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (E7760S; New England Biolabs). Libraries were amplified over 14 cycles. Library quantification and qualification were performed with the Qubit dsDNA HS Assay Kit (Q32854; Thermo Fisher Scientific) and Fragment Analyzer NGS Fragment Kit (DNF-473; Agilent Technologies, Santa Clara, CA, USA). Libraries were equimolarly pooled and sequenced on the Illumina NextSeq 500 in 75PE mode (Rockefeller Genomics Resource Center, New York, NY, USA). Data quality control was performed using FastQC (FastQC v.0.11.5). Adapter and low-quality sequences were trimmed using Trim Galore! (Trim Galore! v.0.4.5). Reads were mapped to the chicken and zebra finch genomes (Gallus_gallus-5.0 and taeGut3.2.4) using Star (v.2.6). Reads were counted using featureCounts (subread-1.6.2) with annotation from Ensembl. Gene orthology between chicken and zebra finch was determined using the biomaRT R package (biomaRt_2.36.1). Only genes found in both annotations with high homology confidence and 1-to-1 correspondence were included in the analysis because these were subject to fewer annotation errors. EdgeR (edgeR_3.22.5; *https://bioconductor.org/packages/release/bioc/html/edgeR.html*) was used to estimate the significance of differentially expressed genes (DEGs). Read counts were normalized by total count and gene length [reads per kilobase of transcript per million (RPKM)] and differential expression significance was estimated with generalized linear models. A false discovery rate–adjusted value (Benjamini-Hochberg method) of $P < 0.05$ was used to determine significance. Gene Ontology (GO) category enrichment analyses were performed using goana in EdgeR.

PGC membrane and transgenic labeling and incorporation in gonads *in vivo*

Zebra finch PGCs cultured for 20–30 d were either labeled with PKH26 fluorescent dye (MilliporeSigma) or a *piggyBac* green fluorescent protein (GFP) expression vector and a helper plasmid designed to ubiquitously express PB transposase, CAGG-PBase (pCyL43). To label the PGCs with PKH26, cells were collected by centrifugation at 280 *g* for 5 min and then resuspended in 500 μl serum-free medium containing 2 μM PKH26 and incubated for 5 min at room temperature. After centrifugation, the cell pellet was resuspended in serum-free medium. Then, about 500 labeled PGCs were injected into the dorsal aorta of each zebra finch embryo at HH13–16. In a parallel study, we labeled feeder cells (GSCs) with PKH26 and injected as a control using the same protocol. After injection, the eggs were sealed with Parafilm and further incubated until HH28. Embryonic gonads from recipient embryos were retrieved, and the number of fluorescent cells in the gonads were counted using a fluorescent microscope (Nikon).

To label the cells with GFP, the *piggyBac* GFP expression vector and CAGG-PBase (pCyL43) (23) were cointroduced into the zebra finch cultured PGCs using lipofection with Lipofectamine 2000 reagent (Thermo Fisher Scientific). Cultured PGCs were collected by centrifugation at 280 *g* for 5 min and then resuspended in serum-free medium containing 3 μg of *piggyBac* GFP expression vector, 2 μg of CAGG-PBase (pCyL43), and 2 μl of Lipofectamine 2000 reagent. Two days after transfection, all floating cells containing GFP-expressing cells were collected and tested as above for their ability to incorporate into host gonads after injection into the dorsal aorta of host embryos at HH13–16.

Statistical analysis

All data are expressed as means ± SD from 3 to 5 independent experiments. A Student's *t* test was used to calculate the significance of differences between experimental groups. Prism software (v.5.0; GraphPad Software, La Jolla, CA, USA) was used to evaluate the data. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Zebra finch has more numerous and widespread PGCs during early embryo development

To characterize the developmental distribution and timing of zebra finch PGCs compared with chicken, we performed *in situ* hybridization for the *DAZL* gene, a PGC-specific marker, across developmental embryonic stages of both species. *DAZL* plays a crucial role in meiotic progression and maintenance of germ cells (24, 25). *DAZL* has been reported as an essential component of stress granules, which prevent male germ cells from undergoing apoptosis upon heat stress (26). Consistent with our previous report (4), *DAZL*-positive PGCs were sparse and concentrated in the central region of the chicken embryo at early EGK.VI and EGK.X stages (**Fig. 1*A, B***). In contrast, PGCs in the zebra finch were more numerous and widely distributed throughout the embryo at these same stages (Fig. 1*F, G*). At HH4, when nearly all PGCs in the chicken had concentrated in the anterior germinal crescent region (Fig. 1*C*), in the zebra finch, concentration occurred around the central disk, including anterior and posterior regions (Fig. 1*H*). It was not until HH6 in the zebra finch that PGCs localized primarily to the germinal crescent, but localization was still not as exclusive as in chicken (Fig. 1*D, I*). At HH7, PGCs were observed not only in the germinal crescent but also anterior to the crescent. At HH9, a small number of PGCs were identified in the anterior region. At HH10, they were found near the anterior vitelline vein, and at HH12 they were absent from extraembryonic tissue and had presumably entered the circulation (Supplemental Fig. S2). Finally, at HH28, PGCs of both species showed nearly complete migration into the embryonic gonads (Fig. 1*E, J*). These species differences in PGC distribution were also verified by *in situ* hybridization for *DDX4*, another PGC-specific marker (Supplemental Fig. S1). These findings indicate that PGCs in zebra finch are present in greater numbers in early embryos and exhibit partial movement to the germinal crescent. To quantify the number of PGCs, we sorted embryo cells by flow cytometry with a fluorescently labeled anti-DAZL antibody. At EGK.VI and EGK.X, zebra finch embryos contained 4–5% DAZL-positive PGCs (**Table 1**). In chicken, the number of PGCs at these stages was too low to quantify reliably by flow cytometry. This is consistent with previous studies that used *in situ* hybridization, which estimated DAZL-positive PGCs in chicken to be very low, about 0.01% (Lee *et al.* (4)). By HH28, when migration into the gonads was complete, chicken gonads contained 0.94% ± 0.06 DAZL-positive PGCs, whereas zebra finch gonads contained 13.2% ± 4.96 (Fig. 1*K, L*). In absolute numbers, zebra finch HH28 gonads contained 11,765 ±

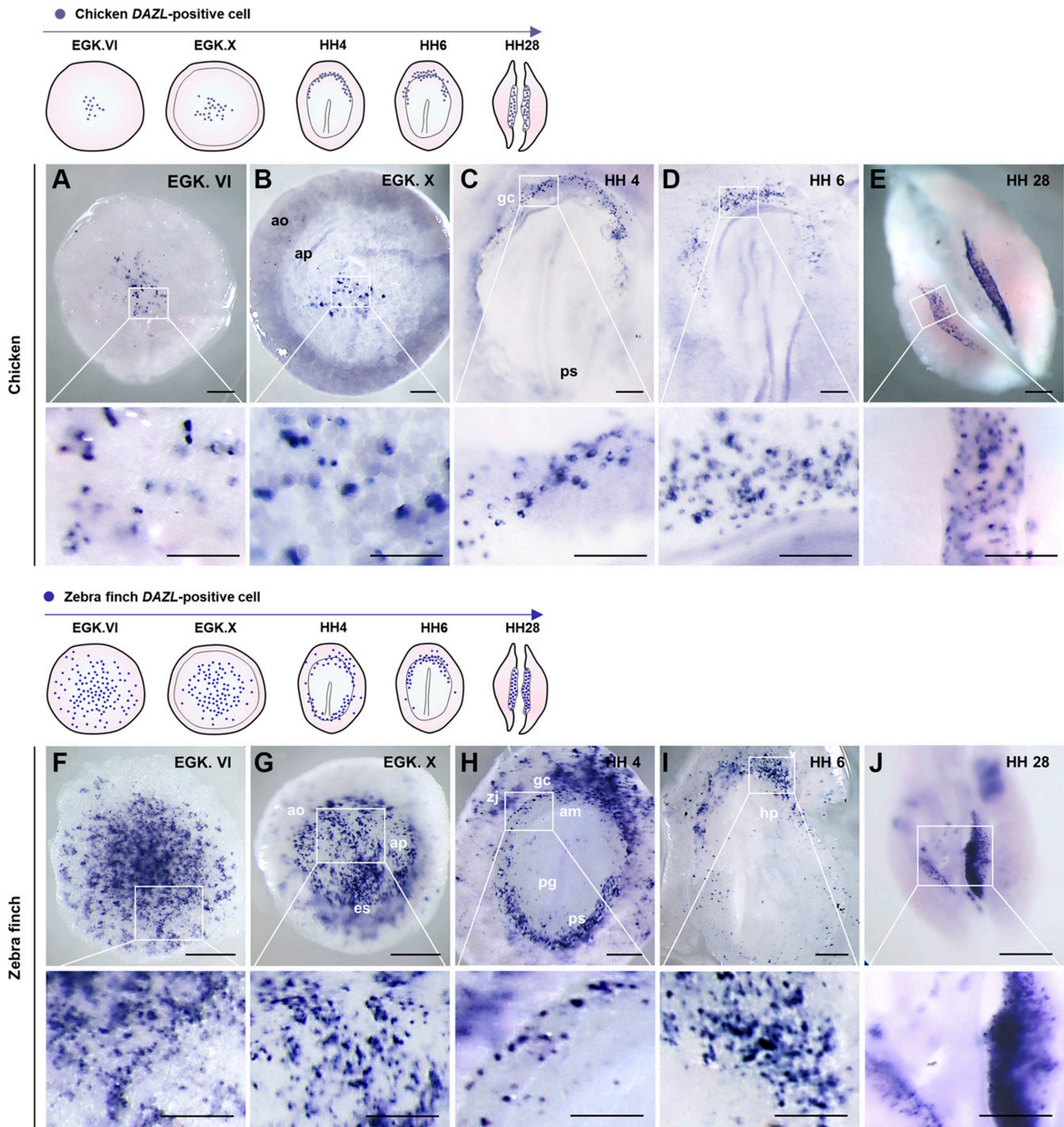
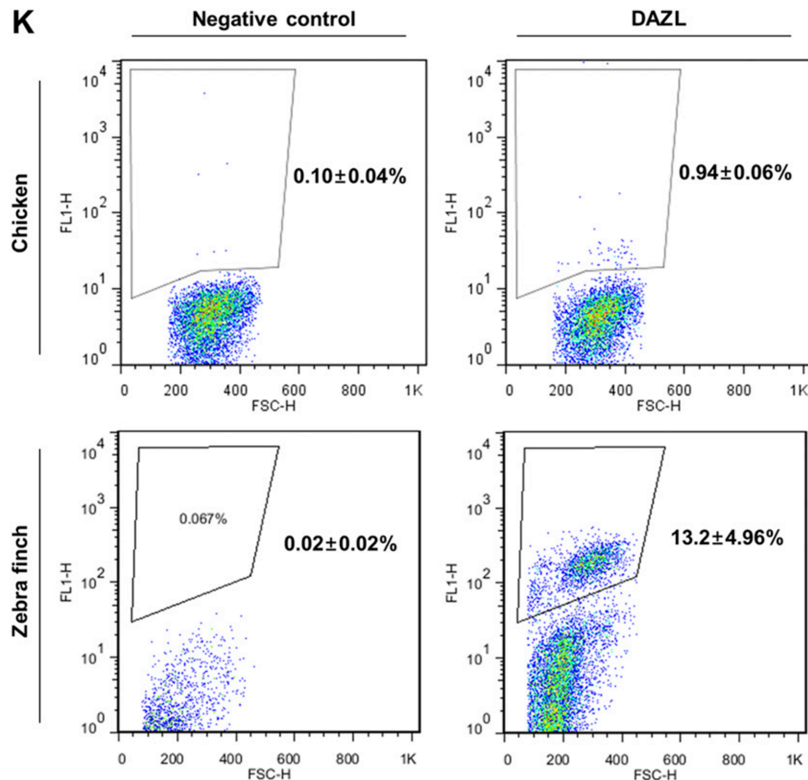


Figure 1. Comparative germ cell development during chicken and zebra finch embryogenesis. *A–E*) Whole-mount *in situ* hybridization with c*DAZL* mRNA in chicken embryos at EGK.VI (*A*), EGK.X (*B*), HH4 (*C*), HH6 (*D*), and HH28 (*E*). Scale bars, 400 (upper row) and 200 μm (bottom row). *F–J*) Whole-mount *in situ* hybridization with z*DAZL* mRNA in zebra finch embryos at the same stages of chickens. Scale bars, 400 μm (upper row); 200 μm (lower row). Boxed areas in the upper rows are magnified in the lower rows. Blue-labeled cells are *DAZL*-positive PGCs. Am, anterior margin of mesoderm; ao, area opaca; ap, area pellucida; es, embryonic shield; gc, germinal crescent; hp, head progress; pg, primitive groove; ps, primitive streak; zj, zone of junction. *K*) Separation of zebra finch and chicken gonadal PGCs using flow cytometry after whole gonadal cells were labeled with anti-DAZL primary antibodies and Alexa Fluor 488–conjugated secondary antibodies. As a negative control, whole gonadal cells were labeled with only Alexa Fluor 488. Right box is the region containing PGCs with higher DAZL expression. *L*) Proportion of DAZL-positive cell in chicken and zebra finch by flow cytometry. $^{**}P < 0.01$.

1664 total cells with 1553 ± 543 being DAZL positive (Table 1), whereas chicken contained 119,898 ± 35,971 total cells with 1099 ± 336 being DAZL positive. These findings indicate clear and striking differences in the total number and distribution of PGCs during early embryonic stages between the 2 species with a more



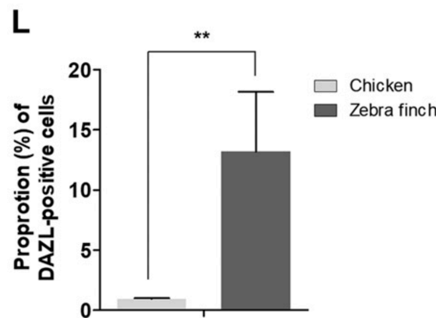


Figure 1a. (*Continued*)

widespread and higher density of PGCs in early zebra finch embryos and eventually their gonads.

Dorsal-ventral localization of PGCs is similar between zebra finch and chicken

We sectioned the above mentioned *in situ*–hybridized embryos in the sagittal plane to examine and determine more precisely the anatomic locations of labeled cells. In both species, *DAZL*-positive PGCs were observed throughout the dorsal-ventral axis of embryos at EGK.VI, again with a higher number of cells in the zebra finch (**Fig. 2*A, D***). At EGK.X, however, when the single-layered blastoderm has separated into a thin, dorsal, epiblast layer and a thick, ventral, hypoblast layer, there was clearly a higher density of PGCs in the ventral layer of both species, but again higher overall for the zebra finch (Fig. 2*B, E*). This pattern was maintained into HH4 (Fig. 2*C, F*). These findings indicate that despite differences between the 2 species in the anterior-posterior anatomic distribution of PGCs, the dorsal-ventral expression distribution is similar.

Gonadal development and PGC localization

To determine an optimal developmental time for isolation of PGCs from zebra finch gonads, embryonic gonads were collected from HH26 to 38 (**Fig. 3*A***), when they could be visualized under a dissecting microscope. Embryo sex was identified by PCR of a sex chromosome–specific gene, chromo-helicase-DNA-binding (Fig. 3*B*). Left and right gonad sizes were estimated by 2-dimensional surface-area measurements from microscope images of the embryos (27). At HH26 (120 h of incubation), the left and right sides of the gonads were similar in size (Fig. 3*C*). However, by HH28 (132–144 h or 5.5–6 d of incubation), we found that in both sexes, the left gonad was consistently larger than the right. In females, this size difference increased further, reaching a 4-fold difference by HH38, whereas in males the difference remained consistent at <2-fold (Fig. 3*A, C*). Given these results, we chose HH28 as a putative optimal stage for isolation of gonadal PGCs in the zebra finch because the gonads are not too far along their differentiation path.

TABLE 1. *DAZL-positive cells in zebra finch determined by flow cytometry*

Stages	Total cells	DAZL-positive cells	Proportion of PGCs (%)
EGK stage VI (embryos)	3397 ± 1675	182 ± 102	5.36 ± 2.12
EGK stage X (embryos)	9848 ± 6072	432 ± 239	4.39 ± 0.68
HH stage 28 (gonads)	11,765 ± 1664	1553 ± 543	13.2 ± 4.96

Values are means ± SD; n = 3.

We also performed immunocytochemistry on the gonads at HH28 with several antibodies made against mammalian proteins known to detect the the following homologous PGC-specific markers in mice and chicken: anti-DAZL, anti–SSEA-1, anti–SSEA-3, anti–SSEA-4, anti-integrin β1, and anti-integrin α6 (4, 28). Only DAZL was highly expressed in zebra finch PGCs followed by a sparse labeling of SSEA-1 (Supplemental Fig. S3). The distributions of DAZL-expressing cells between zebra finch and chicken gonads were similar for both sexes, namely widely distributed in the male gonads and dispersed in the cortex region of female gonads. But, consistent with analyses above, the densities appeared higher in both sexes of the zebra finch (Fig. 3*D*). The activity of anti-cDAZL antibody on zebra finch PGCs was confirmed in duplicate by Western blot analysis of zebra finch whole gonadal cells (Supplemental Fig. S4).

Chicken and zebra finch gonads express different stem-cell gene programs

The above findings on species differences in the timing and distribution of PGCs suggest that the chicken and zebra finch may have differences in their gonadal developmental programs. To test this possibility, we performed RNA-Seq profiling on zebra finch and chicken whole gonads at HH28. Functional categories of DEGs were identified using a GO enrichment analysis over biologic processes, molecular functions, and cellular locations. We found highly significant up-regulation in zebra finch relative to chicken of many genes that were enriched in functions for stem-cell differentiation and growth and overall cell growth and proliferation (**Fig. 4*A***). We did not find highly significant categories of down-regulated genes in zebra finch gonads.

We performed quantitative RT-PCR on gonads from additional embryos for a number of candidate genes and normalized the values to GAPDH. We validated the following 5 of 7 (71%) of the stem cell–regulated genes as significantly up-regulated in the zebra finch: ISL LIM homeobox 1, fibroblast growth factor 2; activin A receptor type 1; semaphorin 3E; and semaphorin 3A. Only the following 2 of 6 (33%) germ cell genes tested showed significant differential expression: up-regulation of WD repeat domain 48 and down-regulation of piwi-like RNA-mediated

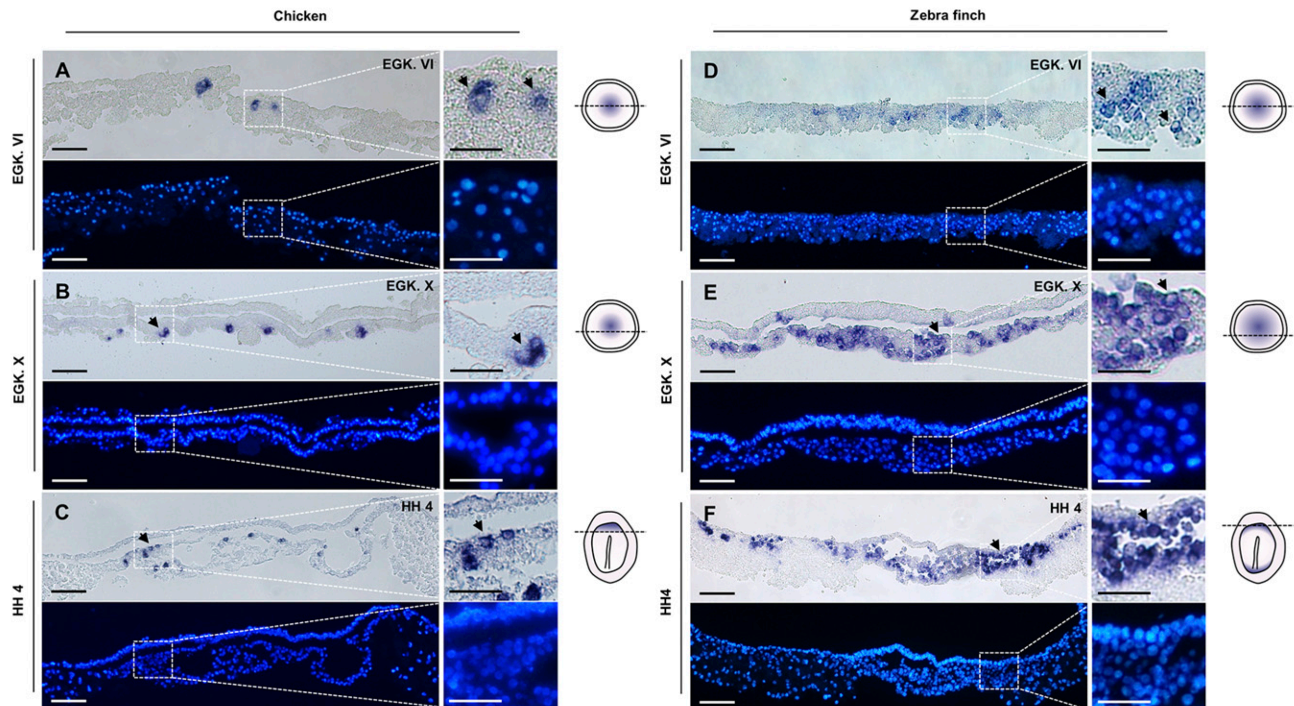


Figure 2. The morphology and location of *DAZL*-positive cells in chicken and zebra finch embryos. *A–C*) Sections of whole-mount *in situ* hybridization to detect c*DAZL* mRNA in chicken embryos at EGK.VI (*A*), EGK.X (*B*), and HH4 (*C*). *D–F*) Sections of whole-mount *in situ* hybridization to detect z*DAZL* mRNA in zebra finch embryos at EGK.VI (*D*), EGK.X (*E*), and HH4 (*F*). Arrows, example *DAZL*-labeled PGCs. Upper image, phase contrast. Lower image, fluoresent with DAPI. Scale bars, 100 μm; 50 μm (magnified images). The illustration at the right shows the sectioned area of each embryonic stage.

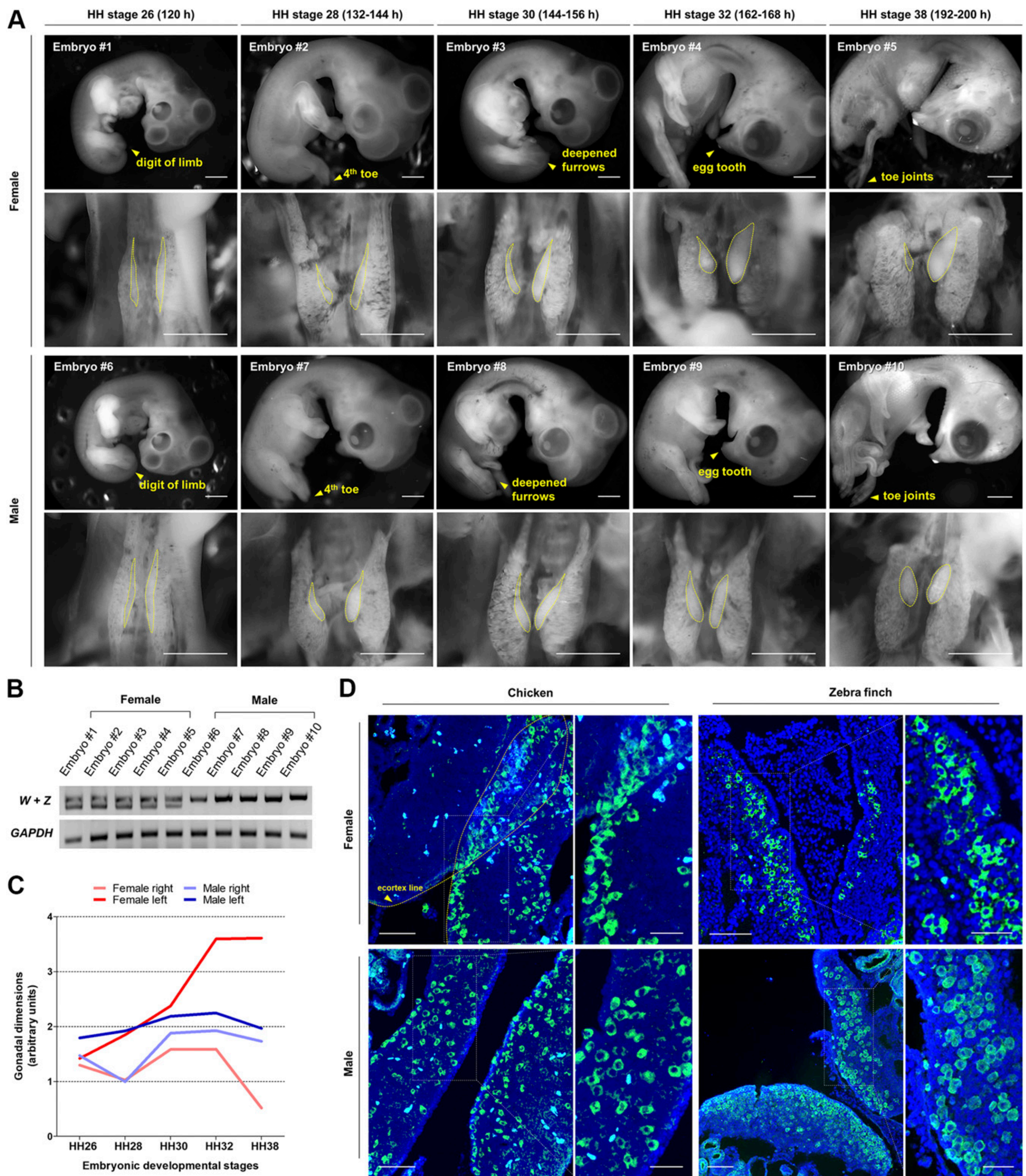


Figure 3. The proportion and distribution of DAZL-positive cells in zebra finch embryonic gonads. *A*) Morphologic differentiation of sex-specific gonads in zebra finch. To determine an optimal time for identification of gonadal PGCs from the zebra finch, fertilized eggs were incubated for 120–200 h (5–8.5 d) and classified based on the HH criteria and developmental features (arrow heads). Morphology of the gonads (dotted line) in the dissected embryos of each stage was observed under a microscope. Scale bars, 1 mm. Embryos, $n = 3$. *B*) Sexing of 10 selected embryos was performed by PCR using W- (female) and Z-chromosome (female and male) –specific primers (amplicon size, 179 and 242 bp, respectively). *C*) Graph showing gonadal dimension changes across embryonic developmental stages (from HH26 to 38, $n = 3$/age). Approximate gonadal dimensions are calculated on the basis of the length and width at midpoint of individual gonads (L × W). *D*) Immunohistochemical analysis of chicken and zebra finch embryonic gonads at HH28 using an anti-DAZL antibody. Scale bars, 100 μm; 200 μm (magnified image).

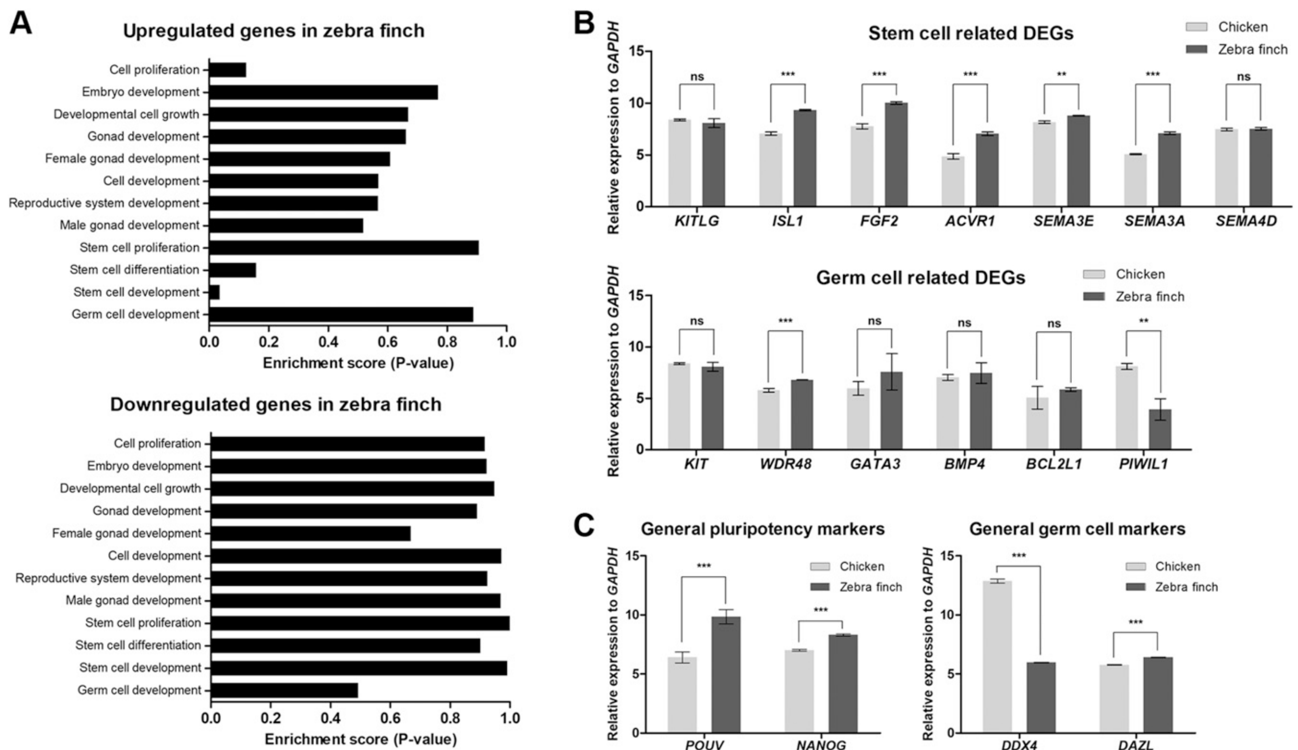


Figure 4. DEGs between chicken and zebra finch gonads. *A*) GO enrichment analysis of differential gene expression determined by RNA-Seq and categories of DEGs between chicken and zebra finch gonads. DEGs are indicated up-regulated or down-regulated in zebra finch gonads relative to chicken gonads. *B*) Quantitative RT-PCR of differentially expressed candidate genes associated with stem cells or germ cells. *C*) Quantitative RT-PCR of pluripotency markers and germ cell markers in chicken and zebra finch gonads. $^{**}P < 0.01$, $^{***}P < 0.001$ (ns, not significant).

gene silencing 1 (*PIWIL1*) in the zebra finch (Fig. 4*B*). In addition, quantitative RT-PCR of general stem-cell markers revealed up-regulation of *POUV* and *NANOG*. For PGC markers, *DAZL* was significantly up-regulated in the zebra finch but only by a small fraction when normalized to *GAPDH*, whereas the *DDX4* was significantly down-regulated by over 2-fold less compared with chicken (Fig. 4*C*), despite there being more PGCs in zebra finch gonads. These findings indicate that the differences between chicken and zebra finch cannot be simply because of differences in only PGC numbers, but also to differences in the gonadal stem-cell gene-expression program.

In vitro culture and characterization of zebra finch PGCs

We next attempted to culture zebra finch PGCs from whole gonads of HH28 zebra finch embryos in culture medium established for chicken (23). But we found that the cells did not proliferate as well as for chicken PGCs. Therefore, we applied several modifications to the culture conditions to encourage zebra finch PGC proliferation and survival for as long as possible, including a reduction in FBS concentration. A fraction of the cells adhered to the plate (presumably GSCs) and were retained as a feeder layer. Floating round cells could be subcultured and passaged at 4–5-d intervals (**Fig. 5*A*, *B***), surviving and proliferating for about a month. These floating cells were all positive for DAZL protein (Fig. 5C), and thus, they were considered to be PGCs. The GSCs did not express DAZL protein (Fig. 5*D*).

RT-PCR analysis showed that round floating cells from zebra finch cultures expressed the germ cell–specific genes *DAZL* and *DDX4* and the pluripotency-specific genes *POUV* and *NANOG* (Fig. 5*E*), further indicating they are PGCs (29). The adherent cells in the cultures did not express these genes (Fig. 5*E*), further supporting that they are GSCs. Scanning electron microscopy revealed that the round floating zebra finch cells were ~10 μm in diameter, and their morphology was very irregular with abundant microvilli (Fig. 5*F*), consistent with cultured chicken PGCs (29).

Molecular labeling and incorporation of cultured zebra finch PGCs into embryonic gonads

Because chicken PGCs are known to migrate to the gonads *via* the bloodstream (30–34), the migration and incorporation capacity of our cultured zebra finch PGCs was examined. Sets of about 500 cultured PGCs (20–30 d) or GSCs as a control were stably labeled with the lipophilic red fluorescent cell membrane marker PKH26 and immediately injected into the bloodstream of zebra finch embryos at HH13–16. Three days later, at HH28, we found labeled PGCs successfully incorporated into the gonads, with greater numbers in the left compared with the right gonad (109.36 ± 31.26 and 79.45 ± 28.47

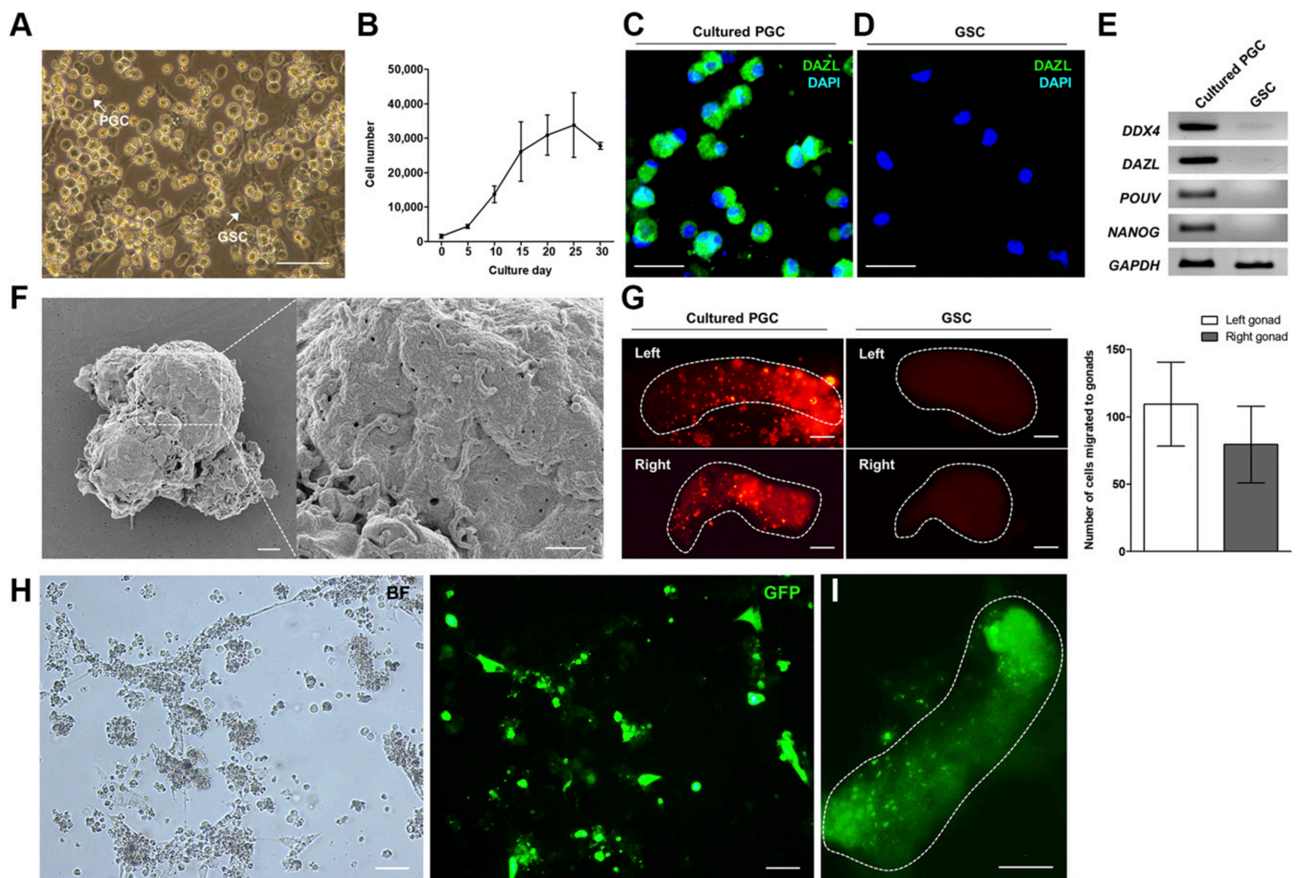


Figure 5. *In vitro* culture, characterization, and transgenetic manipulation of zebra finch PGCs. *A*) Whole gonadal cells of zebra finch embryos at HH28 were cultured in PGC culture medium. The floating cells were subcultured for a month and the adherent cells (GSCs) were partially used as a feeder layer. Scale bar, 100 μm. *B*) Growth curve of cultured PGCs. *C*) Floating cells were immunostained with anti-DAZL antibodies and identified as PGCs. Scale bars, 20 μm. *D*) Adherent cells were immunostained with anti-DAZL antibodies as a negative control. Scale bars, 20 μm. *E*) RT-PCR–based expression of *DDX4*, *DAZL*, *POUV*, and *NANOG* in cultured PGCs along with GSCs. *F*) Scanning electron microscopy of cultured zebra finch PGCs. Scale bars, 2 μm; 1 μm (magnified image). *G*) Approximately 500 cultured PGCs or GSCs were labeled with PKH26 red fluorescence dye and injected separately into the dorsal aorta of zebra finch embryos at HH13–16. The fluorescent cells were observed and counted in recipient embryonic gonads at HH28. Scale bars, 100 μm. *H*) Transposon plasmids were transfected into zebra finch PGCs using lipofection for 2 d. *I*) About 500 transfected cells were injected into zebra finch recipient embryos at HH13–16, and the GFP-expressing cells were observed in recipient embryonic gonads at HH28. Scale bars, 100 μm.

PGCs, respectively) of recipient embryos. In controls, labeled GSCs were not found in the gonads (Fig. 5*G*). These data suggest that our cultured zebra finch PGCs maintained the homing properties characteristic of PGCs *in vivo*.

To test whether our cultured zebra finch PGCs are amenable to genetic modification and whether genetically manipulated PGCs retain their capacity to migrate to the gonads, we transfected PGCs cultured for 20–30 d with a *piggyBac* transposase and a GFP expression vector flanked by the *piggyBac* transposon signals. We found that the cells were transfectable (Fig. 5*H*). Using the same protocol as above, groups of ~500 transfected cells were injected into the bloodstream of zebra finch embryos at HH13–16. When examined at HH28, we found GFP-expressing PGCs successfully incorporated into the gonads of recipient embryos (Fig. 5*I*). These data indicate that our manipulated cultured zebra finch PGCs maintain their germ cell properties and can be used to create chimeric embryos.

DISCUSSION

In this study, we identified, cultured, and characterized zebra finch PGCs and incorporated genetically modified cells into host gonads. In addition, we identified developmental characteristics of zebra finch PGCs that are different from the chicken. Relative to chicken, zebra finch PGCs are much more abundant in early embryos and present at a higher proportion in embryonic gonads relative to other gonadal cell types. Zebra finch PGCs complete their migration to the germinal crescent at later stages but survive in culture for less time than chicken PGCs.

The increased abundance of PGCs in zebra finches indicates that they invest more embryonic resources in PGC development than chickens. What advantage or disadvantage this may confer will require further investigation. The delayed timing of zebra finch PGCs into to the germinal crescent, could be caused by slower migration or incorporation of PGCs in these regions or by some cells located in the posterior region not moving to these regions

and dying off. We speculate the latter hypothesis to be more likely because the migration pattern of zebra finch PGCs at HH7 show PGCs detected both in and above the germinal crescent region, but in the HH9 embryo, a reduced number of PGCs were observed only in the germinal crescent region (Supplemental Fig. S2). From our previous findings using chicken PGCs transplanted into the central region or posterior marginal zone of stage X embryo (35), only PGCs positioned in the central region migrated toward the germinal crescent, because of anterior morphologic movement of the hypoblast. These PGCs then entered the embryonic circulation *via* the anterior vitelline vein. However, PGCs positioned in the posterior region that did not reach the anterior region ultimately never incorporated into the gonads. These results suggest that the fate of PGCs in early stages is significantly affected by their anterior-posterior position in the embryo. On the basis of this phenomenom, we assume that the proportional disparity of zebra finch PGCs between early stages and embryonic gonads is caused by the initial localization of the PGCs in a similar mechanism. Testing this hypothesis may require isolating PGCs migrating through the embryonic bloodstream (30–32, 34) as well as testing the rate of migration and the rate of cell death in the posterior region. Similar to aves, discrepancies in PGC formation and migration properties within the same class are also found in reptiles. The germ cell of turtle and snake embryos shows a different migration route (36). As in reptiles, subsequent studies in zebra finch could make the species differences of PGC properties in birds more distinct and contribute to explaining the evolution of germ cell development in amphibians.

The early gonadal developmental age we chose for PGC collection from the zebra finch, HH28, is similar to the optimal age previously reported for isolation and culture of chicken gonadal PGCs, HH27-28 (33, 37). We found this is the age where both species begin to show clear asymmetry of left and right gonads, similar to what has been previously reported for chicken (27). PGCs cultured from HH28 zebra finch embryos injected into the bloodstream of HH13–16 embryos successfully migrated to the gonads, suggesting that the zebra finch PGCs cultured *in vitro* maintained their migratory potential. Further experiments will be necessary to determine if these chimeric gonads will contribute to germ-line transgenic zebra finch.

According to a quantitative PCR analysis from a previous study (16), germ cell–specific genes were expressed at much higher levels in zebra finch embryos than chicken embryos at oviposition. We found that the likely cause of this difference is that the zebra finch blastoderm at oviposition (EGK.VI), in comparison with the chicken blastoderm at oviposition (EGK.X), has a greater number of *DAZL*-positive cells distributed throughout the embryo. We normalized to *GAPDH* levels, which resulted in little change of the PGC marker *DAZL* expression, and thus we believe our approach mostly normalized out cell number as a factor. The whole gonadal expression differences we found between zebra finch and chicken may help explain why zebra finch PGCs do not proliferate as long in culture conditions established for chicken PGCs (up to a month *vs.* many months). At first, this finding seems counterintuitive because we found that zebra finch gonads are more densely populated with PGCs than chicken and have higher expression of many stem cell–related genes. But they have lower expression of some PGC genes, *PIWIL1* and *DDX4*. This suggests that despite the high density and high stem-cell gene-expression environment, zebra finch PGCs may require culture conditions that boost PGC-specific genes to levels found in chicken.

In order to establish longer-term cultures of zebra finch PGCs, as has been done for chicken, it may be necessary to test modifications of growth factors in the culture medium. However, it may also be useful to modulate gene expression in the zebra finch cells to be more like chicken, such as through increased *PIWIL1 and DDX4* expression. In general, chicken-specific PGC culture conditions have not been transferable to Neoaves bird species. Therefore, the development of culture conditions for the zebra finch may be more broadly applicable to other Neoaves species. In spite of the fact that we have not yet obtained long-term cultures of zebra finch PGCs, we were still able to transfect and inject cultured zebra finch PGCs into host embryos and show that they are able to normally migrate and incorporate into the gonads. We consider this a crucial milestone in the development of transgenic zebra finch.

In summary, we have provided necessary information for the identification, isolation, culture, and characterization of PGCs in the zebra finch, a popularly used model for research on vocal learning and learning-related disorders in humans. Our study lays essential groundwork necessary for the production of germ cell–mediated chimeras and transgenic zebra finches. FJ

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AUTHOR CONTRIBUTIONS

E. D. Jarvis and J. Y. Han participated in study design and coordination; K. M. Jung participated in the design of the study, carried out the experiments, and wrote the first draft of the manuscript; Y. M. Kim participated in the design of the study and was involved in data interpretation and writing final versions of the manuscript; A. L. Keyte, O. Fedrigo, and B. Haase were involved in data analysis and writing final versions of te manuscript; M. T. Biegler, D. Rengaraj, H. J. Lee, C. V. Mello, and T. A. F. Velho were involved in data interpretation and writing final versions of the manuscript; and all authors have read and approved the final manuscript.

REFERENCES

1. Han, J. Y. (2009) Germ cells and transgenesis in chickens. *Comp. Immunol. Microbiol. Infect. Dis.* **32**, 61–80
2. Eyal-Giladi, H., Kochav, S., and Menashi, M. K. (1976) On the origin of primordial germ cells in the chick embryo. *Differentiation* **6**, 13–16
3. Eyal-Giladi, H., and Kochav, S. (1976) From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Dev. Biol.* **49**, 321–337
4. Lee, H. C., Choi, H. J., Lee, H. G., Lim, J. M., Ono, T., and Han, J. Y. (2016) DAZL expression explains origin and central formation of primordial germ cells in chickens. *Stem Cells Dev.* **25**, 68–79
5. Hamburger, V., and Hamilton, H. (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92
6. Ginsburg, M., and Eyal-Giladi, H. (1986) Temporal and spatial aspects of the gradual migration of primordial germ cells from the epiblast into the germinal crescent in the avian embryo. *J. Embryol. Exp. Morphol.* **95**, 53–71
7. Fujimoto, T., Ukeshima, A., and Kiyofuji, R. (1976) The origin, migration and morphology of the primordial germ cells in the chick embryo. *Anat. Rec.* **185**, 139–145
8. Nakamura, M., Kuwana, T., Miyayama, Y., and Fujimoto, T. (1988) Extragonadal distribution of primordial germ cells in the early chick embryo. *Anat. Rec.* **222**, 90–94
9. Chen, Y. C., Lin, S. P., Chang, Y. Y., Chang, W. P., Wei, L. Y., Liu, H. C., Huang, J. F., Pain, B., and Wu, S. C. (2019) *In vitro* culture and characterization of duck primordial germ cells. *Poult. Sci.* **98**, 1820–1832
10. Shin, S. S., Kim, T. M., Kim, S. Y., Kim, T. W., Seo, H. W., Lee, S. K., Kwon, S. C., Lee, G. S., Kim, H., Lim, J. M., and Han, J. Y. (2008) Generation of transgenic quail through germ cell-mediated germline transmission. *FASEB J.* **22**, 2435–2444
11. Park, T. S., Lee, H. C., Rengaraj, D., and Han, J. Y. (2014) Germ cell, stem cell, and genomic modification in birds. *J. Stem Cell Res. Ther.* **4**
12. Jetz, W., Thomas, G. H., Joy, J. B., Hartmann, K., and Mooers, A. O. (2012) The global diversity of birds in space and time. *Nature* **491**, 444–448
13. Wernery, U., Liu, C., Baskar, V., Guerineche, Z., Khazanehdari, K. A., Saleem, S., Kinne, J., Wernery, R., Griffin, D. K., and Chang, I. K. (2010) Primordial germ cell-mediated chimera technology produces viable pure-line Houbara bustard offspring: potential for repopulating an endangered species. *PLoS One* **5**, e15824
14. Doupe, A. J., and Kuhl, P. K. (1999) Birdsong and human speech: common themes and mechanisms. *Annu. Rev. Neurosci.* **22**, 567–631
15. Jarvis, E. D. (2004) Learned birdsong and the neurobiology of human language. *Ann. N. Y. Acad. Sci.* **1016**, 749–777
16. Mak, S. S., Alev, C., Nagai, H., Wrabel, A., Matsuoka, Y., Honda, A., Sheng, G., and Ladher, R. K. (2015) Characterization of the finch embryo supports evolutionary conservation of the naive stage of development in amniotes. *eLife* **4**, e07178
17. Murray, J. R., Varian-Ramos, C. W., Welch, Z. S., and Saha, M. S. (2013) Embryological staging of the Zebra Finch, Taeniopygia guttata. *J. Morphol.* **274**, 1090–1110
18. Lee, H. C., Choi, H. J., Park, T. S., Lee, S. I., Kim, Y. M., Rengaraj, D., Nagai, H., Sheng, G., Lim, J. M., and Han, J. Y. (2013) Cleavage events and sperm dynamics in chick intrauterine embryos. *PLoS One* **8**, e80631
19. Soderstrom, K., Qin, W., and Leggett, M. (2007) A minimally invasive procedure for sexing young zebra finches. *J. Neurosci. Methods* **164**, 116–119
20. Rengaraj, D., Kim, D. K., Zheng, Y. H., Lee, S. I., Kim, H., and Han, J. Y. (2008) Testis-specific novel transcripts in chicken: *in situ* localization and expression pattern profiling during sexual development. *Biol. Reprod.* **79**, 413–420
21. Replogle, K., Arnold, A. P., Ball, G. F., Band, M., Bensch, S., Brenowitz, E. A., Dong, S., Drnevich, J., Ferris, M., George, J. M., Gong, G., Hasselquist, D., Hernandez, A. G., Kim, R., Lewin, H. A., Liu, L., Lovell, P. V., Mello, C. V., Naurin, S., Rodriguez-Zas, S., Thimmapuram, J., Wade, J., and Clayton, D. F. (2008) The Songbird Neurogenomics (SoNG) Initiative: community-based tools and strategies for study of brain gene function and evolution. *BMC Genomics* **9**, 131
22. Stern, C. D. (1998) Detection of multiple gene products simultaneously by *in situ* hybridization and immunohistochemistry in whole mounts of avian embryos. *Curr. Top. Dev. Biol.* **36**, 223–243
23. Park, T. S., and Han, J. Y. (2012) piggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens. *Proc. Natl. Acad. Sci. USA* **109**, 9337–9341
24. Eberhart, C. G., Maines, J. Z., and Wasserman, S. A. (1996) Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. *Nature* **381**, 783–785
25. Haston, K. M., Tung, J. Y., and Reijo Pera, R. A. (2009) Dazl functions in maintenance of pluripotency and genetic and epigenetic programs of differentiation in mouse primordial germ cells *in vivo* and *in vitro*. *PLoS One* **4**, e5654
26. Kim, B., Cooke, H. J., and Rhee, K. (2012) DAZL is essential for stress granule formation implicated in germ cell survival upon heat stress. *Development* **139**, 568–578
27. Guioli, S., Nandi, S., Zhao, D., Burgess-Shannon, J., Lovell-Badge, R., and Clinton, M. (2014) Gonadal asymmetry and sex determination in birds. *Sex Dev.* **8**, 227–242
28. Jung, J. G., Kim, D. K., Park, T. S., Lee, S. D., Lim, J. M., and Han, J. Y. (2005) Development of novel markers for the characterization of chicken primordial germ cells. *Stem Cells* **23**, 689–698
29. Choi, J. W., Kim, S., Kim, T. M., Kim, Y. M., Seo, H. W., Park, T. S., Jeong, J. W., Song, G., and Han, J. Y. (2010) Basic fibroblast growth factor activates MEK/ERK cell signaling pathway and stimulates the proliferation of chicken primordial germ cells. *PLoS One* **5**, e12968
30. Jung, K. M., Kim, Y. M., Ono, T., and Han, J. Y. (2017) Size-dependent isolation of primordial germ cells from avian species. *Mol. Reprod. Dev.* **84**, 508–516
31. Ono, T., and Machida, Y. (1999) Immunomagnetic purification of viable primordial germ cells of Japanese quail (Coturnix japonica). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **122**, 255–259
32. Yasuda, Y., Tajima, A., Fujimoto, T., and Kuwana, T. (1992) A method to obtain avian germ-line chimaeras using isolated primordial germ cells. *J. Reprod. Fertil.* **96**, 521–528
33. Kim, J. N., Kim, M. A., Park, T. S., Kim, D. K., Park, H. J., Ono, T., Lim, J. M., and Han, J. Y. (2004) Enriched gonadal migration of donor-derived gonadal primordial germ cells by immunomagnetic cell sorting in birds. *Mol. Reprod. Dev.* **68**, 81–87
34. Mozdziak, P. E., Angerman-Stewart, J., Rushton, B., Pardue, S. L., and Petitte, J. N. (2005) Isolation of chicken primordial germ cells using fluorescence-activated cell sorting. *Poult. Sci.* **84**, 594–600
35. Kang, K. S., Lee, H. C., Kim, H. J., Lee, H. G., Kim, Y. M., Lee, H. J., Park, Y. H., Yang, S. Y., Rengaraj, D., Park, T. S., and Han, J. Y. (2015) Spatial and temporal action of chicken primordial germ cells during initial migration. *Reproduction* **149**, 179–187
36. Bachvarova, R. F., Crother, B. I., Manova, K., Chatfield, J., Shoemaker, C. M., Crews, D. P., and Johnson, A. D. (2009) Expression of Dazl and Vasa in turtle embryos and ovaries: evidence for inductive specification of germ cells. *Evol. Dev.* **11**, 525–534
37. Mozdziak, P. E., Wysocki, R., Angerman-Stewart, J., Pardue, S. L., and Petitte, J. N. (2006) Production of chick germline chimeras from fluorescence-activated cell-sorted gonocytes. *Poult. Sci.* **85**, 1764–1768

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