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Characterization of primordial germ cells from EG&K stage X chicken embryos

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ABSTRACT

Primordial germ cells (PGCs) derived from Eyal-Giladi and Kochav (EG&K) stage X embryos (XPGCs) represent the earliest germ cells in poultry. However, little is known about the XPGCs due the difficulty in the isolation and propagation of these cells. In this study, we report a method for the stable isolation and efficient in vitro propagation of XPGCs. We conducted a thorough analysis of the characteristics exhibited by XPGCs. First, we identified an average of 42 PGCs at this stage by using an DAZL-2a-mCherry genetically modified chickens. Three stable cell lines, one male and two female, were established from eight isolates using a filter paper ring and differential adhesion. Germ cell-related protein markers, including DAZL, CVH, and SSEA-1, were detected in the established XPGCs. Re-introduction of the XPGCs into the blastoderms of EG&K stage X embryos or the cardiovascular systems of Hamilton and Hamburger (HH) stage 14 embryos showed that the XPGCs retained the ability to migrate to the gonads after long-term culture. XPGCs demonstrated a lower growth rate in vitro but a higher potential of migrating to the gonads than PGCs derived from the embryonic gonads of HH stage 27–31. The ability to isolate, culture, and characterize XPGCs advances our knowledge of early-stage germ cell development and provides a valuable cell tool for genetic conservation and genome editing in chickens.

Introduction

Primordial germ cells (**PGCs**) are germ cell progenitors, crucial for genetic transmission to future generations. In avian species, PGCs originate in the area pellucida of the Eyal-Giladi and Kochav (**EG&K**) stage X embryos and migrate from the epiblast to the hypoblast (Eyal-Giladi and Kochav, 1976; Eyal-Giladi et al., 1981; Ginsburg and Eyal-Giladi, 1987; Swift, 1914). At Hamilton and Hamburger (**HH**) stage 4 (18–19 h of incubation), they move to the germinal crescent (Clawson and Domm, 1969; Ginsburg and Eyal-Giladi, 1986; ; Hamburger and Hamilton, 1951; Tagami and Kagami, 1998; van de Lavoir et al., 2006), and at HH stage 6 (23-25 h), blood islands appear (Sheng, 2010). By HH stage 12 (48-49 h), PGCs enter the vasculature, peaking in density at HH stage 14 (50-53 h) (Tajima et al., 1999), and ultimately settle in the genital ridges by HH stage 28-30 (Bellairs and Osmond, 2014; D'Costa et al., 2001; Fujimoto et al., 1976). PGCs can be isolated from the germinal crescent, embryonic blood, and gonads, allowing in vitro culture and cryopreservation (Chen et al., 2020; Clawson and Domm, 1969; Whyte et al., 2015; van de Lavoir et al., 2012). Transplantation into host embryos enables xenogeneic germline transmission, making PGCs invaluable for germ cell research, genetic resource preservation, and genetic manipulation in poultry (Schusser et al., 2013; Song et al., 2014).

In birds the frequencies of chimera production and the levels of germline transmission of xenogeneic PGCs are low (Kang et al., 2008; Wernery et al., 2010). Only 2 males, out of 65 evaluated, sired more than 1 offspring (Liu et al., 2012). These factors have largely restricted the technical progress and application of constructing gene-edited chickens through PGCs. It has been shown that germline crescent-derived PGCs maintain a higher level of pluripotency than gonad-derived PGCs (Song et al., 2014). Therefore, exploring earlier-stage PGCs that can maintain high levels of pluripotency and possess stronger migratory and homing capabilities toward the gonads during in vitro culture may offer a potential solution to these challenges.

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In poultry, the PGCs (**XPGCs**) at EG&K stage X of chicken embryos are known to be the earliest observable PGCs following embryo be produced (Eyal-Giladi et al., 1976). At this stage, only approximately 30 PGCs are present, accounting for less than 0.1 % of the 60,000 cells in the embryo (Pokhrel et al., 2017). Due to the difficulty of isolating and propagating PGCs, little is known about them at this early stage. In this study, we developed a stable method to isolate and propagate XPGCs derived from EG&K stage X embryos. It was found that XPGCs demonstrated lower proliferation than gonad-derived PGCs (**gPGCs**) in vitro but exhibited greater migration to the developing gonads after they were re-introduced into embryos. The findings in this study demonstrate a procedure for isolating PGCs from early embryos and provide the cellular tools for studying the development and production of genetically edited chickens.

Materials and methods

Animals and ethics statement

The fertilized eggs used to isolate the PGCs were obtained by crossing homozygous DAZL-2a-mCherry roosters with Lohmann hens that were housed at the animal experimental farm of Guangxi University. The PGCs obtained in this manner expressed mCherry fluorescent protein. The fertilized eggs were incubated in an artificial incubator at 60 % humidity and 38°C. The animal experiments conducted in this study were approved by the Animal Experimental Committee of Guangxi University (GXU2018-003) and adhered to the guidelines for the care and use of laboratory animals.

Separation of the EG&K stage X blastoderm

The whole procedure for harvesting blastoderm cells has been widely described in numerous publications since the 90 s (Petitte et al., 1990) for example among others. Therefore, it will not be overly described.

Culture of XPGCs

The blastoderm cells were dissociated, suspended by repeated pipetting, and plated on 24-well plates. After a 12-hour incubation at 37°C under 5 % CO_2, the suspended cells in the supernatant were collected, centrifuged, resuspended in culture medium, and transferred to a new 24-well plate pre-seeded with mouse embryo fibroblast (MEF) feeder layers. Half the culture medium was changed every 2 days. The MEF feeder layers were replaced every 3 days. The XPGCs were continuously cultured and proliferated to 1×10^6 cells to establish the cell lines. The PGCs medium were used as reported in our previous study (Xie et al., 2019)

gPGC isolation and culture

gPGCs were isolated from 7-day-old DAZL-2A-m-Cherry chicken embryos using enzymatic dissociation with 0.05 % trypsin-EDTA (Gibco, 25300062, Carlsbad, CA, USA) at 37°C for 10 min, followed by neutralization in DMEM/F-12 medium (Gibco, A4192001, Carlsbad, CA, USA) containing 10 % fetal bovine serum (HyClone, SH30070.03, Logan, UT, USA). The dissociated gonadal cells were initially seeded in 24-well culture plates and maintained at 37°C with 5 % CO_2 for 4-5 h to facilitate differential adhesion. Non-adherent gPGC-enriched populations were subsequently transferred to over mitomycin C-treated MEF feeder layers in fresh 24-well plates. The culture system was sustained in mKO medium optimized for avian germ cell maintenance, with medium renewal performed every 48 h to ensure metabolic stability.

Cell Counting of PGCs

In this study, the counting method for PGCs was performed using a hemocytometer.

Cryopreservation of XPGCs

The XPGCs were cryopreserved using full culture medium supplemented with 10 % FBS and 10 % DMSO (Solarbio, D8371, Beijing, China). Each cryovial contained 5×10^5 cells. The cryovial was placed in a cryobox and maintained at −80°C overnight before transferring to liquid nitrogen for long-term preservation.

Alkaline phosphatase staining

XPGCs and gPGCs (positive controls) were collected by centrifugation, while MEFs (negative controls) were stained in situ in culture dishes. Prior to staining for alkaline phosphatase (AP) activity, the cells were washed three times with fresh, pre-warmed basal DMEM/F-12 medium (Gibco, A4192001, Carlsbad, CA, USA). A live AP stock staining solution (Thermo Fisher, A14353, Waltham, MA, USA) was diluted 500-fold to a 1 × working solution using basal DMEM/F-12 medium. After dilution, the live AP staining solution was immediately incubated with the cells at 37°C in the dark for 20 min. Subsequently, the cells were washed three times with basal DMEM/F-12 medium to remove excess staining solution. Fresh culture medium was then added, and the fluorescent labeling was observed under a fluorescence microscope.

DNA and RNA extraction and PCR

PCR primers for cell sex determination are listed in Table S1. The primers used for PCR to identify foreign genes in the semen of a G0 chimera rooster are listed in Table S2. The PCR amplification procedure was programmed for 5 min at 95°C; 35 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min. All PCR procedures were performed using a PCR kit (TransGen Biotech, AS111-11, Beijing, China).

Total RNA was extracted from cells using an Omega RNA kit (Omega Bio-Tek Inc., R6934-01, Norcross, GA, USA) and reverse transcribed into cDNA using a reverse transcription reagent kit and gDNA eraser (TransGen Biotech, AU341-02-V2, Beijing, China) following the manufacturer's instructions. To determine the PGC-specific genes, we performed reverse transcription PCR (RT-PCR). The RT-PCR primers are listed in Table S3. To determine the gene expression levels, quantitative real-time PCR (qRT-PCR) was performed using PerfectStart Green qPCR SuperMix (TransGen Biotech, TG-AQ601-02, Beijing, China). The qRT-PCR amplification conditions were as follows: pre-denaturation at 95°C for 30 s, followed by 35 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Chicken *β-actin* (*ACTB*) was used as an endogenous reference to normalize the RNA expression levels in each sample. The PCR primers used in this experiment are listed in Table S4.

Immunocytochemistry Analysis

The PGCs were collected, centrifuged, and resuspended in 4 % paraformaldehyde (Biosharp, BL539A, Hefei, China) for 15 min for fixation. Subsequently, the fixed PGCs were evenly spread on the hydrophobic barrier surface of pathology-grade microscope slides, which were encircled and bordered by a hydrophobic immunohistochemical pen (GeneTech, GT1001, Shanghai, China). The smeared PGCs were then incubated with a blocking solution consisting of PBS with 6 % horse serum (Gibco, 16050-122, Carlsbad, CA, USA) for 45 min and then incubated with primary antibodies for 60 min. After washing three times in PBS, the PGCs were incubated with secondary antibodies for 60 min. The PGCs were then washed three times with PBS, and the cell nuclei were counterstained with Prolong Gold anti-fade reagent containing DAPI (Thermo-Fisher, P36934, Waltham, MA, USA). Finally, the treated slides were examined under a fluorescence microscope and imaged. The

primary antibodies were mouse anti-SSEA-1 (IgM; stage-specific embryonic antigen-1, Hybridoma Bank, MC-480, University of Iowa, Iowa City, IA, USA), rabbit anti-cDAZL polyclonal antibody (prepared in our laboratory), and rabbit anti-CVH polyclonal antibodies (made in our laboratory), which were diluted 1:200 with a blocking solution. The secondary antibodies used were goat anti-mouse IgG coupled with Alexa Fluor 594 (BOSTER, BA1141, Wuhan, China) and goat anti-rabbit IgG coupled with DyLight 488 (BOSTER, BA1127, Wuhan, China), which were diluted 1:1000 with a blocking solution.

Germline Transmission of XPGCs

Prior to injecting PGCs into HH stage 15 embryos to examine germline transmission, the recipient chicken embryos were placed in an incubator for 53–55 h at 38°C and 60 % humidity. To facilitate injection, a 1–2-cm window was created at the blunt end of the egg, and 200 μL of PBS containing 1 % antibiotic–antimycotic (Gibco) was added to moisten the air chamber membrane. An opening was made in the membrane to expose the heart of the HH stage 15 embryo. PGCs, totaling 1×10^4, were then injected into the embryonic heart through a fiberglass needle. The injected embryo was carefully sealed with parafilm and returned to the incubator for 5 days.

Prior to injecting PGCs into EG&K stage X embryos to examine germline transmission, freshly laid fertilized eggs were maintained flat at 16°C for 6–8 h. Under sterile conditions, a fenestration was made on the belly of the eggs near the blunt end to expose the EG&K stage X blastoderm. A total of 500 PGCs were injected into the subgerminal cavity between the chicken blastoderm and yolk. The injected EG&K

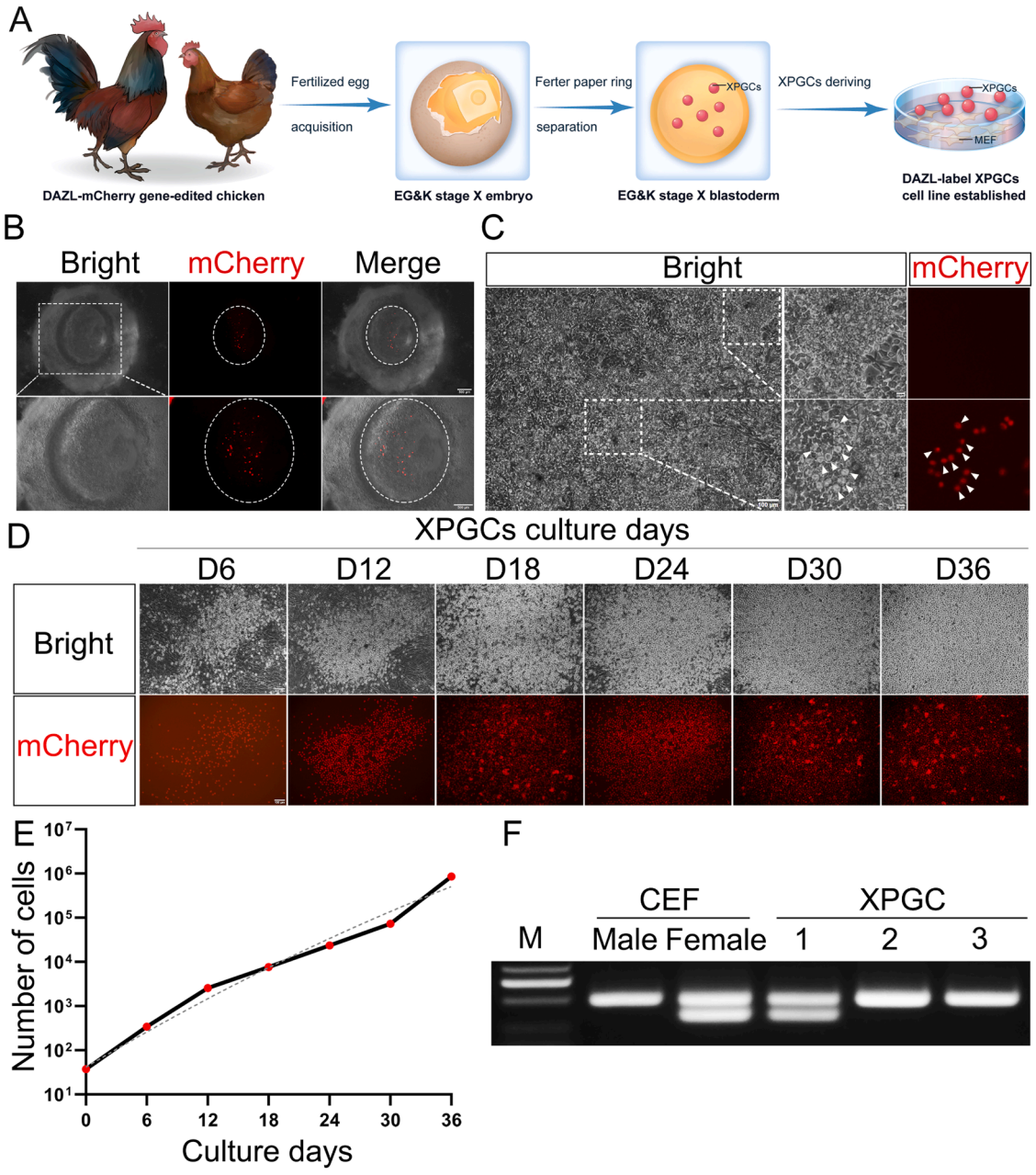


Fig. 1. Isolation and culture of XPGCs. (A) A schematic of the isolation and culture of the XPGCs. (B) A fluorescence image of an EG&K stage X blastoderm from a DAZL-mCherry chicken embryo. (C) A fluorescence image of the mixed cell population from the blastoderm. (D) Images of cells obtained every 6 days during cell line establishment. (E) Cell proliferation curve of XPGCs. (F) Sex identification of XPGC cell lines using PCR. Scale bar: 500 μm (B), 100 μm and 20 μm (C), and 100 μm (D).

stage X embryos were carefully sealed with parafilm and returned to the incubator for 7 days. Following the incubation, the gonads of the embryos at HH stage 28 were examined and imaged using fluorescence microscopy.

Statistical analysis

The experimental data were expressed as mean ± standard deviation, and statistical analysis and plotting were performed using GraphPad Prism 10.0 software. The paired *t*-test was used for comparisons between two groups of data, and one-way ANOVA was used for comparisons among multiple groups of data. $^{*}P < 0.05$ was considered significant, and $^{**}P < 0.01$ was considered extremely significant.

Results

Isolation and culture of XPGCs

As described in Fig. 1A, intact blastoderms were isolated using the filtration membrane technique, and the suspended XPGCs were collected from the blastoderms through the differential adhesion method. The results of the cell count from the fluorescence image of the complete blastoderm showed that each blastoderm contained an average of 42 XPGCs (Fig. 1B; Table S5). Additionally, the isolated XPGCs that grew in suspension were distinctly positive for mCherry, whereas the other cells of the blastoderm were adherent to the MEFs and were negative for mCherry (Fig. 1C). The XPGCs were continuously cultured for 36 days and proliferated up to 1×10^6 cells to establish each cell line. A total of three cell lines of XPGCs were established from eight isolates in this study. The cells were counted every 6 days, and found that these XPGCs proliferated robustly in vitro (Fig. 1D and E). PCR for sex identification was performed on the three cell lines using W chromosome-specific primers, which identified one male and two female cell lines (No. 1 is female, No. 2 and No. 3 are male) (Fig. 1F).

Characterization of germ stem cell-related markers of XPGCs

To characterize the identity of the germ cells derived from the blastoderm, the XPGCs were subjected to alkaline phosphatase detection, RT-PCR, and immunohistochemistry. It was found that all three lines of PGCs were strongly positive for alkaline phosphatase (Fig. 2A). The XPGCs tested positive for the stem cell markers *PouV* and *Nanog* and for the germ cell-related markers *Dazl, Cvh*, and *Cdh* using RT-PCR (Fig. 2B). Furthermore, according to the immunohistochemistry results, XPGCs were positive for the proteins CVH, DAZL, and SSEA-1 (Fig. 2C). These results indicated that the XPGCs cultured in vitro maintained typical germ cell characteristics.

Difference in the proliferation potential between XPGCs and gPGCs

To explore the distinctive features of the XPGCs, we conducted a comparison of the proliferation potential between PGCs isolated from the blastoderm of EG&K stage X chicken embryos and the embryonic gonads of HH stage 27–31 chicken embryos. We performed cell

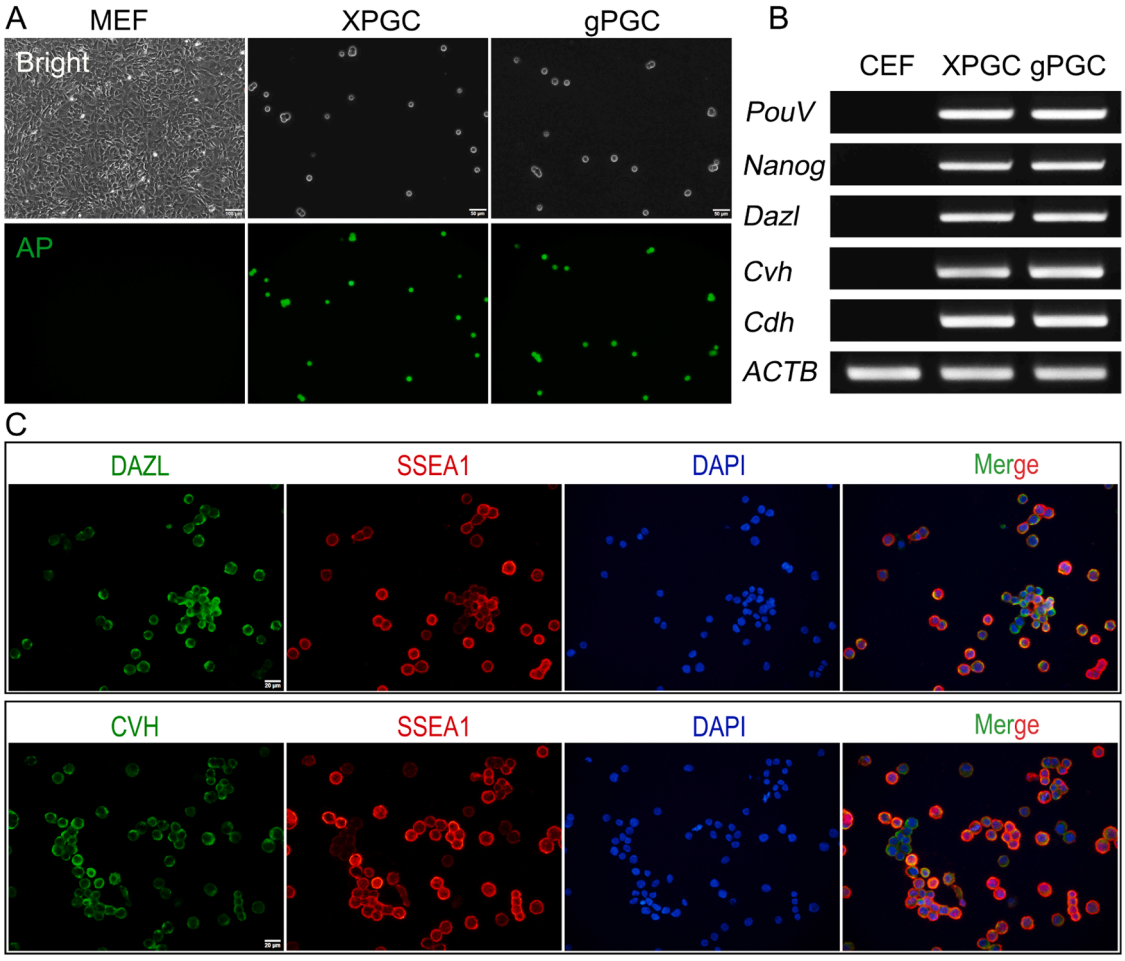


Fig. 2. Characterization of the XPGCs cultured in vitro. (A) Alkaline phosphatase testing of XPGCs. (B) RT-PCR to detect the germline and stem cell-related gene expression of PouV, Nanog, Dazl, Cvh, and Cdh in XPGCs. (C) Immunofluorescence staining for the germ stem cell markers CVH, DAZL, and SSEA1 in XPGCs. Scale bar: 100 μm and 50 μm (A), and 20 μm (C).

proliferation experiments using XPGCs and gPGCs that were established cell lines at the fourteenth passage. The results showed that under the same conditions, 1×10^4 XPGCs proliferated to $4.37 \pm 0.38 \times 10^4$ cells after 3 days, while gPGCs proliferated from 1×10^4 cells to $6.45 \pm 0.51 \times 10^4$ cells, indicating that the proliferation rate of the XPGCs was significantly lower than that of the gPGCs ($P < 0.05$; Fig. 3A). We then tested the expression of genes related to regulating cell proliferation and the cell cycle and found that the expression of *Pcna* and *Ccnd1* was significantly higher in gPGCs than in XPGCs ($P < 0.05$; Fig. 3B). A flow cytometric cell cycle analysis showed that the G1, S, and G2 phase ratios of XPGCs were 51.6 ± 2.69 %, 26.41 ± 0.53 %, and 21.99 ± 2.91 %, respectively, and those of gPGCs were 29.84 ± 0.32 %, 42.33 ± 0.37 %, and 27.83 ± 0.06 %, respectively. A significantly higher percentage of XPGSs than of gPGCs was detected in the G1 phase, while a lower percentage of XPGSs than of gPGCs was detected in the S and G2 phases ($P < 0.05$; Fig. 3C). These results revealed that XPGCs had a lower proliferation potential than gPGCs.

Difference in the migration ability between XPGCs and gPGCs

The ability to migrate to the gonads and colonize there is among the most crucial characteristics of PGCs. We performed injection experiments using XPGCs and gPGCs that were established cell lines at the fifteen to seventeen passages. XPGCs were injected into recipient EG&K stage X blastoderms (after oviposition) at a dose of 500 cells per egg (Fig. 4A). A significant number of mCherry-positive cells were found in the gonads of the recipient embryos after 6.5–7 days of incubation (Fig. 4B). In addition, after transplantation of XPGCs at a dose of 1×10^4 cells per egg into host embryos at HH stages 13–16 (45–56 h of incubation) (Fig. 4C), a significant number of mCherry-positive cells were found in the gonads from host embryos at HH stages 27–31 (6.5–7 days of incubation) (Fig. 4D). The ability to migrate to the gonads as the embryo developed further demonstrated that XPGCs were germline stem cells.

The migration ability was analyzed by injecting PGCs into the vasculature of host embryos at HH stages 13–16. Upon injection of 1×10^3 XPGCs and gPGCs into the vasculature of HH stage 13–16 embryos, a significantly greater number of mCherry-positive cells were observed to migrate to the gonads in XPGC-injected embryos than in gPGC-injected embryos (Fig. 4E). Specifically, the number of mCherry-positive cells migrating to the gonads in XPGC recipient embryos was 1337 ± 117, whereas in gPGC recipient embryos, it was 877 ± 98 ($P < 0.01$; Fig. 4F). *Cxcr4* is involved in guiding PGC migration during chicken embryo development and was significantly more highly expressed in XPGCs than in gPGCs ($P < 0.05$; Fig. 4G). XPGCs and gPGCs were observed using scanning electron microscopy (SEM) (Fig. 4H). First, under SEM, it was found that both XPGCs and gPGCs had typical spherical outlines, and gPGCs were smoother than XPGCs. Second, the surface of the EG&K stage XPGCs had very dense and abundant microvilli, while the gPGCs that had colonized the gonads had very few and short microvilli. These results indicated that XPGCs had a stronger ability to migrate to the gonads than gPGCs.

Difference in germ stem cell-related gene expression between XPGCs and gPGCs

An assay of the expression pattern of germ cell-related genes between XPGCs and gPGCs showed that the expression of *PouV, Nanog, Dazl*, and *Cvh* was significantly greater in XPGCs than in gPGCs ($P < 0.05$; Fig. 5). Expression of *Piwil1*, a gene associated with PGC genome stability, was reduced ($P < 0.05$; Fig. 5) We performed assay of the expression pattern of germ cell-related genes experiments using XPGCs and gPGCs that were established cell lines at the eleventh to eighteenth passage.

Cryopreservation and germline transmission of XPGCs

The three XPGC cell lines were cryopreserved and thawed. Then, the cells were injected into recipient embryos (HH stage 15) to validate their germline transmission ability. The results demonstrated that the thawed XPGCs proliferated rapidly in vitro, with a doubling time of 4.25 days

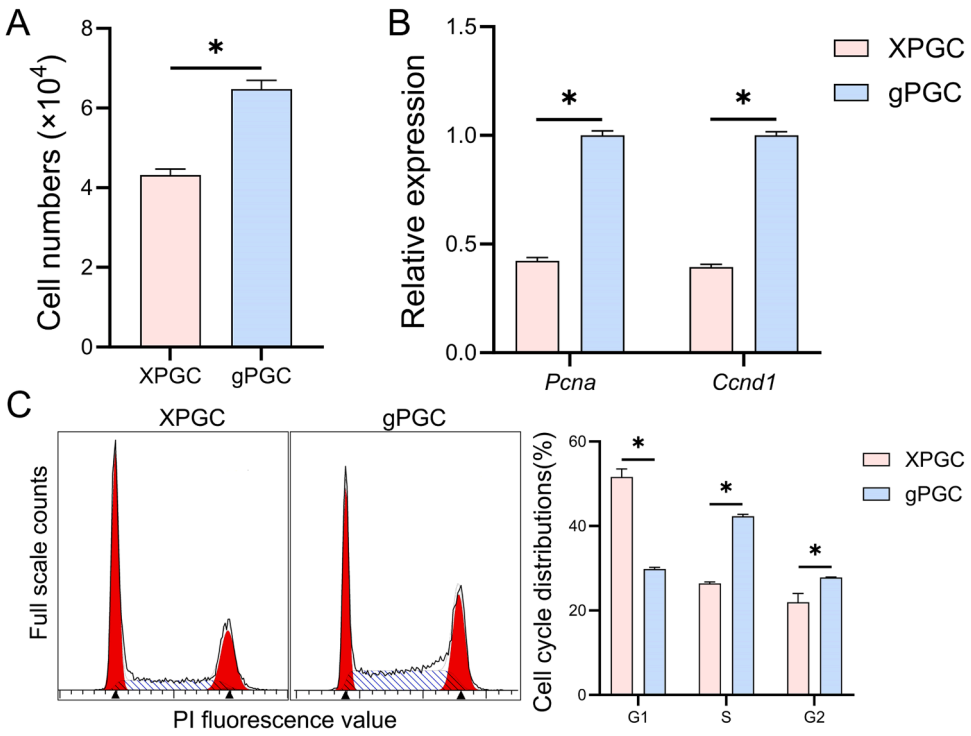


Fig. 3. A comparison of the proliferation potential between XPGCs and gPGCs. (A) A total of 1×10^4 cells each of XPGCs and gPGCs were cultured under identical conditions for 3 days, after which the number of cells was determined. (B) Expression of the cell proliferation-related genes *Pcna* and *Ccnd1* in XPGCs and gPGCs. (C) Analysis of the cell cycle distribution of XPGCs and gPGCs using flowing cytometry. *$P < 0.05$.

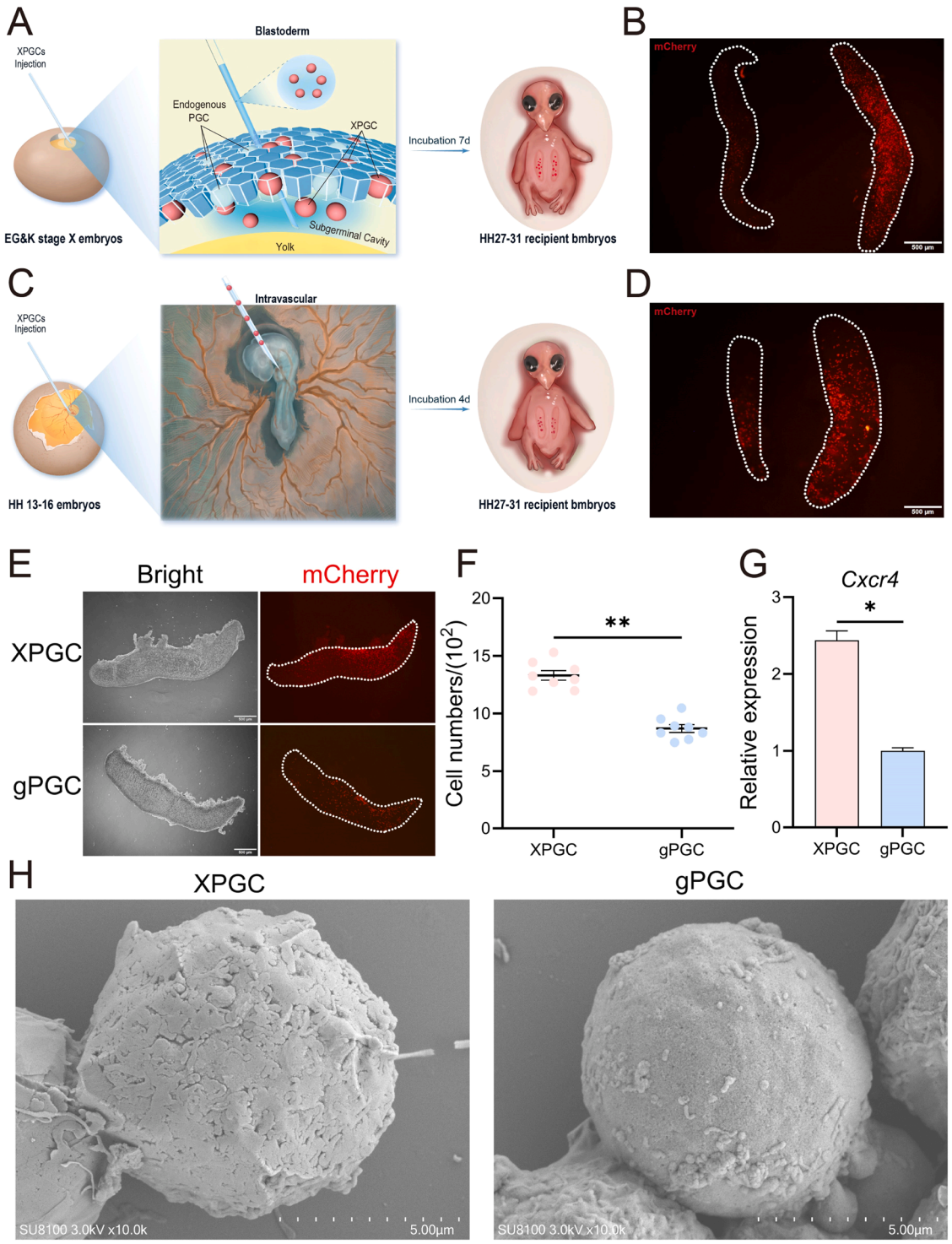


Fig. 4. Migration ability of XPGCs and gPGCs. (A) Schematic of the XPGC transplantation into the subgerminal cavity of the blastoderm of EG&K stage X host embryos. (B) Fluorescence image of the embryonic gonads 6.5–7 days after injecting 500 XPGCs. (C) Schematic of XPGC transplantation into the blood circulation system of HH stage 13–16 host embryos. (D) Fluorescence image of the embryonic gonads 5 days after injecting 1 × 104 XPGCs. (E) Fluorescence image of the embryonic gonads after transplantation of XPGCs and gPGCs. (F) The cell count of mCherry-positive PGCs in the embryonic gonads after transplantation of XPGCs and GPGCs. (G) Expression of the cell migration-related gene Cxcr4 in XPGCs and gPGCs. (H) Scanning electron micrograph of XPGCs and gPGCs. $^{*}P < 0.05$ and $^{**}P < 0.01$. Scale bar: 500 μm (B, D and E), 5 μm (H).

(Fig. 6A and B). Thirty-six chimeric chickens (18 males and 18 females) were hatched from the recipient embryos injected by the thawed mCherry XPGCs at HH stage 15 (Fig. 6C). Five male and five female chicks were dissected at 4 days of age, and a significant number of mCherry fluorescent cells were found in all chicks of ovaries and testes (Fig. 6D). The remaining chicks were grown until sexual maturity. Semen was collected from the chimeric roosters, and PCR analysis revealed that it was positive for the mCherry transgene (Fig. 6E). To obtain the genetic efficiency of the offspring from individual female or male chimeras, we crossed the chimeras with wild-type chickens. Our

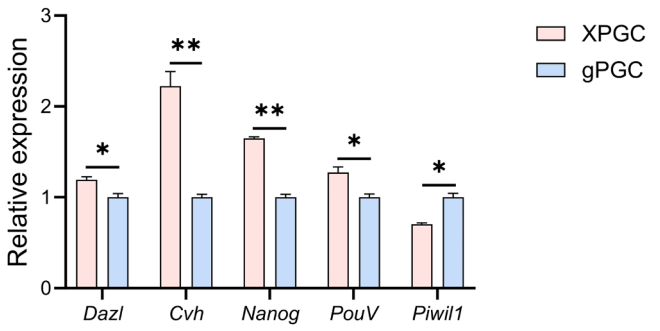


Fig. 5. Germ cell-related gene expression levels of XPGCs and gPGCs. *$P <$ 0.05 and **$P < 0.01$.

results showed that offspring carrying foreign genes occurred in both female and male chimeras (Fig. 6F), with the incidence of carrying the foreign gene "mCherry" in chimeric G1 E6.5 embryos from male G0 chimeras ranging from 6.90 % to 31.43 % and from female G0 chimeras ranging from 8.70 % to 21.43 % (Table S6).

To further assess the germline genetic efficiency of XPGCs, we used XPGCs sourced from Donglan chickens (characterized by black skin and feathers) as donors, with White Leghorn embryos serving as recipients for cell transplantation. After 36 days of in vitro culture and exposure to freeze-thaw cycles, the XPGCs were transplanted into recipient embryos. The resulting chimeric embryos hatched and were nurtured until sexual maturity. The presumed chimeric chickens were paired with wild-type White Leghorn chickens, and the G1 progeny were statistically examined for germline transmission (Fig. 7A). The presence of black skin or feathers in the G1 chicks indicated that the genes were transferred from the donor XPGC. The male and female chimeras were mated and three different genotypes were obtained: homozygous White Leghorn chickens (-/-, 34.48–76.67 %), heterozygous chickens (±, 20.00–48.28 %), and homozygous Donglan chickens (+/+, 3.33–17.24 %) (Fig. 7B and C). To obtain the genetic efficiency of the offspring from individual female or male chimeras, we crossed the chimeras with wild-type White Leghorn chickens. Our results showed that germline transmission occurred in both female and male chimeras, with the incidence of black feathers and skin in chimeric G1 offspring from male G0 chimeras ranging from 8.47 % to 41.43 % and from female G0 chimeras ranging from 17.39 % to 36.36 % (Table S7). These results suggest that XPGCs maintained their germline transmission capacity even after undergoing in vitro culture, cryopreservation, and thawing.

Discussion

XPGCs are known as the earliest germ cells during the development of chicken embryos (Eyal-Giladi and Kochav, 1976; Tsunekawa et al., 2000). They are a valuable cellular tool for studying the early events of germ cell specification and differentiation and for producing gene-edited chickens. However, due to the difficulty in their isolation and cultivation, XPGC lines have not yet been established (Pokhrel et al., 2017). In this study, the DAZL-mCherry gene-edited chicken was used to visualize the tracking, separation, and culture of XPGCs via the filter paper ring and differential adhesion method (Pu et al., 2023). The XPGCs were characterized, and their germline transmission was validated. The results of this study have expanded our understanding of XPGCs and provide a useful tool for studying biological events during the development of germ cells in chicken embryos.

Early studies showed that *Dazl* gene expression is a hallmark of vertebrate germ cells and is a central regulator in the development and differentiation of embryonic germ cells (Chen et al., 2014; Gill et al., 2011; Houston et al., 2000; Johnson et al., 2001; Lin and Page, 2005; Li et al., 2019; Rengaraj et al., 2020; Ruggiu et al., 1997; Schrans-Stassen et al., 2001; Saunders et al., 2003). Lee et al. studied the localization of germplasm and the formation of PGCs by tracking the expression of *Dazl* mRNA and protein in intrauterine-stage embryos and confirmed that PGCs with strong positive expression of DAZL were present in EG&K stage X (Lee et al., 2016). Previous studies analyzed EG&K stage X chicken embryos by in situ hybridization using *Cdh* cRNA as a probe and

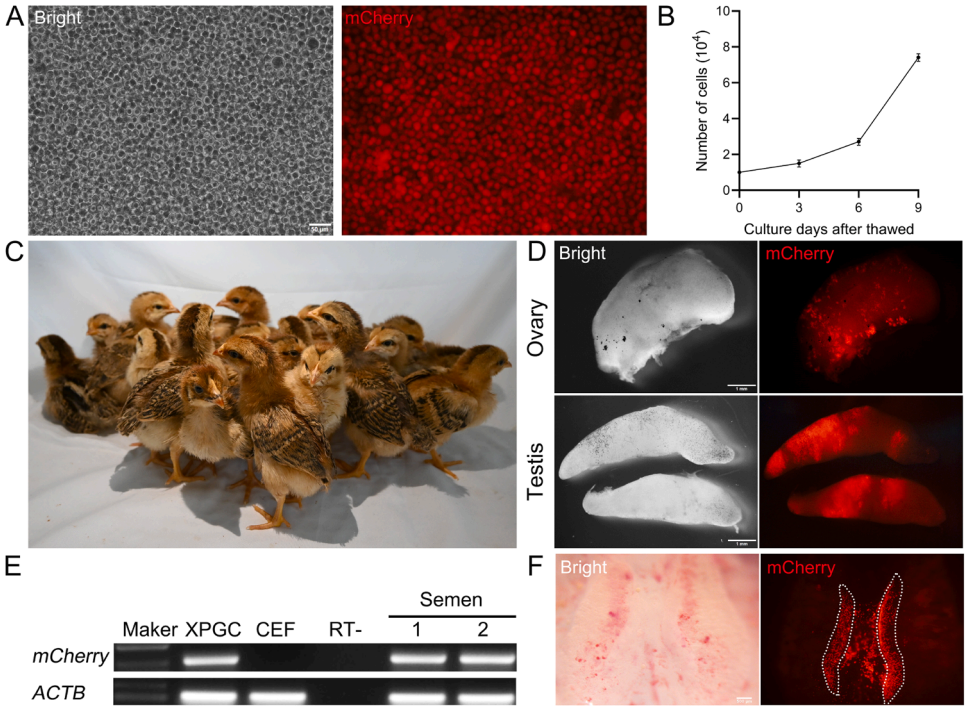
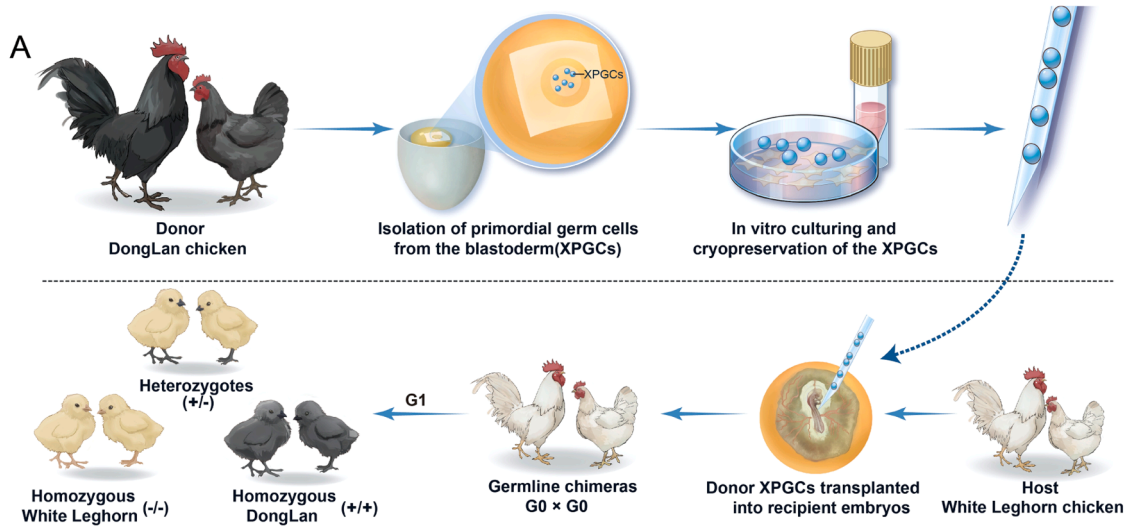


Fig. 6. Cryopreservation and germline transmission of XPGCs. (A) Proliferation of XPGCs after cryopreservation and thawing. (B) Cell proliferation curve of XPGCs after cryopreservation and thawing. (C) G0 chimeric chickens. (D) Fluorescence images of the testes and ovaries of G0 chimeric chickens. (E) PCR analysis of semen from adult G0 chimeric roosters. (F) Fluorescence image of G1 chicken embryos. Scale bar: 50 μm (A), 1 mm (D), and 500 μm (F).



C

G0 Chimera	Number of G1 chicks	White Leghorn (-/-)	Heterozygotes (+/-)	Donglan (+/+)
MEG1×FEG1	35	16(45.71%)	16(45.71%)	3(8.58%)
MEG1×FEG2	29	10(34.48%)	14(48.28%)	5(17.24%)
MEG2×FEG3	31	14(45.16%)	14(45.16%)	3(9.68%)
MEG3×FEG4	30	23(76.67%)	6(20.00%)	1(3.33%)
MEG4×FEG5	31	17(54.84%)	12(38.71%)	2(6.45%)

Fig. 7. Germline genetic efficiency of XPGC. (A) The strategy for determining the XPGC germline transmission efficiency. (B) Three distinct genotypes of G1 offspring derived from chimeric chicken: homozygous White Leghorn chick (-/-, left), heterozygotes (±, middle), and homozygous Donglan chick (+/+, right). (C) Frequency of germline transmission from founder chimeras to G1 chickens of the three genotypes.

found that there were at least 30 *Cdh*-positive cells in an EG&K stage X chicken embryonic disc (Aramaki et al., 2007). Previous studies mechanically isolated and cultured chicken blastodermal cells (cBCs), and they showed that retinoic acid treatment induced the cBCs to differentiate into germ cells (Tang et al., 2017). However, PGCs were already present in the chicken blastodermal disc at stage X, and a true XPGC cell line has not yet been obtained. Therefore, to study the XPGCs specifically, we used DAZL-mCherry gene-edited chickens to isolate XPGCs. As PGCs were fluorescently labeled with mCherry, XPGCs with high expression of DAZL were identified in the center of the bright zone of the EG&K stage X chick embryo blastodisc, with an average number of 42 cells. This was consistent with the study of Eyal-Giladi and Kochav, in which approximately 30 chicken PGCs were first found in the EG&K stage X chick embryo blastodisc (Eyal-Giladi and Kochav, 1976). PGCs isolated from the genital crescent at HH stage 6, blood at HH stages 13-16, gonads at HH stage 19, and gonads at HH stages 28-31 can be cultured in vitro for a long time while maintaining high expression of *Dazl* (Chen et al., 2020; Naito et al., 2015; Whyte et al., 2015). Similarly, the XPGCs isolated by our method were rapidly derived in vitro, reached 1×10^6 cells from about 40 cells in about a month, and continued to express the *Dazl* gene. Previous studies suggested that male PGCs exhibit a greater proliferative capacity and are easier to maintain in long-term culture than female PGCs (Song et al., 2014; Tonus et al., 2016). A study from Wang et al. found that the initial cells were isolated and pooled from both male and female embryos, all derived PGC lines were found to contain only male PGCs (Wang et al., 2017). Notably, our study isolated and established different cell lines, including both male PGC lines and female PGC lines. This suggests that our isolation method and culture system are stable and practical, and thus, the XPGC cell lines proliferate rapidly and can be cultured long term in vitro.

It is generally believed that PGCs have specific migration routes and functions in the developing embryo (Ichikawa and Horiuchi, 2023). Early research found that transferring clumps of cells from unstaged embryos obtained from unincubated eggs produced three phenotypically chimeric embryos from 239 recipients, but none of these embryos survived to hatching (Marzullo, 1970). Petitted et al. obtained somatic mosaicism in 10 % of all recipient embryos by transplanting blastodisc cells obtained from EG&K stage X blastodiscs. By examining semen DNA and the reproductive records of the chimeras, they showed that transferring stage X blastodisc cells could produce germline mosaicism, but only in very rare cases of germline inheritance, and speculated that germline mosaicism might result from injecting pluripotent cells or introducing cells that had previously differentiated into PGCs (Petitte et al., 1990). We injected XPGCs into EG&K stage X recipient embryos and HH 15 recipient embryos, and they migrated to the gonads of HH stage 27-31 chicken embryos and formed high-efficiency germline chimerism, indicating that XPGCs could migrate. These results further revealed that XPGCs had the identity of primordial germ cells and that PGCs were already present in EG&K stage X blastoderms. These results also indicated that the generation of reproductive chimeras by transplanting blastoderm cells may be due to the presence of XPGCs. Interestingly, due to the limited volume of the EG&K stage X blastodisc, only 500 PGCs could be injected into the blastodisc, while 1×10^4 PGCs were injected into the embryonic vasculature (HH stage 15); however, there was no significant difference in the number of PGCs that migrated to the recipient gonads between the two injection methods. This phenomenon suggests that although a small number of PGCs were injected into the blastodisc at EG&K stage X, a large number of exogenous PGCs can migrate to the gonads and colonize there. This result provides us with a faster and more convenient method to produce gene-edited chickens, in which a relatively small number of XPGCs are injected into the blastodisc at the EG&K X stage.

Few previous studies have explored the differences in the cellular properties of PGCs from different stages. A study from Szczerba et al. found that cultured PGCs from embryonic blood circulation (cPGCs) (HH stages 13-16) had significantly greater proliferation and migration

abilities than gPGCs (HH 18) (Szczerba et al., 2020). The CXCR4/CXCL12 axis guides PGCs from their site of formation through different embryonic tissues using chemotaxis mechanisms, with the PGCs eventually migrating to the gonads (Amat-Fernandez et al., 2017; Stebler et al., 2004). The presence of microvilli enhances the reception and response of the CXCL12/SDF-1 signals and directs the migration of the PGCs along the gradient of the chemokines (Cai et al., 2017, 2022; Lee et al., 2017; Lin et al., 1982; Orbach et al., 2020; Ukeshima et al., 1987). Using SEM, Ukeshima et al. found that PGCs lose a large number of cell surface microvilli during extravasation from blood vessels to the genital ridge (Ukeshima et al., 1991). Therefore, differences between PGCs of different sources (blastoderm, embryonic blood, and gonads) are undoubtedly to be expected, particularly for microvilli, and this is also true of the SEM results of this study. We found that XPGCs have a poorer proliferation ability than gPGCs but a greater migration ability. Proliferation markers such as *Pcna* and the cell cycle regulatory gene *Ccnd1* are highly expressed in gPGCs, while the migration-related gene *Cxcr4* is highly expressed in XPGCs, and cell surface microvilli that are critical for migration are more abundant and longer on the surface of XPGCs. In addition, we found that the pluripotency-related genes *Cvh, PouV*, and *Nanog* were expressed at higher levels in XPGCs than in gPGCs. *Vasa* is related to the synthesis of piRNA in mice, and there is an interaction between PIWI protein and *Vasa* in Drosophila oocytes (Thomson and Lin, 2009). In this experiment, compared with gPGCs, the expression of *Cvh* in XPGCs was increased, while the expression of *Piwil1* was decreased, indicating that there may be a regulatory relationship between *Cvh* and *Piwi* genes in poultry. Therefore, we speculated that the migration of PGCs and the development of individuals indicate that PGCs may be heading toward a process of differentiation, which is specifically manifested as decreased pluripotency, a decreased migration ability, and an increased proliferation ability.

Cryopreservation of PGCs is a key means of preserving poultry genetic resources (Woodcock et al., 2019). PGCs isolated from the HH stage 6 germinal crescent, HH stage 14 embryonic blood, HH stage 19 genital ridge formation, and HH stage 28 gonads have been shown to maintain their migration ability and form germline chimeras after long-term culture and cryopreservation and have produced offspring with germline transmission (Hamai et al., 2023; Ibrahim et al., 2024; Ichikawa and Horiuchi., 2023; Whyte et al., 2015; Xie et al., 2019). In our study, the germline transmission experiment of XPGCs showed that XPGCs maintained a strong ability to migrate to the gonads after freezing and thawing and eventually formed germline chimeras. In addition, we injected XPGCs derived from wild-type Donglan black chickens as donors into wild-type White Leghorn chicken embryos as recipients. We found that efficient germline transmission were achieved both through intraspecific breeding of chimeric White Leghorn recipient chickens and through hybridization of chimeric white Leghorn males and females with wild-type White Leghorn chickens. This showed that by preserving XPGCs, it may be feasible to breed endangered rare birds using existing bird populations.

In conclusion, we isolated and propagated PGCs from EG&K stage X chicken embryos. The isolated XPGCs maintained typical germ cell characteristics, exhibited a greater migration ability than gPGCs, and could be cryopreserved for transplantation into recipient embryos to produce offspring from donor sources. These findings contribute significantly to our understanding of early biological events during the development of germ cells in chicken embryos and offer new tools for obtaining genome-edited chickens with desired phenotypes and for preserving endangered bird germplasm resources.

Data availability

Data or resource requests can be made available upon reasonable request by contacting the corresponding author.

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CRediT authorship contribution statement

Dongyang Chen: Methodology, Writing – original draft, Writing – review & editing. **Yifei Zhi:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Xiaoxuan Jia:** Data curation. **Na Ji:** Software. **Jiale Zhang:** Formal analysis. **Jiongming Liang:** Supervision. **Mingxia Ran:** Writing – review & editing. **Xingting Liu:** Writing – review & editing. **Huiyan Xu:** Writing – review & editing. **Yangqing Lu:** Supervision, Resources, Writing – review & editing.

Declaration of competing interest

We would like to submit the enclosed manuscript entitled **"Characterization of primordial germ cells from EG&K stage X chicken embryos"** to be considered for publication in ***Poultry Science***. No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors have approved the manuscript that is enclosed.

We deeply appreciate your consideration of our manuscript, and we look forward to receiving comments from the reviewers. If you have any queries, please don't hesitate to contact me at the address below.

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Supplementary materials

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