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—Original Article—

C-X-C chemokine receptor type 4 (CXCR4) is a key receptor for chicken primordial germ cell migration

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Abstract. In mammals, germ cells originate outside of the developing gonads and follow a unique migration pattern through the embryonic tissue toward the genital ridges. Many studies have attempted to identify critical receptors and factors involved in germ cell migration. However, relatively few reports exist on germ cell receptors and chemokines that are involved in germ cell migration in avian species. In the present study, we investigated the specific migratory function of C-X-C chemokine receptor type 4 (CXCR4) in chicken primordial germ cells (PGCs). We induced loss-of-function via a frameshift mutation in the *CXCR4* gene in chicken PGCs using clustered regularly interspaced short palindromic repeat-CRISPR-associated protein 9 (CRISPR/Cas9) genome editing. The migratory capacity of *CXCR4* knockout PGCs was significantly reduced *in vivo* after transplantation into recipient embryos. However, CXCR4-expressing somatic cell lines, such as chicken DT40 and DF1, failed to migrate into the developing gonads, suggesting that another key factor(s) is necessary for targeting and settlement of PGCs into the genital ridges. In conclusion, we show that CXCR4 plays a critical role in the migration of chicken germ cells.
Key words: Chicken, CXCR4, Genome editing, Primordial germ cell

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Germ cells give rise to sperm in males and oocytes in females after sexual maturation. Germ cell progenitors, primordial germ cells (PGCs), are specified in the epiblast during early development, separately from the somatic lineages, and move through the embryonic tissue to the developing gonads [1]. In mice, germ cell specification is induced by autonomous bone morphogenetic protein 4 signaling, which is secreted from the extraembryonic ectoderm and visceral endoderm [2, 3]. Newly induced germ cells localize primarily outside of the embryo, then migrate through the hindgut endoderm toward the developing genital ridges [1, 4]. Thus, understanding the migratory path and guidance mechanisms involved in PGC migration has been a major research focus in developmental biology.

The steps involved in germ cell migration include attractive (e.g., C-X-C chemokine receptor type 4-stromal cell-derived factor 1 [CXCR4-SDF1]) and repulsive (e.g., wunen and wunen2) guidance signals, resulting in movement through the hindgut toward the lateral mesoderm [1, 5, 6]. Chemotactic molecules guide directional migration toward the gonadal somatic cells, whereas chemorepellent-expressing somatic cells forcibly repel the migrating germ cells, or indirectly induce disruption of the chemoattractant signals in the surrounding somatic cells [1]. Therefore, the combination of CXCR4 and SDF1, also known as C-X-C motif chemokine 12 (CXCL12), mediates the migratory capacity of many different cell types. This receptor-ligand interaction is well known as a master regulator of germ cell migration [1, 7, 8]. In mice and zebrafish, SDF1 is expressed along the migratory path of germ cells, suggesting that SDF1 is a crucial secretory molecule that guides germ cell migration. Furthermore, functional disruption of either SDF1 or CXCR4 results in ectopic germ cell localization outside of the genital ridges [9, 10]. Additionally, when SDF1 is expressed in different ectopic embryonic sites, germ cells alter their migratory path pattern accordingly [9, 10].

Similar to mammals, avian germ cells primarily localize outside of the embryonic gonads and then migrate toward the developing gonads [11, 12]. However, the developmental processes in the avian embryo are different from those of mammals, and derivation of the germ cell lineage is unique. Notably, the migratory route is different from that of mice and zebrafish [8]. Immediately after oviposition at Eyal-Giladi and Kochav Stage X [13], PGCs are evident in the central region of the undifferentiated blastoderm, known as the area pellucida, and includes the epiblast and hypoblast before primitive streak formation. The PGCs then circulate through the embryonic bloodstream to target the developing gonads [14]. To populate the developing gonads, chicken PGCs migrate through three major paths during early embryogenesis. In the initial migratory step, the cells migrate from the central region of the area pellucida to the germinal crescent at Hamburger and Hamilton (HH) Stage 4 [13–15]. We previously reported that passive and active forces sequentially control PGC migration toward the germinal crescent [16]. During the second phase of migration (HH Stages 12–15), PGCs invade the developing blood vessels and circulate until they enter the genital ridges [17, 18]. In the third migratory path, the cells finally settle down into the developing gonads at HH Stage 17 [18]. However,

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the critical regulator(s) involved in germ cell migration in birds have not been identified.

In the present study, we employed clustered regularly interspaced short palindromic repeat-CRISPR-associated protein 9 (CRISPR/Cas9) genome editing technology to knockout the chicken *CXCR4* gene, which is potentially involved in PGC migration. We evaluated the functional consequences of this knockout on PGC migratory capacity *in vivo*, and identified CXCR4 as a key receptor during chicken PGC migration into the developing genital ridges.

Materials and Methods

Cell culture

PGCs were cultured according to previously established procedures [19]. Briefly, PGCs from Day 6 (HH Stage 28) White Leghorn (WL) chicken embryonic gonads were maintained and subpassaged in KnockOut Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 2% chicken serum (Sigma-Aldrich, St. Louis, MO, USA), 1 × nucleosides (0.73 g/l cytidine, 0.85 g/l guanosine, 0.73 g/l of uridine, 0.8 g/l adenosine, and 0.24 g/l thymidine, Millipore, Billerica, MA, USA), 2 mM L-glutamine (Gibco), 1 × nonessential amino acids (10 mM each glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, and L-serine, Gibco), β-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Gibco), and 1 × antibiotic-antimycotic (10,000 units/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B, Gibco). Human basic fibroblast growth factor (10 ng/ml) (Koma Biotech, Korea) was used for PGC self-renewal. Chicken PGCs were maintained in an incubator at 37°C in an atmosphere of 5% CO_2 and 60–70% relative humidity. PGCs were subcultured onto mitomycin-inactivated mouse embryonic fibroblasts (MEF) at 5- to 6-day intervals by gentle pipetting without enzyme treatment.

DF1 chicken fibroblast and DT40 chicken B cell lines (American Type Culture Collection, Manassas, VA, USA) were maintained and subpassaged in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS (Invitrogen) and 1 × antibiotic-antimycotic (Invitrogen). Cells were cultured in an incubator at 37°C in an atmosphere containing 5% CO_2 and 60–70% relative humidity.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA from PGCs, *CXCR4* knockout (KO) PGCs, DT40, DF1, and CXCR4-overexpressing (OE) DF1 cells was isolated using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. RNA was checked using agarose gel electrophoresis, and quantity was determined using a NanoDrop™ 2000 (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized from RNA using a Superscript® III First-Strand Synthesis System (Invitrogen). *CXCR4* mRNA expression was measured using RT-PCR in a 20 μl reaction composed of 2 μl cDNA, 2 μl PCR buffer, 1.6 μl dNTP mixture (2.5 mM), 1 unit Taq DNA polymerase, and 10 pmol forward and reverse primers (CXCR4 RT F: 5'-ttg cct att ggt gat ggt gg-3'; CXCR RT R: 5'-cag acc aga atg gca agg tg-3'). Forward and reverse primers for β-actin amplification were 5'-gtg ctc ctc agg ggc tac tc-3' and 5'-gat gat att gct gcg ctc gt-3', respectively. PCR was performed with an initial incubation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The reaction was terminated by a final incubation at 72°C for 10 min. PCR products were analyzed using agarose gel electrophoresis.

CXCR4 knockout via CRISPR-Cas9

To knockout the *CXCR4* gene in chicken cultured PGCs, a guide RNA (gRNA) expression vector and a Cas9 expression vector carrying the enhanced green fluorescent protein (eGFP) transgene (Sigma-Aldrich) were co-transfected at a ratio of 1:1 (2.5 μg: 2.5 μg) using Lipofectamine® 3000 (Invitrogen) according to the manufacturer's instructions. One day after lipofection, PGCs were harvested and resuspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), and passed through a 40 μm cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for fluorescence-activated cell sorting using a FACSAriaTM III cell sorter (Becton, Dickinson and Company). After enrichment of GFP-positive cells, single GFP-positive PGCs were picked under a microscope and seeded onto individual wells of a 96-well plate containing MEF feeders in PGC complete culture media.

To analyze the knockout mutation, the genomic targeted region of the CRISPR CXCR4 gRNA was amplified using a specific primer set (CXCR4 F: 5'-ggc agc atg gac ggt ttg ga-3'; CXCR4 R: 5'-cat cca cag acc aga atg gc-3') after extraction of genomic DNA from *CXCR4* KO PGC line #3. PCR was performed with an initial incubation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec. PCR amplicons were cloned into a pGEM®-T Easy Vector (Promega, Madison, WI, USA) and sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). To confirm the targeted locus mutation in *CXCR4* KO PGC line #3, RT-PCR amplicons were cloned and sequenced.

Immunofluorescence

Immunofluorescence was performed on wild type PGCs and *CXCR4* KO PGC line #3 after fixation with 10% formaldehyde. Blocking was performed in 5% donkey serum in PBS for 30 min prior to incubation with primary antibodies (1:200); mouse anti-stage-specific embryonic antigen 1 (SSEA1) IgM antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and mouse anti-chicken CXCR4 IgG antibody (Bio-Rad Laboratories, Hercules, CA, USA). Anti-SSEA1 and anti-CXCR4 antibodies were detected using Alexa568 and Alexa488 fluorescent dye-conjugated secondary antibodies (1:100; Invitrogen), respectively. 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) was used to mark the nucleus and stained PGCs were observed under a fluorescent microscope.

Transfection and selection of the chicken CXCR4 expression vector into DF1 cells

The chicken *CXCR4* gene was synthesized (Bioneer, Daejeon, Korea) and a CXCR4 expression vector, controlled by a cytomegalovirus (CMV) immediate-early enhancer/promoter, was constructed and inserted between the 5'-terminal repeat (5'-TR) and 3'-TR *piggy*Bac transposon elements (System Biosciences, Palo Alto, CA, USA). After lipofection of the CXCR4 expression vector into DF1 cells, a stable CXCR-expressing subline was selected using the neomycin-resistant (Neo^R) gene. The subline was selected with 300

μg/ml G418 for more than 2 weeks.

Transfection and selection of a GFP or Discosoma red fluorescent protein (DsRed) expression vector

To generate GFP- or DsRed-expressing PGCs, DF1, and DT40 cells, plasmid DNA containing the *GFP* or *DsRed* gene expressed by a CMV immediate-early enhancer/promoter (System Biosciences) was transfected using Lipofectamine® (Invitrogen) according to the manufacturer's protocol. One day after lipofection, GFP-expressing PGCs were sorted using a FACSAria™ III cell sorter (Becton, Dickinson and Company). Transfected cells were resuspended in PBS containing 1% BSA, and strained through a 40-μm cell strainer (Becton, Dickinson and Company) for FACS. After GFP- or DsRed-positive cell sorting, cells were seeded onto culture dishes containing cell line-specific culture media.

Transplantation and detection in recipient embryos

To inject PGCs, DF1, or DT40 cells into recipient chick embryos, a small window was opened at the pointed end of the egg. A 2 μl aliquot containing a mixture of 1,000 GFP- and 1,000 DsRed-expressing PGCs, or 1,000 somatic cells was microinjected into the dorsal aorta of the recipient embryo at 53 hr. The egg window was sealed with parafilm, and the egg was incubated with the pointed end down until the next experiment. Embryonic gonads were dissected on Day 6 or Day 10 of incubation. The number of migrated cells was counted under a fluorescent microscope.

Statistical analysis

Statistical analysis was performed using the Student *t*-test with SAS software (ver. 9.3; SAS Institute, Cary, NC, USA). Significant differences among the different groups were analyzed using the general linear model in SAS. Differences among treatments were deemed to be significant when $P < 0.05$.

Results

Detection of CXCR4 transcript by RT-PCR

To examine *CXCR4* gene expression in different chicken cell types, RT-PCR analysis was conducted in PGCs and somatic cell lines DT40 and DF1, which were derived from B cells and embryonic fibroblasts, respectively. *CXCR4* transcript was strongly expressed in PGCs and DT40 cells, but was not expressed in DF1 cells (Fig. 1A).

CXCR4 knockout in chicken PGCs via CRISPR-Cas9

To knockout the *CXCR4* gene in chicken PGCs, a gRNA target of CRISPR-Cas9 was targeted to the second exon of the chicken *CXCR4* gene located on chromosome 7 (Fig. 1B). The transfection efficiency of co-transfection of the gRNA expression vector and the Cas9 expression vector carrying the eGFP transgene was approximately 1.0%. After GFP-positive cell sorting by FACS, single PGCs were picked and seeded onto individual wells of a 96-well plate. A single-PGC-derived subline was developed, and mutation analysis was conducted by genomic PCR cloning and sequencing. In *CXCR4* KO PGC line #3, there was 2 nt (GA) deletion that was predicted to cause a frameshift mutation resulting in amino acid changes and/or early termination (Fig. 1C). To confirm the genotype of *CXCR4* KO PGC line #3, RT-PCR products from each *CXCR4* KO PGC subline were re-sequenced; results were identical to the genomic DNA genotypes for each subline (Fig. 1D).

Immunofluorescence detection of CXCR4 in PGCs

Immunofluorescence showed that both wild type and *CXCR4* KO PGC line #3 were positive for SSEA1, which is a specific marker for chicken PGCs (Fig. 2A). However, *CXCR4* KO PGC line #3 was negative for CXCR4, whereas the wild type (WT) PGC line was clearly positive for this marker (Fig. 2B). These results confirm that CXCR4 receptor expression is completely disrupted in *CXCR4* KO PGC line #3.

Transfection and selection of eGFP and DsRed expression vectors

For DF1 cells, a chicken CXCR4 expression vector controlled by the CMV promoter was transfected and selected using G418 (Fig. 3A). Stable expression of the *CXCR4* transcript in CXCR4-overexpressing (OE) DF1 cells was confirmed by RT-PCR (Fig. 3B). Additionally, to efficiently detect PGCs or somatic cells after transplantation into the recipient embryos, a *piggyBac* transposon-mediated eGFP or DsRed transgene was transfected, and eGFP- or DsRed-positive cells were sorted by FACS. All sublines strongly expressed the fluorescent reporter transgenes (Fig. 3C and D).

Transplantation and detection in recipient embryos

To examine the functional role of CXCR4 in PGC migration during early embryonic development, a mixture of *CXCR4* KO GFP PGCs and wild type DsRed PGCs (1,000 cells: 1,000 cells) was transplanted into the bloodstream of recipient embryos. Embryonic gonads were dissected on Day 6 of incubation and the number of migrated cells was counted under a fluorescent microscope (Fig. 4). Numbers of migrated cells were similar when equal numbers of wild type GFP PGCs and wild type DsRed PGCs (1,000 cells: 1,000 cells) were transferred (Fig. 4A upper panel). However, in GFP-expressing *CXCR4* KO line #3 PGCs, migrated cell numbers into recipient embryonic gonads were dramatically fewer compared with wild type DsRed PGCs (Fig. 4 bottom panel). Although the numbers of migrated wild type PGCs were variable among individual recipients (Fig. 4B), the number of transplanted wild type PGCs was significantly higher than that of *CXCR4* KO PGC line #3 cells in recipient embryonic gonads (mean wild type PGCs = 200.4 ± 90.0, and *CXCR4* KO PGC line #3 = 11.2 ± 9.4, respectively. $P < 0.001$). The number of injected wild type PGCs that had migrated into the right and left gonads differed significantly ($P < 0.0001$, Fig. 4C); however, no significant difference was observed between the right and left gonads following transplant of *CXCR4* KO PGCs (Fig. 4C).

Wild type PGCs were also observed in the gonads of the recipients on Day 10 of incubation, but few *CXCR4* KO PGCs were detected at this time (Fig. 5A). To further assess the migration capacity of *CXCR4* KO PGCs, a mixture of 10,000 *CXCR4* KO PGCs and 1,000 wild type PGCs (i.e., 10-fold more *CXCR4* KO PGCs) was transplanted, and recipient gonads were observed on Day 6 of incubation. The number of wild type GFP PGCs was significantly increased compared with wild type DsRed PGCs (10,000 GFP PGCs: 1,000 DsRed PGCs); however, only a few *CXCR4* KO PGCs could be detected, even

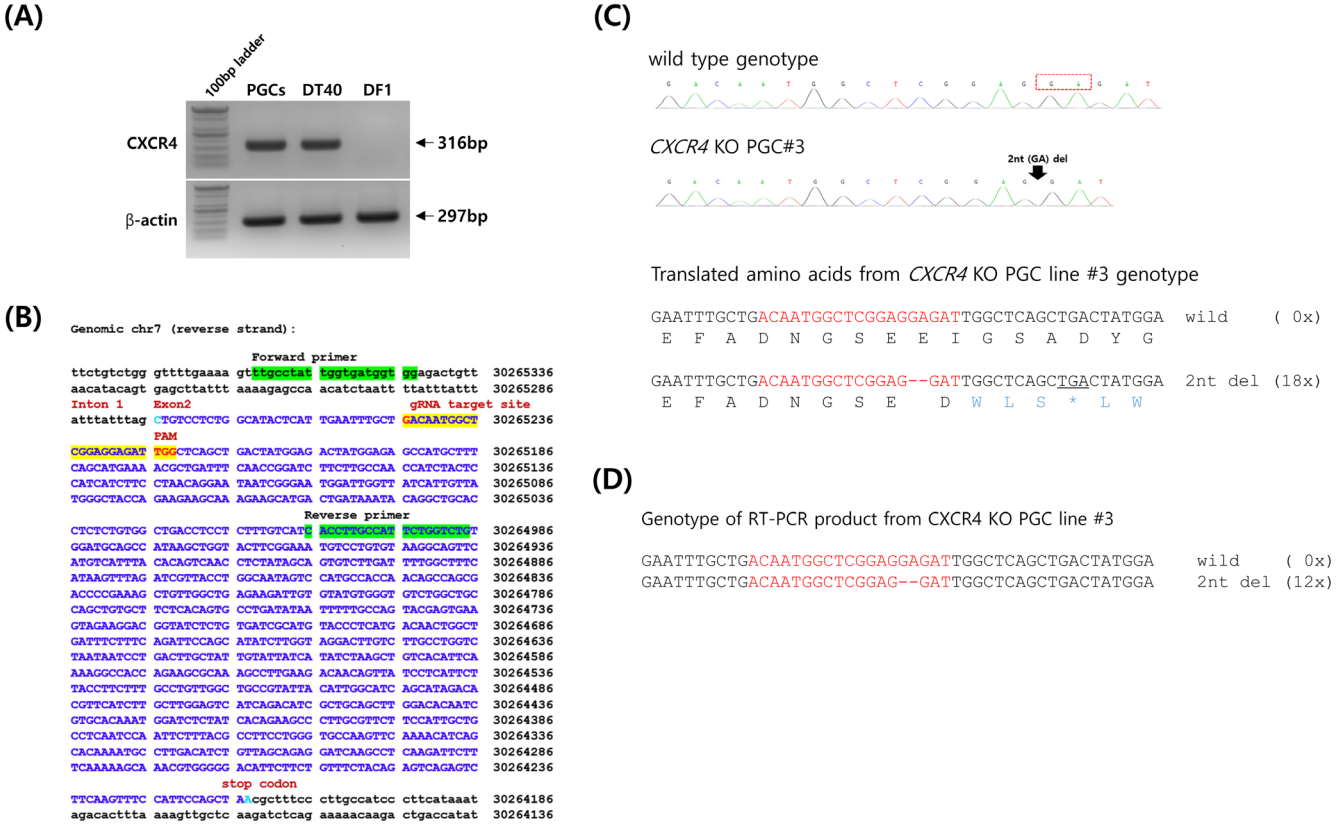
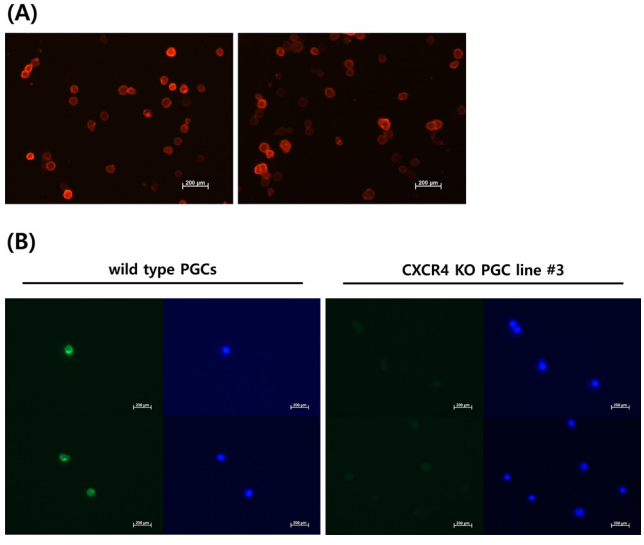


Fig. 1. Experimental design of chicken *C-X-C chemokine receptor type 4* (*CXCR4*) gene targeting. (A) Reverse transcription polymerase chain reaction (RT-PCR) analysis of *CXCR4* transcript in different chicken cell types (primordial germ cells [PGCs], DT40, and DF1). PGCs and DT40 cells strongly express CXCR4, but DF1 cells do not. (B) Schematic of CRISPR targeting of the chicken *CXCR4* gene on chromosome 7 and genomic mutation analysis. Sequences of the guide RNA target site are located in the second exon (yellow box). TGG in red is a protospacer adjacent motif (PAM). Lowercase and capital letters are intron and exon sequences, respectively. Green boxes denote primer annealing sites. (C) Mutated target DNA sequences of chicken *CXCR4* KO PGC line #3 induced by CRISPR/Cas9. Dashes denote deleted nucleotides. Mutant genotypes show a frameshift in translated proteins that induces amino acid changes and a stop codon (asterisk). (D) Genotyping analysis of *CXCR4* RT-PCR product amplified from *CXCR4* KO PGC line #3.



after transferring a 10-fold greater number than wild type DsRed PGCs (Fig. 5B).

To determine whether CXCR4 in somatic cells other than PGCs plays a crucial role in migration and targeting into embryonic gonads, CXCR4-overexpressing DF1 cells and DT40 cells that express intact CXCR were transplanted into the embryonic gonads. After transferring GFP-expressing DT40 or DF1 cells, recipient embryonic gonads were dissected on Day 6 or 10. No GFP-positive cells were detected following either DF1 or DT40 injection (Fig. 6).

Fig. 2. Immunofluorescence analysis of wild type and *CXCR4* KO PGC#3 PGCs. (A) Both wild type and *CXCR4* KO PGC line #3 are positive for SSEA1 (red). (B) *CXCR4* KO PGC line #3 is completely negative for CXCR4, whereas wild type PGCs are positive (green). 4',6-diamidino-2-phenylindole (DAPI) labels the nucleus (blue) and stained PGCs were observed under a fluorescent microscope (bars, 200 μm).

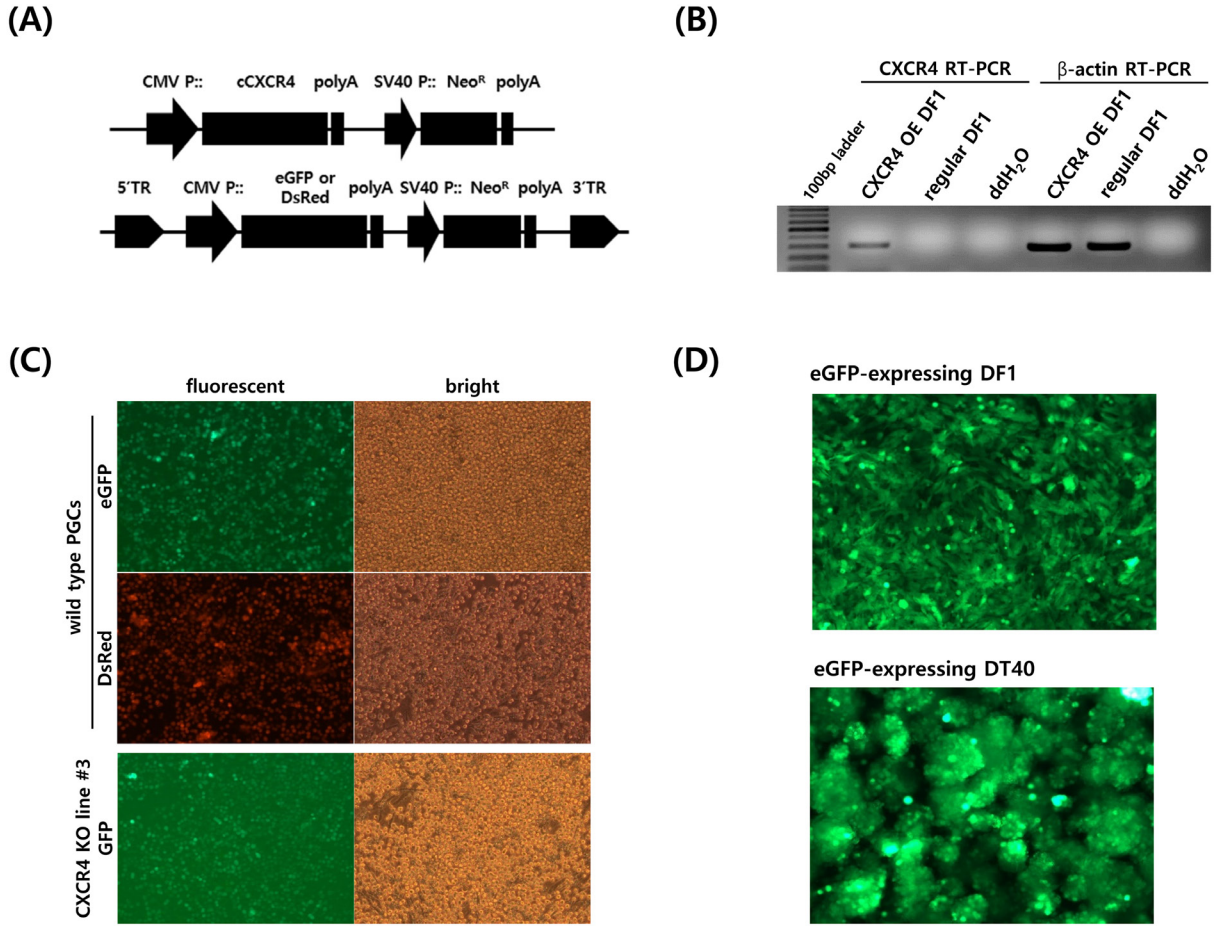


Fig. 3. Establishment of stably-transfected cells. (A) Schematic diagrams of transgene expression vectors for enhanced green fluorescent protein (eGFP), Discosoma red fluorescent protein (DsRed), and chicken *CXCR4* controlled by the cytomegalovirus (CMV) promoter. Constructs were inserted between 5'-terminal repeat (5'-TR) and 3'-TR *piggy*Bac transposon elements. The neomycin-resistant (Neo^R) gene was used as a selection marker. (B) RT-PCR analysis after transfection and selection of *piggy*Bac CMV-chicken CXCR4-overexpressing (OE) DF1 cells. (C) Fluorescent images of GFP or DsRed-expressing wild type and GFP-expressing *CXCR4* KO PGCs. All PGC sublines strongly expressed the fluorescent reporters (magnification, 100 ×). (D) GFP-expressing DF1 and DT40 cells after transfection and selection of the *piggy*Bac CMV-eGFP expression vector (magnification, 100 ×).

Discussion

In vertebrates, germ cells are a unique cell type that transfer genetic information to the next generation [1, 2]. Germ cells first appear outside of the embryonic gonads and localize to the developing genital ridges during early embryonic development. Many studies have been conducted to understand germ cell migratory behavior and guidance mechanisms. Transgenic mouse lines that express germ cell-specific fluorescent reporters, such as octamer-binding transcription factor 4 (Oct 4)-GFP and stella-GFP, have made it possible to detect and track migratory PGCs *in vivo* [4, 20].

In a previous study, Kang *et al*. described the patterns of the first migratory phase of chicken PGCs from the central region of the area pellucida toward the germinal crescent at HH Stage 4 [16]. Using GFP-expressing chicken PGCs and live-imaging, spatiotemporal migration patterns during primitive streak formation were examined. Chicken PGCs passively migrate from the central to the anterior region, then actively move toward the germinal crescent [16]. These results indicate that chicken PGCs use sequential passive and active forces to migrate toward the germinal crescent. In the second migratory phase, chicken PGCs penetrate the developing blood vessels and circulate until they finally settle down into the genital ridges [17, 18]. Motono *et al*. first reported expression of the *CXCR4* gene in chicken PGCs during migration [21]. The G-protein-coupled receptor CXCR4, also known as fusin or cluster of differentiation 184 (CD184), is an alpha-chemokine receptor specific to chemotactic SDF-1, also known as CXCL12 [8]. The CXCR4-SDF1 interaction is known to play a role in chemoattraction during the directed migration of several cell types, including immune and germ cells [7]. Quantitative RT-PCR revealed that *CXCR4* is relatively highly expressed in chicken PGCs in blood vessels at 2.5 days, then decreases after the circulating PGC stages [21]. Additionally, Stebler *et al*. reported that ectopic expression of SDF-1 causes chicken PGCs to accumulate at those embryonic sites,

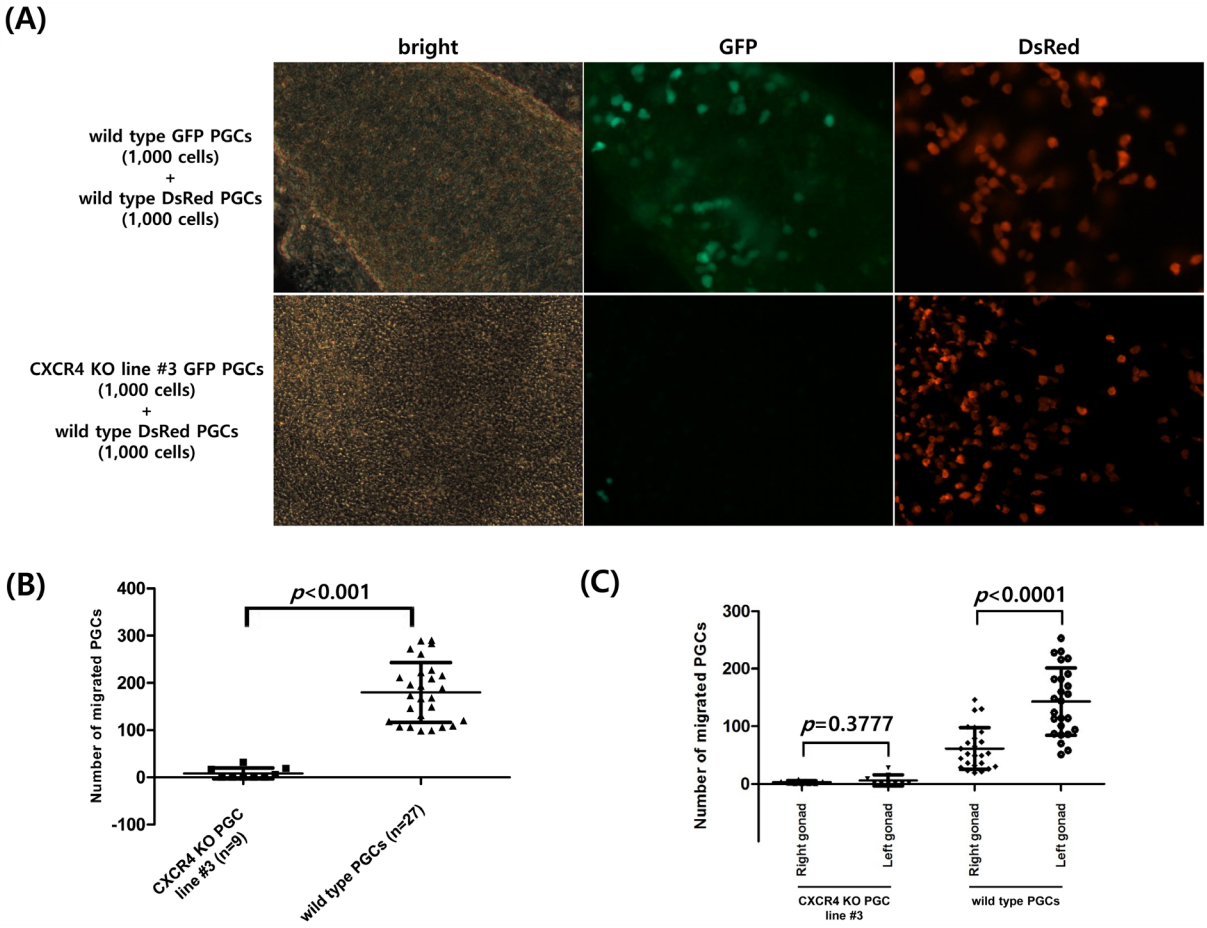


Fig. 4. *In vivo* migration assay of wild type and *CXCR4* KO PGCs after transplantation into recipient embryos. (A) Dissected embryonic gonads on Day 6 of incubation after transferring a combination of wild type and *CXCR4* KO PGCs. A combination of wild type GFP and DsRed PGCs was used as a control, and the migration capacity of GFP-expressing *CXCR4* KO PGCs was compared after co-injection with wild type DsRed-expressing PGCs. An equal number of cells (1,000: 1,000) was mixed and injected into the bloodstream of recipient embryos (magnification, 100 ×). (B) Comparison of total number of migrated PGCs in recipient embryonic gonads on Day 6 after transplantation of wild type PGCs or *CXCR4* KO PGC line #3 (number of recipient embryos; n = 27 and n = 9, respectively). Each dot indicates the sum of migrated PGC numbers in the right and left gonads of individual recipient embryos. (C) Comparison of the number of migrated PGCs in the right and left gonads of recipient embryos on Day 6. Each dot indicates the number of migrated PGCs in individual recipient embryonic right or left gonads.

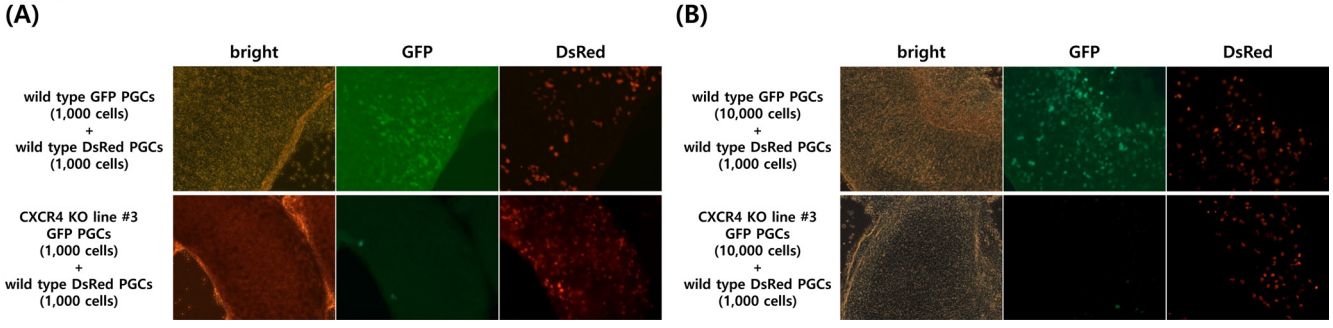


Fig. 5. Detection of wild type and *CXCR4* KO PGCs in 10-day old recipient embryonic gonads. (A) Embryonic gonads on Day 10 of incubation after transfer of wild type and *CXCR4* KO PGCs. A combination of wild type GFP and DsRed PGCs was used as a control, and migration capacity of GFP-expressing *CXCR4* KO PGCs was compared after co-injection with wild type DsRed-expressing PGCs. An equal number of cells (1,000: 1,000) was mixed and injected into the bloodstream of recipient embryos (magnification of second panel, 50 ×; all other panels, 100 ×). (B) Embryonic gonads on Day 6 of incubation after transferring a combination of *CXCR4* KO and wild type PGCs (10,000: 1,000) (magnification of middle panel, 50 ×; all other panels, 100 ×).

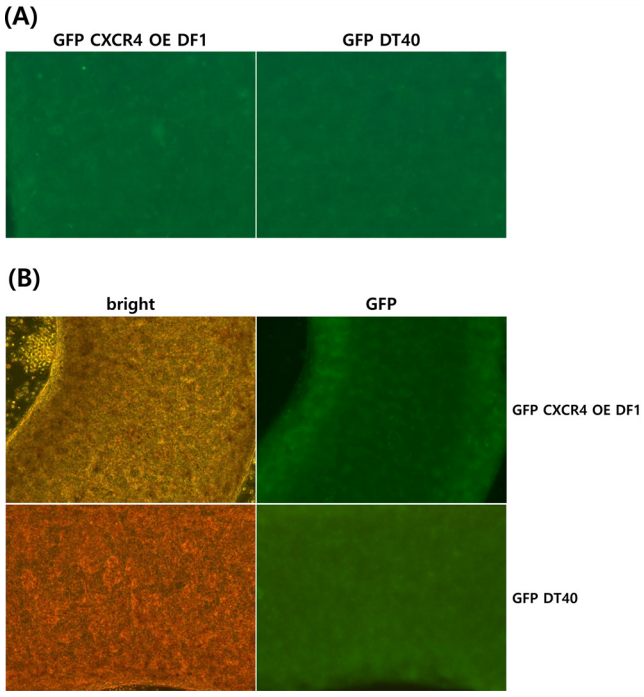


Fig. 6. *In vivo* migration assay of GFP-expressing DT40 and *CXCR4* OE DF1 cells. GFP-expressing DT40 or DF1 cells carrying the CMV-controlled chicken CXCR4-overexpressing transgene were transplanted into the bloodstream of recipient embryos. Embryonic gonads were dissected on Day 6 (A) and Day 10 (B). GFP-expressing cells were examined using a fluorescent microscope.

suggesting that SDF-1 controls the final migration steps [22]. Previous reports also suggest that gonadal PGCs have a lower efficiency of germline transmission than the high CXCR4-expressing circulating PGCs [23, 24]. However, the key factor(s) involved in chicken PGC migration have yet to be identified.

Current genomic editing technology has revolutionized biological research, and can be applied to agriculture and bio-industry. In this study, we generated *CXCR4* knockout chicken PGCs using CRISPR-Cas9 genomic editing, and used them to investigate functional migratory activity *in vivo*. Migratory capacity toward the developing gonads was significantly reduced in *CXCR4* KO PGCs, indicating that CXCR4 plays a critical role in the migration of chicken PGCs. However, no migration of CXCR4-expressing somatic, chicken DT40, and DF1 cells was observed. Therefore, inclusion of additional key factor(s) and/or receptor(s) for guiding PGCs along their migratory path will be necessary in future experiments. In addition, we cannot rule out variations in migratory capacity between single cell-derived PGC lines. In the future, the development of mutant PGC lines in germ cell-specific genes and an *in vitro* model system under a controlled environment will be necessary to fully investigate the regulatory mechanism(s) that underlie chicken PGC migration. In the current study, the number of migrated transplanted *CXCR4* KO PGCs was similar between the right and left gonads, which could be due to low numbers of migrated *CXCR4* KO PGCs into each gonadal ridge. However, the number of migrated transplanted wild type PGCs differed significantly between the right and left gonad in individual recipients. This asymmetric distribution of germ cells is consistent with previous reports [25–27], and a recent study from Hen *et al*. suggested that EMA1 epitope, which is expressed specifically in PGCs, could underlie this asymmetric migration between the right and left gonads [28]. The complete mechanisms that underlie PGC migration and their asymmetric distribution remain to be elucidated.

Acknowledgments

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