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PGC-Mediated Establishment of a Chicken Transgenic Line with Mendelian Inheritance and No Detectable Growth or Reproductive Effects

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Abstract

Background: Stable germline transmission is a central requirement for avian transgenesis, yet the inheritance behavior and organism-level consequences of a defined transgene integration remain insufficiently characterized in chickens. **Methods:** Here, we established a chicken transgenic line by introducing enhanced green fluorescent protein (eGFP) into cultured primordial germ cells (PGCs) using a lentiviral vector. The resulting eGFP-positive PGCs retained morphological and molecular characteristics consistent with cultured chicken PGCs and colonized recipient gonads after transplantation. We performed inheritance analysis across F_2 and F_3 generations, combined whole-genome resequencing, junction PCR, Sanger sequencing, and Southern blot to characterize transgene insertion sites and transmission stability. Growth and reproductive traits were compared between transgenic and wild-type chickens. **Results:** Donor-derived germline transmission was confirmed by recovery of eGFP-positive F_1 offspring. Inheritance analysis showed that the transgene segregated in accordance with Mendelian expectations in both F_2 (17/34 positive; $\chi^2 = 0.000$, $P = 1.000$) and F_3 (68/96 positive; $\chi^2 = 0.889$, $P = 0.346$) generations. Whole-genome resequencing, junction PCR, Sanger sequencing, and Southern blot analysis supported stable transmission of the same integration event across generations, with the insertion mapped to chromosome 10 in reverse orientation. Reporter expression was detectable from early embryonic stages and in multiple post-hatch tissues. No significant differences were detected between transgenic and wild-type birds in the measured growth or reproductive traits under the conditions tested. **Conclusions:** Together, these

findings establish a PGC-derived avian transgenic line in which a defined eGFP integration showed stable multigenerational inheritance without detectable adverse effects on the measured phenotypes. This study provides a tractable model for examining germline inheritance and organism-level consequences of transgene integration in birds.

Keywords: primordial germ cells; transgenesis; germline transmission; Mendelian inheritance; growth and reproductive traits

Introduction

Avian species represent a major component of vertebrate biodiversity and remain important to global agriculture [1, 2]. In birds, germline engineering has value not only for developing genetically modified lines, but also for addressing the more fundamental question of whether an introduced genomic variant can be predictably transmitted across generations without detectable organism-level costs. This question is relevant to both genotype-to-phenotype studies and avian germplasm manipulation, because the broader utility of any germline modification strategy depends on stable inheritance and preservation of reproductive competence [3].

Primordial germ cells (PGCs) provide a particularly suitable platform for addressing this question in birds. Chicken PGCs originate early in embryogenesis, migrate through the circulation, and ultimately colonize the developing gonads, where they give rise to functional gametes [4, 5]. Because PGCs can be isolated, cultured, genetically manipulated *in vitro*, and reintroduced into recipient embryos, they have become the principal cellular vehicle for avian germline modification [6–8]. This combination of developmental potential and experimental accessibility makes PGCs a tractable system for evaluating the inheritance behavior of engineered genomic insertions.

Several strategies have been developed to introduce exogenous sequences into chicken PGCs. Liposome-mediated transfection, electroporation, transposon-based systems, and lentiviral vectors have all been used to deliver foreign genes, and some studies have further demonstrated germline transmission after transplantation into

recipient embryos [9–13]. Among these methods, lentiviral vectors offer several practical advantages, including efficient genomic integration and stable long-term expression in transduced cells [9, 14, 15]. However, although previous studies have established the feasibility of avian PGC transgenesis, fewer studies have systematically examined within the same transgenic line the identity of the integration event, its multigenerational inheritance pattern, and its organism-level phenotypic consequences.

To address this gap, we established a chicken transgenic line using lentivirus-transduced PGCs carrying enhanced green fluorescent protein (*eGFP*). In this study, *eGFP* was used as a visually tractable reporter rather than a trait-improving transgene, allowing us to examine the inheritance behavior and phenotypic consequences of a defined exogenous insertion without confounding effects from a specific production-related gene. We therefore asked whether an *eGFP*-marked integration event could be stably transmitted through the germline across generations, whether its segregation followed Mendelian expectations, and whether it was associated with detectable effects on growth or reproductive performance. By combining genomic validation with multigenerational phenotypic evaluation, this study provides an avian model for examining stable germline inheritance and the organism-level consequences of transgene integration.

Materials and Methods

Ethics statement

Fresh, fertilized, unincubated eggs of Shouguang chickens and White Plymouth Rock (WPR) chickens were obtained from the Poultry Science Institute, Chinese Academy of Agricultural Sciences. All animal care and experimental procedures were conducted in accordance with the National Institutes of Health guidelines (NIH Pub. No. 85-23, revised 1996) and were approved by the Experimental Animal Management and Ethical Committee of Yangzhou University, China.

Isolation and culture of gonadal PGCs

Chicken PGC culture medium was prepared essentially as described previously [16, 17]. FAcs medium was prepared by diluting calcium-free DMEM (21068028, Gibco, USA) with sterile water at a 3:1 ratio and supplementing it with 0.15 mmol/L CaCl2 (C7902, Sigma, Germany), 1× non-essential amino acids (11140050, Gibco), 0.1 mmol/L β-mercaptoethanol (31350010, Gibco), 1× B-27 supplement (17504044, Gibco), 0.1 mg/mL sodium heparin (HY-17567A, MCE, USA), 25 ng/mL human Activin A (HY-P70311, MCE), 0.2% chicken serum (16110082, Gibco), 1× nucleosides (E8-008-D, Sigma, Germany), 2 mmol/L GlutaMax (35050061, Gibco), 1 mmol/L sodium pyruvate (11360070, Sigma), 0.20% ovalbumin (A5503, Sigma), and 4 ng/mL human FGF2 (HY-P70600, MCE).

Fertilized Shouguang chicken eggs were incubated at 37.8 °C for 5.5 days to obtain HH stages 27-28 embryos. Embryonic gonads were dissected, washed in PBS (P1020, Solarbio, China) containing 0.1% BSA (A8020, Solarbio), and dissociated in 0.25%

trypsin-EDTA (25200056, Gibco) for 1 min with gentle trituration. Digestion was terminated with DMEM containing 5% FBS (A5670701, Gibco), and the cells were collected by centrifugation. The cell pellet was resuspended in FAcs medium and seeded into 48-well plates. Half of the medium was replaced every 3 days, and expanding cultures were transferred to larger dishes for continued propagation.

Lentiviral infection of PGCs and enrichment through drug selection

Lentiviral transduction of PGCs was performed using a protocol adapted from our previous GFP-PGC work [18], with the vector, viral titer, and selection conditions used in the present study specified below.

The recombinant lentiviral vector pLenti-CMV-CBH-eGFP-2A-Puro-WPRE was co-transfected with the packaging plasmids psPAX2 and pMD2.G into 293T cells using Lipofectamine 3000 reagent (Invitrogen, USA) at a molar ratio of 3:2:1. Transfected cells were cultured in DMEM supplemented with 10% FBS for 48-72 h. Viral supernatants were collected, passed through a 0.45 μm filter, and concentrated by ultracentrifugation at 25,000 rpm for 2 h at 4 °C. The viral pellet was resuspended in PBS, and viral titer was determined by TCID50 assay on HEK293T cells, yielding a final titer of 1.2×10^8 TU/mL.

For transduction, 1 × 105 PGCs were seeded into 24-well plates and cultured to logarithmic growth phase. Polybrene (sc-134220, Santa Cruz Biotechnology, USA) was added to a final concentration of 10 μg/mL, followed by addition of lentiviral particles to achieve a multiplicity of infection (MOI) of 40. After 12 h, the viral medium was replaced with fresh culture medium. Green fluorescence was examined 72 h after

transduction. Positive cells were enriched by culture in medium containing 0.3 μg/mL puromycin (P8230, Solarbio) for 24 h, and the surviving eGFP-positive PGCs were subsequently expanded in fresh culture medium.

Periodic Acid-Schiff (PAS) staining

PAS staining was performed essentially as previously described [18], following the manufacturer's instructions. Briefly, eGFP-PGCs were fixed in 4% paraformaldehyde, mounted on glass slides, and stained using a PAS staining kit (G1360, Solarbio). After oxidation and Schiff staining, the slides were washed, mounted with neutral resin (G8590, Solarbio), and examined microscopically.

RNA extraction and reverse transcription

Total RNA extraction and reverse transcription were carried out essentially as in our previous studies [19], with sample types and target genes adjusted for the present work.

eGFP-PGCs and chicken embryonic fibroblasts (CEFs) were collected separately (1×10^6 cells per sample). Total RNA was extracted using TRIzol reagent (R411-01, Vazyme, China) according to the manufacturer's protocol. RNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA was synthesized using the HiScript III RT SuperMix for qPCR (+gDNA wiper) kit (R323-01, Vazyme) according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed as previously described [19], with primer sets specific to the present study listed in Table 1. Reactions were carried out using ChamQ Universal

SYBR qPCR Master Mix (Q711, Vazyme) in a total volume of 20 μL containing 10 μL 2× master mix, 2 μL cDNA, 0.4 μL each of forward and reverse primers, and DNase/RNase-free water. Amplification conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 15 s, with a final extension at 72 °C for 5 min. Target genes included *CXCR4*, *DAZL*, *CVH*, *POUV*, *SOX2*, and *NANOG*, and *GAPDH* was used as the reference gene. Relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Immunofluorescence and confocal microscopy

Immunofluorescence staining was performed largely as previously described [18]. Briefly, eGFP-PGCs were fixed on slides with 4% paraformaldehyde, permeabilized with PBS containing 0.5% Triton X-100, and blocked with PBS containing 1.0% BSA. The cells were incubated overnight at 4 °C with primary antibodies against SSEA-1 (1:100; MC-480, DSHB, USA), EMA-1 (1:100; DSHB, USA), and CVH (1:100; orb5967, Biorbyt, UK). After washing, the cells were incubated with Alexa Fluor-conjugated secondary antibodies, counterstained with DAPI (C0065, Solarbio), and mounted with antifade medium (S2100, Solarbio). Fluorescence images were acquired using a Zeiss LSM 710 Meta confocal microscope (Germany).

DNA extraction, PCR, agarose gel electrophoresis, and Sanger sequencing

Genomic DNA of eGFP-PGCs and blood from offspring chickens was extracted using the TIANamp Genomic DNA Kit (YDP304, Tiangen, China) according to the manufacturer's protocol. PCR amplification was performed using PrimeSTAR Max DNA Polymerase (R045Q, Takara, Japan). The amplification program consisted of

98 °C for 5 min, followed by 35 cycles of 98 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. Target amplicons included eGFP, KI-1, and KI-2 (primer sequences listed in Table 1). PCR products were separated on 1.5% agarose gels, stained with RedSafe nucleic acid stain (21141, INtRON Biotechnology, South Korea), and visualized under UV illumination.

For junction validation, the KI-1 and KI-2 amplicons were purified using the Universal DNA Purification Kit (DP214, Tiangen, China), ligated into the pUC57 Simple TA/Blunt-end Cloning Vector (D2401, HaiGene, China), transformed into Trelief 5α chemically competent cells (DLC101, Tsingke Biotechnology, China), and sequenced by Sanger sequencing at Tsingke Biotechnology (China).

Detection of PGC migration

Detection of PGC migration after dorsal aorta injection was carried out essentially as previously described [19], with donor cell concentration and observation time specified below.

Recipient WPR embryos were incubated to HH stages 13-16. eGFP-PGCs were resuspended at 3000 cells/μL, and 1 μL of the suspension was microinjected into the dorsal aorta under a stereomicroscope (Leica, Germany). After injection, the shell opening was treated with penicillin-streptomycin, sealed with sterile breathable tape, and the eggs were returned to the incubator. At 7.5 days of incubation, a subset of embryos was dissected and examined under fluorescence microscopy to assess migration of eGFP-positive cells to the gonads. The remaining embryos were incubated to hatching and raised to sexual maturity.

Detection of *eGFP*-positive sperm ratio in F_0 recipient roosters

To estimate donor-derived germline contribution in F_0 recipient males, semen was collected by manual ejaculation from sexually mature chimeric roosters. Genomic DNA was extracted from semen samples and subjected to PCR using eGFP-specific primers to confirm the presence of the transgene. For quantitative analysis, semen samples from F_0 recipient males and wild-type controls were washed three times with PBS and analyzed by flow cytometry using an LSRFortessa cytometer (BD Biosciences, USA). The proportion of eGFP-positive sperm was calculated using FSC and FITC channels in FlowJo software (v10.8.1).

Production and mating of transgenic chickens

Putative chimeric males and females were artificially inseminated with WPR chickens. Hatched F_1 chicks were screened for eGFP expression by fluorescence observation. eGFP-positive F_1 females were crossed with recessive white WPR males to generate F_2 offspring. eGFP-positive F_2 chickens were raised to sexual maturity and intercrossed to generate F_3 offspring. The proportion of eGFP-positive chickens in the F_2 and F_3 generations was determined by fluorescence observation.

Observation of chicken embryos and tissue dissection of chicks

F_2 fertilized eggs were collected and examined for eGFP expression at 0, 15, 24, and 180 h of incubation under a stereomicroscope (Leica, Germany). eGFP-positive and wild-type F_2 chicks were also examined at 15 days post-hatch. Tissues including skeletal muscle, feather follicles, bone, liver, heart, gastrointestinal tract, pancreas, lung, spleen, kidney, and ovary were dissected under a stereomicroscope, washed with PBS,

and examined for eGFP fluorescence.

Growth performance analysis of transgenic chickens

For the F_2 generation, six female and six male eGFP-positive chickens were randomly selected, and six females and six males from the wild-type group were used as controls. All birds were reared to 6 months of age. Body weight was measured individually using an electronic scale (precision 0.1 g). Body size traits, including body oblique length, keel bone length, breast width, breast circumference, breast depth, shank length, and shank circumference, were measured using a vernier caliper (precision 0.1 mm) and flexible measuring tape (precision 1 mm).

For the F_3 generation, 15 female and 15 male chickens were randomly selected from each of the eGFP-positive and wild-type groups at hatching. Body weight was recorded weekly for 7 consecutive weeks. The same body size traits were measured at the corresponding time points using the instruments described above.

Routine semen parameter analysis

For semen analysis, six-month-old F_2 eGFP-positive roosters and age-matched wild-type roosters were used, with three birds per group. All roosters were healthy, free of reproductive disease, and maintained under identical housing conditions. Semen was collected once from each rooster by abdominal massage and used immediately for analysis.

For fluorescence detection, semen was transferred to 1.5 mL tubes and examined under a handheld blue-light excitation source (Model 3410, LUYOR, USA). Semen volume was recorded directly using a graduated collection tube. Sperm concentration

was determined by diluting semen 200-fold and counting sperm using a hemocytometer (Model BE6138, Shanghai Jingqing, China). Sperm motility was measured using a MAILANG automatic sperm analyzer (Model ML-608JZ, China) based on images from five random microscopic fields per sample, with at least 200 sperm counted. Sperm abnormality rate was determined on Giemsa-stained smears by counting at least 200 sperm across five random fields. Total motile sperm (TMS) was calculated as semen volume (mL) × sperm concentration (×109/mL) × sperm motility (%).

High-throughput sequencing

Blood samples from eGFP-positive F_1 offspring were collected for whole-genome sequencing. Library preparation and sequencing were performed by GENE DENOVO Ltd. (Guangzhou, China). Libraries were constructed using the TruSeq Nano DNA LT Sample Preparation Kit (Illumina, USA). Genomic DNA was fragmented using a Covaris S220 ultrasonicator (Covaris, USA) to approximately 500 bp, followed by adapter ligation, amplification, purification, and sequencing on the Illumina HiSeq X Ten platform to generate paired-end reads. Raw reads were quality-filtered using fastp (v0.19.5), and clean reads were aligned to the chicken reference genome (GCF_000002315.5) using BWA (v0.7.12).

Southern blot

Southern blot analysis was performed using a DIG-labeled probe targeting the CMV promoter fragment. Genomic DNA from F_1-F_3 transgenic chickens was digested with HindIII (1060B, Takara), separated on 0.7% agarose gels, and transferred to nylon membranes (RPN303B, Amersham, UK) by capillary transfer. Membranes were

hybridized overnight at 37 °C with the denatured DIG-labeled probe in Hyb-100 buffer, washed with SSC buffer, and detected using anti-DIG-AP antibody (11093274910, Roche) and CSPD substrate, followed by chemiluminescence imaging.

Statistical analysis

Statistical analysis was performed using SPSS version 19 (SPSS Inc., Chicago, IL, USA). Group comparisons for growth performance, body size traits, and semen parameters were conducted using Student's t-test. Percentage data were arcsine-transformed before analysis when appropriate. Results are presented as mean ± SEM, and $P < 0.05$ was considered statistically significant. Segregation ratios in F_2 and F_3 offspring were evaluated using Pearson's chi-square test against the expected Mendelian ratios.

Results

Establishment and characterization of a germline-competent *eGFP*-PGC line

PGCs were successfully isolated from the gonads of HH stage 27 chicken embryos. After 45 days of *in vitro* culture, the cells expanded to more than 1×10^6 while maintaining typical PGC morphology, including a round shape and a high nuclear-to-cytoplasmic ratio (Figure. 1A), indicating successful establishment of a stable cultured PGC line. To generate *eGFP*-expressing PGCs, the *Aequorea victoria eGFP* coding sequence was inserted into the pLenti-CMV-CBH-*eGFP*-2A-Puro-WPRE vector (Figure. 1B). Following lentiviral transduction and puromycin selection, stable *eGFP*-positive PGCs were obtained, with at least 95% of cells showing fluorescence (Figure. 1C). PCR further confirmed the presence of the *eGFP*-specific fragment in the genome of the transduced cells (Figure. 1D).

To determine whether these cells retained key biological features of chicken PGCs after transduction, we examined their molecular and cytological characteristics. PAS staining showed positive glycogen accumulation (Figure. 1E). qRT-PCR detected strong expression of the germ cell markers CVH and DAZL, the pluripotency-associated genes POUV, SOX2, and NANOG, and the migration-associated gene CXCR4, all of which were significantly higher than in chicken embryonic fibroblasts (CEFs), which showed little or no expression of these markers ($P < 0.0001$; Figure. 1F). Immunofluorescence further confirmed expression of CVH, SSEA-1, and EMA-1 at the protein level (Figure. 1G). Together, these data indicated that the eGFP-positive cells retained characteristics consistent with cultured chicken PGCs and remained

297 suitable for germline transplantation.

298 **Donor PGCs contributed to the germ line and generated transgenic offspring**

299 To evaluate the developmental potential of the transduced cells in vivo,
300 approximately 3000 eGFP-PGCs were injected into the dorsal aorta of HH13-16
301 recipient embryos. At HH32, eGFP-positive cells were detected in the gonads of
302 recipient embryos, with a gonadal colonization rate of at least 80% (Figure. 2A),
303 indicating that the transplanted cells retained migratory and gonadal colonization ability.

304 After the recipient males reached sexual maturity, flow cytometric analysis of
305 semen from F_0 chimeras showed that eGFP-positive sperm accounted for 1.16% of the
306 total sperm population (Figure. 2B), and fluorescence microscopy confirmed eGFP
307 expression in the sorted sperm fraction (Figure. 2C). Testcrossing of F_0 chimeric males
308 with recessive White Plymouth Rock chickens yielded eGFP-positive F_1 offspring
309 (Figure. 2D; Table 2), demonstrating that the donor-derived eGFP-PGCs had entered
310 the germ line and achieved germline transmission.

311 **The *eGFP* integration was stably inherited across generations**

312 To examine inheritance of the transgene after establishment of the transmitting
313 line, eGFP-positive F_1 females were crossed with White Plymouth Rock males,
314 producing 34 F_2 offspring, of which 17 were eGFP-positive (Figure. S1A). This
315 segregation pattern did not deviate from the expected 1:1 Mendelian ratio for a single
316 heterozygous locus ($\chi^2 = 0.000$, df = 1, P = 1.000). Intercrossing of eGFP-positive F_2
317 birds subsequently generated 96 F_3 offspring, of which 68 were eGFP-positive (Figure.
318 S1B). This result did not deviate significantly from the expected 3:1 Mendelian ratio

(χ^2 = 0.889, df = 1, P = 0.346). These data support Mendelian inheritance of the transgene as a single locus across generations.

Whole-genome resequencing of eGFP-positive F_1 females identified the integration site on chromosome 10 between nucleotides 6,528,773 and 6,528,774, with the exogenous sequence inserted in reverse orientation (Figure. 3A). PCR amplification across the junctions using flanking primers (KI-1 and KI-2) generated products of the expected sizes (Figure. 3B), and the amplified fragments were confirmed by Sanger sequencing (Figure. 3C). Southern blot analysis of eGFP-positive birds from the F_1 to the F_3 generations showed consistent hybridization patterns (Figure. 3D), supporting stable transmission of the same integration event across generations.

In addition to genetic transmission, reporter expression was detectable throughout development. eGFP fluorescence was observed from the embryonic disc stage (EGK.X) and remained detectable during gastrulation (HH3-HH4) and later organogenesis, including in the mesonephroi and gonads at HH32, whereas no fluorescence was detected in wild-type controls (Figure. 4A-C). In 15-day-old post-hatch F_2 chickens, eGFP expression was detected in multiple tissues, including skeletal muscle, feather follicles, liver, heart, gastrointestinal tract, pancreas, lung, spleen, kidney, and ovary (Figure. 4D). Together, these findings showed that the defined eGFP integration was functionally expressed and stably inherited over multiple generations.

The transgene produced reporter expression without detectable adverse effects on measured growth or reproduction

To assess whether the eGFP integration was associated with detectable effects on

341 growth-related traits, eGFP-positive F_2 males and females were randomly selected and
342 raised to 6 months of age. No significant differences were observed between transgenic
343 and wild-type birds in body weight or body size measurements, including body oblique
344 length, keel bone length, breast width, breast circumference, breast depth, shank length,
345 and shank circumference ($P > 0.05$; Figure. 5A-B). Similarly, dynamic monitoring of
346 body weight and body size in eGFP-positive F_3 males and females up to 7 weeks of age
347 showed no significant differences relative to wild-type controls ($P > 0.05$; Figure. 5C-
348 J).

349 We next evaluated reproductive traits in the transgenic line. Semen collected from
350 eGFP-positive F_2 males showed green fluorescence, and sperm volume, sperm density,
351 sperm motility, abnormal sperm rate, and total motile sperm count did not differ
352 significantly from those of wild-type males (Figure. 6A-I). Likewise, egg production,
353 fertility, and hatchability of eGFP-positive F_2 females were comparable to those of
354 wild-type controls (Figure. 6J-L). Under the conditions tested, these results indicated
355 that the defined eGFP integration was not associated with detectable adverse effects on
356 the measured growth or reproductive traits.

Discussion

In this study, we established a chicken transgenic line by lentiviral transduction of cultured PGCs and showed that the resulting eGFP-marked integration event could be transmitted through the germ line across multiple generations (Figure. 7). The transgene was detected in F_1, F_2, and F_3 offspring, and its segregation patterns in F_2 and F_3 were consistent with Mendelian expectations for a single heterozygous locus. In addition, whole-genome resequencing, junction PCR, Sanger sequencing, and Southern blot analysis supported stable transmission of the same integration event across generations. Importantly, within the growth and reproductive traits examined in this study, no detectable adverse effects were observed in the transgenic line. Together, these findings support the use of cultured chicken PGCs as a practical platform for generating avian transgenic lines with stable germline transmission.

Efficient germline contribution of donor-derived PGCs is a key determinant of success in PGC-mediated transgenesis. In the present study, eGFP-positive donor cells successfully colonized recipient gonads, but only 1.16% of sperm from F_0 chimeric males were eGFP-positive. This relatively low contribution likely reflects competition between donor-derived PGCs and endogenous recipient germ cells in embryos that retained their native germ line, as reported previously in avian transplantation studies [20, 21]. Nevertheless, the recovery of eGFP-positive F_1 offspring demonstrated that donor-derived PGCs were able to complete gametogenesis and achieve germline transmission. Thus, although founder-level germline contribution was limited, establishment of a transmitting line enabled subsequent inheritance analyses at the

organismal level.

A major strength of this study is that the transgene was not only transmitted, but also genetically tracked as a defined integration event. The F_2 and F_3 segregation patterns were consistent with inheritance of a single locus, and this interpretation was further supported by stable Southern blot patterns and identical junction validation across generations. Whole-genome resequencing localized the insertion to chromosome 10, where the exogenous sequence was integrated in reverse orientation. The long-term stability of this event may reflect, at least in part, features of the lentiviral system used here. Compared with some retroviral systems in which transgene silencing has been reported in chicken embryos, lentiviral vectors can support more stable genomic integration and long-term expression [22, 23]. In addition, the insertion site identified in this study was located in a noncoding genomic region, which may have reduced direct disruption of nearby genes. However, the regulatory basis of the stable expression and inheritance observed here was not directly examined, and additional work would be required to determine whether local chromatin state, DNA methylation, or other epigenetic factors contributed to transgene stability.

The present study also addresses a more basic genotype-to-phenotype question in avian transgenesis by examining whether a defined inherited genomic insertion is associated with detectable organism-level consequences. Here, eGFP was used as a reporter transgene rather than a trait-improving gene, which allowed us to evaluate the biological consequences of the integration event itself with relatively limited confounding from a specific production-related function. eGFP expression was readily

detectable during embryonic development and in multiple post-hatch tissues, indicating functional expression of the inserted sequence. At the same time, no significant differences were detected between transgenic and wild-type birds in the measured growth traits, body size parameters, semen quality traits, egg production, fertility, or hatchability. These findings suggest that, within the traits and sample sizes examined here, the defined eGFP integration was not associated with detectable adverse effects on growth or reproductive performance. At the same time, our phenotypic evaluation was limited to the measured traits in this study, and broader physiological or molecular effects were not assessed.

The broader significance of these findings lies in their relevance to future avian germline engineering. From a conservation perspective, the results show that genetically manipulated PGCs can generate transmitting and reproductively competent offspring, which is relevant to future efforts in avian germplasm preservation and surrogate-host-based genetic resource management [3, 24]. From a more general genotype-to-phenotype perspective, this transgenic line provides a tractable model in which a defined genomic insertion can be followed across generations and evaluated at the organismal level. Similar questions have also been examined in other transgenic animal systems, including livestock species in which stable inheritance and the physiological or reproductive consequences of transgene integration have been assessed across generations. For example, studies in sheep and cattle have reported stable transmission or long-term phenotypic stability together with reproductive, physiological, or health-related evaluations, indicating that biological assessment

beyond simple transgene transmission is important in large-animal transgenesis [25, 26]. In this context, the present study extends such evidence to a PGC-mediated avian system, in which a defined integration event could be followed together with measured growth and reproductive traits. In addition, although eGFP itself is not an agricultural trait gene, the framework established here may be useful for future studies aimed at introducing and evaluating agronomically relevant alleles in poultry. Overall, this study provides a practical avian model for linking a defined transgene integration event to multigenerational inheritance and measured organism-level phenotypes.

Conclusion

In summary, this study established a chicken transgenic line derived from cultured PGCs in which a defined eGFP integration was transmitted through the germ line across three generations. The transgene behaved as a single Mendelian locus and was not associated with detectable adverse effects on the measured growth or reproductive traits under the conditions tested. These findings provide an avian model for studying stable germline inheritance and the organism-level consequences of transgene integration, and may inform future work on avian germline engineering and genetic resource manipulation.

Declarations

Ethics Approval and Consent to Participate

All experimental procedures were approved by the Experimental Animal Ethics Committee of Yangzhou University.

Consent for Publication

The manuscript has been given publication permission by all authors.Not applicable.

Availability of data and materials

The data that support the findings of this study are obtainable from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Guangzheng Liu: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. Nan Zhang: Investigation, Project administration, Software. Yixin Diao: Investigation, Project administration, Software. Gaozhan Yuan: Investigation, Project administration. Jun Wu: Investigation, Project administration, Software. Mingyang Zou: Investigation, Software. Xinrui Wang: Project administration, Software. Kai Jin: Supervision, Validation, Visualization. Qisheng Zuo: Supervision, Validation. Guohong Chen: Validation, Visualization. Ying-Jie Niu: Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. Bichun Li: Funding acquisition, Investigation, Resources, Writing – review & editing.

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552 **Table 1** PCR primer sets.

Gnen	Sequence	Annealing Temp.	Product Size (bp)	Reference
CVH F	CAGACCGCATGCTTGATATG	60°C	135	[17]
CVH R	CAGCCAGCCTCTGAACTTCT			
DAZL F	TCACTGACAGGACTGGTGTTTC	60°C	127	[17]
DAZL R	ATTGCTGGTCCCAGTTTCAG			
POUV F	GTTGTCCGGGTCTGGTTCT	60°C	189	[17]
POUV R	GTGGAAAGGTGGCATGTAGAC			
SOX2 F	GTGAACCAGAGGATGGACAGTTACG	60°C	185	[16]
SOX2 R	TGCGAGCTGGTCATGGAGTTG			
NANOG F	GGTTTCAGAACCAACGGATG	60°C	122	[17]
NANOG R	GTGGGGGGTCATATCCAGGTA			
CXCR4 F	GCCATTCTGGTCTGTGGATG	60°C	151	
CXCR4 R	GGCATGGACTATTGCCAGGT			
GAPDH F	GAGGGTAGTGAAGGCTGCTG	60°C	113	[17]
GAPDH R	CATCAAAGGTGGAGGAATGG			
eGFP F	CTCTGACTGACCGCGTTACT	60°C	740	
eGFP R	CATGTGATCGCGCTTCTCG			
KI-1 F	CCTTTCCTAATTAGTTTTTAATAGAC	52°C	317	
KI-1 R	CTGGGAGCTCTCTGGCTAA			
KI-2 F	GGGTCTGAGGGATCTCTA	52°C	310	
KI-2 R	CCAAACTGGATCTTTTCTTTC			
CMV1 F	AAGTCCCGTTGATTTTGGTG	55°C	360	
CMV1 R	CAATAATGACGTATGTTCCC			

Table 2 Production of germline chimeric chickens and test mating.

PGC lines			No. of recipients incubated (Sex)	No. of $eGFP^+$ recipient roosters in semen (%)	No. of offspring chickens	No. of recipients producing $eGFP^+$ offspring (%)	No. of $eGFP^+$ offspring (%) *
Breed	Sex	Days in culture					
Shouguang Chicken	male	106	8 (Male)	1 (12.5)	37	1 (12.5)	1 (3.3)
			7 (Female)	-	134	-	-

-, not detected. *, Number and proportion of $eGFP^+$ offspring produced by each germline-transmitting recipient chicken.

List of Figure Captions

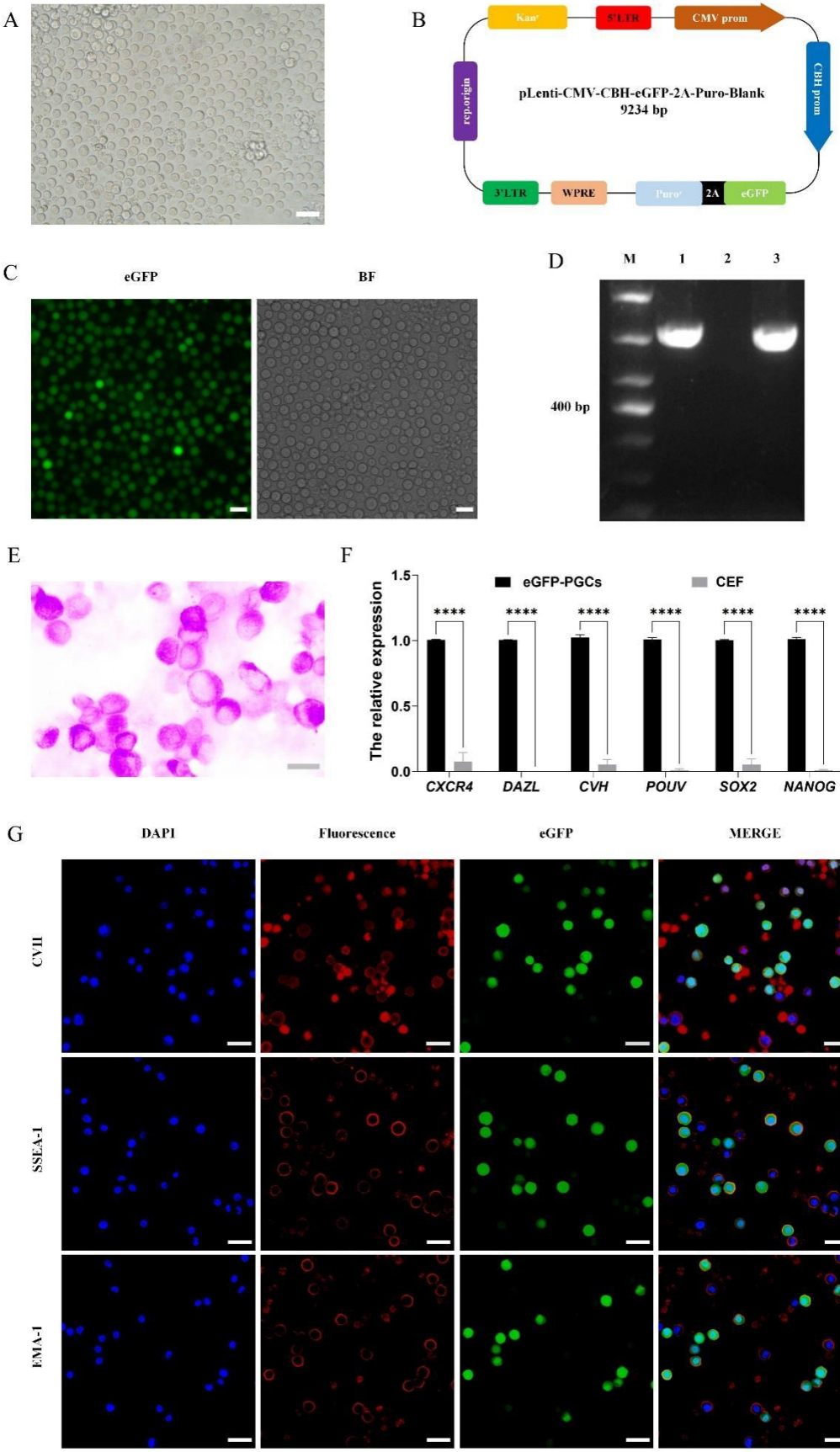


Figure 1. Isolation, transgenesis, and characterization of chicken PGCs. (A) Morphology of gonadal PGCs isolated from HH stage 27 chicken embryos after establishment of the cell line. Scale bar = 60 μm. (B) Schematic diagram of the pLenti-CMV-CBH-*eGFP*-2A-Puro-WPRE lentiviral vector. (C) Stable *eGFP*-expressing PGCs after lentiviral transduction, puromycin selection, and subsequent expansion. Scale bar = 50 μm. (D) PCR detection of *eGFP* integration. M, DNA marker; lane 1, *eGFP*-PGCs; lane 2, wild-type PGCs (negative control); lane 3, vector plasmid (positive control). (E) PAS staining of *eGFP*-PGCs. Scale bar = 25 μm. (F) qRT-PCR analysis of specific gene expression in *eGFP*-PGCs, with CEFs as the negative control. **** $P < 0.0001$. (G) Immunofluorescence analysis of *eGFP*-PGCs. Scale bar = 20 μm.

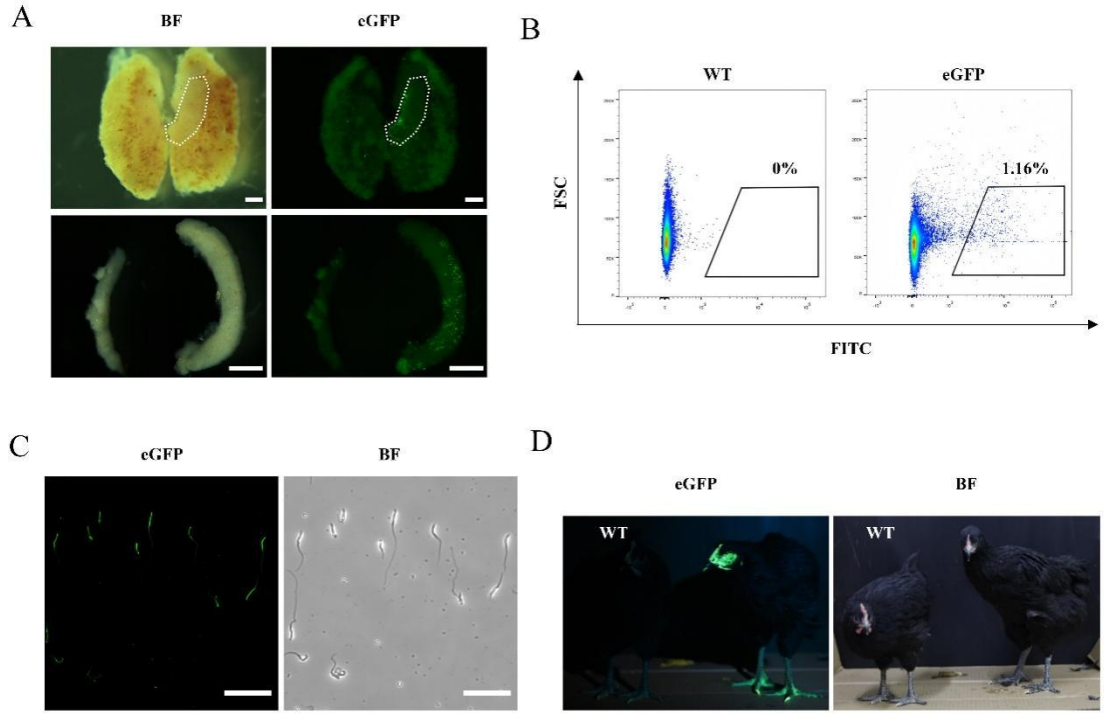


Figure 2. Generation of transgenic chickens and assessment of germline transmission efficiency. (A) Colonization of *eGFP*-PGCs in recipient embryonic gonads. Upper

panel, mesonephros and gonad, with the gonad outlined by a dashed line; lower panel, isolated gonad. Scale bar = 500 μm. (B) Flow cytometric analysis of the proportion of *eGFP*$^{+}$ sperm in semen from chimeric roosters. (C) Fluorescence microscopy of sorted *eGFP*$^{+}$ sperm. Scale bar = 50 μm. (D) Fluorescence observation of a 2-month-old F_1 *eGFP*-positive female chicken. WT, 2-month-old wild-type chicken.

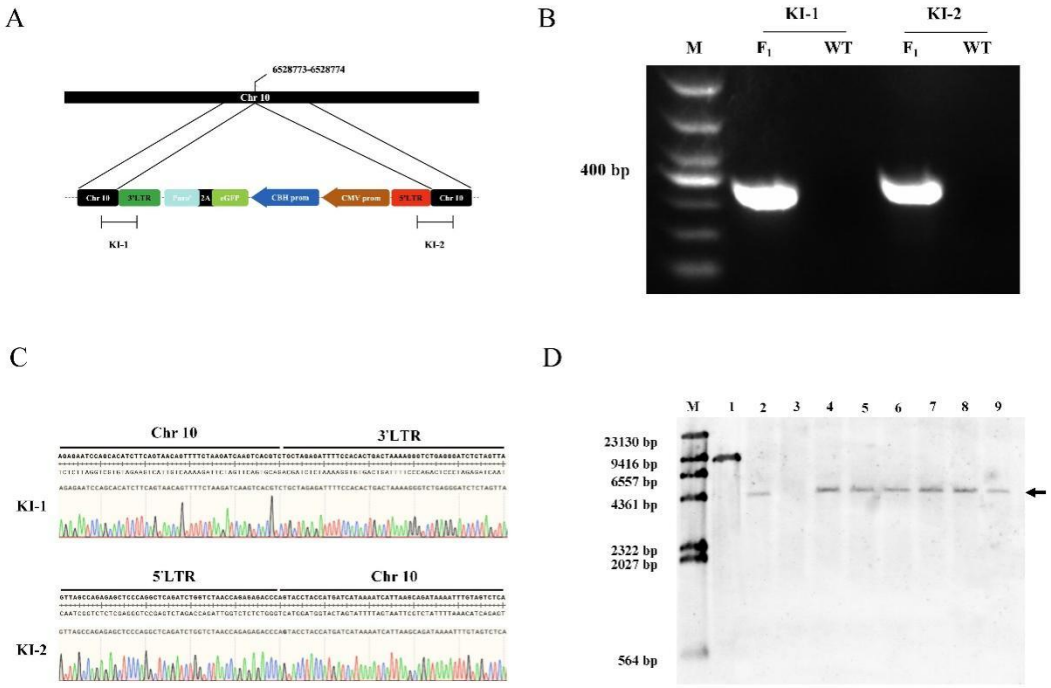


Figure 3. Identification of the *eGFP* integration site and validation of genetic stability. (A) Schematic diagram of reverse integration of the lentiviral sequence into chicken chromosome 10 (positions 6,528,773–6,528,774) based on whole-genome sequencing (GRCg7b assembly). (B) PCR amplification and agarose gel electrophoresis of KI-1 and KI-2 fragments flanking the integration site. M, DNA marker. Primer positions are indicated in panel A. (C) Sanger sequencing validation of the integration site. (D) Southern blot analysis of F_1–F_3 *eGFP*-positive chickens after HindIII digestion using a

CMV probe. Lane 1, vector plasmid; lane 2, F_1 *eGFP*-positive female; lane 3, wild-type chicken; lanes 4–6, F_2 *eGFP*-positive chickens; lanes 7–9, F_3 *eGFP*-positive chickens; M, DNA molecular-weight marker.

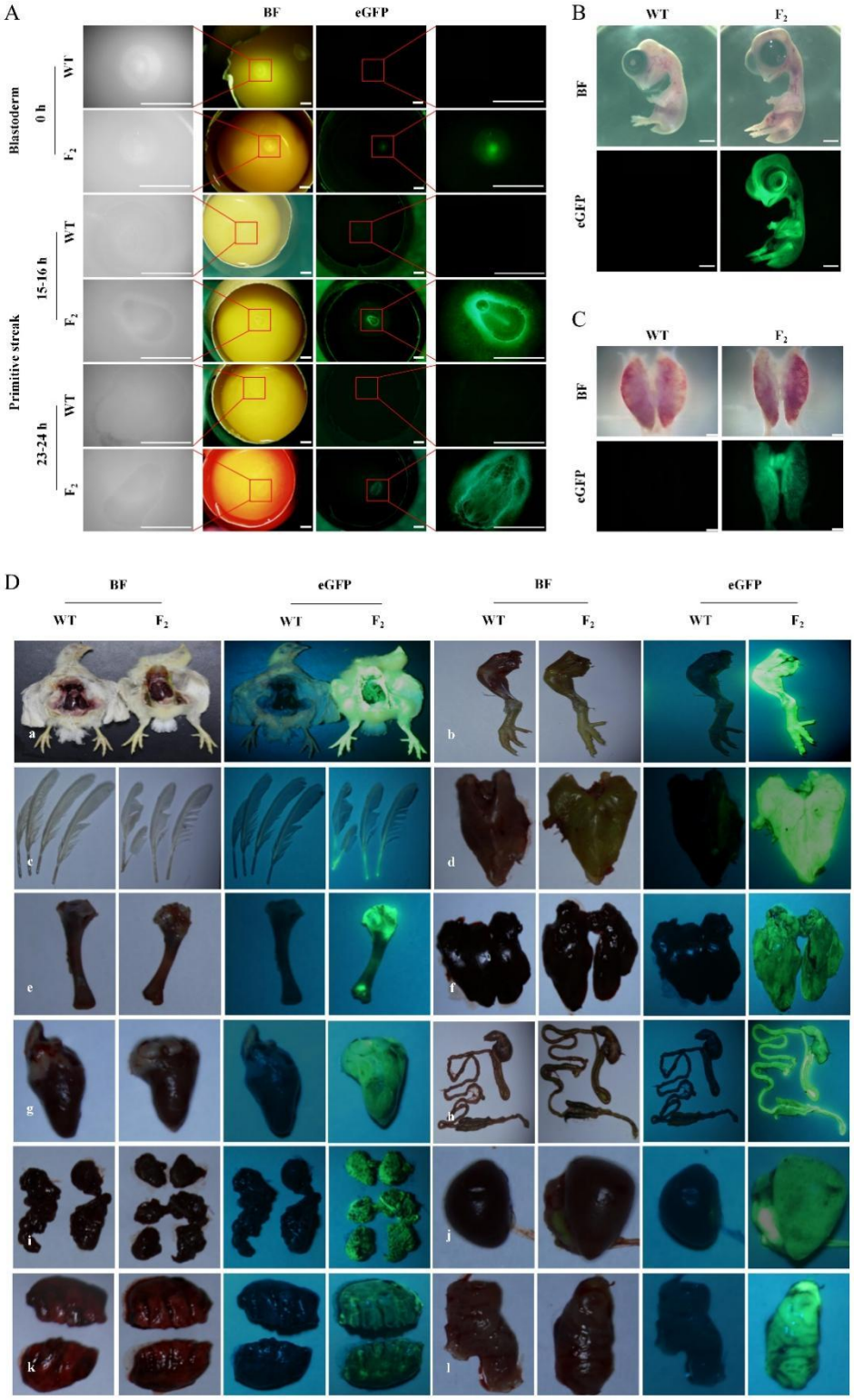


Figure 4. Expression pattern analysis of *eGFP*. (A) *eGFP* expression at the EGK.X stage and primitive streak stage. Scale bar = 2 mm. *eGFP* expression in 7.5-day-old embryos (B) and gonads (C). Scale bar = 500 μm. (D) Anatomical observation of a 15-day-old F_2 *eGFP*-positive chicken. a, dissected abdominal cavity; b, skin-removed hindlimb; c, feathers; d, pectoral muscle; e, bone and associated tissues; f, liver; g, heart; h, gastrointestinal tract and pancreas; i, kidney; j, spleen; k, lung; l, ovary.

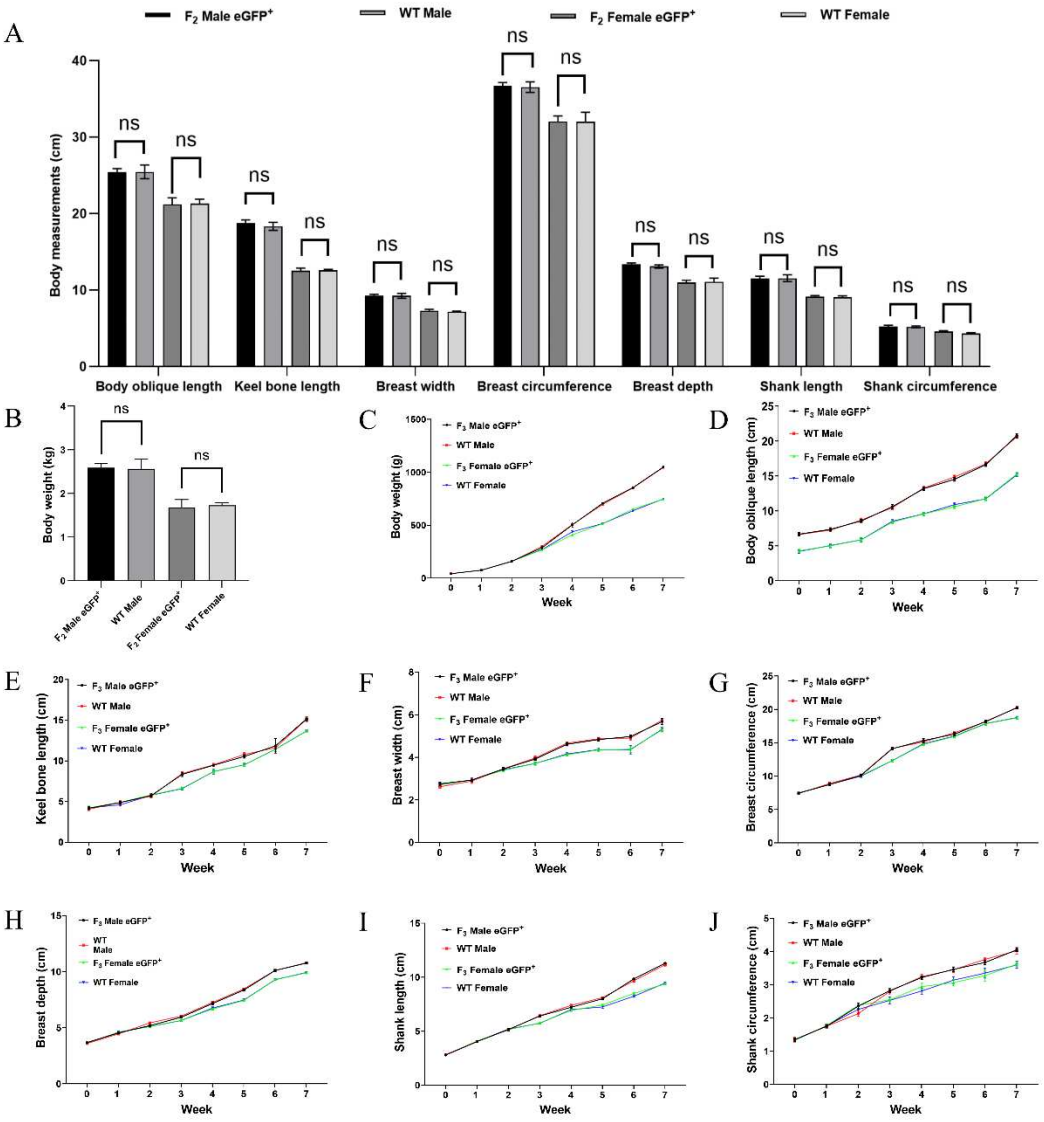


Figure 5. Assessment of growth performance in transgenic chickens. (A, B) Comparison of body measurements and body weight between 6-month-old F_2 *eGFP*-

positive chickens and wild-type chickens. (C–J) Growth-related traits of F_3 *eGFP*-positive chickens from hatching to 7 weeks of age compared with wild-type chickens, including body weight (C), body oblique length (D), keel bone length (E), breast width (F), breast circumference (G), breast depth (H), shank length (I), and shank circumference (J). No significant differences were detected ($P > 0.05$).

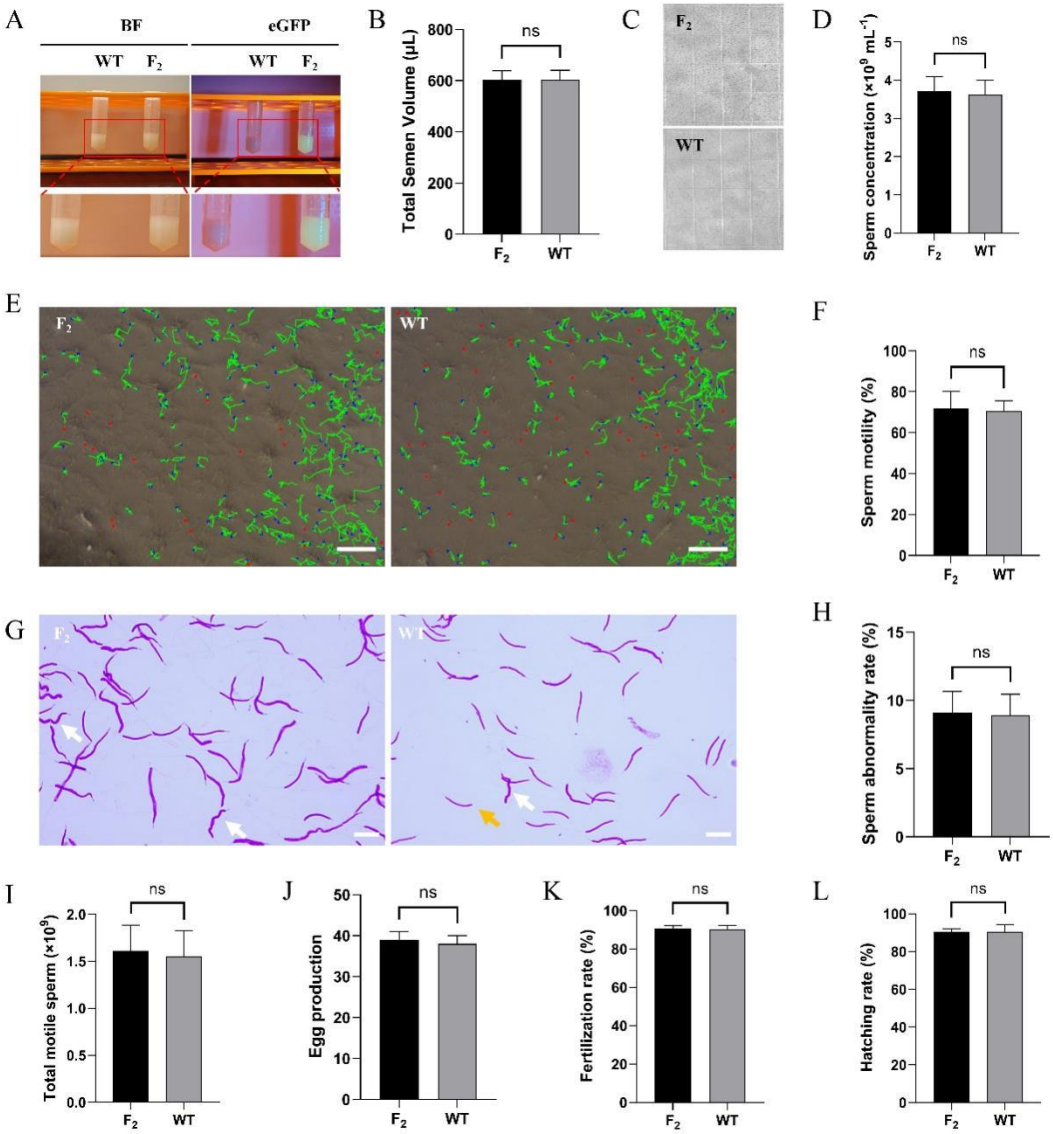


Figure 6. Assessment of reproductive performance in F_2 transgenic chickens. (A–I) Semen quality parameters of F_2 transgenic roosters compared with wild-type controls,

including semen volume (A–B), sperm concentration (C–D), sperm motility (E–F), sperm abnormality rate (G–H), and total motile sperm count (I). Scale bars = 50 μm in panel E and 10 μm in panel G. (J–L) Reproductive performance of F_2 transgenic hens compared with wild-type controls, including total egg production per hen over 2 months (J), fertility rate (K), and hatchability (L). No significant differences were detected between transgenic and wild-type chickens ($P > 0.05$).

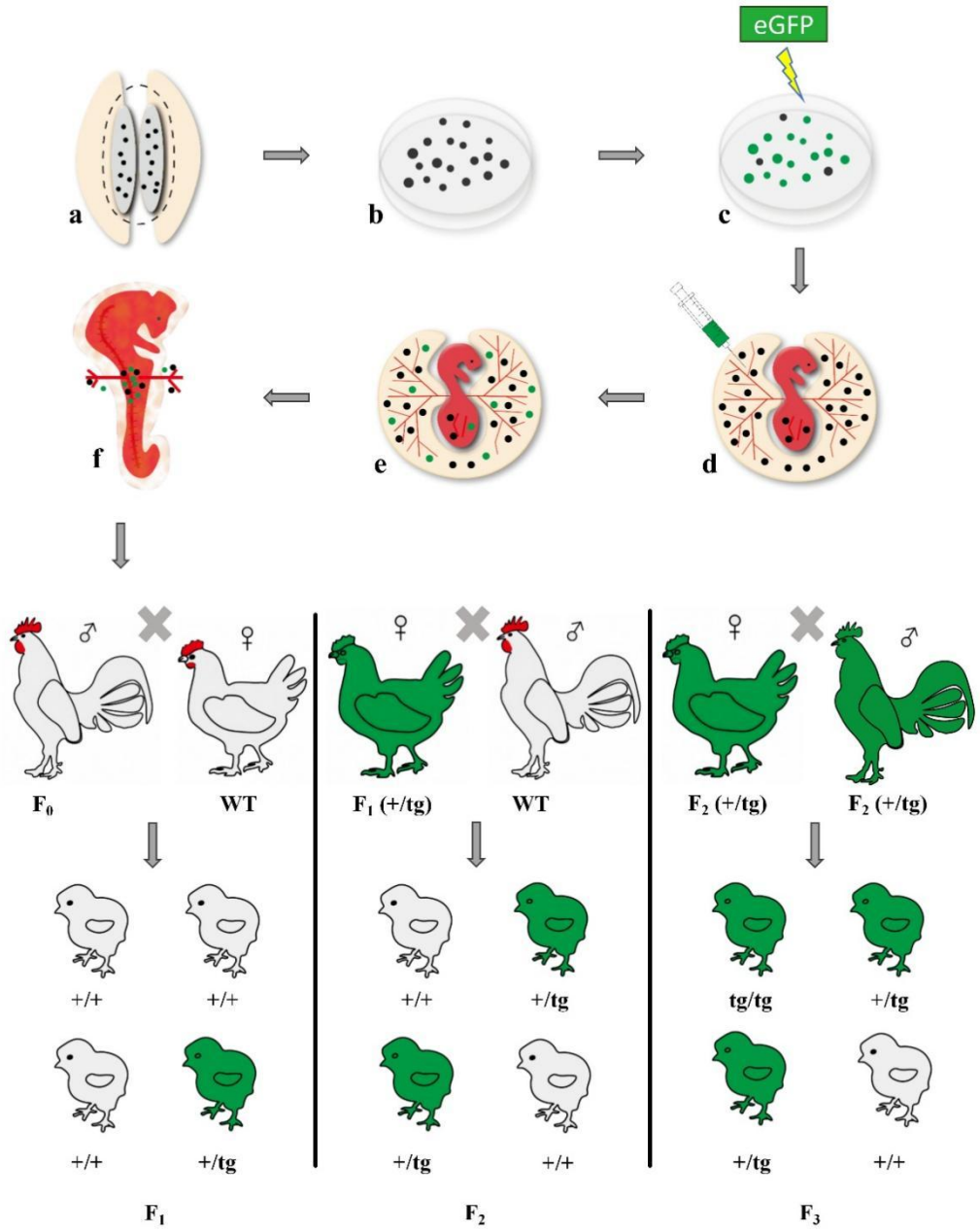


Figure 7. Schematic diagram of *eGFP* transgenic chicken production and breeding. (a) Mesonephros of a 5.5-day-old chicken embryo, with bilateral gonads outlined by dashed lines. (b) *In vitro* culture of PGCs. (c) Lentiviral transduction of PGCs to generate *eGFP*-PGCs. (d) Microinjection of *eGFP*-PGCs into the blood vessels of HH stages 13–16 chicken embryos. (e-f) Migration of *eGFP*-PGCs to embryonic gonads via the circulation. F_0, chimeric chickens carrying *eGFP*-PGCs in the gonads; +/+, offspring without *eGFP* insertion; +/tg, offspring with a heterozygous *eGFP* insertion; tg/tg, offspring with a homozygous *eGFP* insertion; WT, recessive White Plymouth Rock chickens used in testcrosses.

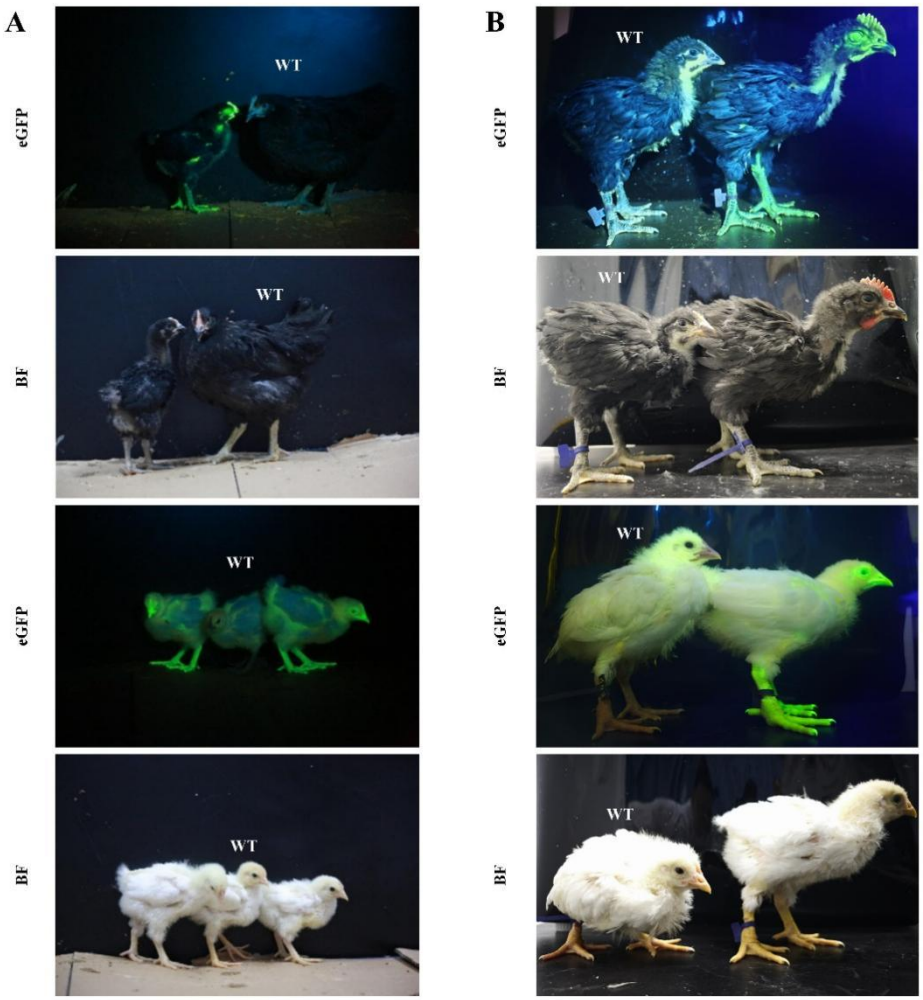


Figure S1. Observation of eGFP-positive chickens. F_2 generation (A) and F_3 generation (B) eGFP-positive chickens.

Supplementary Files

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