Sequential verification of exogenous protein production in *OVA* gene-targeted chicken bioreactors

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ABSTRACT The chicken has potential as an efficient bioreactor system because of its outstanding protein production capacity and low cost. The CRISPR/Cas9-mediated gene-editing system enables production of highly marketable exogenous proteins in transgenic chicken bioreactors. However, because it takes approximately 18 mo to evaluate the recombinant protein productivity of the bioreactor due to the generation interval from G0 founders to G1 egg-laying hens, to verification of the exogenous protein at the early stage is difficult. Here we propose a system for sequential validation of exogenous protein production in chicken bioreactors as in hatching female chicks as well as in egg-laying hens. We generated chicken *OVALBUMIN* (***OVA***) *EGFP* knock-in (**KI**) chicken (*OVA EGFP* KI) by CRISPR/Cas9-mediated nonhomologous end joining at the chicken *OVA* gene locus. Subsequently, the estrogen analog, diethylstilbestrol (**DES**), was subcutaneously implanted in the abdominal region of 1-wk-old *OVA EGFP* KI female chicks to artificially increase *OVALBUMIN* expression. The oviducts of DES-treated *OVA EGFP* KI female chicks expressed OVA and EGFP at the 3-wk-old stage (10 d after DES treatment). We evaluated the expression of EGFP protein in the oviduct, along with the physical properties of eggs and egg white from *OVA EGFP* KI hens. The rapid identification and isolation of exogenous protein can be confirmed at a very early stage and high-yield production is possible by targeting the chicken oviduct.

Key words: chicken bioreactor, oviduct specific expression, *OVALBUMIN*, *EGFP*, diethylstilbestrol

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INTRODUCTION

In recent decades, there has been a significant increase in demand worldwide for the production of recombinant proteins for therapeutic, diagnostic, and industrial uses. A platform for lower-cost large-scale production of recombinant proteins is therefore needed; this has led to the development of animal bioreactors including cattle, goat, and sheep, as well as insect bioreactors (Ivarie, 2003; Carneiro et al., 2018; Li et al., 2021). The targeting of mammary glands in livestock or the silk gland in silkworm (Long et al., 2015; Li et al., 2021) has allowed cost-effective production of exogenous proteins. The production of recombinant proteins from transgenic chicken eggs is a practical alternative. A single female chicken lays >300 eggs per year; each egg contains abundant protein, half of which is coded by a single gene, *OVALBUMIN* (***OVA***). Therefore, the production of foreign proteins has been attempted using a viral vector (Lillico et al., 2007; Kamihira et al., 2009; Herron et al., 2018) or *piggyBac* transposon system (Kim et al., 2018). However, the foreign protein production efficiency in transgenic hen eggs has been insufficient for commercial use.

The mass production of a foreign protein in chicken was recently achieved by targeting *OVA* using the clustered regularly interspaced short palindromic repeat (**CRISPR**) and CRISPR-associated protein 9 (**Cas9**) system (Oishi et al. 2018). CRISPR/Cas9 requires only a single-stranded guide RNA (**gRNA**) to direct Cas9 to the target site, where it induces a double-strand break. Cells attempt to repair the double-strand break by homology-directed repair or nonhomologous end joining. CRISPR/Cas9 has been used for genome editing in various species (Kimura et al., 2014; Suzuki et al., 2016; Kesavan et al., 2017; Lee et al., 2019; Shi et al., 2020; Cui et al., 2021). Oishi et al. and Mukae et al. reported

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therapeutic protein production in transgenic chicken by *OVA* targeting using the CRISPR/Cas9 system (Oishi et al., 2018; Mukae et al., 2020). However, despite the potential for industrialization, transgenic chicken bioreactor systems lack a method to verify the production of exogenous proteins in the intermediate stage until the egg laying of G1 transgenic chicken. This verification is performed after approximately 18 mo (from generation of the founder to the egg-laying stage of the G_1 transgenic hen) (Lillico et al., 2005), thus hampering industrialization; this ineffectiveness results in economic loss (Li et al., 2010).

Here, we devised a method to observe the expression and pattern of foreign proteins in the early and egg-laying phases in an *OVA*-targeted knock-in (**KI**) chicken bioreactor. Transgenic chickens were produced in which the enhanced green fluorescent protein (***EGFP***) gene was inserted into the *OVA* locus using the CRISPR/Cas9 system. We observed exogenous protein expression in 17-day-old chick oviducts by implanting a diethylstilbestrol (**DES**) pellet into the abdominal region of G_1 *OVA EGFP* KI female chicks for 10 d. We confirmed magnum-specific expression of the exogenous protein in *OVA EGFP* KI hens at the egg-laying stage and high productivity in the KI chicken egg. This validation system could be used in chicken bioreactors for the production of economically or medically important proteins.

MATERIALS AND METHODS

Experimental Animals and Animal Care

The care and use of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University. Chickens were maintained in accordance with a standard management program at the University Animal Farm, Seoul National University, South Korea. All experimental procedures and care of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University. All methods were carried out in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and were approved by the Institutional Animal Care and Use Committee (SNU−190401−1-1) of Seoul National University, South Korea.

Construction of OVA-Targeting CRISPR/Cas9 Expression Plasmids and EGFP Donor Plasmids

A CRISPR/Cas9 vector targeting intron 1 of the chicken *OVA* gene was constructed using the pX459 vector (Addgene, MA). To insert gRNA sequences into the CRISPR/Cas9 plasmid, sense and antisense oligonucleotides were designed and synthesized (Bioneer, Daejeon, South Korea). These oligonucleotides were annealed under the following conditions: 30 s at 95°C, 2 min at 72°C, 2 min at 37°C, and 2 min at 25°C. For gene insertion into chicken OVA, codons of the EGFP gene were optimized for expression in the hens' oviduct using the Gallus gallus codon usage database (http://www.kazusa.or.jp/codon). To allow secretion of EGFP from the oviduct cells, a chicken lysozyme signal peptide sequence was placed right before EGFP gene. The donor cassette containing a portion of the intron 1 region, a portion of exon 2 including the translation start site of chicken OVA, EGFP, the cytomegalovirus (CMV) promoter, and a puromycin resistance gene were synthesized in the pBHA vector backbone (Bioneer) (Daejeon, Korea). The gene sequence including the flanking region and the corresponding sequence was added to Supplementary Figure 1 and oligonucleotides used for the construction of the CRISPR/Cas9 vector are listed in Supplementary Table S1.

Culture of Chicken PGCs

The cultivation, transfection, and puromycin selection of white leghorn male PGC cells were performed as previously described (Kim et al., 2021). Briefly, male PGCs were cultured on mitotically inactivated mouse embryonic fibroblasts in knockout Dulbecco's modified Eagle's medium (Invitrogen, Life Technologies, Carlsbad, CA) supplemented with 20% (v/v) fetal bovine serum (Invitrogen), 2% (v/v) chicken serum (Sigma-Aldrich, St. Louis, MO), 1 × nucleoside mix (EMD Millipore, Temecula, CA), 2 mM L-glutamine, 1 × nonessential amino acid mix, β-mercaptoethanol, 10 mM sodium pyruvate, 1 × antibiotic antimycotic mix (Invitrogen), and human basic fibroblast growth factor (10 ng/mL; Koma Biotech, Seoul, South Korea). PGCs were cultured at 37°C in an atmosphere of 5% (v/v) CO_2 and 60 to 70% relative humidity. For *OVA*-targeted gene insertion in chicken PGCs, donor plasmids (2 μg) and CRISPR/Cas9 expression plasmids (2 μg) were co-introduced into 1×10^5 cultured PGCs with 4 mL of Lipofectamine 2000 reagent suspended in 1 mL of Opti-MEM. Four hours after transfection, the transfection plasmid mixture was replaced with PGC culture medium. One day after transfection, the transfected PGCs were transferred into a new culture plate along with 1 μg/mL puromycin-containing medium that lacked a mouse embryonic fibroblast feeder layer. After selection for 24 h, transfected PGCs were cultured in fresh medium with a mouse embryonic fibroblast feeder layer.

Production of OVA EGFP KI Chickens

A window was cut at the sharp end of the Korean Ogye (*i*/*i*) recipient egg and a 2-μL aliquot containing at least 3,000 *OVA EGFP* KI PGCs was microinjected into the dorsal aortas of Korean Ogye recipient embryos at Hamburger and Hamilton stages 14-17. The egg window was sealed with paraffin film and the eggs were incubated until hatching. After sexual maturation, sperm of the male recipient chickens were evaluated by breed-specific PCR, and the chickens with white leghorn sperm were mated with WT female chickens. Germline-

chimeric chickens were identified based on offspring feather color and genomic DNA analysis.

Sequencing of OVA KI PGCs and Chickens

To verify puromycin-selected PGCs and transgenic chickens, PCR was performed using primers specific for the 5' and 3' junctions of genomic DNA (Supplementary Table S1); the PCR results were confirmed by sequencing analysis. For sequencing analysis, the amplicons were annealed to the pGEM-T Easy Vector and sequenced using an ABI Prism 3730XL DNA Analyzer (Thermo Fisher Scientific, MA). The sequences were compared against assembled genomes using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov).

DES Treatment of 1-Week-old Female Chicks

DES treatment of female chicks and oviduct retrieval were conducted as previously described (Song et al., 2011). Briefly, 100 mg of DES powder (Sigma-Aldrich) were placed into the mold of a laboratory press and exposed to 5 tons of pressure for 5 min. A 15-mg DES pellet was subcutaneously implanted in the abdominal region of 1-wk-old female chicks for 10 days and the oviducts were removed.

Immunohistochemistry

Chicken oviduct tissues from WT and *OVA EGFP* KI chickens (DES-treated or adult hens) were fixed in 4% paraformaldehyde at 4°C overnight. Tissues were dehydrated using a methanol gradient, cleared in xylene, and embedded in paraffin. Embedded tissues were sectioned (thickness, 9 μm) and deparaffinized. The sections were washed three times with 1 × phosphate-buffered saline (**PBS**) and blocked with a blocking buffer (5% goat serum and 1% bovine serum albumin in PBS) for 1 h at room temperature. The sections were then incubated at 4°C overnight with a rabbit anti-GFP primary antibody (A11122, Thermo Fisher Scientific) or anti-OVA primary antibody (NB600-922, Novus, CO). After sections had been washed 3 times with PBS, they were incubated with fluorescence-conjugated secondary antibodies (Alexa Fluor 594 or 488, Invitrogen) for 1 h at room temperature. After sections had been washed three additional times with PBS, they were mounted with 4',6 diamidino-2-phenylindole (Vector Laboratories, CA) and imaged by confocal microscopy.

Short-Term Culture of 17-day-old Chick Oviduct Cells

Oviduct tissue was isolated, dissected, and washed twice with PBS. As previously reported (Kasperczyk et al., 2012), the tissue was digested in a solution of collagenase P (11213857001, Sigma-Aldrich) for 30 min in 5% CO_2 and 37°C with gentle pipetting at 5-min intervals, then passed through a 40-μm nylon cell strainer (F352340, Falcon, NY). The resulting suspension was centrifuged; the cells were then seeded at a density of 3×10^5 per well in a 12-well culture plate and cultured under serum-free conditions (Stadnicka et al., 2019).

RT-PCR and Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) and reverse-transcribed using the SuperScript Ⅲ Reverse Transcription Kit (Thermo Fisher Scientific). The resulting complementary DNA (cDNA) was amplified using a target gene-specific primer set. Gene expression was measured in triplicate using EvaGreen Dye (Biotium, CA) and a CFX96 Real-Time PCR Detection System (Bio-Rad, CA). Gene expression was quantified using the formula: DCt = Ct of the target gene − Ct of *GAPDH*. The primer sets are listed in Table S1.

Western Blotting and ELISA Analysis

Total protein was prepared in radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific) and denatured at 95°C for 5 min in an equal volume of 2 × Laemmli sample buffer (Bio-Rad). Proteins were separated in 10% SDS-polyacrylamide gels, transferred onto a polyvinylidene fluoride membrane, and blocked for 1 h at room temperature. The membrane was incubated with a suitable primary antibody, followed by an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz, TX, USA). The primary antibodies were anti-β-actin (sc-47778, Santa Cruz), -OVA (NB600-922, Novus), -GFP (A11122, Thermo Fisher Scientific), -ovotransferrin (MBS715799, MyBioSource, CA), -ovomucoid (MBS715888, MyBioSource), -lysozyme (GTX48846, GeneTex, CA), and -avidin (GTX19507, GeneTex). Immunoreactive proteins were visualized using the ECL Select Western Blotting Detection Reagent (GE Healthcare Bio-Science, NJ). Signals were detected using a ChemiDoc XRS imaging system (Bio-Rad). The quantities of OVA and EGFP were measured using OVA ELISA kit (CSB-E13315C, Cusabio, TX) and GFP ELISA kit (ab171581, Abcam, Cambridge, UK) according to the manufacturer's instructions.

Structural and Morphological Analysis of ESM Nanofibers

The morphological features of the WT and *OVA EGFP* KI egg shell membrane (**ESM**) were characterized by field-emission scanning electron microscopy (Carl Zeiss Sigma, Germany). Briefly, the specimens were fixed with Karnovsky's fixative at 4°C overnight. Specimens were dehydrated using an alcohol gradient, then subjected to critical point drying (Leica EM CPD300). Each specimen surface was coated with gold

for 180 s and observed under vacuum conditions by field-emission scanning electron microscopy. The membrane thickness was measured using a micrometer to the nearest 0.001 mm.

Sample Preparation and two-Dimensional Electrophoresis

Sample preparation and 2-dimensional electrophoresis were performed as described by (Bahk et al., 2004). Egg-white protein concentration was determined by the Bradford method (Bio-Rad). For 2-dimensional electrophoresis analysis, pH 3 to 10 immobilized pH gradient (**IPG**) strips (GE Healthcare Life Sciences, PA) were rehydrated in swelling buffer containing 7 M urea, 2 M thiourea, 2.5% (w/v) dithiothreitol, and 4% (w/v) CHAPS. Protein lysates (600 μg) were loaded into the rehydrated IPG strips using an IPGphor Ⅲ (GE Healthcare Life Sciences) and the 2-dimensional separation was performed on 12% SDS-polyacrylamide gels. After the gels had been subjected to fixation, they were stained with Colloidal Coomassie Blue G-250 solution (ProteomeTech, Seoul, South Korea). The gels were then destained using deionized water and imaged using a scanner (Bio-Rad). Major egg-white proteins were identified as previously described (Guerin-Dubiard et al., 2006).

In-gel Trypsin Digestion and Extraction of Peptides

Protein bands from SDS-polyacrylamide gels were excised and subjected to in-gel trypsin digestion, in accordance with established procedures (Bahk et al., 2004). Briefly, protein bands were excised from stained gels, cut into pieces, and washed for 1 h at room temperature in 25 mM ammonium bicarbonate buffer, pH 7.8, containing 50% (v/v) acetonitrile (**ACN**). After gel pieces had been dehydrated for 10 min in a centrifugal vacuum concentrator (Biotron, Inc., Incheon, South Korea), they were rehydrated in 50 ng of sequencing-grade trypsin solution (Promega, WI). After the gel pieces had been incubated in 25 mM ammonium bicarbonate buffer, pH 7.8, at 37°C overnight, tryptic peptides were extracted with 100 μL of 1% formic acid (**FA**) containing 50% (v/v) ACN for 20 min with mild sonication. The extracted solution was concentrated using a centrifugal vacuum concentrator. Prior to mass spectrometric analysis, the peptide solution was subjected to a desalting process using a reversed-phase column (Gobom et al., 1999). After the peptide solution had been equilibrated with 10 μL of 5% (v/v) FA, it was loaded on the column and washed with 10 μL of 5% (v/v) FA. The bound peptides were eluted using 8 μL of 70% ACN with 5% (v/v) FA.

Identification of Proteins by LC-MS/MS

LC-MS/MS was performed using a nano-ACQUITY UPLC and LTQ-orbitrap mass spectrometer (Thermo Electron, CA) with a BEH C18 1.7 μm, 100 μm × 100 mm column (Waters, MA). Mobile phase A for LC separation was 0.1% FA in deionized water, while mobile phase B was 0.1% FA in ACN. The chromatography gradient increased linearly from 10% B to 40% B for 16 min. The flow rate was 0.5 μL/min. For tandem mass spectrometry, mass spectra were acquired using data-dependent acquisition with full mass scan (300−2000 m/z), followed by MS/MS scans. Each MS/MS scan was a mean of one microscan on the LTQ-orbitrap. The temperature of the ion transfer tube was set to 275°C and the spray was set to 2.3 kV. The normalized collision energy was set to 35% for MS/MS. The individual spectra from MS/MS were processed using SEQUEST software (Thermo Quest, CA) and the generated peak lists were used to query an in-house database via MASCOT software (Matrix Science Ltd., London, UK). We used the carbamidomethyl (**C**), deamidated (**NQ**), and oxidation (**M**) modifications for MS analysis; the peptide mass tolerance was set to 10 ppm. The MS/MS ion mass tolerance was 0.8 Da, the missed cleavage allowance was 2, and the charge state (+2, +3) was considered for data analysis. We used only significant hits, as defined by the MASCOT probability analysis.

Statistical Analysis

Statistical analysis was performed using Prism software (GraphPad Software, CA). Significant differences between groups were determined by Student's t-test. A value of $P < 0.05$ was considered indicative of statistical significance.

RESULTS

CRISPR/Cas9-Mediated Targeted Insertion of EGFP into the OVA Locus and Production of OVA-Targeted KI Chicken

To visualize the exogenous protein, EGFP, we inserted *EGFP* into the *OVA* locus. We constructed a Cas9-expressing plasmid with a single gRNA that targeted the *OVA* intron located between exons 1 and 2, along with a donor plasmid carrying *EGFP* (Figure 1A). After co-transfection of these plasmids into primordial germ cells (**PGC**s), we performed puromycin selection to establish genome-edited PGCs in vitro. Next, we confirmed donor plasmid insertion in PGCs by genomic DNA polymerase chain reaction (**PCR**) with a 5'and 3' junction-specific primer (Figure 1B). Sequencing showed that the donor plasmid was inserted into the *OVA* locus with genetic insertion or deletion mutations at each junction (Figure 1C). By transplanting the *OVA EGFP* KI PGCs into recipient embryos, we produced 2 germline chimeras (S0454 and S0455). The two germline chimeras generated donor-derived progenies, which could

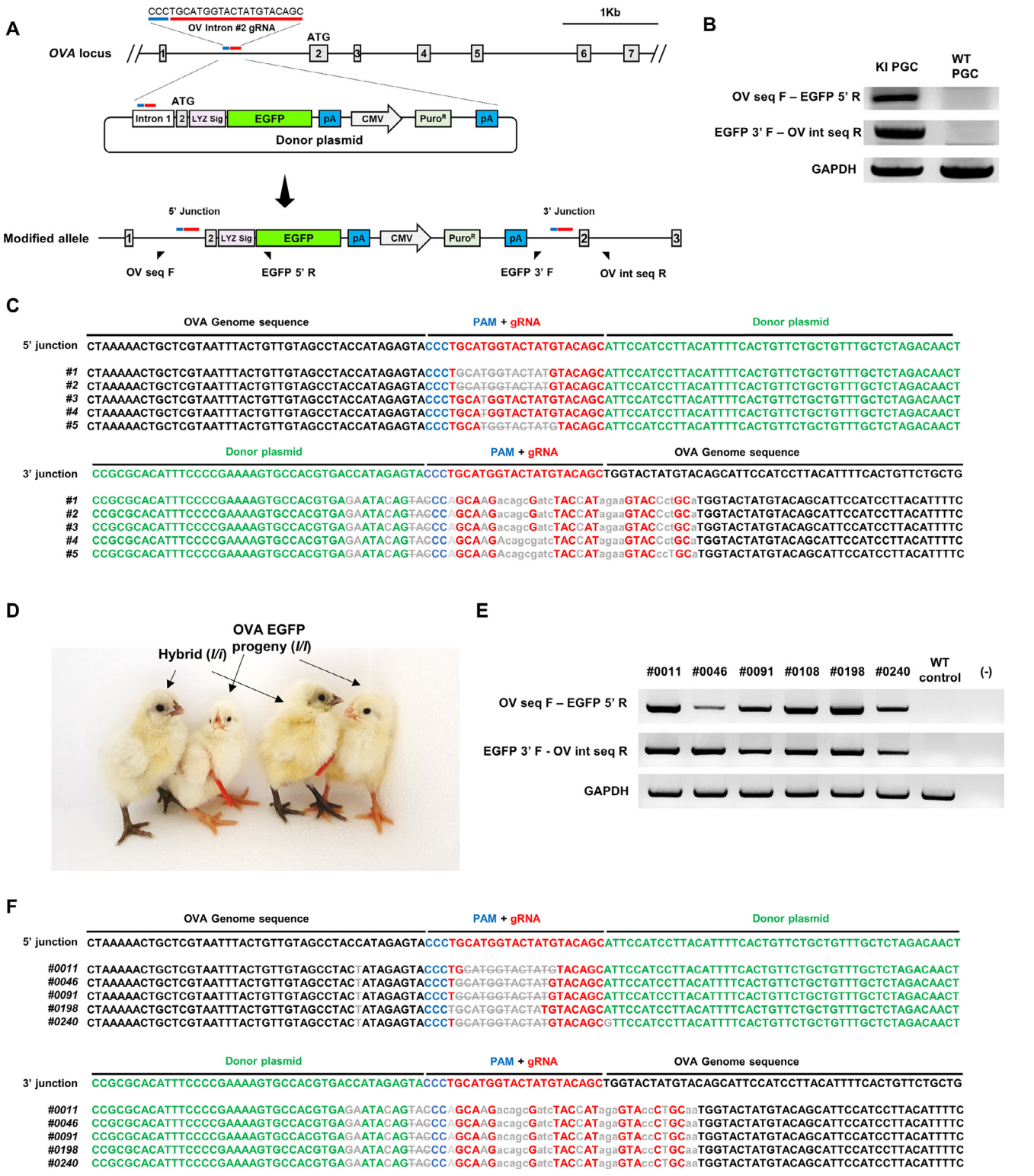

Figure 1. *EGFP* insertion at the *OVA* locus in PGCs and establishment of transgenic chickens. **(A)** Illustration of the *OVA*-targeting single guide RNA-expressing vector with *EGFP*-containing donor plasmid by non-homologous end joining repair. The *OVA*-targeting single gRNA expression vector targets the intron region between exons 1 and 2 of *OVA* (blue bar, PAM sequence; red bar, guide RNA recognition region). The donor plasmid contains *EGFP* fusion DNA, including the start codon region of *OVA* exon 2 and a *CMV Puro*^{R} (puromycin resistance gene) cassette. **(B)** The modified gene includes the synthesized exon 2 partial region, *EGFP*, and *CMV Puro*^{R}, and verified the integrated DNA is 5′ and 3′ junction specific primer. **(C)** Sequencing of genomic DNA to confirm donor cassette gene insertion in chicken PGCs using 5' junction and 3' junction sequence-specific PCR primers. **(D)** Images of donor PGC-derived *OVA EGFP* KI transgenic chicks (*I/I*) and hybrid chicks (*I/i*) **(E)** Genomic DNA PCR of *OVA EGFP* KI chickens using 5' junction and 3' junction sequence-specific primers, and **(F)** sequencing of *OVA EGFP* KI chickens. **(C, F)** Black text, *OVA* sequence; red text, gRNA sequence; blue text, PAM sequence; green text, donor plasmid sequence. Deleted nucleotides are indicated by underlined gray uppercase text, substituted nucleotides are indicated by non-underlined gray uppercase text, and inserted nucleotides are indicated by gray lowercase text.

Table 1. Efficiency of germ-line transmission and genome-edited chick production for *OVA EGFP* donor PGCs.

Germline chimera ID	No. of hatched chicks	No. of endogenous germ cell-derived chicks (%)*	No. of donor germ-cell-derived chicks (%)†	No. of KI chicks (%)‡
S 0454	75	29 (38.66)	46 (61.33)	16 (34.78)
S 0455	62	16 (25.80)	46 (74.19)	11 (23.91)

*Test-cross analysis was conducted by mating between WL (I/I) and germ-line chimeric KO (I/i); that is, transplanted *OVA EGFP* KI donor PGCs of WL (I/I).
†The phenotype of offspring derived from donor PGCs of WL (*I/I*).
‡The percentage of *OVA EGFP* KI chicks in donor germ-cell−derived chicks.

Table 2. Production efficiency of G_2 *OVA EGFP* KI chickens after testcrossing of G_1 *OVA EGFP* KI chicken with wild-type chickens.

KI chicken ID	Sex	No. of incubated eggs	No. of hatched chicks (%)†	No. of KI chicks (%)‡
0011	Male	92	74 (80.43)	49 (66.21)
0091	Male	103	77 (74.75)	41 (53.24)
0198	Male	134	124 (92.53)	70 (56.45)
0108	Female	184	0 (0.0)	0 (0.0)

†The percentage of the number of chicks hatched after incubation.
‡The percentage of *OVA EGFP* KI chicks in hatched chicks.

be distinguished by feather color (Figure 1D and Table 1). The production efficiencies of donor germ cell-derived progenies were 61.33% and 74.19% from S0454 and S0455, respectively (Table 1). From the genomic DNA of the produced donor-derived progenies, junction-specific PCR was performed to select *OVA EGFP* KI chicks (Figure 1E); 34.78% and 23.91% of the donor-derived offspring were validated as *OVA*-targeted KI chicks (Table 1). The genotypes of G_1 *OVA EGFP* KI chicks were confirmed by sequencing of genomic DNA junction-specific PCR amplicons (Figure 1F). Subsequently, we tried to produce G_2 homozygous KI offspring. In the case of G_1 males, the progenies were normally produced, however, the offspring did not hatch from G_1 females' eggs (Table 2). Therefore, the subsequent results of this paper were conducted with only heterozygous *OVA EGFP* KI chickens.

Induction of OVA and EGFP in Immature Oviducts of OVA EGFP KI Chicks by DES

Steroid hormones, especially estrogen, induce the cytodifferentiation of progenitor cells to tubular gland cells, in which OVA synthesis occurs (Palmiter and Wrenn, 1971; Dougherty and Sanders, 2005). Based on these characteristics, we induced early expression of EGFP protein by inserting a DES pellet under the abdominal hypodermic region of wild-type (**WT**) and *OVA EGFP* KI female chicks, 7 d after hatching (Figure 2A). After 10 d of stimulation, the chicks were euthanized; DES-untreated and -treated oviduct samples were collected. In WT and *OVA EGFP* KI chicks, oviduct growth was promoted by DES; DES-treated oviducts of *OVA EGFP* KI chicks showed strong EGFP fluorescence (Figure 2B). We conducted immunohistochemistry to detect EGFP in the oviduct (Figure 2B). Development of the chick oviduct was promoted by DES; tubular gland DES-treated *OVA EGFP* KI chicks strongly expressed OVA and EGFP protein (Figure 2C). EGFP expression was not observed in the liver, heart, gizzard, muscle, or intestine; nonspecific expression was not detected by reverse transcription (**RT**)-PCR (Supplementary Figure 2). Next, we investigated the transcript levels of *OVA* and *EGFP* in DES-treated *OVA EGFP* KI chicks by RT-PCR; *OVA* was induced in DES-treated WT and *OVA EGFP* KI chicks (Figure 2D), as was *EGFP*. Quantitative RT-PCR showed that the *OVA* expression level was significantly lower in *OVA EGFP* KI oviducts than in WT oviducts (Figure 2E). However, *EGFP* expression was high in *OVA EGFP* KI chicks. The protein concentrations showed patterns similar to the transcript levels. Western blotting showed induction of OVA protein expression in the DES-treated group; it showed specific induction of EGFP protein expression in *OVA EGFP* KI chicks (Figure 2F). The OVA concentration in DES-treated WT chicks was 96.57 ± 9.90 μg/mL, compared to 15.62 ± 1.78 μg/mL in *OVA EGFP* KI chicks. The EGFP concentration was 1.10 ± 0.07 μg/mL in *OVA EGFP* KI chicks (Figure 2G). Oviduct cells from DES-treated *OVA EGFP* KI chicks strongly expressed EGFP, while cells from DES-untreated chicks did not (Figure 2H). Western blotting detected EGFP in cell lysates and culture media of DES-treated *OVA EGFP* KI chicks (Figure 2I). Collectively, these results indicate that *OVA* induction by DES in newborn chicks can verify the expression and secretion of foreign proteins.

Analysis of Mature Oviduct from OVA EGFP KI hens and Magnum-Specific Expression of EGFP

We next analyzed the oviducts of adult *OVA EGFP* KI chickens. In *OVA EGFP* KI hens, EGFP was expressed in the magnum (Figure 3A and B).

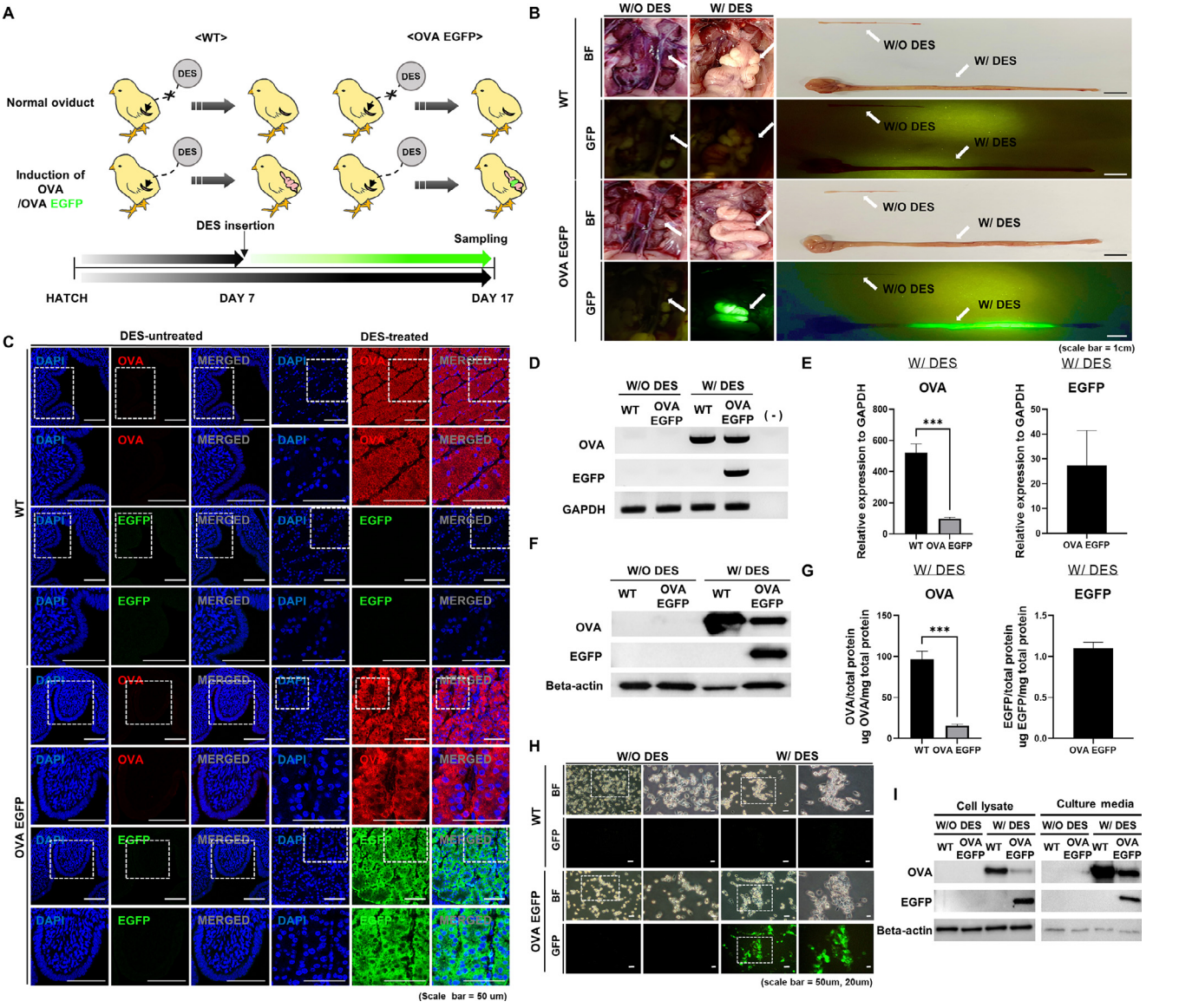

Figure 2. Induction of OVA and EGFP by DES in *OVA EGFP* KI chickens. **(A)** Diagram of DES treatment in WT and *OVA EGFP* KI chicks. **(B)** Oviduct development in 17-day-old DES-treated and -untreated chicks. Scale bar, 1 cm. **(C)** Immunohistochemistry of OVA and EGFP in DES-treated and -untreated chick oviducts. Scale bars, 50 μm. **(D)** RT-PCR of *OVA* and *EGFP* in oviducts of DES-treated and -untreated chicks. **(E)** Quantitative RT-PCR of *OVA* and *EGFP* in oviducts of DES-treated chicks. $^{***}P < 0.001$. **(F)** Western blotting of OVA and EGFP in oviducts of DES-treated and -untreated chicks. **(G)** Enzyme-linked immunosorbent assay of OVA and EGFP in oviducts of DES-treated chicks. **(H)** Morphologies of short-term cultured oviduct cells from DES-treated and -untreated chicks, as determined by bright field and fluorescence microscopy. Scale bars, 50 μm (right panels) and 20 μm (left panels). **(I)** Western blotting of OVA and EGFP in cell lysate and cell culture medium of short-term cultured DES-treated and -untreated chick oviduct cells. Quantitative data are means ± standard deviations. BF, Bright field; GFP, GFP fluorescence; W/DES, with DES; W/O DES, without DES.

Prominent EGFP protein expression was observed in the magnum, but not in other parts of the oviduct (infundibulum, isthmus, and shell gland). RT-PCR showed that *EGFP* was expressed in the infundibulum and isthmus, as well as the magnum (Figure 3C). *OVA* expression in the isthmus was much weaker in *OVA EGFP* KI chickens than in WT chickens. Indeed, *EGFP* expression was weaker in the isthmus than in the infundibulum or magnum of *OVA EGFP* KI hens (Figure 3C). As in DES-treated *OVA EGFP* KI chicks, *EGFP* was not expressed in the heart, liver, lung, muscle, spleen, pancreas, or intestine of hens (Supplementary Figure 3). Western blotting (Figure 3D) showed that EGFP protein expression was much more prominent in the magnum than in other parts of the oviduct (infundibulum, isthmus, and shell gland) was restricted to the magnum (Figure 3A and B). This is consistent with previous reports that OVA protein is synthesized in the tubular glands of the magnum (Draper et al., 1972; Jung et al., 2011). In our *OVA EGFP* KI chickens, transgene expression is controlled by the endogenous *OVA* promoter; thus, we predicted that the EGFP and OVA protein expression patterns would overlap. Indeed, immunohistochemistry analysis showed that EGFP and OVA were highly expressed in the tubular gland of *OVA EGFP* KI hens (Figure 3E).

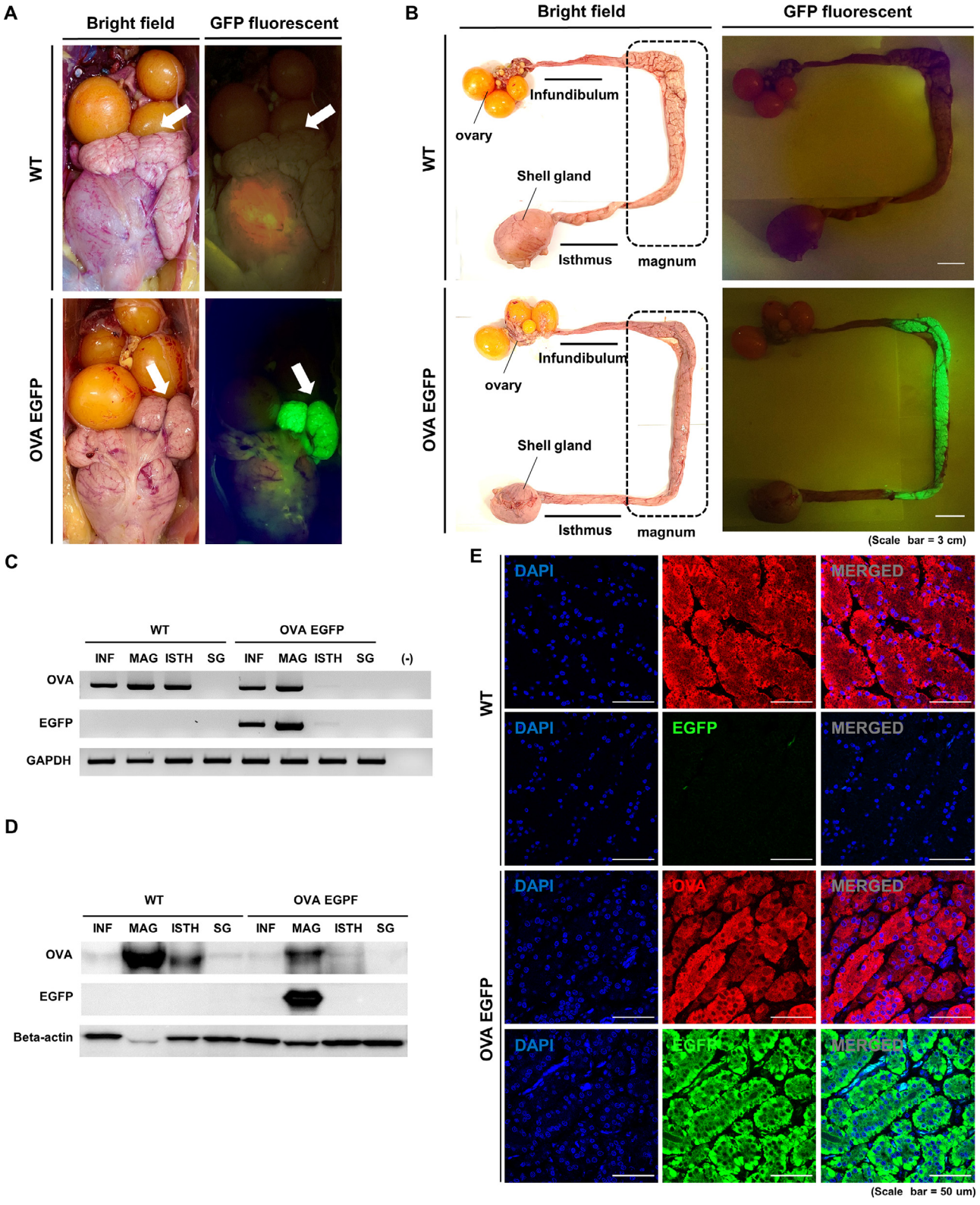

Figure 3. Analysis of the oviduct in *OVA EGFP* KI chickens. **(A)** Intraperitoneal dissection of *OVA EGFP* KI hens and EGFP fluorescence in oviduct tissue. White arrow, magnum part. **(B)** Morphology and EGFP expression in the reproductive tract of an adult *OVA EGFP* KI chicken. **(C)** RT-PCR of *OVA* and *EGFP* in the infundibulum, magnum, isthmus, and shell gland. **(D)** Western blotting of OVA and EGFP in the infundibulum, magnum, isthmus, and shell gland. **(E)** Immunohistochemistry of OVA and EGFP in the magnum of an *OVA EGFP* KI chicken. Scale bars, 50 μm. INF, infundibulum; ISTH, isthmus; MAG, magnum; SG, shell gland.

Characteristics of Eggs and egg White From OVA EGFP KI hens

In the *OVA EGFP* KI hens, because the *EGFP* gene was integrated into the *OVA* locus, we confirmed the EGFP expression in the egg white. We confirmed that GFP fluorescence was strongly expressed in the egg white, and it can be detected outside the egg shell (Figure 4A). In addition, the mean egg weight (27.80 ± 0.63 g) and egg-white volume (8.02 ± 1.83 mL) from

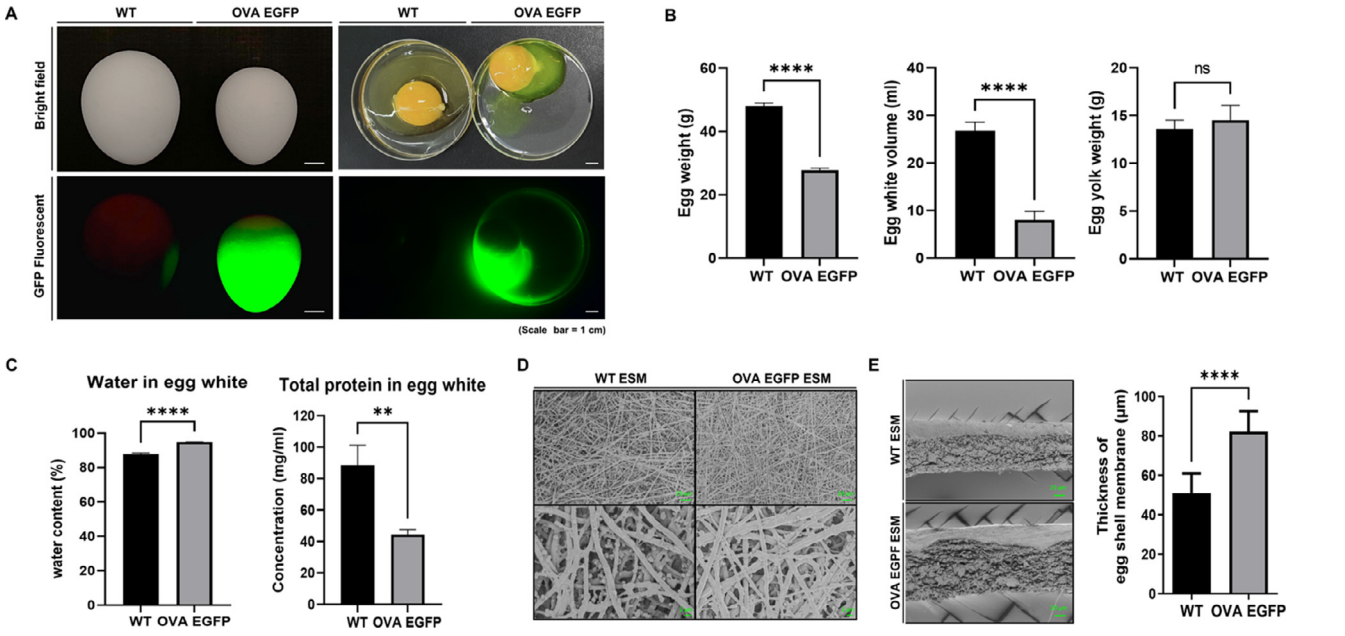

Figure 4. Physical properties of eggs from *OVA EGFP* KI chickens**. (A)** Appearance of *OVA EGFP* KI chicken egg and fluorescence in eggshell and egg white. **(B)** *OVA EGFP* KI and wild-type egg weight, egg-white mass, and yolk weight. $^{***}P < 0.001$; ns, not significant **(C)** *OVA EGFP* KI and wild-type egg water content and egg-white protein concentration. $^{**}P < 0.01$ and $^{***}P < 0.001$. **(D)** Scanning electron micrographs of wild-type and *OVA EGFP* KI ESM. Scale bar, 20 μm, and 2 μm. **(E)** Thicknesses of *OVA EGFP* KI and WT ESM. $^{***}P < 0.001$ (n = 10). Scale bar, 20 μm. WT and *OVA EGFP* KI chickens were 31 to 32 wks of age. Quantitative data are presented as means ± standard deviations.

OVA EGFP KI hens were significantly lower than those values for WT eggs (48.04 ± 0.96 g, and 26.75 ± 1.85 mL, respectively) (Figure 4B); thus, exogenous protein expression presumably reduced the egg-white volume and egg weight, without influencing the yolk weight. Water and protein constitute 88.5% and 9.7 to 10.6%, respectively, of egg white (Mine 1995). The water content of egg white was significantly greater in eggs from *OVA EGFP* KI hens than in eggs from WT hens (87.77 ± 0.67%−94.87 ± 0.06%) (Figure 4C). In contrast, the concentration of total egg-white protein was significantly higher in WT eggs (79.59 ± 4.53 mg/mL) than in *OVA EGFP* KI eggs (44.51 ± 3.06 mg/mL). Consistent with this finding, scanning electron microscopy showed that the eggshell membrane (**ESM**) structure was contracted and dense (Figure 4D). Cross-sectional thickness was significantly greater in the *OVA EGFP* KI ESM (82.10 ± 10.48 μm) than in the WT ESM (51.00 ± 9.97 μm) (Figure 4E). Therefore, the expression of exogenous protein in the *OVA* locus increases the water content, while significantly reducing the egg-white volume and egg-white protein concentration. These changes cause *OVA EGFP* KI chicken eggs to become smaller, with altered ESM structure and thickness.

Composition of OVA EGFP KI egg White

We performed 2-dimensional electrophoresis of *OVA EGFP* KI eggs (Figure 5A). There were significant decreases in the concentrations of egg-white proteins, such as SERPIN, OVOTRANSFERRIN, OVOMUCOID, and LYSOZYME. Additionally, three distinct spots near 27 kDa were detected only in *OVA EGFP* KI eggs; they were identified as EGFP by liquid chromatography with tandem mass spectrometry (**LC-MS/MS**) ($P < 0.05$). The distinct expression of EGFP protein in *OVA EGFP* KI eggs was detected through Western blotting analysis. On the other hand, it was confirmed that Ovalbumin itself was reduced compared to that in WT egg white (Figure 5B). Enzyme-linked immunosorbent assay (**ELISA**) analysis showed that *OVA EGFP* KI chicken eggs contained 165.25 ± 19.82 μg/mL EGFP (Figure 5C).

DISCUSSION

Transgenic chickens can supply a large amount of protein with high egg-laying ability, and egg protein is easy to purify and has glycosylation suitable for anticancer antibodies, so it is considered an efficient animal bioreactor (Kim et al., 2018; Mukae et al., 2020). The CRISPR/Cas9-mediated *OVA* gene-targeted chicken bioreactor overcomes the limitations of transgenic chicken bioreactors in terms of protein yield (mg/mL) (Oishi et al., 2018; Mukae et al., 2020). However, existing systems require considerable time to detect, isolate, and identify foreign proteins. Cell culture-based bioreactors enable verification at each step: from the introduction of exogenous genes and expansion of host cells, to the identification, isolation, and purification of foreign proteins (Li et al., 2010). Currently, transgenic chicken bioreactors require approximately 18 mo from production of G_0 germline chimera to production and sexual maturity of G_1 chickens (Lillico et al., 2005).

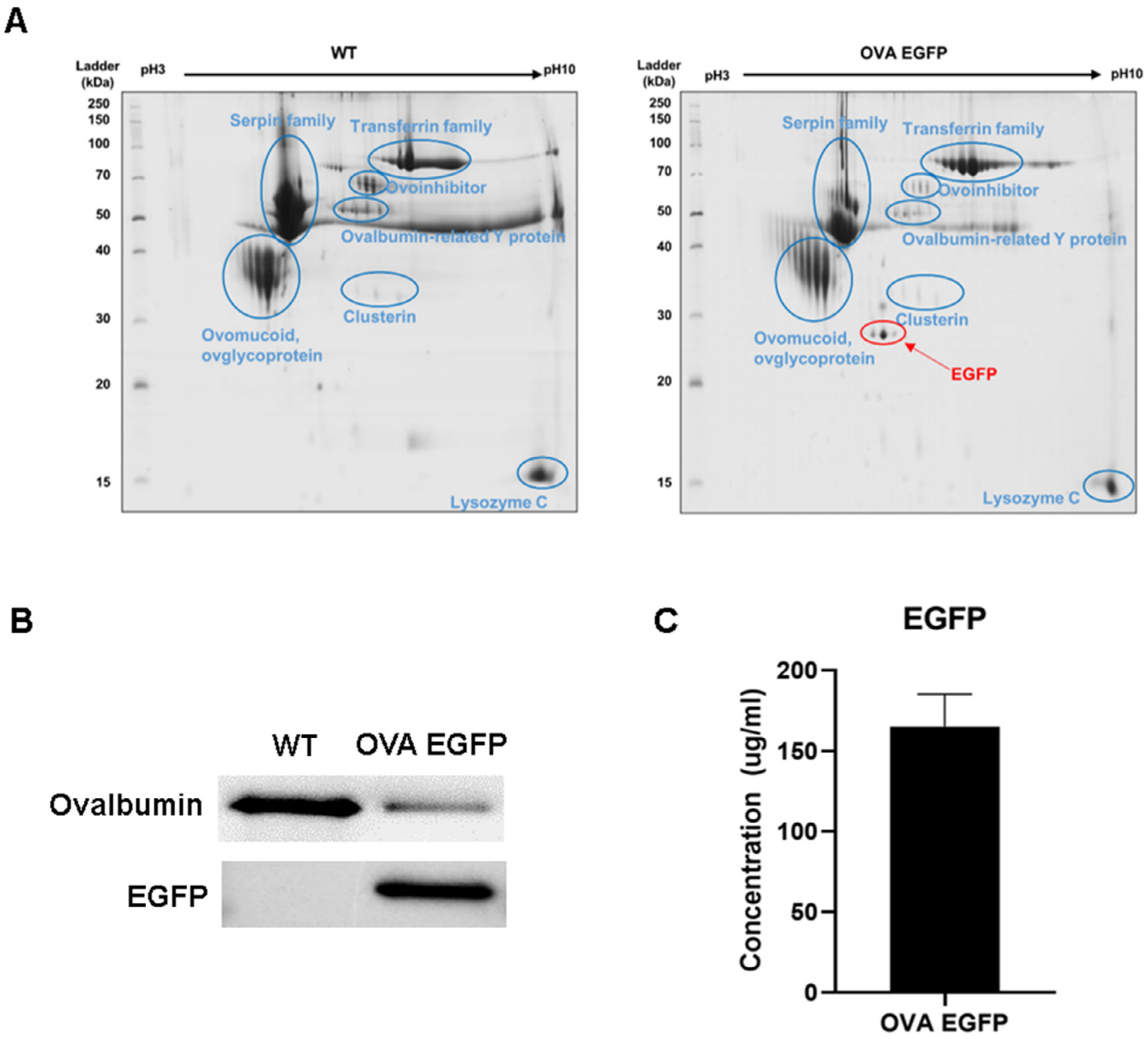

Figure 5. Compositions of egg-white protein from *OVA EGFP* KI and WT chickens. **(A)** Two-dimensional gel analysis of *OVA EGFP* KI chicken egg-white protein. Red box, area of EGFP detection. **(B)** Western blotting of OVA and EGFP in oviducts of *OVA EGFP* KI chicken egg white. **(C)** EGFP protein concentration in *OVA EGFP* KI chicken egg, as determined by enzyme-linked immunosorbent assay.

We devised a method for verifying foreign gene expression in newborn chicks by DES transplantation. Because estrogen induces the proliferation and differentiation of immature chicken oviducts, DES increases the expression levels of major egg-white proteins such as ovalbumin and lysozyme, as well as the development of the oviduct (Kohler et al., 1969; Oka and Schimke, 1969; Song et al., 2011; Jeong et al., 2012, 2018). Therefore, we hypothesized that DES would allow verification of the expression of foreign proteins in young chicks. To test this hypothesis, we produced transgenic chickens in which *EGFP* was inserted into the *OVA* locus (*OVA EGFP* KI chickens). As in our previous report of oviduct development-regulating gene discovery (Song et al., 2011), a DES pellet transplanted into the abdomen of 7-day-old chicks resulted in rapid oviduct development, along with the expression of both *EGFP* and *OVA*. This strategy allows the confirmation of exogenous protein expression at 3 wks of age. In other studies with chicken bioreactors (Kwon et al., 2018; Oishi et al., 2018; Mukae et al., 2020), the confirmation of exogenous protein expression was achieved in the eggs of G_1 hens after G_0 germline chimeras had been mated with WT hens; this required approximately 1 y. In addition, the expression of EGFP in cell lysate and culture medium was confirmed, suggesting the potential for use of this strategy to produce chicken cell culture-based bioreactors. Furthermore, EGFP was well expressed in the oviducts and eggs of OVA EGFP KI hens as in the oviducts of DES-treated chicks. These results suggest that the expression of exogenous proteins in chicken bioreactors can be confirmed from young chicks similar to adult, so it will be effective for shortened the period of exogenous protein verification.

High exogenous protein expression in KI eggs resulted in sterile offspring of G_1 females, diminished egg size, and reduced OVA protein concentration. No offspring were produced from 184 eggs of the G_1 females that had been produced through artificial insemination (Table 2). This low fertilization rate of *OVA* KI chicken is similar

to the findings in a previous report (Oishi et al., 2018). It is unlikely that EGFP was responsible for this phenomenon because it is nontoxic and has been used in previous chicken bioreactors (Kwon et al., 2004; Koo et al., 2006; Park and Han, 2012; Lee et al., 2019; Wang et al., 2019). Instead, changes in the water content and secretion of other egg major white proteins may have led to the poor fertilization rate. Further research is needed to determine how foreign protein production affects the levels of other egg-white proteins. Notably, the decrease in total protein content was proportional to the decrease in egg size, but inversely related to ESM thickness. Meanwhile, because production of OVA KI female-derived offspring is difficult, sustainable bioreactors can be maintained by breeding OVA KI males with wild-type females to produce OVA KI offspring (Woodfint et al., 2018).

Despite these limitations, chicken bioreactors have advantages such as a glycosylation pattern similar to the pattern in humans (Sheridan, 2016; Kim et al., 2018), as well as the presence of antibacterial, antimicrobial, and proteinase-inhibitor proteins in egg white (Nagase et al., 1983; Korpela et al., 1984; Mine et al., 2004; Pellegrini et al., 2004; Legros et al., 2021). There is a need to investigate the relationship between exogenous protein overexpression and the reduction of total protein concentration in egg white. Nevertheless, our system could be applied to chicken bioreactors for the production of therapeutic proteins, which require stepwise verification and accurate analysis of physical properties. The system will enable early evaluation, including efficacy and toxicity testing, of exogenous proteins in chicken bioreactors.

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DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

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