RESEARCH ARTICLE

Improving germline transmission efficiency in chimeric chickens using a multi-stage injection approach

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Abstract

Although different strategies have been developed to generate transgenic poultry, low efficiency of germline transgene transmission has remained a challenge in poultry transgenesis. Herein, we developed an efficient germline transgenesis method using a lentiviral vector system in chickens through multiple injections of transgenes into embryos at different stages of development. The embryo chorioallantoic membrane (CAM) vasculature was successfully used as a novel route of gene transfer into germline tissues. Compared to the other routes of viral vector administration, the embryo’s bloodstream at Hamburger-Hamilton (HH) stages 14–15 achieved the highest rate of germline transmission (GT), 7.7%. Single injection of viral vectors into the CAM vasculature resulted in a GT efficiency of 2.7%, which was significantly higher than the 0.4% obtained by injection into embryos at the blastoderm stage. Double injection of viral vectors into the bloodstream at HH stages 14–15 and through CAM was the most efficient method for producing germline chimeras, giving a GT rate of 13.6%. The authors suggest that the new method described in this study could be efficiently used to produce transgenic poultry in virus-mediated gene transfer systems.

Introduction

Genetic modification of avian species provides great benefits for industrial and medical purposes by enabling resistance to zoonotic diseases, improving meat and egg productivity, and generating effective bioreactor models [1,2]. Despite these great benefits, the unique reproductive system of avian species has restricted the application of efficient transgenesis protocols routinely employed in mammals, so different strategies have been developed to produce transgenic birds [3,4]. Although great advances have been made in gene transfer methods, avian transgenic technology has not been widely used until recently mainly because of the relatively low and variable rates of germline transmission (GT) [2,5]. In addition to this limitation, many other challenges lie ahead, such as high embryonic lethality following injection of transgenes [5], the need to wait for two generations before having ubiquitously expressing
transgenic birds [3], insertional mutagenesis due to the lack of precise control over transgene integration site (especially in direct and virus-mediated gene transfer systems), and uncontrolled overexpression of transgenes [5,6]. Despite these above-mentioned limitations, a scientometric analysis was conducted and the results showed a recent increase in publications on transgenic poultry research [7], indicating the real possibility of application of this technology in various fields in future.

Numerous studies have attempted to produce genetically-modified transgenic birds by transducing blastoderm embryos at Eyal-Giladi and Kochav (EGK) stage X [8] using retroviruses. However, the transduction efficiency of G₀ founders has been reported to be low with a limited rate of GT [9–13]. To further improve the efficiency of this system, some research groups have used lentiviral vectors [14–21]. The GT efficiency using lentiviral vectors was low in these reports, ranging between 0.6 and 4.0%, although a relatively higher GT (average 17.8%) has also been reported by a research group following injection of lentiviral vectors into the subgerminal cavity of newly laid eggs [22].

To optimize the GT, some researchers have attempted to determine the ideal developmental stage for viral vector injection in chicken embryos. For example, Kawabe et al. [23] reported that the highest rate of transduction of germline cells could be obtained when the viral solution was injected directly into the blood stream of chicken embryo after 55 h of incubation at Hamburger-Hamilton (HH) stages 14–15 [24]. Likewise, through direct injection of a lentiviral vector into the blood vessels of quail embryos at similar stages (HH 13–15), Zhang et al. [25] achieved a GT efficiency of 13.0%, nearly 8 times higher than that achieved through the conventional method of blastoderm injection. Both studies proposed that circulating primordial germ cells (PGC) in early embryos are effective viral targets for generating transgenic birds using retroviral vectors.

PGC-mediated gene transfer has been suggested to be the most appropriate strategy to overcome the low GT efficiency in avian species [26–29]. Different studies have reported that ex vivo manipulated chicken PGC show much more improvement in GT efficiency than do the viral-mediated systems when introduced into the bloodstream of recipient embryos [30–35]. However, the processes of isolation, culture, and genetic modification of PGC, as well as screening of genome-edited PGC, are time-consuming and complicated procedures for routine use which require highly skilled operators [36].

In this study, we present the first successful use of chick embryo chorioallantoic membrane (CAM) vasculature as an accessible route for germline gene transfer. We also assessed the germline targeting efficiency of a multi-stage injection through three different routes of lentiviral vector administration. Since the multi-stage injection approach requires the eggshells to be opened at different times during the development process without compromising embryo survival, here we established a novel procedure for eggshell windowing at the desired time points during embryonic development.

Materials and methods

Ethics statement

All the procedures for the handling and treatment of chickens in this study were approved by the Royan Institute Animal Ethics Committee (No. R-084-2003). All experimental chickens were housed under optimum conditions of light and temperature with regular cleaning. In addition, chickens were provided ad libitum access to fresh food and water.
Virus particles production and titration

To make HIV-based lentiviral vector particles encoding enhanced green fluorescent protein (EGFP), confluent human embryonic kidney (HEK293T) cells were co-transfected with pWPXL (Addgene, Cambridge, MA, USA), and a mixture of packaging helper plasmids (psPAX, pMD2G, Addgene). Transfection was achieved using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. The supernatants containing viral particles were harvested at 48 and 60 h after transfection and filtered through 0.45 μm cellulose acetate filters (Millipore, USA). Afterwards, the lentiviral particles were concentrated by ultracentrifugation at 21000 rpm for 2 h at 4°C. The resulting viral pellet was resuspended in an adequate volume of Dulbecco’s modified Eagle’s medium (DMEM)/high glucose (Sigma-Aldrich, St. Louis, MO, USA) with 10% inactivated serum, aliquoted and stored at −80°C.

The biological titration of lentiviral vectors expressing EGFP was carried out by flow cytometry as previously described [37]. Lentiviral stocks with titers of 1×10⁹ transducing units per milliliter (TU/ml) were used for experimentation.

Chicken embryo injection and hatching

Fertilized chicken eggs (Iranian native Golpayegani breed) were obtained from a local poultry farm at the peak production period. Each embryo was injected thrice (Group BAC) at different stages of its development and through different routes as follows: at stage X, into the sub-germinal cavity of blastoderm embryo, at HH stages 14–15, into the dorsal aorta of the embryos, and at HH stage 37 (on the 11th day of incubation), into the CAM vasculature. For the first injection, fresh and fertile eggs were placed vertically with their blunt end down at room temperature (RT) for 4 hours before manipulation, allowing the embryos to float to the highest point inside the shell. A round window with a diameter of approximately 1.5 cm was opened at the pointed end of each egg using a handheld rotary tool (Dremel, model number: 4000-4/65) to reach the blastoderm embryo. Before removing the cut eggshell piece, it was marked according to its position with respect to the cutting edge so that it could easily be put back to its original location. The eggshell pieces were labeled and stored in phosphate buffered saline solution (PBS), containing 10% egg albumen and 1% penicillin-streptomycin and a cell culture incubator at 37°C until further use. Two microliters of the concentrated lentiviral vector stock (S1 Fig) with a titer of 1×10⁹ TU/ml were injected into the sub-germinal cavity using a pulled-glass capillary needle (inner diameter: 40 μm; World Precision Instruments, Sarasota, FL, USA) attached to an oil hydraulic injection system (CellTram oil, Eppendorf). Before the injection, phenol red (Sigma-Aldrich, St. Louis, MO, USA) was added to the viral solution at a final concentration of 0.1% to facilitate visualization during microinjection. Correct injections were confirmed by visual inspection verifying the spreading of the viral solution across the entire area pellucida but not outside the borders of the area opaca (Fig 1a). After the injection, eggshells were completely filled with fresh albumen collected from other freshly laid eggs (Fig 1b). Having an overflow of egg albumen is necessary to prevent air bubble formation after sealing the window. A small piece of plastic wrap (PVC cling film, Pilgon, Iran) was stretched over the window (Fig 1c) and its ends were secured with a hot glue gun (Shanghai Techway Industrial Co., China) to the eggshell. The sealed eggs were incubated in a vertical position (with the window uppermost) in a humidified incubator (Rcom Maru 380 Deluxe, Rcom, Korea) at 37.7°C and 55% relative humidity without turning.

After 55 h of incubation, the manipulated eggs were removed from the incubator and the plastic wrap was peeled away carefully to avoid damaging the embryo vasculature. Two microliters of the lentiviral vector solution (at the same concentration of the first injection) were
Fig 1. The three-stage injection method in chicken embryos from the blastoderm stage to the hatching using original eggshells. (a) A round window with a diameter of approximately 1.5 cm was opened at the pointed end of the non-incubated fertilized eggs (stage X); the viral solution, containing phenol red, was injected into the sub-germinal cavity. The injected solution had to be spread across the area pellucida but it should not surpass the borders of the area opaca. (b) Thin albumen was added to fill the shells so that they would slightly overflow when the cover was put on the window. (c) Plastic wrap was stretched over the window and its ends were secured with a hot glue gun to the eggshell. (d) After 55 h of incubation, the plastic wrap was removed and the second injection was performed into the dorsal aorta at HH stages 14–15 embryo. (e) The distribution of the injection solution, containing phenol red, throughout the
injected into the dorsal aorta of each embryo at HH stages 14–15 using a pulled-glass capillary needle (inner diameter: 40 μm; World Precision Instruments, Sarasota, FL, USA) attached to an oil hydraulic injection system under a stereo-microscope. In order to visualize and confirm the distribution of the injection solution throughout the embryonic vasculature, phenol red was added to the viral solution at a concentration similar to that of the first injection (Fig 1d and 1e). After the injection, each eggshell fragment was removed from the storage, put back into its respective place across the window, and fixed with a hot glue gun (Fig 1f and 1g). The manipulated eggs were then incubated in an incubator (Rcom Maru 380 Deluxe, Rcom, Korea) at 37.7˚C and 55% relative humidity in a vertical position with the air sac uppermost and egg turning every 2 h.

On the 11th day of incubation (HH stage 37), the manipulated eggs were candled at their blunt end to identify blood vessels of CAM. A small window was carefully drilled over the site of a prominent blood vessel and the drilled shell piece was gently removed, leaving the underlying white shell membrane intact. A drop of mineral oil was used to render the underlying blood vessel transparent and 4 μl of the viral solution, at the same concentration used previously, were injected through the transparent eggshell membrane into the blood vessels using a pulled-glass capillary needle (inner diameter: 80 μm; World Precision Instruments, Sarasota, FL, USA) attached to an oil hydraulic injection system. Before injection, the plunger was aspirated slightly to ensure that the needle was successfully inserted into a blood vessel (Fig 1h). After the eggshell window was sealed with a hot glue gun (Fig 1i), the eggs were returned to the incubator (Rcom Maru 380 Deluxe, Rcom, Korea). At the end of the 18th day of incubation, egg turning was stopped and the eggs were placed in hatching trays at 37.2˚C and 65% relative humidity until hatching (Fig 1j).

**Experimental groups**

The eggs were grouped according to the route of injection (B: Blastoderm injection at EGK stage X; A: Aorta injection at HH stages 14–15; and C: Chorioallantoic membrane injection at HH stage 37).

- Group BAC: a triple injection into the blastoderm, aorta, and CAM
- Group BA: a double injection into the blastoderm and aorta
- Group BC: a double injection into the blastoderm and CAM
- Group AC: a double injection into the aorta and CAM
- Group B: a single injection into the blastoderm
- Group A: a single injection into the aorta
- Group C: a single injection into the CAM
- Group Co (Control group): no injection
- Group Sh (Sham group): a triple injection of PBS

**Genomic DNA analysis**

Genomic DNA samples were extracted from the semen of mature G₀ roosters and the blood of G₁ hatchlings using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. For genomic PCR analysis, a primer pair was designed to
include the upper strand (5´-TCCGATCACGAGACTAGCC-3´) and lower strand (5´-CATGGACAGCTGTACAA-3´) sequences and used for amplification of a 780 bp DNA fragment encoding EGFP gene. PCR amplification was performed using the subsequent cycles: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 45 sec, primer annealing at 56°C for 45 sec, and polymerization at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The plasmid DNA was used as the positive control, and the genomic DNA isolated from the wild-type chickens was used as the negative control. Finally, 5 μl of each genomic PCR amplicon was analyzed by agarose gel electrophoresis.

**Reverse transcription quantitative PCR (RT-qPCR) analysis**

Total RNA was isolated from the testes of G₀ newly hatched chicks using TRIzol reagent (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. The isolated RNA samples were treated with DNase I (Thermo Scientific, Waltham, MA, USA) and reverse-transcribed using random hexamer-primers and M-MuLV reverse transcriptase (Takara, Japan). The resulted cDNA was used to perform quantitative PCR in an Applied Biosystems Step One Plus real-time PCR system (Applied Biosystems, CA, USA) using SYBR Green PCR Master Mix (Takara, Japan). The relative expression levels of the target gene (EGFP) were calculated using the 2^−ΔΔCt method. For EGFP, the following primers were used for the real-time PCR: forward, 5´-AGCAGAAGAACGGCATCAAG-3´ and reverse, 5´-GGTGCTCAGGGTAGGTTGTC-3´. The following primers were also used to amplify chicken glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene as the internal control: forward 5´-GATTCTACACACGGACACTTCAAGG-3´ and reverse 5´-ACAATGCCAAAGTTGTCATGGATGAC-3´. All primers designed by Beacon Designer 7.9 software (Premier Biosoft International, Palo Alto, CA, USA).

Real-time PCR reactions were performed in triplicates with the following cycling conditions: 95°C for 2 min followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec.

**Visualization of EGFP in organs of transgenic chickens**

The tissue localization of EGFP in transgenic chickens was examined either by the microscopic detection of native EGFP-fluorescence in frozen sections or immunohistochemistry with an anti-GFP antibody in paraffin-embedded sections. Briefly, organ tissues were excised and fixed in 4% paraformaldehyde in PBS at 4°C overnight. For frozen sections, after the overnight cryo-protection of tissues in a sucrose solution (30% sucrose in PBS), the tissues were embedded in OCT compound (Tissue-Tek, Sakura, Torrance, CA). Sections at 7 μm thickness were cut on a cryostat (Leica Biosystems, Wetzlar, Germany), mounted on positively charged slides, and stored at −80°C until assay. For immunohistochemistry staining, the fixed samples were embedded in paraffin and sectioned at 4 μm thickness. After deparaffinization, antigen retrieval and blocking of nonspecific antigenic sites, the sections were incubated with a rabbit anti-GFP antibody (Abcam, Cambridge, UK; 1:1000) overnight at 4°C and then with the goat anti-rabbit Alexa Fluor 488 secondary antibody (Life Technologies, Ltd., Paisley, UK; 1:500) for 2 h at RT, followed by counterstaining with 4’,6-diamidino-2-phenylindole (DAPI). The immunostained sections were imaged under a fluorescence microscope (BX51; Olympus, Japan), which was equipped with appropriate filter sets and coupled to a digital camera (DP72, Olympus). A negative tissue specimen was used for each tissue type to ensure the elimination of autofluorescence.

**Flow cytometry analysis**

Single-cell suspension of testicular cells was prepared by a one-step enzymatic digestion protocol [38]. The resulting single cells were then diluted to 10^6 cells/ml in PBS and directly
analyzed for EGFP fluorescence on a FACSCalibur instrument using CellQuest software (BD Biosciences, San Jose, CA, USA). EGFP-positive cells were identified by comparison with single-cell suspensions obtained from the testes of wild-type chicks.

**Western blot experiments**

Testicular EGFP expression at the protein level was further confirmed by western blot analysis of the whole testis proteins using an anti-GFP antibody. The samples were incubated for 15 minutes at −20°C after the addition of acetone to allow protein precipitation. Afterwards, the solutions were centrifuged for 10 min at 14,000 g and 4°C and the precipitates were resuspended in a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, pH 8.5, 0.002% bromophenol blue, 65 mM DTT, 1 mM EDTA, and 1 mM PMSF). Equal amounts (30 μg) from each protein sample were separated by SDS-PAGE and transferred to PVDF membranes (PVDF; Biorad, USA). The membranes were blocked with 10% (W/V) skim milk (Merck, USA) and subsequently incubated with primary antibodies for 2 h at RT. Specific primary antibodies were monoclonal purified anti-GFP (Biolegend, San Diego, CA, USA; 1:1000) and monoclonal anti-β-actin (Clone AC-74, Sigma-Aldrich, St. Louis, MO, USA; 1:500) antibodies. After being washed with TBST buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% tween 20), the membranes were incubated with secondary antibodies for 45 min. The secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000, Dako, Japan) and HRP goat anti-rat IgG antibody (Biolegend, San Diego, CA, USA; 1:4000). The protein bands were visualized using an Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, Germany).

**Statistical analysis**

Data were analyzed using SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA) by one-way ANOVA, followed by the Tukey’s post-hoc test. The hatching rates, hatching mortality rates, transgenic frequencies, and germline transmission rates of different experimental treatments were compared using the chi-squared test. Values are expressed as mean ± SEM (standard error of the mean) and P < 0.05 was considered statistically significant.

**Results**

**Effect of injection method on viability and transgenic frequency in G₀ presumptive founders**

As shown in Table 1, the triple injection (BAC and sham groups) had the most adverse effect on hatchability compared with other types of injection, including single and double injections. Among the injection routes, the lowest hatchability was observed in the blastoderm group (B vs. A and C). However, neither the injection frequency nor the injection route affected the sex ratio. Similar to hatchability, the number of injections and the route of injection also affected the hatching mortality rate, with the highest rates observed in groups with triple injection and those with injection into the blastoderm. Fig 2a shows DNA amplification (EGFP region) from semen samples collected from G₀ roosters. Regarding the transgenic frequency, based on the percentage of EGFP-positive semen samples (Table 1), the lowest frequency was observed when the injection was performed into the blastoderm (P < 0.05), whereas no significant difference was observed between the dorsal aorta and CAM route groups (P = 0.67). Both the double injection into the dorsal aorta and CAM route (AC group) and the triple injection (BAC group) achieved higher germline-transgenic frequencies in the G₀ generation, compared
to the single injection groups \((P < 0.05)\), while no significant difference was found between the double- and triple-injection groups \((P > 0.41)\).

RT-qPCR and flow cytometry results

We quantitatively assessed the EGFP expression at both mRNA (Fig 2b) and protein (Fig 3) levels in the testes of G₀ newly hatched chicks. The RT-qPCR data revealed that the triple-injection group had the highest EGFP expression, which was significantly higher than that of
the double- and single-injection groups ($P < 0.05$) (Fig 2b). Among the double-injection groups, the relative expression of EGFP was significantly lower in the BC group than BA and AC groups ($P < 0.05$) (Fig 2b). Among double- and single-injection groups, only two groups (BC and A) showed no significant difference ($P > 0.05$) (Fig 2b). No significant differences were observed among the single-injection groups ($P > 0.05$) (Fig 2b).

As depicted in Fig 3a, over 90% of the testis samples which were collected from the double- and triple-injection groups and analyzed by flow cytometry contained EGFP positive cells, while this percentage drastically reduced in testis samples collected from the single injection groups, 33.3% (B), 45.4% (A), and 61.5% (C). The results of flow cytometry analysis also revealed that the percentage of testicular cells positive for EGFP was significantly higher in the triple-injection group (BAC) than that of the double injection groups ($P < 0.05$) and in the double injection groups than that of the single injection groups ($P < 0.05$) (Fig 3b and 3c). Among the double injection groups, no significant difference was observed between BA and
BC groups ($P > 0.05$); however, the percentages of EGFP positive testicular cells in the aforementioned groups were significantly lower than that of the AC group ($P < 0.05$) (Fig 3b and 3c). We also detected no significant difference between groups A and C in terms of the percentage of EGFP positive cells ($P > 0.05$), but the mean values for these two groups were significantly higher than that of the group B ($P < 0.05$) (Fig 3b and 3c).

**Western blot analysis and fluorescence microscopy**

We assessed the EGFP and $\beta$-actin expression at the protein level by Western blot analysis in the testicular tissues of $G_0$ hatched chicks (Fig 4a) in which their EGFP expression was previously confirmed by the RT-qPCR (Fig 2b) and flow cytometry (Fig 3) assays. Our results showed that all the seven vector-injected groups were positive for EGFP protein. Despite similar levels of $\beta$-actin expression, the level of EGFP expression varied between groups. Fig 4b shows the immunostaining for EGFP protein in the pancreas, spleen and testis tissue sections from a $G_0$ chick in group C, indicating mosaic expression of the transgene in these tissues. The assessment of the EGFP expression in cryosections of different tissues showed that native EGFP fluorescence was present in most of the examined tissues; as shown in the pancreas, spleen and testis tissues taken from a $G_0$ chick in group C (Fig 4c).
Germline transmission evaluation in G₁ generation

To assess the germline transmission (GT) efficiency, three germline chimeric roosters from each group were randomly selected and mated with wild-type hens. DNA derived from the blood of G₁ chicks, obtained by the above crossing, was assessed for the presence of EGFP using PCR (Fig 5a), and the results were expressed as transgenic ratios in G₁ progeny (Table 1). The best result for this parameter was observed in the AC group, which received a double injection into the dorsal aorta at HH stages 14–15 and CAM, with a GT efficiency of 13.6%. Although the ratio for the triple-injection group (BAC) was lower than that of the AC group, the difference was not significant ($P = 0.18$). Comparison of the results between the three independent injection routes revealed that the lowest rate of GT was achieved when the injection was carried out into the blastoderm (0.4%), whereas injection into the dorsal aorta at HH stages 14–15 resulted in the highest GT rate (7.7%). Injection of viral vectors into the CAM vasculature resulted in a GT efficiency of 2.7%, which was significantly higher than that obtained by injection into embryos at the blastoderm stage ($P < 0.05$). Transgene inheritance in G₁ chicks was further confirmed by the sequencing of PCR products obtained from the amplification of blood DNA (S2 Fig). Ubiquitous EGFP fluorescence was detected in various tissues of transgenic G₁ chicks, as shown for the liver tissue in Fig 5b.

Discussion

A variety of strategies have been used for the generation of transgenic poultry; however, the efficiency of chimera production and transgene inheritance is still low [5]. To improve the efficiency of generating germline-transmitting chimeric chickens, we aimed to assess the multi-injection of viral vectors into embryos at three different stages of development using the advantages of HIV-1 derived lentiviral vectors (S1 Fig) and modified manipulation of chicken eggs at the desired developmental stages (Fig 1). In this modified approach, we used the original eggshells instead of the surrogate eggshells [39] to maintain embryo development from laying eggs to hatching. Up to our knowledge, this is the first report presenting this novel modified approach for improving germline transmission efficiency in chicken chimeras.
The minimum hatchability of this technique was 52.9%, which is higher than that reported (less than 37%) in most of the previous studies using a surrogate eggshell system [6,11,13,15,18,22,40–43]. Although some improvements have been reported in the culture of chicken embryos [44–47], culture methods using surrogate eggshells or artificial vessels suffer from several disadvantages, such as high economic costs, long operating time, inconsistency between the morphology of the original eggs and the surrogate shells, and the risk of injury to or contamination in the embryo during the process of transferring egg content [48]. In addition, it is important to note that the rates of hatchability achieved in this study is following single-, double- and triple- injection and it is clear that over-manipulation could be more detrimental to embryo survival than a single manipulation. Therefore, the new approach described in this study could have potentially important consequences for future research on the production of recombinant proteins in highly efficient poultry systems rather than other expression systems such as bacteria or mammalian cells [21,49].

The prevention of the transfer of embryos from their shells to surrogate shells can be considered as one of the reasons for the improved efficiency of the modified approach in this study. Other reasons for this improved efficiency could be the reduced dead-in-shell mortality rate, which routinely occurs during the last 3 days of incubation in the classical surrogate eggshell system due to yolk sac infection and egg turning limitation [46,48], and also maintaining optimal gas exchange across the eggshell pores during incubation by keeping the developing embryos in their original eggshells. It is imperative to underscore that the aim of this study was not to compare two methods; rather, the study was designed to investigate the efficiency of generating germline transgenic chickens using multiple injections at three different developmental stages.

The tissue composition and accessibility of the CAM for experimental manipulation, have attracted many researchers. The highly vascularized network of the CAM has been widely used as an experimental model to evaluate tumor cell metastasis and dissemination in vivo, as well as to study anticancer drug effects on tumor inhibition [50–53]. Several studies have demonstrated that many tumor cells can be metastasized into various tissues (e.g. liver, spleen, lung, pancreas, brain, bursa of fabricius, bone marrow, ovary, etc.) after cell inoculation or intravasation into the CAM vasculature [53–57]. Hen and colleagues [58] were the first researchers who used the CAM as a route of gene delivery into the somatic tissues of chickens with the highest levels of expression in the liver and spleen. In the current study, for the first time, we demonstrated the possibility of using CAM as an accessible route of gene transfer to germline cells. However, the mechanistic basis of the above observations and our findings remains unclear due to the lack of enough knowledge about the developmental characteristics of CAM such as its lymphatic and vascular system remodeling and permeability [52], which could affect the pattern of biodistribution of viral vector particles. Based on the pilot experience, we found that a minimum volume of 4 μl of concentrated viral stock (1×10⁹ TU/ml) had to be injected into the CAM for consistent detection of EGF gene in the semen samples. However, due to the limited capacity of the receiving vessels, the amount injected into the dorsal aorta of embryos at HH stages 14–15 did not exceed 2 μl. Therefore, caution should be exercised when comparing the two injection routes in terms of GT efficiency as similar volumes of viral vectors were not injected. We acknowledge that this may be seen as a shortcoming of our approach, but improving the overall GT efficiency was the main focus of this study.

Germline chimeric roosters (G₀) which were identified as positive based on the genomic PCR screen were mated with wild type hens in order to assess the rates of GT of the transgene to the resultant G₁ offspring. We didn’t measure the level of chimerism for each individual G₀ rooster, because the main focus of this study was to assess the overall germline transgenesis for each method. However, we acknowledge this as a shortcoming of our analysis, making it
impossible to assess the correlation between the degree of chimerism in each selected G₀ rooster and the level of germline transgenesis. PCR analysis was performed on blood DNA from G₁ chicks to identify transgenic individuals. Subsequently, DNA sequencing of the PCR products was carried out to confirm transgene integration in the G₁ chicks. The injection of lentiviral vectors into the dorsal aorta at HH stages 14–15 yielded a GT efficiency of 7.7%, which was significantly higher than those when the injection was administered into the blastoderm (0.4%) or CAM (2.7%). Despite the significant improvement observed in the GT efficiency when using the dorsal aorta and CAM routes compared to the blastoderm route, in the double-injection system, when the injection was administered into the blastoderm and then either dorsal aorta (BA: GT efficiency 3.1%) or CAM (BC: GT efficiency 0.4%), the GT efficiency was significantly reduced. These results indicate that the injection into the blastoderm is inversely correlated with the GT efficiency. Similar results were observed when the triple-injection group (BAC: GT efficiency 9.5%) was compared with the double-injection group (AC: GT efficiency 13.6%), further emphasizing the inverse correlation mentioned above. This observation raises the possibility that early injection into chick embryos at blastoderm stage may lead to a decrease in germ cells pool which is considered as the target of gene transfer at later stages.

The results regarding the EGFP expression at the mRNA level were not totally in line with the above findings. The relative expression of EGFP at the mRNA level was similar between the groups with a single injection despite to the different injection routes. However, in the double- and triple-injection groups, the relative expression of EGFP at the mRNA level was significantly higher than that of the single injection groups (Fig 2b). These findings indicate that although the mRNA expression level was higher in the BA, BC, and BAC groups, which received an additional injection into the blastoderm, their GT efficiency did not increase compared to A, C, and AC groups, respectively. This incompatibility may be related to the differential ability of PGC in the blastoderm, compared to that of the circulating PGC at HH stages 14–15 or gonadal germ cells at the HH stage 37, to allow for transgene integration into their genome. This differential ability could be attributed to the high heterochromatin content and chromatin epigenetic modifications in early PGC in the blastoderm relative to the late PGC [59]. It is also of note that the number of PGC at the blastoderm stage was reported to be significantly lower compared to the later stages [60]. In addition, PGC at the blastoderm stage might be more vulnerable to manipulation than those at the later stages. These two latter possibilities can also account for the differential gene targeting ability of PGC in the blastoderm. Furthermore, the increase in the EGFP mRNA expression could be related to other testicular cells as the efficiency of GT is affected by the efficiency of gene targeting in PGC. Another possibility is that although the PGC in the blastoderm were targeted successfully by the viral vectors, they were not able to form functional gametes that can produce offspring. Similar results were also observed at the protein level assessed by flow cytometry (Fig 3b and 3c). The percentage of testis samples positive for EGFP also indicates that the injection into the blastoderm is not the best route to obtain transgene-expressing testis cells with high repeatability (Fig 3a). The finding of low germline transgenesis observed in this study was consistent with that of the previous studies which used blastoderm-stage embryos for germline gene transfer [14,15,17–21,25]. The results of this study and those of earlier studies suggest that early blastoderm PGC in chick embryos may not be a suitable target for transgene integration and hence gene targeting in chicken germ cells should be aimed at later stages [23,25,61–64].

The groups receiving at least one injection into the blastoderm, including the B, BA, BC, BAC, and Sh groups, showed the highest G₀ hatchling mortality and the lowest hatchability rates. These results might be due to the direct effect of early viral injection on embryo survival
and post-hatching development or, alternatively, the effect of air entrance on the embryo survival during the blastoderm stage associated with egg windowing [65].

Conclusions

The results of this study showed that the embryo’s bloodstream at HH stages 14–15 was the most effective route of viral vector administration in terms of germline gene transfer. The embryo CAM vasculature was successfully used as a novel route of gene transfer into germline tissues. Thus, CAM can be considered as a possible route for the germline transmission of in vitro modified PGC in future studies. In addition, double injection of transgenes into the dorsal aorta, at HH stages 14–15, and CAM vasculature, at HH stage 37, was found to be the most efficient method in terms of hatchability rates of the manipulated eggs, hatchling viability and GT efficiency. This new double-injection strategy combines the simplicity and reliability, so that can be employed in place of the classical less efficient method of using a single injection into the sub-germinal cavity of blastoderm embryos. Accordingly, we believe that this new strategy has the potential to become a versatile and effective tool for germline gene transfer.

Supporting information

S1 Fig. The schematic representation of the pWPXL lentiviral vector. LTR, long terminal repeat; RRE, ref-responsive element; cPPT, central polypurine tract; pEF1a, human elongation factor-1 alpha promoter; EGFP, enhanced green fluorescent protein; WPRE, woodchuck hepatitis virus posttranscriptional control element. (PDF)

S2 Fig. A sample sequencing of the genomic PCR product that contained the EGFP sequence amplified from a genomic sample of transgenic G1 chick. Here, the sequence of genomic PCR product is compared with the EGFP sequence in pWPXL viral vector using SnapGene software. The result of sequence analysis confirmed the integration of the vector into the chicken genome. Upper sequence: EGFP ORF amplified from the genome-integrated pWPXL. Lower sequence: EGFP ORF in pWPXL viral vector. (PDF)

S1 Raw images. (PDF)

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References

1. Oishi I, Yoshii K, Miyahara D, Kagami H, Tagami T. Targeted mutagenesis in chicken using CRISPR/Cas9 system. Sci Rep. 2016; 6(1): 23980. https://doi.org/10.1038/srep23980 PMID: 27050479

2. Lee HJ, Kim YM, Ono T, Han JY. Genome modification technologies and their applications in avian species. Int J Mol Sci. 2017; 18(11): 2245. https://doi.org/10.3390/ijms18112245 PMID: 29072628

3. Cooper CA, Challagulla A, Jenkins KA, Wise TG, O’Neil TE, Morris KR, et al. Generation of gene edited birds in one generation using sperm transfection assisted gene editing (STAGE). Transgenic Res. 2017; 26(3): 331–347. https://doi.org/10.1007/s11248-016-0003-0 PMID: 27896535

4. Han JY, Park YH. Primordial germ cell-mediated transgenesis and genome editing in birds. J Anim Sci Biotechnol. 2018; 9(1): 81–89. https://doi.org/10.1186/s40104-018-0234-4 PMID: 29372515

5. Bednarczyk M, Kozlowska I, Lakota P, Szczepińska A, Krawczuk T. Generation of transgenic chickens by the non-viral, cell-based method: effectiveness of some elements of this strategy. J Appl Genet. 2018; 59(1): 81–89. https://doi.org/10.1007/s13353-018-0429-6 PMID: 29372515

6. Koo BC, Kwon MS, Kim D, Kim SA, Kim NH, Kim T. Production of transgenic chickens constitutively expressing human erythropoietin (hEPO): Problems with uncontrollable overexpression of hEPO gene. Biotechnol Bioprocess Eng. 2017; 22(1): 22–29.

7. Farzanmanesh, Hassan SN, Mozdziak P, Baharvand H. Avian embryos and related cell lines: A convenient platform for recombinant proteins and vaccine production. Biotechnol J. 2017; 12(5): 1600598. https://doi.org/10.1002/biot.201600598 PMID: 28371379

8. Eyal-Giladi H, Kochav S. From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick: I. General morphology. Dev Biol. 1976; 49(2): 321–337. https://doi.org/10.1016/0012-1606(76)90178-0 PMID: 944662

9. Harvey AJ, Speksnijder G, Baugh LR, Morris JA, Ivarie R. Consistent production of transgenic chickens using replication-deficient retroviral vectors and high-throughput screening procedures. Poult Sci. 2002; 81(2): 202–212. https://doi.org/10.1093/ps/81.2.202 PMID: 11873828

10. Rapp JC, Harvey AJ, Speksnijder GL, Hu W, Ivarie R. Biologically active human interferon α-2b produced in the egg white of transgenic hens. Transgenic Res. 2003; 12(5): 569–575. https://doi.org/10.1023/a:1025854217349 PMID: 14601655

11. Mozdziak PE, Borwornpinyo S, McCoy DW, Petitte JN. Development of transgenic chickens expressing bacterial β-galactosidase. Dev Dyn. 2003; 226(3): 439–445. https://doi.org/10.1002/dvdy.10234 PMID: 12619130

12. Koo BC, Kwon MS, Choi BR, Kim JH, Cho SK, Sohn SH, et al. Production of germline transgenic chickens expressing enhanced green fluorescent protein using a MoMLV-based retrovirus vector. FASEB J. 2006; 20(13): 2251–2260. https://doi.org/10.1096/fj.06-5866com PMID: 17077302

13. Kwon MS, Koo BC, Choi BR, Park YY, Lee YM, Suh HS, et al. Generation of transgenic chickens that produce bioactive human granulocyte-colony stimulating factor. Mol Reprod Dev. 2008; 75(7): 1120–1126. https://doi.org/10.1002/mrd.20860 PMID: 18196568

14. Chapman SC, Lawson A, MacArthur WC, Wiese RJ, Loechel RH, Burgos-Trinidad M, et al. Ubiquitous GFP expression in transgenic chickens using a lentiviral vector. Development. 2005; 132(5): 935–940. https://doi.org/10.1242/dev.01652 PMID: 15673573

15. Lillko SG, Sherman A, McGrew MJ, Robertson CD, Smith J, Haslam C, et al. Oviduct-specific expression of two therapeutic proteins in transgenic hens. Proc Natl Acad Sci U S A. 2007; 104(6): 1771–1776. https://doi.org/10.1073/pnas.0610401104 PMID: 17259305

16. Kwon SC, Choi JW, Jang HJ, Shin SS, Lee SK, Park TS, et al. Production of bifunctional recombinant human interleukin 1 receptor antagonist (II RN) from transgenic quail egg white. Biol Reprod. 2010; 82(6): 1057–1064. https://doi.org/10.1095/biolreprod.109.081687 PMID: 20147736

17. Cao D, Wu H, Li Q, Sun Y, Liu T, Fei J, et al. Expression of recombinant human lysozyme in egg whites of transgenic hens. PLoS One. 2015; 10(2): e0118626. https://doi.org/10.1371/journal.pone.0118626 PMID: 25706123

18. Liu T, Wu H, Cao D, Li Q, Zhang Y, Li N, et al. Oviduct-specific expression of human neutrophil defensin 4 in lentivirally generated transgenic chickens. PLoS One. 2015; 10(5): e0127922. https://doi.org/10.1371/journal.pone.0127922 PMID: 26020529
19. Byun SJ, Yuk SS, Jang YJ, Choi H, Jeon MH, Erdene-Ochir TO, et al. Transgenic chickens expressing the 3D8 single chain variable fragment protein suppress avian influenza transmission. Sci Rep. 2017; 7(1): 1–11.

20. Kwon MS, Koo BC, Kim D, Nam YH, Cui XS, Kim NH, et al. Generation of transgenic chickens expressing the human erythropoietin (hEPO) gene in an oviduct-specific manner: Production of transgenic chicken eggs containing human erythropoietin in egg whites. PLoS One. 2018; 13(5): e0194721. https://doi.org/10.1371/journal.pone.0194721 PMID: 29947554

21. Herron LR, Pridans C, Turnbull ML, Smith N, Lillico SG, Gilhooley HJ, Kingsman AJ, et al. Efficient production of germline transgenic chickens using lentiviral vectors. EMBO Rep. 2004; 5(7): 728–733. https://doi.org/10.1038/sj.embor.7400171 PMID: 15192698

22. Kawabe Y, Naka T, Komatsu H, Nishijima KI, Iijima S, Kamihira M. Retroviral gene transduction into chicken embryo gonads through blood circulation. J Biosci Bioeng. 2008; 106(6): 598–601. https://doi.org/10.1263/jbb.106.598 PMID: 19134558

23. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. Dev Dyn. 1992; 195(4): 231–272. https://doi.org/10.1002/aja.1001950404 PMID: 1304821

24. Zhang Z, Sun P, Yu F, Yan L, Yuan F, Zhang W, et al. Transgenic quail production by microinjection of lentiviral vector into the early embryo blood vessels. PLoS One. 2012; 7(12): e50817. https://doi.org/10.1371/journal.pone.0050817 PMID: 23251391

25. Park TS, Jeong DK, Hong YH, Park TS, Moon YK, Ohno T, et al. Production of germline chimeric chickens by transfer of cultured primordial germ cells. Cell Biol Int. 1997; 21(8): 495–499. https://doi.org/10.1006/cbir.1997.0173 PMID: 9451806

26. Lee JY, Park TS, Hong YH, Jeong DK, Kim JN, Kim KD, et al. Production of germline chimeras by transfer of chicken gonadal primordial germ cells maintained in vitro for an extended period. Theriogenology. 2002; 58(8): 1531–1539. https://doi.org/10.1016/s0093-691x(02)01061-0 PMID: 12374123

27. Park TS, Jeong DK, Kim JN, Song GH, Hong YH, Lim JM, et al. Improved germline transmission in chicken chimeras produced by transplantation of gonadal primordial germ cells into recipient embryos. Biol Reprod. 2003; 68(5): 1657–1662. https://doi.org/10.1095/biolreprod.102.006825 PMID: 12606438

28. Van de Lavor MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, et al. Germline transmission of genetically modified primordial germ cells. Nature. 2006; 441(7094): 766–769. https://doi.org/10.1038/nature04831 PMID: 16760981

29. Park TS, Han JY. PiggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens. Proc Natl Acad Sci U S A. 2012; 109(24): 9337–9341. https://doi.org/10.1073/pnas.1203823109 PMID: 22645326

30. Park TS, Lee HG, Moon JK, Lee HJ, Yoon JW, Yun BN, et al. Deposition of bioactive human epidermal growth factor in the egg white of transgenic hens using an oviduct-specific minisynthetic promoter. FASEB J. 2015; 29(6): 2386–2396. https://doi.org/10.1096/fj.14-264739 PMID: 25690652

31. Dimitrov L, Pedersen D, Ching KH, Yi H, Collarini EJ, Izquierdo S, et al. Germline gene editing in chickens by efficient CRISPR-mediated homologous recombination in primordial germ cells. PLoS One. 2016; 11(4): e0154303. https://doi.org/10.1371/journal.pone.0154303 PMID: 27099923

32. Taylor L, Carlson DF, Nandi S, Sherman A, Fahrenkrug SC, McGrew MJ. Efficient TALEN-mediated gene targeting of chicken primordial germ cells. Development. 2017; 144(5): 928–934. https://doi.org/10.1242/dev.145367 PMID: 28174243

33. Lee J, Kim DH, Lee K. Current approaches and applications in avian genome editing. Int J Mol Sci. 2020; 21(11): 3937. https://doi.org/10.3390/ijms21113937 PMID: 32486292

34. Salomon P, Trono D. Production and titration of lentiviral vectors. Curr Protoc Hum Genet. 2007; 54(1): 12–10. https://doi.org/10.1002/0471142905.hg1210s54 PMID: 18428406

35. Lee YM, Jung-JG, Kim JN, Park TS, Kim TM, Shin SS, et al. A tests-mediated germline chimera production based on transfer of chicken testicular cells directly into heterologous testes. Biol Reprod. 2006; 75(3): 380–386. https://doi.org/10.1095/biolreprod.106.052084 PMID: 16723507

36. Perry MM. A complete culture system for the chick embryo. Nature. 1988; 331(6151): 70–72. https://doi.org/10.1038/331070a0 PMID: 3340149
Koo BC, Kwon MS, Lee H, Kim M, Kim D, Roh JY, et al. Tetracycline-dependent expression of the human erythropoietin gene in transgenic chickens. Transgenic Res. 2010; 19(3): 437–447. https://doi.org/10.1007/s12148-009-9327-3 PMID: 19795218

Byun SJ, Kim SW, Kim KW, Kim JS, Hwang IS, Chung HK, et al. Oviduct-specific enhanced green fluorescent protein expression in transgenic chickens. Biosci Biotechnol Biochem. 2011; 75(4): 646–649. https://doi.org/10.1271/bbb.100721 PMID: 21512248

Kwon MS, Koo BC, Roh JY, Kim M, Kim JH, Kim T. Production of transgenic chickens expressing a tetracycline-inducible GFP gene. Biosci Biotechnol Biochem. 2011; 410(4): 890–894. https://doi.org/10.1016/j.bbrc.2011.06.088 PMID: 21708138

Naito M, Nirasawa K, Oishi T. Development in culture of the chick embryo from fertilized ovum to hatching. J Exp Zool. 1990; 254(3): 322–326. https://doi.org/10.1002/jez.1402540311 PMID: 2345349

Kamihira M, Oguchi S, Tachibana A, Kitagawa Y, Iijima S. Improved hatching for in vitro culture using surrogate eggshell and artificial vessel. Dev Growth Differ. 1998; 40(4): 449–455. https://doi.org/10.1046/j.1440-169x.1998.101-2-00010.x PMID: 9727359

Borwompinyo S, Brake J, Mozdziak PE, Petitte JN. Culture of chicken embryos in surrogate eggshells. Poult Sci. 2005; 84(9): 1477–1482. https://doi.org/10.1093/ps/84.9.1477 PMID: 16206571

Tahara Y, Obara K. A novel shell-less culture system for chick embryos using a plastic film as culture vessel. Poult Sci. 2014; 51(3): 307–312.

Ishak N, Chan M, Ang SC, Cheung C, Teoh SC. Bioengineered three-dimensional transparent eggshell as a chicken embryo experimentation platform for biomedical research. Eng Rep. 2020; 2(1): e12092.

Bahrampour A, Daneshpour A, Jazayeri SH, Mozdziak PE, Sanati MH, et al. Designing a transgenic chicken: applying new approaches toward a promising bioreactor. Cell J. 2020; 22(2): 133–139. https://doi.org/10.22074/cellj.2020.6738 PMID: 31721526

Deryugina EI, Quigley JP. Chick embryo chorioallantoic membrane model systems to study and visualize human tumor cell metastasis. Histochim Cell Biol. 2008; 130(6): 1119–1130. https://doi.org/10.1007/s10456-008-0536-2 PMID: 19005674

Klingenberg M, Becker J, Eberth S, Kube D, Wilting J. The chick chorioallantoic membrane as an in vivo model for Burkitt lymphoma. BMC Cancer. 2014; 14(1): 1–12.

Nowak-Sliwinska P, Segura T, Iruela-Arispe ML. The chicken chorioallantoic membrane model in biology, medicine and bioengineering. Angiogenesis. 2014; 17(4): 779–804. https://doi.org/10.1007/s10456-014-9440-7 PMID: 25138280

Vu BT, Shahin SA, Croissant J, Fatieiev Y, Matsumoto K, Doan TL, et al. Chick chorioallantoic membrane assay as an in vivo model to study the effect of nanoparticle-based anticancer drugs in ovarian cancer. Sci Rep. 2018; 8(1): 1–10.

Nishida Y, Miyamori H, Thompson EW, Takino T, Endo Y, Sato H. Activation of matrix metalloproteinase-2 (MMP-2) by membrane type 1 matrix metalloproteinase through an artificial receptor for proMMP-2 generates active MMP-2. Cancer Res. 2008; 68(21): 9096–9104. https://doi.org/10.1158/0008-5472.CAN-08-2522 PMID: 18974156

Grinberg I, Reis A, Ohana A, Taizi M, Cipok M, Tavor S, et al. Engraftment of human blood malignancies to the turkey embryo: a robust new in vivo model. Leuk Res. 2009; 33(10): 1417–1426. https://doi.org/10.1016/j.leukres.2009.02.009 PMID: 19297019

Zhao Z, Bauer N, Aleksandrowicz E, Yin L, Gladkich J, Gross W, et al. Intraductal papillary mucinous neoplasm of the pancreas rapidly xenografts in chicken eggs and predicts aggressiveness. Int J Cancer. 2018; 142(7): 1440–1452. https://doi.org/10.1002/ijc.31160 PMID: 29143337

Hu J, Ishihara M, Chin AI, Wu L. Establishment of xenografts of urolithic cancers on chicken chorioallantoic membrane (CAM) to study metastasis. Precis Clin Med. 2019; 2(3): 140–151. https://doi.org/10.1007/s10456-014-9440-7 PMID: 25138280

Hen G, Yosefi S, Shinder D, Or A, Mygdal S, Conditti R, et al. Gene transfer to chicks using lentiviral vectors administered via the embryonic chorioallantoic membrane. PLoS One. 2012; 7(5): e36531. https://doi.org/10.1371/journal.pone.0036531 PMID: 22606269

Kress C, Montillet G, Jean C, Fuet A, Pain B. Chicken embryonic stem cells and primordial germ cells display different heterochromatic histone marks than their mammalian counterparts. Epigenetics Chromatin. 2016; 9(1): 1–18. https://doi.org/10.1186/s13072-016-0056-6 PMID: 26865862

Nakamura Y, Yamamoto Y, Usui F, Mushika T, Ono T, Setioke AP, et al. Migration and proliferation of primordial germ cells in the early chicken embryo. Poult Sci. 2007; 86(10): 2182–2193. https://doi.org/10.1093/ps/86.10.2182 PMID: 17878448
61. Kamihira M, Ono KI, Esaka K, Nishijima KI, Kigaku R, Komatsu H, et al. High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector. J Virol. 2005; 79(17): 10864–10874. https://doi.org/10.1128/JVI.79.17.10864-10874.2005 PMID: 16103139

62. Tyack SG, Jenkins KA, O’Neil TE, Wise TG, Morris KR, Bruce MP, et al. A new method for producing transgenic birds via direct in vivo transfection of primordial germ cells. Transgenic Res. 2013; 22(6): 1257–1264. https://doi.org/10.1007/s11248-013-9727-2 PMID: 23807321

63. Yu F, Ding LJ, Sun GB, Sun PX, He XH, Ni LG, et al. Transgenic sperm produced by electrotansfection and allogeneic transplantation of chicken fetal spermatogonial stem cells. Mol Reprod Dev. 2010; 77(4): 340–347. https://doi.org/10.1002/mrd.21147 PMID: 20063420

64. Li B, Sun G, Sun H, Xu Q, Gao B, Zhou G, et al. Efficient generation of transgenic chickens using the spermatogonial stem cells in vivo and ex vivo transfection. Sci China C Life Sci. 2008; 51(8): 734–742. https://doi.org/10.1007/s11427-008-0100-2 PMID: 18677601

65. Andacht T, Hu W, Ivarie R. Rapid and improved method for windowing eggs accessing the stage X chicken embryo. Mol Reprod Dev. 2004; 69(1): 31–34. https://doi.org/10.1002/mrd.20155 PMID: 15278901