Targeting lentiviral vectors to primordial germ cells (PGCs): An efficient strategy for generating transgenic chickens

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ABSTRACT

Recent advances in avian transgenic studies highlight the possibility of utilizing lentiviral vectors as tools to generate transgenic chickens. However, low rates of gonadal chimerism and germ line transmission efficiency still limit the broad usage of this method in creating transgenic chickens. In this study, we implemented a simple strategy using modified lentiviral vectors targeted to chicken primordial germ cells (PGCs) to generate transgenic chickens. The lentiviral vectors were pseudotyped with a modified Sindbis virus envelope protein (termed M168) and conjugated with an antibody specific to PGC membrane proteins. We demonstrated that these optimized M168-pseudotyped lentiviral vectors conjugated with SSEA4 antibodies successfully targeted transduction of PGCs *in vitro* and *in vivo*. Compared with the control, 50.0%–66.7% of chicken embryos expressed green fluorescent protein (GFP) in gonads transduced by the M168-pseudotyped lentivirus. This improved the targeted transduction efficiency by 30.0%–46.7%. Efficient chimerism of exogenous genes was also observed. This targeting technology could improve the efficiency of germ line transmission and provide greater opportunities for transgenic poultry studies.

INTRODUCTION

Germ-line insertion of viral DNA by injection of recombinant avian leukosis virus (ALV) into chicken blastoderms was first described by Salter et al. (1986, 1987). Thereafter, transgenic chickens were generated by injecting replication-defective retroviral vectors into chicken embryos (Bosselman et al., 1989). Retroviruses have been widely used in gene editing and in creating genetically modified chickens. Lentiviruses, which are a kind of retrovirus, can deliver genes into both dividing and non-dividing cells. However, because of nonspecific transduction, the efficiency of using lentiviruses to prepare gonadal chimeric transgenic chickens is low, leading to low efficiency of transgenic progeny production (Cooper et al., 2019). To solve this problem, we adopted targeted transduction by pseudotyping viral vectors with an alternative envelope protein.

Several approaches to change viral tropism have been explored, e.g., inserting ligands (Boerger et al., 1999;

Kasahara et al., 1995; Valsesia-Wittmann et al., 1996), peptides (Bupp et al., 2005; Sarangi et al., 2007), or single-chain antibody fragments that recognize and bind to specific cell-surface molecules (Ahani et al., 2016; Aires Da Silva et al., 2005). However, these methods can suffer from low viral titers and fusion efficiencies after changes to the viral envelope. Therefore, it is important to develop new and improved methods of targeting specific cells with lentiviruses.

The Sindbis virus, a member of the *Alphavirus* genus, contains two transmembrane envelope proteins, E1 and E2, which form a trimer of E1/E2 heterodimers that function as a unit. However, the E1 protein can mediate the fusion of viruses with cells, independent of the receptor-binding protein E2 (Smit et al., 1999). Lentiviral vectors can be pseudotyped with Sindbis virus E2 envelope proteins modified by inserting a protein A immunoglobulin G recognition domain (ZZ domain), which enables them to bind to monoclonal antibodies that recognize surface antigens of specific cells (Morizono et al., 2001). However, the research showed that the infectivity of the viruses to liver and spleen cells remained high when intravenously injecting ZZ SINDBIS pseudotypes into mice. Thereafter, this method was improved by mutating several key sites of ZZ SINDBIS (M168), which reduced the endogenous tropism of the Sindbis envelope and allowed more viruses to infect the target cells (Morizono et al., 2005). Recent successful improvements to this lentiviral targeting system enabled it to recognize its target cells by conjugated antibodies (Allen et al., 2018; Gruell & Klein, 2018; Mason et al., 2016). In the current study, we employed a transduction system that allows entry of M168-pseudotyped lentiviruses into primordial germ cells (PGCs) by conjugating the viruses with the antibody that recognizes SSEA4, a surface molecule of PGCs. We provide a new and feasible method for generating transgenic chickens by improving the efficiency of transgenic-positive chicken production.

MATERIALS AND METHODS

Monoclonal antibodies

Immunofluorescence staining of PGCs and antibody-mediated targeted transduction of PGCs were performed using the following primary antibodies: anti-SSEA1 (Abcam, MC-480, UK), anti-SSEA3 (Abcam, MC-631, UK), anti-SSEA4 (Abcam, MC-813, UK), anti-EMA1 (Abcam, GP1.4, UK), and anti-DAZL (Abcam, EPR21028, UK). Secondary antibodies used were Alexa Fluor 488 goat anti-mouse IgM, Alexa Fluor 594 goat anti-rabbit, and goat anti-mouse antibodies (Invitrogen, Thermo Fisher Scientific, USA). Mouse anti-human HLA-ABC (Sigma, HLA class I, clone W6/32, USA) was used to mediate the targeted infection by lentiviruses and in flow cytometry analysis.

Lentivirus production

All lentiviral particles were produced in HEK 293T cells using FuGENE® HD (Promega, PRE2311, USA) transfection reagents. The HEK 293T cells ($1.8×10^7$) were transfected with either three (pWPXL, psPAX2, VSV-G or M168) or four plasmids (FUGE, pMDLg-pRRE, pRSV-Rev, VSV-G or M168) to produce lentiviruses. The vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentivirus, which has a wide range of host cell receptors, thus allowing transfection of most cell types, was used as a control. The viral particles were harvested from the culture medium after 48 h of incubation and then filtered through a 0.45 μm filter. The filtered viral particles were centrifuged at 25 000 g for 8–9 h at 4 ℃ and then centrifuged at 50 000 g for 2 h at 4 ℃. The viral particles were then resuspended in virus storage buffer and stored at −80 °C. Lentiviral titers were assayed using HIV-1 p24 ELISA Kits (XpressBio, USA) following the manufacturer's instructions. The M168 plasmid was provided by the lab of Dr. Irvin S.Y. Chen (University of California, USA); other plasmids were purchased from the Addgene website.

Lentivirus transduction of HEK 293T and BHK fibroblast cells

Different amounts of M168-lentiviruses were incubated with 1 μg of HLA antibody for 1 h on ice prior to infection. The same quantities of VSV-G lentiviruses were used as a control. HEK 293T cells ($0.5×10^5$) were infected with these vectors for 48 h at 37 ℃ with 5% CO_2. Transduction efficiency was detected via green fluorescent protein (GFP) expression in target cells using flow cytometry 2 d after infection.

A mixed population of HEK 293T cells and BHK fibroblast cells (ratio of 1:1) were infected with HLA-M168 lentiviruses or VSV-G lentiviruses for 8 h at 37 ℃ with 5% CO_2. The viruses were subsequently removed and replaced with 1 mL of DMEM supplemented with 10% fetal bovine serum (FBS), and the cells were cultured for another 48 h at 37 ℃ with 5% CO_2. After infection, the percentage of GFP-positive cells was measured by flow cytometry. Real-time polymerase chain reaction (RT-PCR) was performed using primers: GFP-F: AAACGGCCACAAGTTCAGCG and GFP-R: ATGGTG CGCTCCTGGACGTA; GAPDH-F: GGAGCGAGATCCCTCC AAAAT and GAPDH-R: GGCTGTTGTCATACTTCTCATGG.

Derivation and analysis of PGCs from white leghorn chickens

Freshly fertilized white leghorn eggs were obtained from the China Agricultural University and incubated at 37 ℃ and 60%–70% humidity, with rocking at an angle of 90° every 30 min for 6 d, after which the embryos were isolated (Song et al., 2014). After the embryos were rinsed with PBS, they were dissected, and the gonads were separated from the medial section of the abdomen with sharp tweezers under a stereo microscope (Leica, SMZ 1000, Germany). The gonadal tissues were dissociated with 0.25% trypsin (Gibco, 25200, USA) for 10–15 min at 37 ℃, and the reaction was stopped by the addition of 10% FBS (Gibco, 10099, USA). The cell suspension was centrifuged at 500 g for 5 min at 25 ℃, and then the cells were seeded into a 48-well culture plate previously coated with buffalo rat liver (BRL) cells. The cells were cultured in DMEM supplemented with 7.5% FBS, 2.5% chicken serum, 46% knockout-DMEM, 40% BRL-condition

medium, 1×NEAA, 1 mmol/L Sodium Pyruvate, 0.5 mmol/L GlutaMAX, 1% penicillin-streptomycin, 0.1 mmol/L ß-mercaptoethanol, 4 ng/mL rhFGF (R&D, 234-FSE, USA), and 6 ng/mL rmSCF (R&D, 455-MC/CF, USA). The culture medium was changed every 2 d, and the cells were passaged on day 7 and every 3–4 d thereafter. All cell culture reagents were purchased from Gibco.

To analyze PGCs, immunohistochemistry and RT-PCR were performed. The cultured PGCs were fixed with 4% paraformaldehyde for 30 min, rinsed twice with PBS and blocked with a blocking reagent for 1 h. The cells were then incubated with a primary antibody overnight at 4 ℃. After washing with PBS twice, the cells were incubated with the secondary antibody at 25 °C in the dark for 30 min and then washed with PBS three times. The cells were incubated with DAPI for 2 min under the same conditions described above. The treated cells were observed under an inverted microscope (Nikon, TS100, Japan).

Total RNA was isolated from cells using a RNeasy Mini Kit (Qiagen, 74104, Germany), and then cDNA was synthesized according to the instructions of the Reverse Transcription System (Qiagen, 205311, Germany). The RT-PCR amplification conditions were as follows: 94 ℃ for 5 min, followed by 35 cycles (94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s) and one cycle at 72 °C for 7 min. The sets of primers were: CVH-F: GCTCGATATGGGTTTTGGAT and CVH-R: TTCTCTTGGGTTCCATTCTGC; Actin-F: AACACCCAGCCA TGTATGTA and Actin-R: TTTCATTGTCTAGTGCCA.

Targeted infection of PGCs *in vitro*
Flow cytometry was used to analyze the cell surface markers commonly found on PGCs: i.e., SSEA1, SSEA4, and EMA1. The PGCs ($1×10^5$) were then incubated with M168-lentiviruses conjugated with the specific antibody for 8 h at 37 ℃. Control cells were infected with VSV-G lentiviruses. The expression of GFP was detected by fluorescence microscopy.

Targeted infection of chicken gonads *in vivo*
Following the surrogate eggshell method (Perry, 1988), we microinjected 1–2 µL of SSEA4 M168 lentiviruses (1 µg of virus normalized to levels of p24 incubated with 1 µg of SSEA4) into the sub-germinal cavity beneath the blastoderm, after which the embryos were placed into surrogate eggshells and incubated for 3 d. The embryos were then transferred into new, larger surrogate eggshells until they hatched.

To confirm the targeted infection of gonads, we isolated heart, liver, gizzard, kidney, gonad, and muscle tissue from the chicken embryos incubated for 6–10 d, and then observed them under a microscope (Nikon, TS100, Japan). DNA was extracted from each tissue sample using HiPure Tissue DNA Mini Kits (Magen, D3121, USA), and PCR was performed with 2×Taq MasterMix (CWBIO, CW0682, China). The following primers were used: GWPRE-F: TCACATGGTCCTGCTGGA GT and GWPRE-R: GGGCCACAACTCCTCATAAAG.

Flow cytometry
Flow cytometry was performed with a FACSCalibur platform

(Becton Dickinson, USA). The data were analyzed using FlowJo 10 software.

Ethics approval

RESULTS

M168-pseudotyped lentivirus production
As lentiviruses pseudotyped with a Sindbis envelope M168 can target specific cells for infection via conjugated antibodies (Morizono et al., 2005), we used these modified lentiviral vectors to generate transgenic chickens. The VSV-G-pseudotyped lentivirus control used in this study has become a benchmark for assessing the efficiency of transduction by other viral envelope pseudotypes (Mochizuki et al., 1998; Reiser et al., 1996). Lentiviral packaging systems mainly include systems with three or four plasmids. Therefore, we examined which packaging system would generate M168-pseudotyped viruses with the highest transduction efficiency (Figure 1A), measured by GFP expression. The VSV-G-pseudotyped viruses produced with four plasmids transduced 36.8% of 293T cells, less than the three-plasmid system, which had a transduction efficiency of 68.7% (Figure 1B and Supplementary Figure S1). The three-plasmid system similarly showed higher transduction efficiency for the M168-pseudotyped lentiviral vectors conjugated with HLA antibodies (Figure 1B and Supplementary Figure S1). A small amount of background infection in the non-antibody group was also observed. Therefore, the GFP-expressing M168-pseudotyped viruses generated by the three-plasmid packaging system were used in subsequent experiments.

As the ratios of the three plasmids in the viral packaging system could influence the production of viral particles, we next aimed to maximize viral particle assembly in the 293T cells. The ratios of the three plasmids (GFP-expressing vector pWPXL, lentivirus packaging plasmid psPAX2, and M168 envelope protein-expressing plasmid) were adjusted. The viral amounts were normalized to the levels of HIV-1 capsid protein p24. The most viral particles were obtained using a three-plasmid ratio of 1:2:1, with a total of 10 µg of plasmids used to package the virus (Figure 1C). This ratio was therefore used for further lentiviral production.

Optimization of gene transduction using M168-pseudotyped lentiviral vectors in 293T cells
Poor viral transgene expression was observed in the 293T cells infected by the M168-pseudotyped lentiviruses conjugated with HLA antibodies. Therefore, we adjusted the transduction conditions to increase viral infection and

therefore transgene expression in 293T cells. Different concentrations of M168-pseudotyped viral particles conjugated with 1 µg of HLA antibodies were used to transduce 293T cells. Fluorescence microscopy (Supplementary Figure S2) and flow cytometry (Figure 2A) were used to compare GFP expression to assess the efficiency of gene transduction. The cells transduced with VSV-G lentiviruses showed strong GFP expression in a dose-dependent manner; 1 µg of M168-pseudotyped viruses (normalized to p24 amount) conjugated with 1 µg of anti-HLA generated maximal GFP expression. Due to the saturation of antibody and virus conjugation, no significant differences in GFP expression were found between the cells infected with 2 µg or with 1 µg of M168 virus normalized to p24 amount. Similar results were observed using flow cytometry (33.7% GFP+ and 33.6% GFP+) and RT-PCR analyses (Figure 2A, B). Subsequently, 1 µg of viral particles normalized to p24 amount, conjugated with 1 µg of antibody, was used.
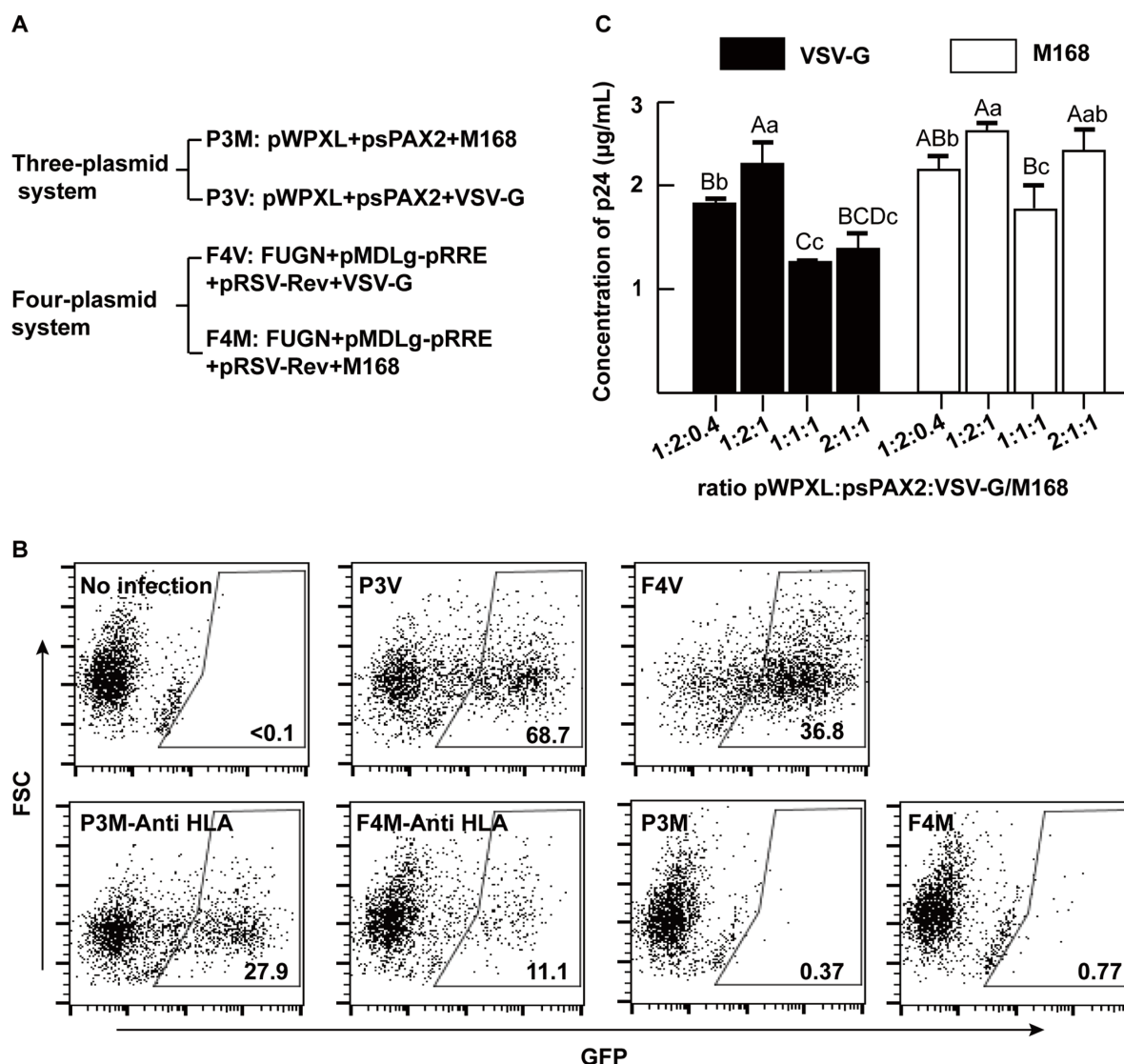

Figure 1 Production of M168-pseudotyped lentivirus

A: Lentiviral packaging systems were used for optimization of lentivirus production. VSV-G and M168 pseudotypes produced with three- and four-plasmid packaging systems are labeled as P3V, P3M, F4V, and F4M, respectively. B: Flow cytometry was used to detect GFP expression to assess transduction efficiency of different viruses after 2 d of infection. 293T cells ($1×10^5$) were infected with 1 µg p24 of P3M or F4M viruses with or without 1 µg of HLA antibody. Same amounts of P3V and F4V viral particles were used as controls. C: Ratios of plasmids (pWPXL, psPAX2, and VSV-G or M168) were adjusted to optimize conditions of virus production in 293T cells. Resulting virus stocks were titrated by p24. Data are shown as mean±standard deviation (*SD*). Values followed by same letter are not significantly different. Uppercase letters indicate significant differences at $P<0.01$. Lowercase letters indicate significant differences at $P<0.05$.

Targeted transduction of 293T cells via antibody-conjugated M168-pseudotyped lentiviral vectors

To investigate whether the M168-pseudotyped lentiviral vectors were able to specifically transduce 293T cells via the specific monoclonal antibody, HLA-ABC expression in 293T and BHK cells was analyzed. Flow cytometry results showed that 98% of 293T cells expressed HLA-ABC, and <1% of BHK cells were labeled with HLA antibodies (Supplementary Figure S3). We next determined whether transduction of 293T cells could be targeted using the M168-pseudotyped lentivirus conjugated with anti-HLA. Briefly, 293T cells, BHK cells, and a mixed population of the two cell types were transduced overnight with M168 or VSV-G lentiviruses. After 2 d, the cells were analyzed by HLA-ABC staining, followed by flow cytometry. The VSV-G lentiviruses transduced both 293T and BHK cells with efficiencies of 57.6% and 58.4%, respectively (Figure 3B). In contrast, lentiviral vectors pseudotyped with M168 and conjugated with HLA antibodies specifically infected 293T cells, but not BHK cells. These transduction results were similar to those obtained by fluorescence microscopy (Figure 3A). Thus, M168-pseudotyped lentiviruses could infect target cells under mediation of specific antibodies.

Derivation, culture, and characterization of PGCs

To further study the targeted infection efficiency of antibody-conjugated M168 lentiviruses, we first isolated gonads from 5.5- to 6-day-old chicken embryo (E5.5–E6) kidneys and cultured PGCs as described in the Materials and Methods (Figure 4A). The newly separated PGCs displayed a round morphology with a diameter of about 20 μm and were clear with bright edges (Figure 4B). After 8 d of cultivation, the cells clustered and grew in the suspension (Figure 4C). One of the markers specifically expressed in the germ cells was the chicken *vasa* homolog (*CVH*), which is typically used to characterize PGCs (Tsunekawa et al., 2000). RT-PCR analysis showed the expression of *CVH* in PGCs, but not in DF-1 cells (Figure 4C). Moreover, PGCs expressed surface markers of pluripotent stem cells, such as *SSEA1*, *SSEA3*, and *SSEA4*, and germ cell marker *DAZL*. The antibodies SSEA1, SSEA3, and SSEA4 are known to specifically bind to chicken PGCs (Jung et al., 2007; Raucci et al., 2015). Immunofluorescence staining showed that the PGC membranes were positively stained by SSEA1, SSEA3, SSEA4, and DAZL antibodies (Figure 4D). Therefore, we successfully derived PGCs from chicken gonads and characterized them with germ cell and pluripotent cell markers.

Targeted transduction of PGCs by M168-pseudotyped lentiviral vectors *in vitro*

Once we established the system for specific transduction of 293T cells using HLA-mediated targeted infection, we next investigated whether we could achieve specific transduction of PGCs with M168-pseudotyped lentiviruses via mediation of a specific monoclonal antibody. Flow cytometry was used to detect three cell surface markers commonly expressed in PGCs: i.e., SSEA1 (65.9%), SSEA4 (43.2%), and EMA1 (5.12%; Figure 5A). Transduction efficiency was determined by GFP expression of the infected PGCs (Figure 5B). As a control, GFP expression was detected in PGCs and BRL
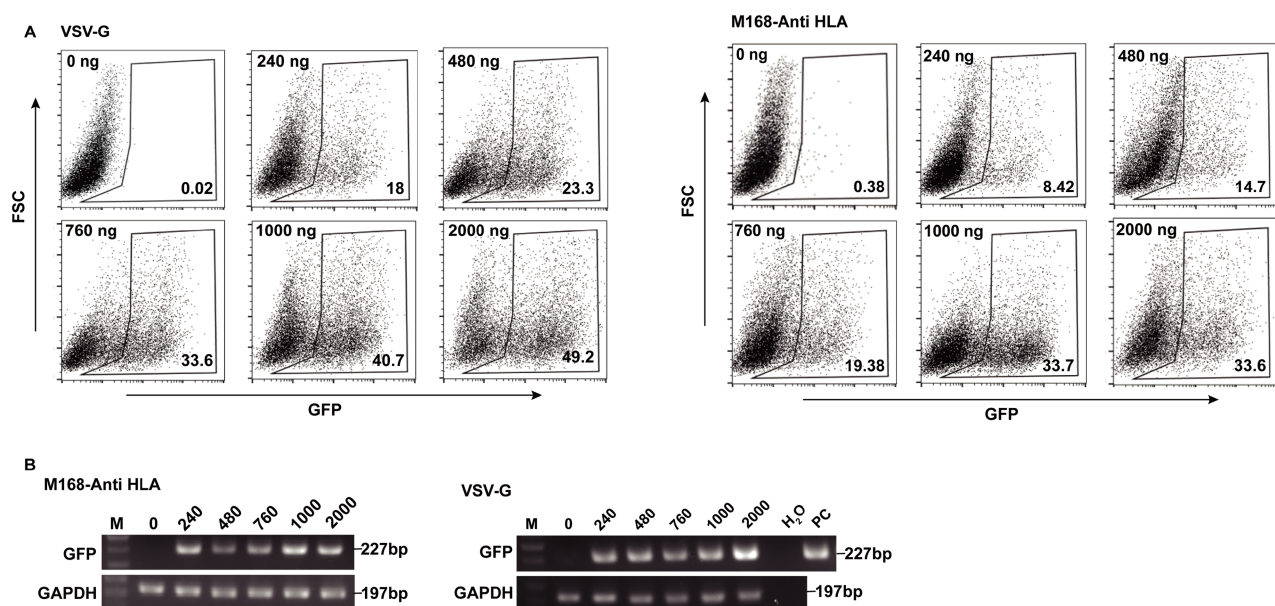

Figure 2 Optimization of gene transduction using M168-pseudotyped lentiviral vectors

A: Flow cytometry was used to detect GFP expression after 48 h of viral infection. Different concentrations of viral particles were used to infect 1×10^5 293T cells; VSV-G lentiviruses were used as the control. A greater percentage GFP-positive cells was obtained when infected with 1 μg p24 of M168 lentiviruses conjugated with 1 μg of HLA antibody. B: RT-PCR was used to analyze expression of GFP in infected cells. PC: Positive control.

feeder cells transduced with VSV-G-pseudotyped lentiviruses. Although GFP expression was detected in both PGCs and BRL cells infected with VSV-G lentiviruses, only PGCs expressed GFP after infection of M168 lentiviruses conjugated with the SSEA4 antibody. Fluorescent counting was used to quantify successfully transduced PGCs. The infection rate of PGCs by SSEA4-mediated M168-pseudotyped lentiviruses was 7.5%, which was substantially higher than the 1.7% infection rate found for VSV-G lentiviruses (Supplementary Figure S4). We also found that the SSEA4-mediated M168-pseudotyped lentiviruses only transfected PGCs, not DF-1 or feeder cells (Supplementary Figure S5). As the ZZ domain
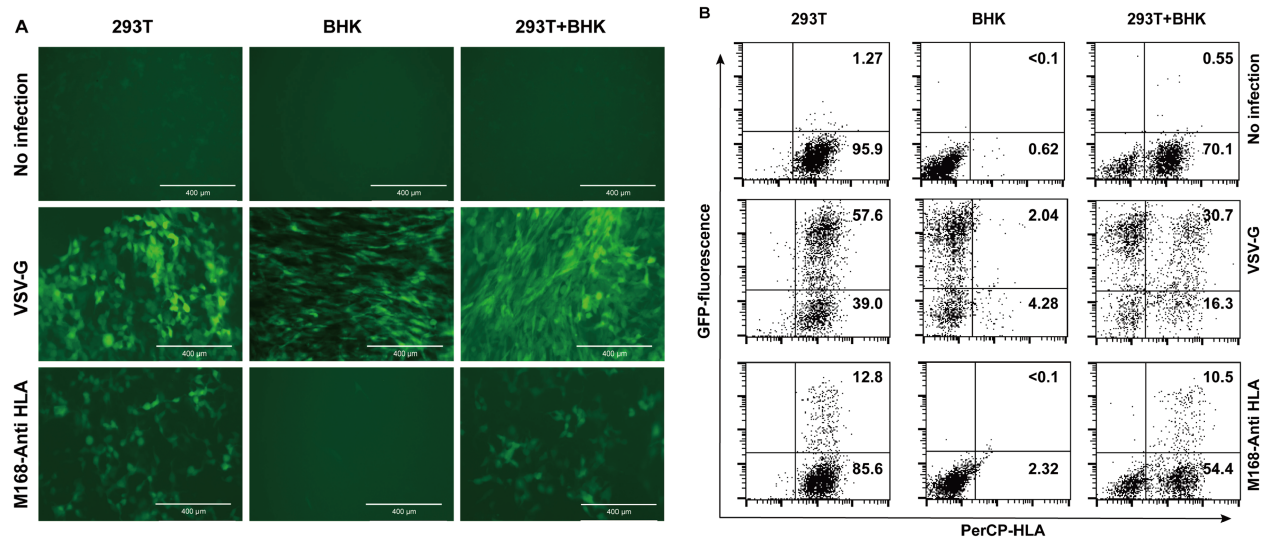

Figure 3 Targeted transduction of M168-pseudotyped lentiviruses conjugated with anti-HLA in mixed population of 293T and BHK cells
GFP expression was analyzed by fluorescence microscopy (A) and flow cytometry (B) after infection with anti-HLA-conjugated viral particles. HLA-M168 lentiviruses infected 293T cells, but not BHK cells. Scale bars: A=400 μm.
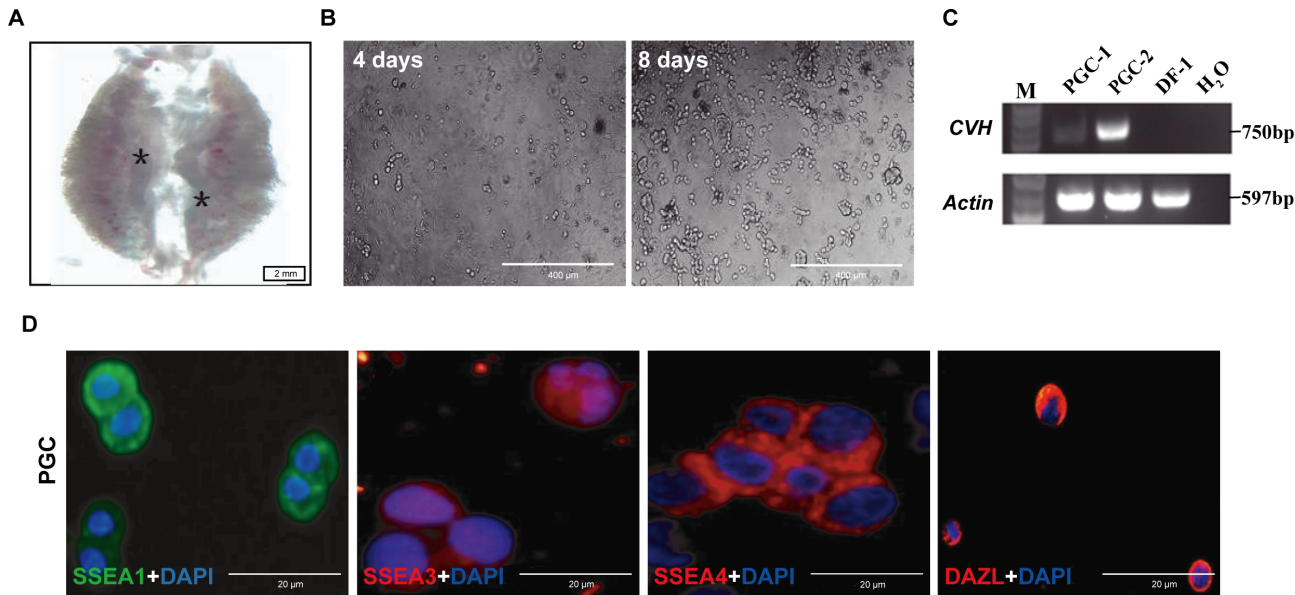

Figure 4 Culture and characterization of PGCs
A: Picture of gonads attached to the middle of kidney. *: Gonad. B: Morphology of PGCs cultured on a feeder layer of BRL cells at 4 and 8 d, respectively. C: RT-PCR was used to detect *CVH* expression in PGCs and DF-1 cells. M: Marker. D: Immunofluorescence staining was used to detect cell markers expressed on membrane of PGCs: stage-specific embryonic antigen-1 (*SSEA1*): stage-specific embryonic antigen-3 (*SSEA3*): stage-specific embryonic antigen-4 (*SSEA4*); and deleted-in-azoospermia-like (*DAZL*), germ cell marker. Scale bars: A=2 mm; B=400 μm; D= 20 μm.

was inserted into the modified Sindbis virus E2 envelope protein, the predominant antibody molecule it binds to is IgG. Therefore, the IgM antibody of SSEA1 was not effective as a means of targeted infection, although more PGCs were positive for SSEA1 than for SSEA4 (Figure 5A, B). These results indicated that M168-pseudotyped lentiviral particles conjugated with the SSEA4 antibody could be used to transduce target PGCs *in vitro*.

Targeted transduction of PGCs by M168-pseudotyped lentiviral vectors *in vivo*

We injected M168 lentiviruses conjugated with SSEA4 antibodies into the subgerminal cavity beneath the blastoderm of chicken embryos using the surrogate eggshell method to target PGCs for infection. We observed the expression of GFP in developing embryos via fluorescence microscopy. Results showed that the VSV-G lentiviruses infected various chicken embryo tissues to different degrees, and the M168 lentiviruses bearing SSEA4 antibodies induced a higher level of GFP expression in the gonads. This is a possible consequence of a lower rate of nonspecific infection (Figure 6A). Six tissues (i.e., heart, liver, kidney, muscle, gizzard, and gonad) were isolated from the embryos and then analyzed using PCR. We found that the M168-pseudotyped lentiviruses conjugated with SSEA4 antibodies targeted the gonads for infection more specifically than the VSV-G-pseudotyped lentiviruses

(Figure 6B). Statistical analysis showed that more than 62.5% of embryos obtained by VSV-G-pseudotyped lentivirus infection were chimeric, but only 20% were gonadal chimeras. However, the percentage of gonadal chimeras obtained by M168-pseudotyped lentivirus infection was 50.0%–66.7%, which improved upon the rate of VSV-G lentiviruses by 30.0%–46.7% (Table 1). Therefore, M168-pseudotyped lentiviruses conjugated with SSEA4 antibodies can efficiently target PGCs for transfection *in vivo*, resulting in an increased rate of gonadal chimerism.

DISCUSSION

At present, many strategies have been used to produce transgenic chickens. Lentiviruses and PGCs appear to be the most effective and widely used tools (Collarini et al., 2019; Kwon et al., 2018). Compared with lentiviral approaches, considerable progress has been made with the use of PGCs in recent years (Han et al., 2017). PGCs are precursor cells that can develop into spermatozoa and ova. They first appear in the X stage of chicken embryo development, then migrate to the germinal ridges through the vascular system. The establishment of *in vitro* culture technology has enabled genetic modification and screening. Recently, transcription activator-like (TAL) effector nucleases (TALENs) have been successfully used in chicken PGCs for the introduction of
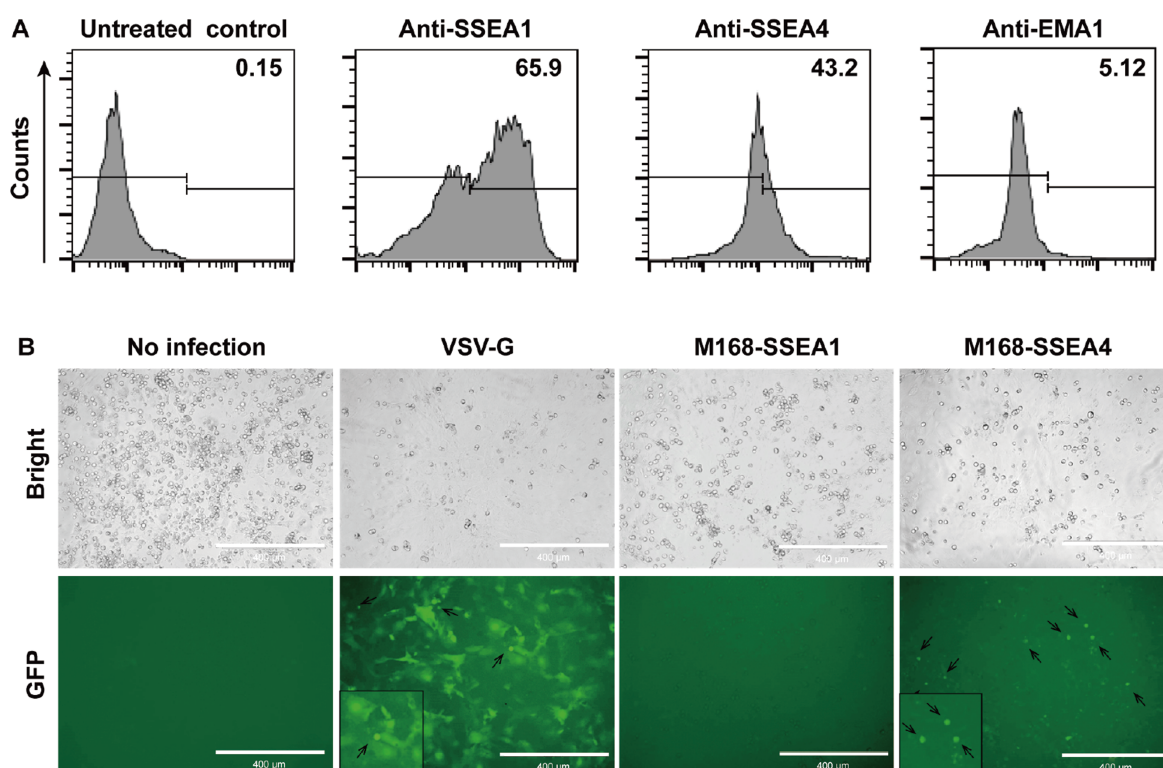

Figure 5 Targeted transduction of PGCs by M168-pseudotyped lentiviral vectors *in vitro*
A: Flow cytometry was used to analyze expression of SSEA1, SSEA4, and EMA1 in PGCs. B: M168 lentiviruses conjugated with SSEA4 antibody targeted infection to PGCs; VSV-G lentiviruses were used as a control. Scale bars: 400 μm.

subtle mutations and gene targeting (Glover et al., 2013; Lee et al., 2016; Park et al., 2014; Taylor et al., 2017). However, the long-term culture and stable transfection of PGCs, as well as embryo infection, require proficient skills and high costs.

The rate of chimeric embryo production is not stable under the lentiviral method; the positive rate in our group is less than 1% (Cao et al., 2015; Liu et al., 2015). Other researchers show positive rates of G1 transgenic chickens of only
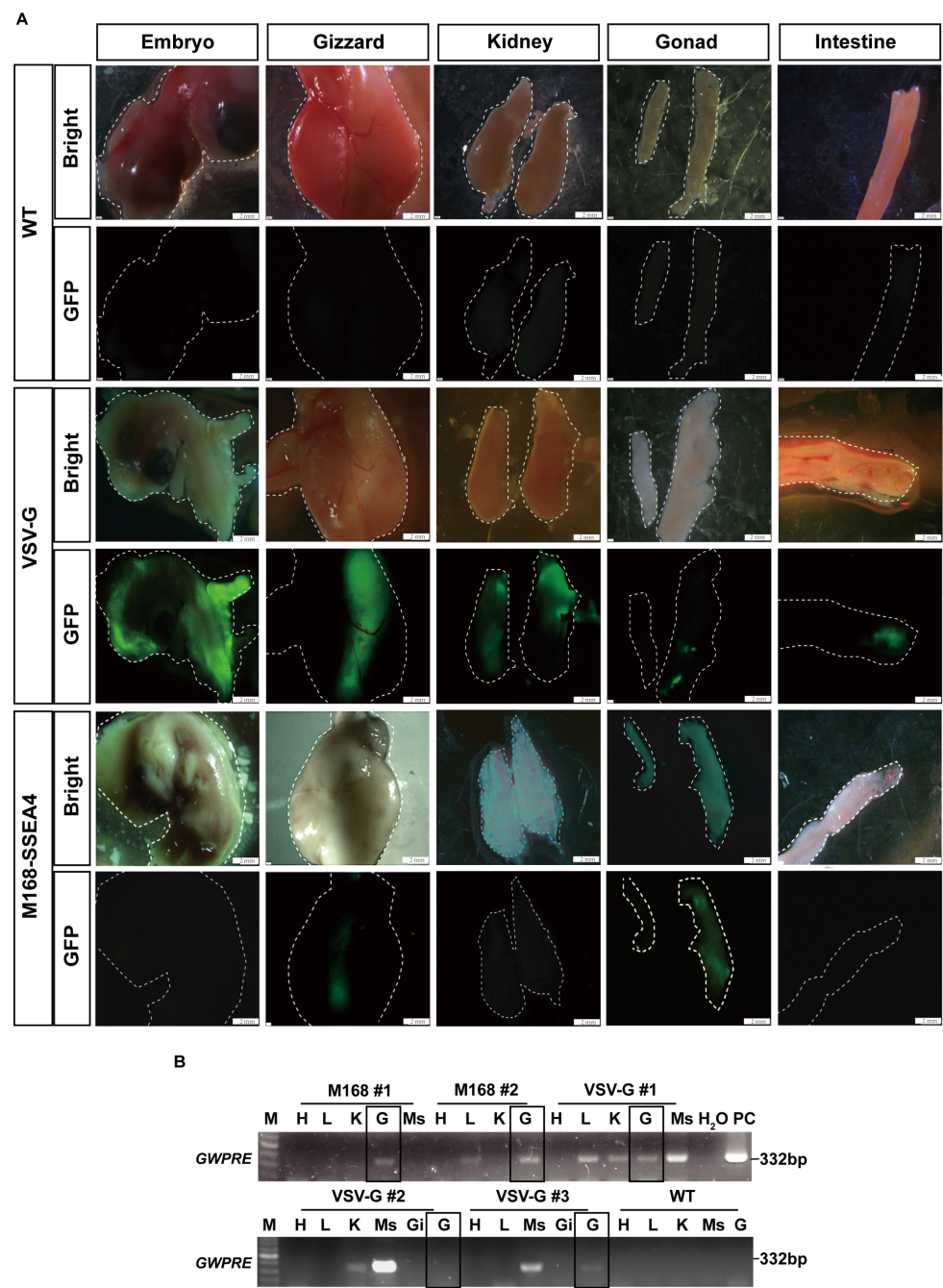
Figure 6 Targeted transduction of PGCs by M168-pseudotyped lentiviral vectors *in vivo*

A: Fluorescence microscopy was used to observe expression of GFP in chicken embryos. Scale bars: 2 mm. B: PCR was used to analyze chimeric embryos. DNA was extracted from heart (H), liver (L), kidney (K), gonad (G), gizzard (Gi), and muscle (Ms) from SSEA4 antibody-conjugated M168-lentivirus-infected embryos (M168 #1 and M168 #2); VSV-G-pseudotyped lentivirus-infected embryos (VSV-G #1, VSV-G #2, and VSV-G #3); and wild-type embryos (WT). PC: Positive control.

0.3%–2.1% (Cooper et al., 2019). Therefore, we designed this study using M168-pseudotyped lentiviral vectors conjugated with specific antibodies to target PGCs for *in vivo* infection in order to improve the chimerism of gonads and the production of transgenic chickens.

A number of strategies for targeted infection of cells by lentiviruses have been reported in the past few years, including the use of lentiviral vectors pseudotyped with envelope proteins of other viruses whose natural tropisms include the target cell type (Girard-Gagnepain et al., 2014; Palomares et al., 2013), or genetic fusion of a cell-binding protein (Buchholz et al., 2015; Kasaraneni et al., 2017). The mutant Sindbis envelope glycoprotein M168 was obtained through the insert of the ZZ domain (IgG binding domain of protein A) and the introduction of several mutations which inactivate the receptor binding sites (Morizono et al., 2005). M168 is widely used in lentiviral vector pseudotyping and produces high-titer viruses that are stable to ultracentrifugation and freeze-thaw cycles (Morizono et al., 2001; Smit et al., 1999). In this study, we tested different conjugation ratios of viral particles and antibodies to optimize the delivery of transgenes to specific cells using M168. We showed that 1 μg of viral particles normalized to p24 levels, conjugated with 1 μg of antibody, was the optimal ratio. Hamster BHK cells are HLA antigen-negative and are therefore ideal negative controls for studying targeted infections mediated by HLA antibody-conjugated M168 lentiviruses. We demonstrated that our system could achieve targeted infection in a mixed population of 293T and BHK cells.

One of our main objectives was to explore the feasibility of using antibodies to mediate lentiviral infections in order to create transgenic chickens. Therefore, PGCs were first collected from chicken gonads at E5.5–E6 days. The embryonic stem cell-related markers *SSEA1*, *SSEA3*, *SSEA4*, and *DAZL*, which are expressed in various undifferentiated progenitor cells and germ cells (Durcova-Hills et al., 2008), were also detected in our derived PGCs. RT-PCR analyses of PGCs also showed *CVH* expression. Thus, our results indicated that we successfully separated PGCs.

We next performed targeted infection of PGCs using antibody-mediated M168 lentiviruses *in vitro*, with VSV-G lentiviruses used as a control. We detected GFP expression in both PGCs and BRL cells infected with VSV-G lentiviruses; however, only PGCs expressed GFP after infection by M168 lentiviruses conjugated with SSEA4 antibody (Figure 5B). The

trimeric protein VSG-G is commonly used to pseudotype lentiviral vectors; because of its extremely broad tropism, the VSV-G lentivirus can transduce many cell types via binding to phosphatidylserine and low-density lipoprotein receptors (LDLR; Finkelshtein et al., 2013). Our study showed that the infection efficiency of VSV-G lentiviruses was low in PGCs. This may be related to its tendency toward nonspecific infection, which is amplified in PGC culture, as many virus particles infect feeder cells. The Sindbis virus, which is in the *Alphavirus* genus, can be prepared at high titers, expresses high levels of fusogenic proteins, and can fuse to cells independently of receptor binding proteins (Smit et al., 1999). Morizono applied lentiviral vectors pseudotyped with M168 to target infection by binding to a monoclonal antibody that recognizes surface antigens of specific cells (Morizono et al., 2001). We achieved similarly successful results using M168-pseudotyped lentiviruses conjugated with SSEA4 antibodies to target PGCs.

However, because of competitive binding to the ZZ domain between serum and recombinant monoclonal antibodies, and the fact that the noncovalent bonds between antibody and virus are easily broken, nonspecific infections are prone to occur, thus lowering transduction efficiency (Morizono et al., 2005; Wu et al., 2012). We observed similar results in our experiments (Figures 2A, 3B, 5B). To reduce interference of serum, we used the surrogate eggshell method to produce transgenic chickens. Several methods have been developed to optimize the binding of antibodies to viruses, such as inserting a single-chain antibody fragment into the Sindbis virus E2 protein (Aires et al., 2005); substituting envelope proteins (Bender et al., 2016); adding biological agents (Morizono et al., 2010); or using other affinity reagents, such as streptavidin. The latter would be more suitable for *in vivo* applications, where plasma antibodies exist (Situ et al., 2018). The binding status of antibodies or ligands to antigens also affects the efficiency of virus transduction. It has been reported that efficient binding can be attained when viruses conjugated with certain ligands target the membrane-distal site of the HER2/neu receptor, while when the membrane-proximal site of the same receptor is the target, it cannot (Kasaraneni et al., 2018). Additionally, the E1 protein is a structural protein of the Sindbis virus, and can effectively promote envelope fusion with the endosomal membrane at low pH, thereby enabling the virus to penetrate the cytoplasm and thus improving virus transduction efficiency (Glomb-

Table 1 Rates of gonadal chimeric embryos after infection with VSV-G or M168

Virus	Experiment	No. of embryos at 6 d	[a]No. of chimeric embryos (% of total embryos)	[b]No. of gonadal chimeric embryos (% of total embryos)
VSV-G	1	8	5 (62.5)	1 (20)
	2	15	10 (66.7)	2 (20)
M168	1	13	6 (46.2)	3 (50)
	2	17	6 (35.3)	4 (66.7)

[a]: Any embryonic tissue expressing green fluorescence is categorized as a chimeric embryo. [b]: Any gonadal expression of green fluorescence is categorized as a gonadal chimeric embryo.

Reinmund & Kielian, 1998). Efficient and targeted transduction of primary B cells in non-fractionated peripheral blood cells has been achieved by combining targeting vectors with low pH treatment (Morizono et al., 2006). These results indicate that precise pH manipulation can increase the efficiency of targeted transduction *in vitro.*

In conclusion, the antibody-mediated lentivirus method was used to successfully create transgenic chimera chicken embryos by targeted infection of PGCs. A higher rate of chimeric embryonic gonads was obtained using M168-pseudotyped lentiviruses, which was 30.0%–46.7% greater than that obtained using VSV-G-pseudotyped lentiviruses. This finding will be of great significance in improving the efficiency of germ-line transmission. Lentivirus-targeted transduction has also developed into one of the most important technologies in modern medicine for the treatment of genetic diseases and various cancers (Barrett et al., 2014; Lévy et al., 2015). Further improvement of this targeting technology will promote applications not only in transgenic research but also in clinical fields and disease therapy.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

X.X.H. and N.L. proposed the ideas. Z.Q.J. collected the data, reviewed the literature, and drafted the manuscript. H.Y.W. and X.X.H. commented on and revised the paper. J.T. was responsible for transferring the embryos into the surrogate eggshells. All authors read and approved the final version of the manuscript.

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REFERENCES

Ahani R, Roohvand F, Cohan RA, Etemadzadeh MH, Mohajel N, Behdani M, Shahosseini Z, Madani N, Azadmanesh K. 2016. Sindbis virus-pseudotyped lentiviral vectors carrying VEGFR2-specific nanobody for potential transductional targeting of tumor vasculature. *Molecular Biotechnology*, **58**(11): 738−747.

Aires Da Silva F, Costa MJ, Corte-Real S, Goncalves J. 2005. Cell type-specific targeting with sindbis pseudotyped lentiviral vectors displaying anti-CCR5 single-chain antibodies. *Human Gene Therapy*, **16**(2): 223−234.

Allen ER, Krumm SA, Raghwani J, Halldorsson S, Elliott A, Graham VA,

Koudriakova E, Harlos K, Wright D, Warimwe GM, Brennan B, Huiskonen JT, Dowall SD, Elliott RM, Pybus OG, Burton DR, Hewson R, Doores KJ, Bowden TA. 2018. A protective monoclonal antibody targets a site of vulnerability on the surface of rift valley fever virus. *Cell Reports*, **25**(13): 3750−3758.e4.

Barrett DM, Singh N, Porter DL, Grupp SA, June CH. 2014. Chimeric antigen receptor therapy for cancer. *Annual Review of Medicine*, **65**: 333−347.

Bender RR, Muth A, Schneider IC, Friedel T, Hartmann J, Plückthun A, Maisner A, Buchholz CJ. 2016. Receptor-targeted Nipah virus glycoproteins improve cell-type selective gene delivery and reveal a preference for membrane-proximal cell attachment. *PLoS Pathogens*, **12**(6): e1005641.

Boerger AL, Snitkovsky S, Young JAT. 1999. Retroviral vectors preloaded with a viral receptor-ligand bridge protein are targeted to specific cell types. *Proceedings of the National Academy of Sciences of the United States of America*, **96**(17): 9867−9872.

Bosselman RA, Hsu RY, Boggs T, Hu S, Bruszewski J, Ou S, Kozar L, Martin F, Green C, Jacobsen F, Nicolson M, Schultz JA, Semon KM, Rishell W, Stewart RG. 1989. Germline transmission of exogenous genes in the chicken. *Science*, **243**(4890): 533−535.

Buchholz CJ, Friedel T, Büning H. 2015. Surface-engineered viral vectors for selective and cell type-specific gene delivery. *Trends in Biotechnology*, **33**(12): 777−790.

Bupp K, Sarangi A, Roth MJ. 2005. Probing sequence variation in the receptor-targeting domain of feline leukemia virus envelope proteins with peptide display libraries. *Journal of Virology*, **79**(3): 1463−1469.

Cao DN, Wu HY, Li QY, Sun YM, Liu TX, Fei J, Zhao YF, Wu S, Hu XX, Li N. 2015. Expression of recombinant human lysozyme in egg whites of transgenic hens. *PLoS One*, **10**(2): e0118626.

Collarini EJ, Leighton PA, Van de Lavoir MC. 2019. Production of transgenic chickens using cultured primordial germ cells and gonocytes. *Methods in Molecular Biology*, **1874**: 403−430.

Cooper CA, Tizard ML, Stanborough T, Moore SC, Chandry PS, Jenkins KA, Wise TG, O'Neil TE, Layton DS, Morris KR, Moore RJ, Fegan N, Doran TJ. 2019. Overexpressing ovotransferrin and avian β-defensin-3 improves antimicrobial capacity of chickens and poultry products. *Transgenic Research*, **28**(1): 51−76.

Durcova-Hills G, Tang FC, Doody G, Tooze R, Surani MA. 2008. Reprogramming primordial germ cells into pluripotent stem cells. *PLoS One*, **3**(10): e3531.

Finkelshtein D, Werman A, Novick D, Barak S, Rubinstein M. 2013. LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. *Proceedings of the National Academy of Sciences of the United States of America*, **110**(18): 7306−7311.

Girard-Gagnepain A, Amirache F, Costa C, Lévy C, Frecha C, Fusil F, Nègre D, Lavillette D, Cosset FL, Verhoeyen E. 2014. Baboon envelope pseudotyped LVs outperform VSV-G-LVs for gene transfer into early-cytokine-stimulated and resting HSCs. *Blood*, **124**(8): 1221−1231.

Glomb-Reinmund S, Kielian M. 1998. The role of low ph and disulfide shuffling in the entry and fusion of semliki forest virus and sindbis virus. *Virology*, **248**(2): 372−381.

Glover JD, Taylor L, Sherman A, Zeiger-Poli C, Sang HM, McGrew MJ. 2013. A novel piggybac transposon inducible expression system identifies a role for Akt signalling in primordial germ cell migration. *PLoS One*, **8**(11):

e77222.

Gruell H, Klein F. 2018. Antibody-mediated prevention and treatment of HIV-1 infection. *Retrovirology*, **15**(1): 73.

Han JY, Lee HJ. 2017. Genome editing mediated by primordial germ cell in chicken. *Methods in Molecular Biology*, **1630**: 153−163.

Jung JG, Lee YM, Park TS, Park SH, Lim JM, Han JY. 2007. Identification, culture, and characterization of germline stem cell-like cells in chicken testes. *Biology of Reproduction*, **76**(1): 173−182.

Kasahara N, Dozy AM, Kan YW. 1995. Tissue-specific targeting of retroviral vectors via ligand-receptor interactions. *Advanced Drug Delivery Reviews*, **17**(3): 227−234.

Kasaraneni N, Chamoun-Emanuelli AM, Wright G, Chen ZL. 2017. Retargeting lentiviruses via SpyCatcher-SpyTag chemistry for gene delivery into specific cell types. *mBio*, **8**(6): e01860−17.

Kasaraneni N, Chamoun-Emanuelli AM, Wright GA, Chen ZL. 2018. A simple strategy for retargeting lentiviral vectors to desired cell types via a disulfide-bond-forming protein-peptide pair. *Scientific Reports*, **8**(1): 10990.

Kwon M S, Koo B C, Kim D, Nam Y H, Cui X S, Kim N H, Kim T. 2018. 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*, **13**(5): e194721.

Lee HJ, Lee HC, Kim YM, Hwang YS, Park YH, Park TS, Han JY. 2016. Site-specific recombination in the chicken genome using Flipase recombinase-mediated cassette exchange. *The FASEB Journal*, **30**(2): 555−563.

Lévy C, Verhoeyen E, Cosset FL. 2015. Surface engineering of lentiviral vectors for gene transfer into gene therapy target cells. *Current Opinion in Pharmacology*, **24**: 79−85.

Liu TX, Wu HY, Cao DN, Li QY, Zhang YQ, Li N, Hu XX. 2015. Oviduct-specific expression of human neutrophil defensin 4 in lentivirally generated transgenic chickens. *PLoS One*, **10**(5): e0127922.

Mason RD, Welles HC, Adams C, Chakrabarti BK, Gorman J, Zhou TQ, Nguyen R, O'Dell S, Lusvarghi S, Bewley CA, Li H, Shaw GM, Sheng ZZ, Shapiro L, Wyatt R, Kwong PD, Mascola JR, Roederer M. 2016. Targeted isolation of antibodies directed against major sites of SIV Env vulnerability. *PLoS Pathogens*, **12**(4): e1005537.

Mochizuki H, Schwartz JP, Tanaka K, Brady RO, Reiser J. 1998. High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. *Journal of Virology*, **72**(11): 8873−8883.

Morizono K, Bristol G, Xie YM, Kung SK, Chen ISY. 2001. Antibody-directed targeting of retroviral vectors via cell surface antigens. *Journal of Virology*, **75**(17): 8016−8020.

Morizono K, Xie YM, Ringpis GE, Johnson M, Nassanian H, Lee B, Wu L, Chen ISY. 2005. Lentiviral vector retargeting to P-glycoprotein on metastatic melanoma through intravenous injection. *Nature Medicine*, **11**(3): 346−352.

Morizono K, Ringpis GE, Pariente N, Xie YM, Chen ISY. 2006. Transient low pH treatment enhances infection of lentiviral vector pseudotypes with a targeting Sindbis envelope. *Virology*, **355**(1): 71−81.

Morizono K, Ku A, Xie YM, Harui A, Kung SKP, Roth MD, Lee B, Chen ISY. 2010. Redirecting lentiviral vectors pseudotyped with Sindbis virus-derived envelope proteins to DC-SIGN by modification of N-linked glycans of envelope proteins. *Journal of Virology*, **84**(14): 6923−6934.

Palomares K, Vigant F, Van Handel B, Pernet O, Chikere K, Hong P, Sherman SP, Patterson M, An DS, Lowry WE, Mikkola HKA, Morizono K, Pyle AD, Lee B. 2013. Nipah virus envelope-pseudotyped lentiviruses efficiently target ephrinB2-positive stem cell populations *in vitro* and bypass the liver sink when administered in vivo. *Journal of Virology*, **87**(4): 2094−2108.

Park TS, Lee HJ, Kim KH, Kim JS, Han JY. 2014. Targeted gene knockout in chickens mediated by TALENs. *Proceedings of the National Academy of Sciences of the United States of America*, **111**(35): 12716−12721.

Perry MM. 1988. A complete culture system for the chick embryo. *Nature*, **331**(6151): 70−72.

Raucci F, Fuet A, Pain B. 2015. *In vitro* generation and characterization of chicken long-term germ cells from different embryonic origins. *Theriogenology*, **84**(5): 732−742.e2.

Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M. 1996. Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proceedings of the National Academy of Sciences of the United States of America*, **93**(26): 15266−15271.

Salter DW, Smith EJ, Hughes SH, Wright SE, Fadly AM, Witter RL, Crittenden LB. 1986. Gene insertion into the chicken germ line by retroviruses. *Poultry Science*, **65**(8): 1445−1458.

Salter DW, Smith EJ, Hughes SH, Wright SE, Crittenden LB. 1987. Transgenic chickens: insertion of retroviral genes into the chicken germ line. *Virology*, **157**(1): 236−240.

Sarangi A, Bupp K, Roth MJ. 2007. Identification of a retroviral receptor used by an envelope protein derived by peptide library screening. *Proceedings of the National Academy of Sciences of the United States of America*, **104**(26): 11032−11037.

Situ K, Chua BA, Bae SY, Meyer AS, Morizono K. 2018. Versatile targeting system for lentiviral vectors involving biotinylated targeting molecules. *Virology*, **525**: 170−181.

Smit JM, Bittman R, Wilschut J. 1999. Low-pH-dependent fusion of sindbis virus with receptor-free cholesterol- and sphingolipid-containing liposomes. *Journal of Virology*, **73**(10): 8476−8484.

Song YH, Duraisamy S, Ali J, Kizhakkayil J, Jacob VD, Mohammed MA, Eltigani MA, Amisetty S, Shukla MK, Etches RJ, Van De Lavoir MC. 2014. Characteristics of long-term cultures of avian primordial germ cells and gonocytes. *Biology of Reproduction*, **90**(1): 15.

Taylor L, Carlson DF, Nandi S, Sherman A, Fahrenkrug SC, McGrew MJ. 2017. Efficient TALEN-mediated gene targeting of chicken primordial germ cells. *Development*, **144**(5): 928−934.

Tsunekawa N, Naito M, Sakai Y, Nishida T, Noce T. 2000. Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. *Development*, **127**(12): 2741−2750.

Valsesia-Wittmann S, Morling FJ, Nilson BH, Takeuchi Y, Russell SJ, Cosset FL. 1996. Improvement of retroviral retargeting by using amino acid spacers between an additional binding domain and the N terminus of Moloney murine leukemia virus SU. *Journal of Virology*, **70**(3): 2059−2064.

Wu DT, Seita Y, Zhang X, Lu CW, Roth MJ. 2012. Antibody-directed lentiviral gene transduction for live-cell monitoring and selection of human iPS and hES cells. *PLoS One*, **7**(4): e34778.