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Efficient Gene Transfer into Chicken Gonads by Combining Transposons with Polyethylenimine

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Abstract

Transposon mediated transfection is a promising, safe, and convenient way to generate transgenic chicken compared with virus-mediated technology and the in vitro modification of primordial germ cells (PGCs). To establish a simple method for in vivo transfection of chicken PGCs, we applied four different transposon systems (PB, SB, Tol2, and ZB) to investigate the gene transfer efficiency of chicken gonads via direct injection of a mixture of transposon and transposase plasmids and transfection reagent (polyethylenimine, PEI) into the subgerminal cavity of Hamburger and Hamilton stage 2-3 chick embryos. We also compared the effect of the amount of plasmids injected on the gene transfer efficiency of chicken gonads. We found that over 70% of the gonads were green fluorescent protein (GFP)-positive across all four transposon groups, and that the proportion of GFP-positive gonads was not significantly different between different transposons. Some GFP positive cells in gonads were confirmed as germ cells by co-labeling with the germ cell specific antibody. We also found that the proportions of GFP-positive gonads decreased significantly with a decrease of plasmid dose from 100 ng to 20 or 50 ng. Here we revealed that a combination of transposons with PEI is a simple and efficient method for gene transfer into chicken gonads and able to transfect PGCs in vivo that could be used for the production of transgenic chickens.

Keywords: transposon, primordial germ cells, polyethylenimine, transfection efficiency, chicken, transgenesis

1. Introduction

The chick embryo, as a model system, has many applications in developmental biology research (Rashidi & Sottile, 2009; Vergara & Canto-Soler, 2012) and as a bioreactor for pharmaceutical proteins (Lillico et al., 2005). This model becomes increasingly popular because of peculiar advantages, such as a short incubation period, the accessibility of embryos, and the availability of experimental embryology (Stern, 2005). According to contemporary transgenic methods, viral injection has proven to have the highest efficiency in birds (McGrew et al., 2004), and has been used to generate transgenic hens that synthesize functional recombinant pharmaceutical proteins in a tightly regulated, tissue-specific manner (Lillico et al., 2007); however, viral integration methods have size and sequence restraints (Scott et al., 2010). The most important effect is that transgene expression with viral vector may be silenced in the germline. Because of these potential drawbacks, transposons have become an alternative to viruses for integration and expression of transgenes. Transgenic offspring generated by transposon vectors (such as Minos, Tol1, Tol2, piggyBac [PB], and sleeping beauty [SB]) have been achieved in invertebrates, fish, and mammals (Dupuy et al., 2002; Kawakami et al., 2000; Sasakura et al., 2003; Sumiyama et al., 2010; Wilber et al., 2006). Three commonly used transposons—PB, SB, and Tol2—have also been utilized to transfer foreign genes to the avian genome. The SB transposon system was the first used to try and increase the transgene efficiency in chicken and turkey cells (Kong et al., 2008), while a germ-line-competent chicken PGC line was developed with the PB transposon and transposase, and both of the efficiency of transgenesis and the expression level of transgene were improved with these modified PGCs (Park & Han, 2012). A transgenic chick can be generated with the PGCs modified in vitro using both PB and Tol2 transposons (Macdonald et al., 2012). Lu et al. (2015) first demonstrated that a PB transposon mediated the insertion of a transgene into chicken embryos during developmental stages. Later, both PB and Tol2 were shown effective for chimera production and

germline transgenesis by direct injection into the vasculature of developing chick embryos or the subgerminal cavity of newly laid eggs (Liu et al., 2012; Tyack et al., 2013). In addition, transfection reagents such as Lipofectamine 2000 and jetPEI, combined with transposons, have also been applied in the direct injection of chick embryos (Jordan et al., 2014; Tyack et al., 2013), and were proven to be effective and promising for chicken transgenesis.

Although all three transposons (PB, SB, and Tol2) have been tested in avian models, to our knowledge, there is no report on the parallel comparison of the efficiency of these transposons using the same protocol. In the present study, we investigated the gene transfer efficiency into the chicken gonads mediated with three classic transposons (PB, SB, and Tol2) and one new transposon (ZB), which was isolated in our laboratory, by direct injection into the subgerminal cavity of early chicken embryos. We also tested PEI to deliver DNA. PEI was the second polymeric transfection agent discovered after poly-L-lysine (Boussif et al., 1995), and has been used to deliver DNA and RNA both in vivo and in vitro (Lu et al., 2015; Lungwitz et al., 2005; Morishita et al., 2015; Sramkova et al., 2014). This research aimed to establish a simple and efficient method for PGC transfection by direct injection into embryos and to select the best transposon system for further application in avian transgenesis.

2. Materials and Methods

2.1 Animals

Newly fertilized eggs of Rucao chickens were purchased from the Institute of Poultry Science, Chinese Academy of Agricultural Science. The eggs were kept in an incubator at 37.8 °C for 10 h, and embryos from Hamburger and Hamilton (HH) stage 2 to HH 3 were used for gene transfection in vivo. The protocol for animal use was approved in accordance with the University Council on Animal Care guidelines.

2.2 Plasmid DNA

Two vector systems harboring four transposons were used to transfect the PGCs. One vector system constructed in our laboratory is a trinity of PB, SB, and Tol2 transposons named pT3-CAG-GFP. This system consists of the terminal inverted repeat sequences (TIRs) of three transposons and a green fluorescence protein (GFP) expression box, including the chicken β-actin promoter, GFP ORF, and rabbit β-globin poly A (Figure 1A). The other vector system contains the same GFP expression box, but is flanked with the TIRs of a new Tc1-like transposon that was isolated from zebrafish in our laboratory (named ZB, unpublished data) and named pZB-CAG-GFP (Figure 1B). The PB, SB, ZB, and Tol2 transposase ORF were cloned downstream of the CMV promoter and located upstream of the rabbit β-globin poly A, respectively. Those vectors were named pCMV-PB, pCMV-SB, pCMV-Tol2, and pCMV-ZB (Figure 1C). Both of the PB and SB transposases (pCMV-PB and pCMV-SB) are new versions of vectors that were recently optimized (Mates et al., 2009; Yusa et al., 2011).

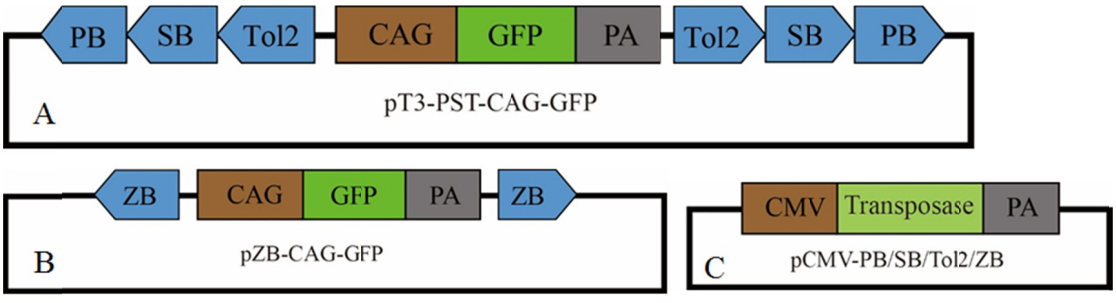


Figure 1. Structure of vectors

Note. A: pT3-CAG-GFP; B: pZB-CAG-GFP; C: pCMV-PB, pCMV-SB, pCMV-Tol2, and pCMV-ZB.

2.3 Formulation of a Plasmid Complex for Microinjection

We mixed a transposon plasmid with transposase plasmid in a 2:1 ratio, and then mixed PEI with plasmid in a 2:1 ratio. The plasmid and PEI complex was prepared according to the polyethylenimine manufacturer's instructions (PEI, 25 kDa branched PEI, Sigma-Aldrich). Briefly, transposon vector (pT3-CAG-GFP or

pZB-CAG-GFP) and transposase vector (pCMV-PB, pCMV-SB100X, pCMV-Tol2, or pCMV-ZB) were mixed at 2:1, respectively, and incubated at room temperature for 5 min. Then, an appropriate amount of stock PEI (10 μg/μL) was added to the DNA mixture at 2:1 and incubated for 15 min at room temperature. Finally, sterile Trypan blue was added to the mixture at final concentration of 10% (vol/vol) and dissolved completely. The final DNA concentrations of 100, 50, or 20 ng/μL were used for injection. This mixture is stable at room temperature for several hours.

2.4 Microinjection of Chick Embryos

PGCs are enriched in the transparent region of chick embryos at HH stage 2-3 (6-12 h of incubation) (Kang et al., 2015), which suggests that this stage is an appropriate time for PGC transfection. A window was cut in the pointed end of a recipient egg to allow access to the HH stage 2-3 embryo. The prepared plasmid-PEI complex (1 μL) was injected into the central part of subgerminal cavity using a micropipette (90 mm, Japan) and a microinjector (Tritech Research, America). The injection pipette was drawn from a Narishige glass tube with an inner diameter of 30 μm at the tip. After injection, the opening in the egg was sealed with a type of adhesive medical rubberized fabric plaster tape. The eggs were incubated at 37.8 °C and 60% relative humidity with a rocking motion every 2 h through a 90° angle for 18 days, after which they were further incubated at 37 °C and 70% relative humidity without rocking until they hatched.

2.5 Detection of Green Fluorescence

To assess the success of the technique, all embryos were hatched for 21 days, then eight organs, including the heart, liver, spleen, lung, kidney, brain, gonads, and intestine, were dissected to detect the GFP signal under a fluorescence microscope. At the same time, the viability data at embryo day 7 (ED 7) and ED 14 and the hatching rate at ED 21 were collected.

2.6 Immunohistochemistry

We used immunostaining of chicken Vasa homologue gene (CVH) to identify GFP-positive germ cells. Briefly, dissected gonads were dissociated using trypsin and fixed in 4% paraformaldehyde for 10 min. The resulting cells were centrifuged for 5 min and the supernatant was discarded. The cells were washed two times in phosphate-buffered saline (PBS) and nonspecific binding sites were blocked with 1% bovine serum albumin in PBST for 30 min, then centrifuged for 5 min per wash, and the supernatant discarded. The cells were subsequently incubated with the primary rabbit polyclonal anti-DDX4 at 1:100 (Beijing Biosynthesis Biotechnology Co.) overnight at 4 °C and washed three times in PBST for 5 min per wash. The cells were then incubated with secondary Cy3-conjugated goat anti-rabbit antibody (Beijing Biosynthesis Biotechnology Co.) at 1:200 for 1 h at room temperature and then washed three times with PBST for 5 min per wash. Finally, the cells were stained with DAPI (1 μg/mL) and visualized using a fluorescence microscope.

2.7 Statistical Analysis

A chi-square test was used to determine the significance of differences between the GFP-positive proportion of gonads, GFP-positive proportion of embryos, viability at ED 7 and ED 14, and hatching rates at ED 21 between groups. $P < 0.05$ was considered significant.

3. Results

3.1 Efficient Gene Transfer into Chicken Gonads Using Different Transposons

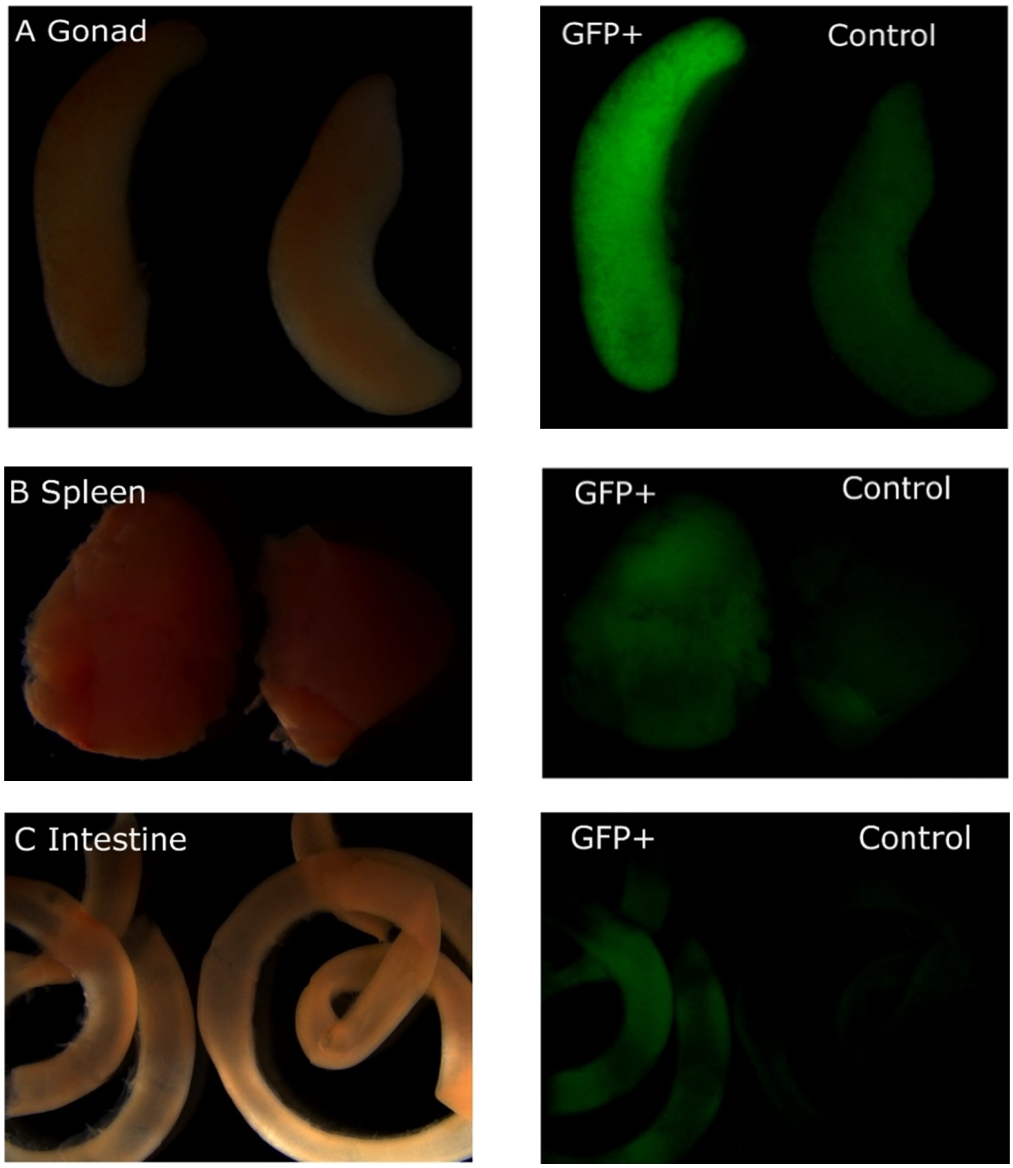
Four transposons systems were used to evaluate the gene transfer efficiency into chicken gonads. The transposon and transposase plasmids were combined and formulated with PEI to produce the injection mixture. Then, the mixture was injected into the subgerminal cavity of HH 2-3 embryos to transfect the PGCs, as previously described. All injected embryos were incubated for 21 days. Then, the GFP-positive proportions of gonads and embryos, viability, and hatching proportions were analyzed. High gene transfer efficiencies into the gonads were obtained (Table 1). Most gonads of injected embryos were GFP-positive in all four transposon groups. The GFP-positive proportions of gonads were 70.97%, 78.26%, 77.42%, and 72.00% in PB, SB, Tol2, and ZB, respectively. We found no significant difference between the four transposons ($P > 0.05$) based on a chi-square test. The GFP signals were detected in diverse tissues, including gonads (Figure 2A), spleen (Figure 2B), intestine (Figure 2C), and brain (Figure 2D). Green fluorescence was observed extensively throughout the gonads (Figure 2A). We found that the proportion of GFP-positive embryos (including embryos with any GFP-positive tissue) was about 80% for all transposon groups. No significant differences were found between transposons ($P > 0.05$).

Table 1. Hatching rates and transfection efficiency of manipulated eggs

Group	Viability at ED 7 (%)	Viability at ED 14 (%)	Hatching rate at ED 21 (%)	Proportion of GFP$^+$ embryos (%)	Proportion of GFP$^+$ gonads (%)
PB	75.56 (68/90)	56.67 (51/90)^B	34.44 (31/90)	80.65 (25/31)	70.97 (22/31)
SB	71.11 (64/90)	40.00 (36/90)^A	25.56 (23/90)	78.26 (18/23)	78.26 (18/23)
TOL2	80.90 (72/89)	58.43 (52/90)^B	34.83 (31/89)	80.65 (25/31)	77.42 (24/31)
ZB	83.13 (69/83)	68.12 (47/83)^B	30.12 (25/83)	80.00 (20/25)	72.00 (18/25)

Note. Various letters in the same row indicate significant differences ($P < 0.05$).

The toxic effect of these transposons combining with PEI was then checked. We found that the viability at ED 7 and the hatching rate at ED 21 were generally similar across the four transposon groups ($P > 0.05$), but the viability at ED14 showed a significant difference between the four ($P < 0.05$) (Table 1). Compared with previous studies (Jordan et al., 2014; Tyack et al., 2013), the viability at ED 7 and ED 14 and the hatching rate at ED 21 (25-35%) were acceptable.



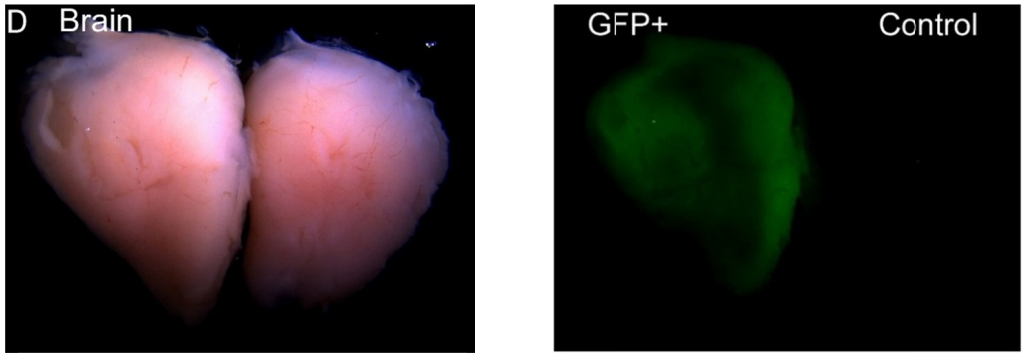


Figure 2. Analysis of fluorescent tissues from directly injected embryos and negative controls

Note. GFP and corresponding bright field images of representative gonads (A), spleen (B), intestine (C), and brain (D).

3.2 Confirmation of the Ability of Transposons to Transfect Germ Cells Using Immunohistochemistry

To confirm the ability of these transposons to transfect germ cells, we identified GFP-positive gonad cells by immunohistochemistry for the germ cell-specific antigen, CVH. Some GFP-expressing cells were positive for CVH, which is generally used as a marker for chicken germ cells (Figure 3). We found that we had successfully transfected germ cells in vivo with the transposon-mediated technique.

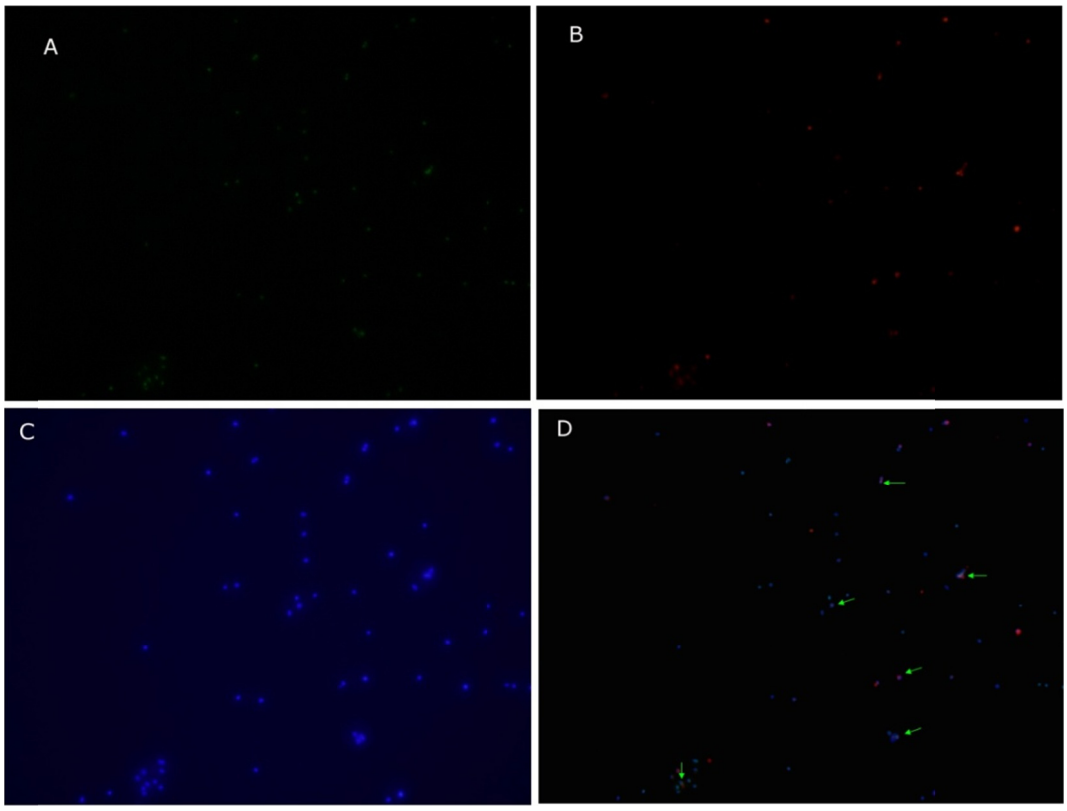


Figure 3. Colocalization of some GFP-positive cells with germ cell-specific antigens in cells from gonads

Note. Cells were observed by fluorescence microscopy (A: GFP; B: anti-CVH; C: DAPI; D: merged images). Arrows indicate colocalization of GFP and immunoreactivity. Magnification ×40.

3.3 Effect of the Amount of Plasmid on the in vivo Transfection Efficiency of PGCs

Next, we examined the effect of the amount of injected plasmid on the in vivo transfection efficiency of PGCs. We decreased the dose of the injected plasmids to 50 ng or 20 ng for each embryo in the SB transposon group. We found that the proportion of GFP-positive gonads and embryos decreased significantly ($P < 0.05$) with the decrease of plasmid dose (Figures 4A and 4B), while the viability and hatching rate of the embryos did not increase significantly ($P > 0.05$) (Figures 4C, 4D and 4E).

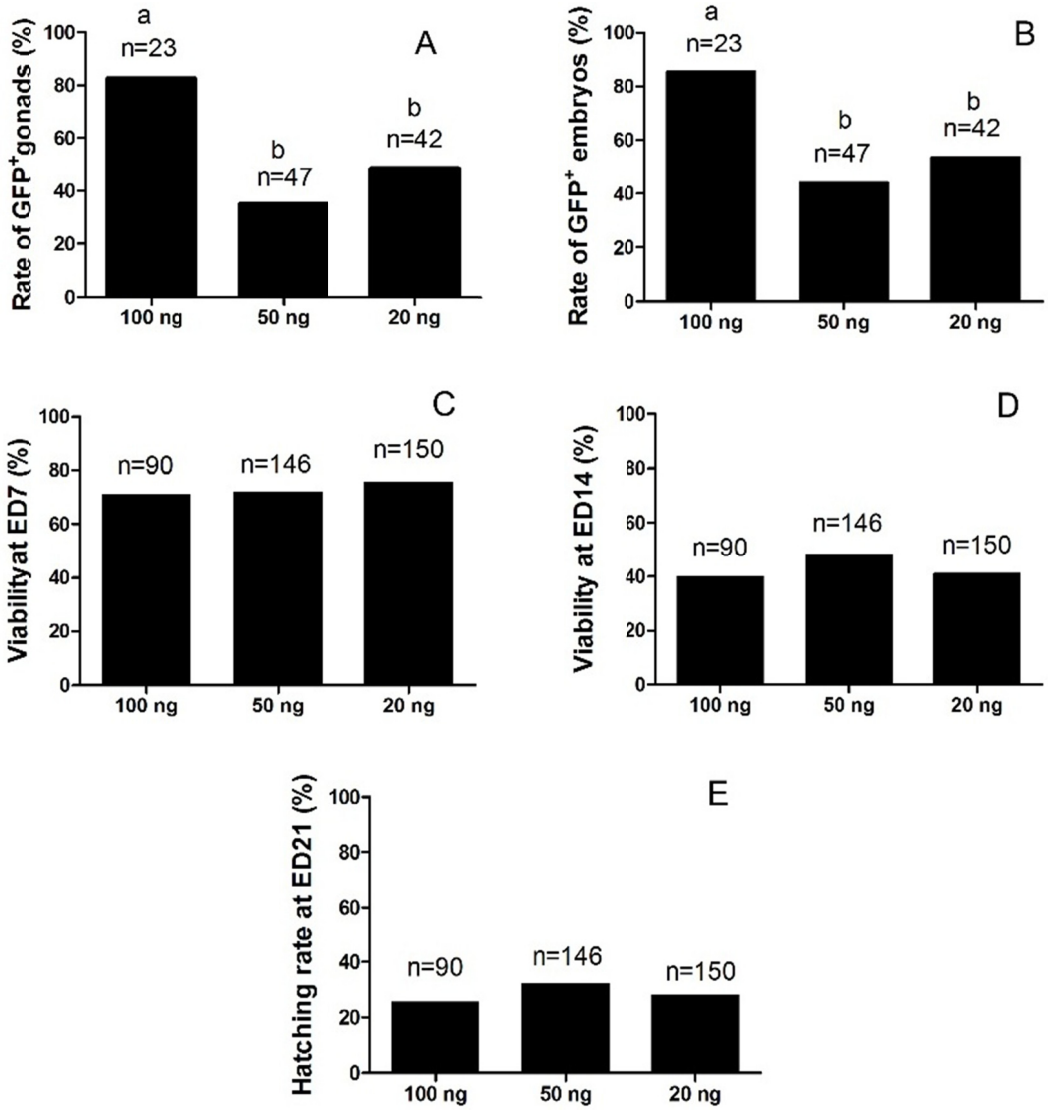


Figure 4. The effect of the amount of plasmid on the proportion of GFP-positive gonads (A) and embryos (B), and the viability and hatching rate of embryos (C, D, E)

Note. The various letters indicate significant differences ($P < 0.05$).

4. Discussion

The current study demonstrated that all four transposons (PB, SB, Tol2, and ZB) combined with the transfection reagent (PEI) were capable of transferring foreign genes into the chicken gonads with similarly high efficiency (over 70% of the gonads being GFP positive) by direct injection into the subgerminal cavity of HH 2-3 embryos. The gene transfer efficiency of chicken gonads with a high injection dose (100 ng) of plasmid packed with PEI was higher than that of the low dose (20 and 50 ng).

Although the transfection of PGCs can be achieved by injecting plasmid DNA and liposome complexes into the bloodstream of stage HH 14 embryos (Watanabe et al., 1994), this method is inefficient and unstable without a transposon medium. Combining transposons with transfection reagents has been shown to be effective for chimera production and germline transgenesis by direct injection into the vasculature of developing chick embryos or stage X embryos (Jordan et al., 2014; Tyack et al., 2013). The combination of Tol2 transposon with Lipofectamine 2000 has been confirmed to transfect the PGCs efficiently in vivo by direct injection into the vasculature of developing chick embryos and to generate stable germ-line transgenic male chickens who passed the transgene onto their offspring (Tyack et al., 2013). That study (Tyack et al., 2013) revealed that all gonads sampled at ED 14 were GFP positive (10 out of 10 embryos), five out of the 11 F0 male semen were transgene positive, five out of the 419 offspring from the F0 individuals with transgene positive semen were GFP positive, and the transgene rate of their offspring was about 1.5%, which is substantially lower than that using lentiviral vectors (4-45%) (McGrew et al., 2004).

Other nonviral vehicles, such as PEI, have also been used in combination with the transposon to improve gene transfer efficiency in chickens (Jordan et al., 2014). PEI, a highly water-soluble cationic polymer, has been widely used for nonviral transfection of DNA and RNA in vitro and in vivo (Lu et al., 2015; Lungwitz et al., 2005; Morishita et al., 2015; Sramkova et al., 2014), and the advantage of PEI over other polycations is that it combines strong DNA compaction capacity with an intrinsic endosomolytic activity (Tang & Szoka, 1997). PEI has two structural forms, linear and branched, with various molecular masses (Fischer et al., 1999; Huh et al., 2007). The linear PEI derivative (jetPEI) combined with the PB transposon, which has been tried in chickens, can transfect multiple chick cell types, including germline stem cells, by direct injection into the vitelline artery of HH stage 23 chick embryos (Jordan et al., 2014). This technique has also been applied for long-term expression of a transgene in chickens by direct injection into stage X embryos. Stable expression of GFP was seen in multiple tissue types, including the heart, brain, liver, intestine, kidney, and gonads (Jordan et al., 2014). Here we demonstrated that the 25 kDa branched PEI, combined with transposons, can be used to deliver genes into chicken gonads and transfect PGCs efficiently in vivo. To find the most efficient transposon, we compared the transfection efficiency of PGCs across four transposons by screening the GFP-positive gonads. All of these transposons were highly efficient, and no significant difference was observed in the proportion of GFP-positive gonads, which also suggests that all four of these transposons are applicable in chicken transgenesis and that their efficiency is consistent with that previously reported (Tyack et al., 2013). We also found that combining PEI with a high dose (100 ng) of plasmid can generate a high proportion of GFP-positive gonads, while combining PEI with a low dose (20 or 50 ng) of plasmid can generate a low proportion of GFP-positive gonads, but with a limited increase in the hatching rates and viability of embryos, which indicates that the hatching rates and viability of embryos may be affected mainly by the physical injury of the injection, not the dose of plasmid or transfection reagent. Transposons combined with other techniques, such as electroporation, have also been attempted in chickens (Liu et al., 2012; Sato et al., 2007). However, the gene transfer efficiency, hatching rates, and viability of embryos were substantially lower than that of transposons combined with the transfection reagents reported in this and previous studies (Tyack et al., 2013).

Another common approach to the production of transgenic chickens mediated with transposons is based on PGC culture in vitro. Chicken PGCs are cultured and modified in vitro and then injected into embryos to generate transgenic chickens. Investigators have generated transgenic chickens and offspring efficiently using PB transposons (Macdonald et al., 2012; Park & Han, 2012). However, as is widely known, it is difficult to isolate many PGCs from embryos, and culturing PGCs in vitro is technically challenging and expensive. Meanwhile, the in vivo transfection of PGCs by combining transposons with a transfection reagent using direct embryo injection is much simpler and more convenient, and the efficiency of PGC transfection is high, which has been proven by this and previous studies (Tyack et al., 2013; Jordan et al., 2014). The protocol described in this study could be used to produce germline transgenics, and may offer a new solution for precision genome manipulation involving CRISPR/Cas9 (Cong et al., 2013), zinc finger nucleases (ZFNs) (Kim et al., 1996), and TALENs (Bedell et al., 2012).

We found that direct embryo injection with the combination of transposons and PEI is a simple and readily adoptable method for the gene transfer into chicken gonads. All four transposons investigated in this study (PB, SB, ZB, Tol2) are highly efficient for the gene transfer into chicken gonads and able to transfect PGCs in vivo, and applicable in chickens. Furthermore, the rates of GFP-positive gonads decreased significantly with the decrease of plasmid dose from 100 ng to 20 or 50 ng.

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