

## Effective Transfection of Chicken Primordial Germ Cells (PGCs) using Transposon Vectors and Lipofection

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Accepted February 28, 2019

Published online March 26, 2019

Issue online March 29, 2019

Original article

SAWICKA D., CHOJNACKA-PUCHTA L. 2019. Effective transfection of chicken primordial germ cells (PGCs) using transposon vectors and lipofection. *Folia Biologica (Kraków)* **67**: 45-52.

This study had two aims. First, we optimized conditions for the lipofection of CHO-K1 cells with transposon vectors. We used flow cytometry to examine the effects of different cell densities, plasmid constructs and lipofection protocols on *in vitro* transgene expression. Second, we transfected purified chicken primordial germ cells (PGCs) with the most effective lipofection conditions identified in CHO-K1 cells. The use of the CHO-K1 cell model reduced the number of chicken embryos required as PGC donors. CHO-K1 cells ( $1 \times 10^5$  cells/ml or  $5 \times 10^5$  cells/ml) were transfected with transposon vectors (pCMV-Tol2 with pL-miniTol2-OVA5IFN/pL-miniTol2-OVA5Egfp) via protocol A, B, C or D, the last of which is a control with the nontransposon vector pL-OG-OVAIFNEnh-Egfp. The highest transfection efficiency for transposon vectors was obtained after lipofection of cells with method A (60.0-62.0%), which suggested that this method would be appropriate for transfecting PGCs. Therefore, we performed lipofection of purified PGCs ( $1 \times 10^5$  cells/ml or  $5 \times 10^5$  cells/ml) using transposon vectors via method A and nontransposon vectors via method D. The most appropriate conditions for introducing transposon vectors into PGCs was lipofection at a density of  $5 \times 10^5$  cells/ml with method A (transfection efficiency of 67%). We developed an effective protocol for transfecting PGCs using transposon vectors combined with lipofection. The method elucidated in this work will facilitate the production of transgenic chickens in future projects.

Key words: primordial germ cells, CHO-K1 cells, lipofection, transposons, chicken.

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The methods used to produce transgenic animals are still being improved. One new approach for generating transgenic animals is based on mobile genetic elements (transposons), which are integrated into the host genome by transposase. Transposon systems, such as Minos, Tol1, Tol2, piggyBac and Sleeping Beauty, have been used to modify invertebrates, fish, frogs and mammals (KAWAKAMI *et al.* 2000; DUPUY *et al.* 2002; SASAKURA *et al.* 2003; WILBER *et al.* 2006; SUMIYAMA *et al.* 2010). Among these systems, piggyBac, Tol2 and Sleeping Beauty have been used to transfer foreign genes into the avian genome, but only piggyBac and Tol2 transposons have been applied to produce transgenic chickens (MACDONALD *et al.* 2012; WANG *et al.* 2016).

Transgenic chickens are generated primarily through chimeric intermediates via the transfer of modified primordial germ cells (PGCs) into recipient embryos (KUWANA 1993; NAITO 2003; ZHU *et al.* 2005; VAN DE LAVOIR *et al.* 2006; NAKAMURA *et al.* 2010; CHOJNACKA-PUCHTA *et al.* 2015; NAITO 2015). A previous study (MACDONALD *et al.* 2012) showed that application of the piggyBac or Tol2 transposon systems improved DNA transfer into PGCs. Furthermore, this system has important advantages, as it effectively inserts exogenous DNA into the host chromosome without transgene expression silencing (MACDONALD *et al.* 2012; PARK & HAN 2012; GLOVER *et al.* 2013; NAITO 2015). Therefore, transposable elements are efficient vectors for the genetic manipulation

of PGCs and the chicken genome. The transfection method also plays a critical role during the transfer of DNA into PGCs. Previous studies (MACDONALD *et al.* 2012; PARK & HAN 2012; GLOVER *et al.* 2013; LIU *et al.* 2013) showed that the use of transposon vectors in combination with lipofection is a promising approach for cell modification.

Here, we optimized transfection conditions with new Tol2 transposon vectors carrying gene expression cassettes, insulators and enhancer elements using the Chinese Hamster Ovary cell line (CHO-K1). We investigated the effects of cell density, lipofection conditions and plasmid constructs on *in vitro* transgene expression, as determined by FACS (Fluorescence-activated cell sorter). CHO-K1 cells were a suitable model for evaluating the effects of these factors; thus, we reduced the number of chicken embryos used as donors of PGCs. We modified chicken PGCs by using the most effective transfection protocol identified for CHO-K1 cells.

## Materials and Methods

### Plasmid construction

The miniTol2 plasmid system used in this study was described by BALCIUNAS *et al.* (2006). The vectors pL-miniTol2-OVA5IFN and pL-miniTol2-OVA5Egfp were constructed on the miniTol2 backbone. The first plasmid contains Tol2 transposon with mini ITRs and the terminal Tol2 sequences flanking the pOVA promoter driving the enhanced human IFN $\alpha$ 2a gene. The second plasmid expresses EGFP (GFPmut1 variant, Promega) under the control of the chicken ovalbumin promoter, including the enhancer and regulatory sequences. The third plasmid contains the Tol2 transposase sequence under the control of the CMV promoter (plasmid from Stephen Ekker, Addgene plasmid # 31823 <http://n2t.net/addgene:31823>; RRID:Addgene\_31823). Based on the pSF-CMV-RSV-Neo\_G418 OXFORD vector we constructed a donor vector: pL-OG-OVAIFNEnh-Egfp. This vector contains a Neomycin/G418 resistance expression cassette under the control of the RSV promoter. The human IFN $\alpha$ 2a gene with SV40 enhancer is under ovalbumin promoter. This plasmid also contains the mammalian CMV promoter to drive EGFP expression. Plasmids used in this study are presented in Fig. 1.

### CHO-K1 cell culture

Chinese hamster ovary cells (CHO-K1, ATCC CCL-61) were maintained in Iscove's Modified

Dulbecco's Medium (IMDM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 4 mM L-glutamine (Sigma-Aldrich) and 0.5% antibiotic antimycotic solution (100x Penicillin-Streptomycin-Amphotericin B, Sigma) in a humidified 5% CO<sub>2</sub> incubator at 37°C. The cells were cultured in 100 mm plates (Corning, N.Y, USA) and passaged before reaching full confluency. To detach cells from the culture plates, 5 mM EDTA solution (Gibco, Invitrogen, Carlsbad, CA, USA) was used.

### PGC culture

PGCs were isolated from the bloodstream of Ross 308 embryos (Malec H. Poultry Farm, Góra Kalwaria, Poland) at stage 14-16 (Hamburger Hamilton, H.H.) and incubated in a FEST incubator (Gostyń, Poland) at 37.8°C with 60-62% relative humidity. The isolated PGCs were purified via Percoll gradient density centrifugation, as previously described by OISHI (2010). Purified PGCs were seeded in 24-well plates (Nunc, Roskilde, Denmark) in OPTIMEM C medium [OPTIMEM (Gibco Invitrogen) supplemented with 10% FBS, 2% chicken serum (Gibco Invitrogen), 9 ng/ml murine leukemia inhibitory factor (Sigma-Aldrich), 20 ng/ml basic fibroblast growth factor (Sigma-Aldrich), 5 ng/ml human stem cell factor (Sigma-Aldrich) and 1× penicillin/streptomycin (Sigma-Aldrich) (CHOJNACKA-PUCHTA *et al.* 2015; SAWICKA *et al.* 2015)] and incubated at 37°C with 5% CO<sub>2</sub>.

### Optimization of the lipofection protocol using CHO-K1 cells

One day before transfection, cells were seeded in 24-well or 6-well plates (Nunc) at a density of 1 x 10<sup>5</sup> or 5 x 10<sup>5</sup> cells/ml, respectively. Transfections were performed with the Xtreme HP DNA transfection reagent (Roche Diagnostics, Rotkreuz, Switzerland) using different protocols, with the transposon vector pCMV-Tol2 and pL-miniTol2-OVA5IFN or pL-miniTol2-OVA5Egfp at the following ratios: 0.5 µg: 1 µg (A), 1 µg: 0.5 µg (B) and 1 µg: 1 µg (C) in the presence of 1 µl of lipofectant for 1 x 10<sup>5</sup> cells/ml or 2 µl of lipofectant for 5 x 10<sup>5</sup> cells/ml. Lipofections were also performed using the nontransposon vector pL-OG-OVAIFNEnh-Egfp (as a control) at a ratio of 1 µg: 3 µl of lipofectant (protocol D). Complexes of DNA- liposomes were added to cells in a dropwise manner after 20 min of incubation at room temperature (RT). After culturing the cells for 24 h, antibiotic for selection (G418, 0.4 mg/ml, Sigma-Aldrich) was added. The transfection reagent was removed after 48 h of culture, and the cells were

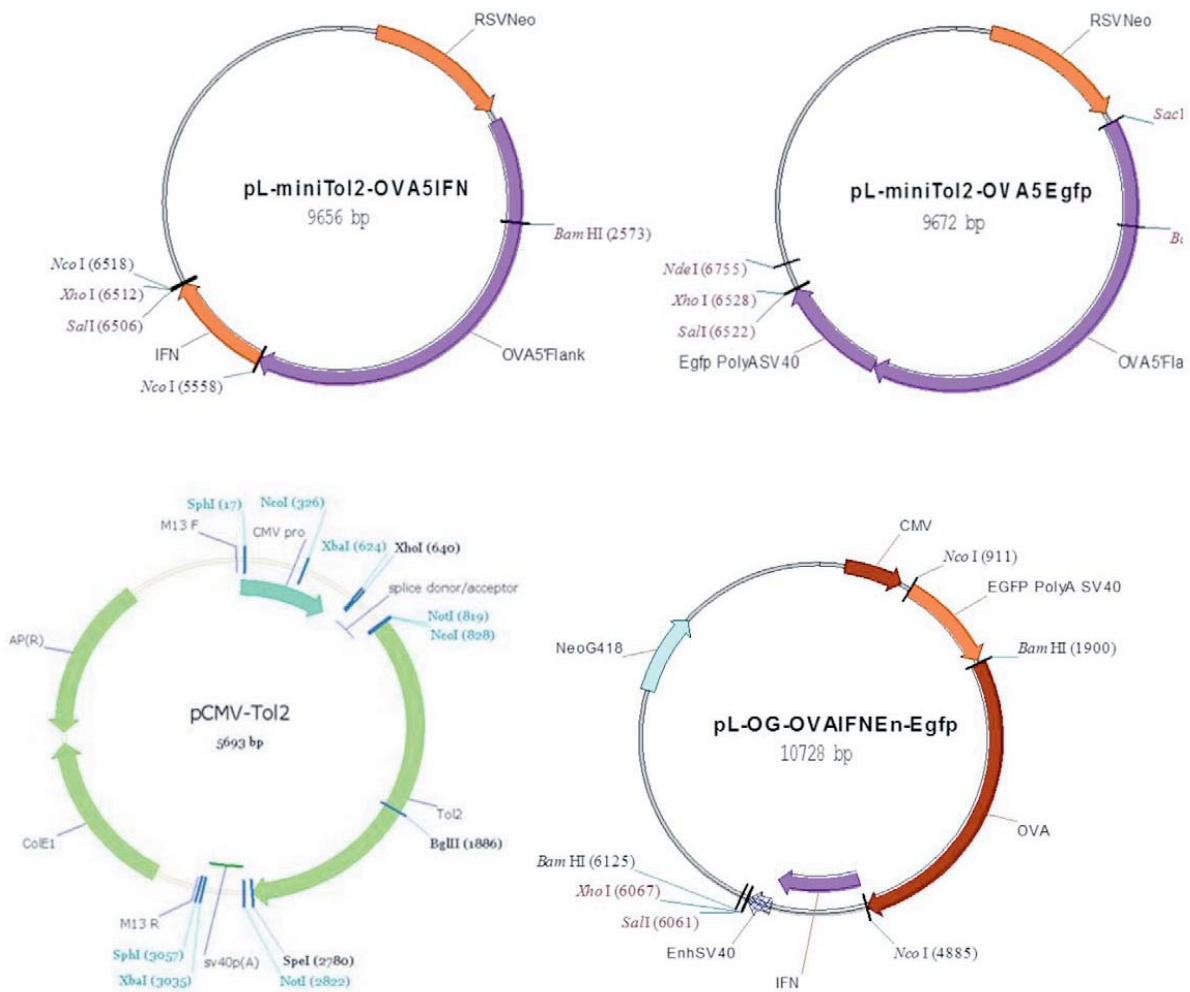


Fig. 1. The schemes of the expression vectors used in this study: pL-miniTol2-OVA5IFN, pL-miniTol2-OVA5Egfp, pCMV-TOL2, pL-OG-OVAIFNEnh-Egfp.

maintained for the next 24 h in selective medium. The successfully transfected cells compared to nontransfected produced the enzyme digesting G418, therefore cells survived. The cells were analyzed using flow cytometry (FACS Aria, BD Biosciences, San Jose, CA, USA) after staining with PI (propidium iodide, BD Biosciences). We performed four replicates for each combination of applied factors.

#### Transfection of PGCs

Purified PGCs were transfected with the most effective lipofection conditions identified in CHO-K1 cells. Briefly, purified PGCs were seeded in 24-well plates at a density of  $1 \times 10^5$  or  $5 \times 10^5$  cells/ml and cotransfected with method A (0.5  $\mu$ g of pCMV-Tol2 with 1  $\mu$ g of pL-miniTol2-OVA5IFN/pL-miniTol2-OVA5Egfp and 1  $\mu$ l of

lipofectant). The cells were also transfected using the nontransposon vector pL-OG-OVAIFNEnh-Egfp (as a control) at a ratio of 1  $\mu$ g: 3  $\mu$ l of lipofectant (protocol D). Lipofections were performed as described above. After 24 h of culturing cells in OPTIMEM C medium, G418 (0.4 mg/ml) was added. The medium was changed after 24 h, and the cells were cultured in selective medium until FACS analysis. Every experiment was carried out in triplicate.

#### Determination of transfection efficiency via FACS analysis

Cell transfection efficiencies (CHO-K1 and PGCs) were determined based on viability. The cells were centrifuged (5 min at 1,000 rpm at 21°C) and stained with PI (BD Biosciences) according to the manufacturer's recommendations. Briefly, the

cells were washed twice with cold 1x PBS (phosphate-buffered saline, Gibco Invitrogen) and subsequently suspended in 1x Binding Buffer. Then,  $1 \times 10^5$  cells/ml were transferred to a 5 ml culture tube, and 5  $\mu$ l of PI was added. The specimens were gently vortexed and incubated for 15 min at RT (25°C) in the dark. Next, 400  $\mu$ l of 1x Binding Buffer was added to each tube, and the specimens were analyzed by FACS. The fluorescence of PI was detected at an excitation wavelength of 488 nm using the 605/75 filter. PGCs were also observed under a confocal microscope (TE2000-E, Nikon Corporation, Tokyo, Japan).

### Statistical analysis

We calculated differences in the viability of CHO-K1 cells transfected with various cell densities, plasmid constructs and lipofection protocols with three-factor analysis of variance followed by the Bonferroni and Games-Howell tests. To examine differences in the viability of PGCs after lipofection with different plasmid constructs at various cell densities, two-way analysis of variance was performed. Analysis of variance took into account interactions between applied factors. Figures include empirical values. Statistical Package for the Social Sciences (SPSS) 17.0 software was used for statistical analyses. Values were considered statistically significant at  $p < 0.05$ . Percentage data are presented as means  $\pm$  SD.

## Results

### Flow cytometric analysis of CHO-K1 cell transfection efficiency

The CHO-K1 cells analyzed by FACS before lipofection exhibited high viability ( $95.5 \pm 1.5\%$ ). These cells were transfected under various conditions. We used cell viability as a measure of transfection efficiency. The effectiveness of cell lipofection was examined by flow cytometric analysis after the cells had been subjected to G418 selection. The percentage of transfected cells was significantly higher ( $p < 0.05$ ) after transfection at a density  $1 \times 10^5$  cells/ml than after transfection at a density of  $5 \times 10^5$  cells/ml. There were significant differences ( $p < 0.05$ ) in viability between cells transfected with nontransposon vectors via protocol D and cells transfected with transposon vectors using protocols B and C (Figs 2-3). However, among the transposon vectors, the highest transfection efficiency was achieved after lipofection of cells at a density of  $1 \times 10^5$  cells/ml with the pCMV-Tol2 and pL-miniTol2-OVA5IFN vectors using protocol A ( $62 \pm 1\%$ ) (Fig. 2). The combination of a cell density of  $5 \times 10^5$  cells/ml, pCMV-Tol2 with pL-miniTol2-OVA5Egfp and protocol C yielded the lowest percentage of transfected cells ( $13 \pm 1.5\%$ ) (Fig. 3).

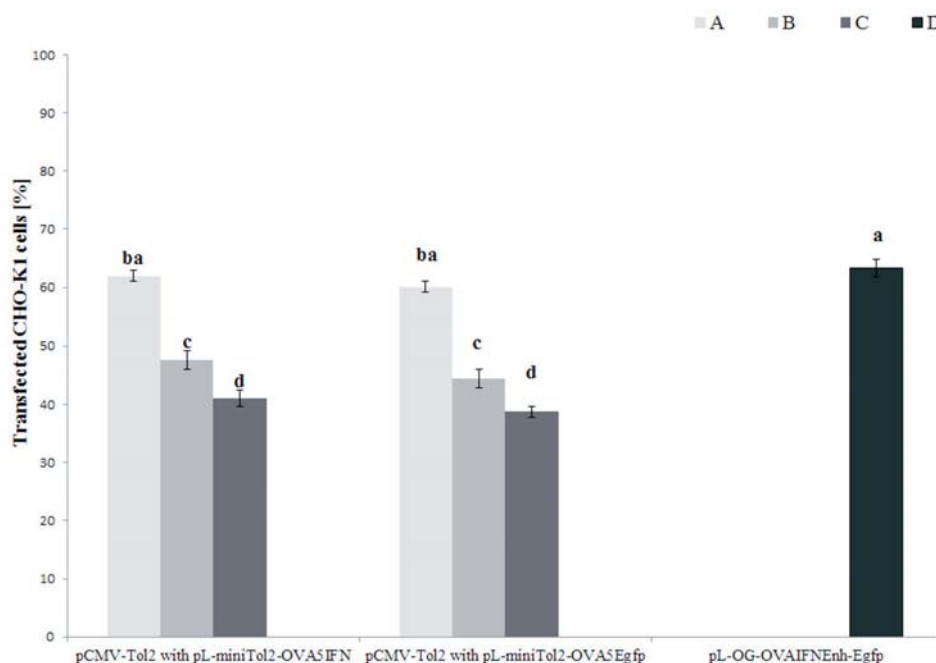


Fig. 2. Efficiency of CHO-K1 cell lipofection with transposon vectors and different protocols (A, B or C) or pL-OG-OVAIFNEnh-Egfp and protocol D. Lipofections were performed at a cell density of  $1 \times 10^5$  cells/ml. Letters a-d indicate significant differences at  $p < 0.05$ .

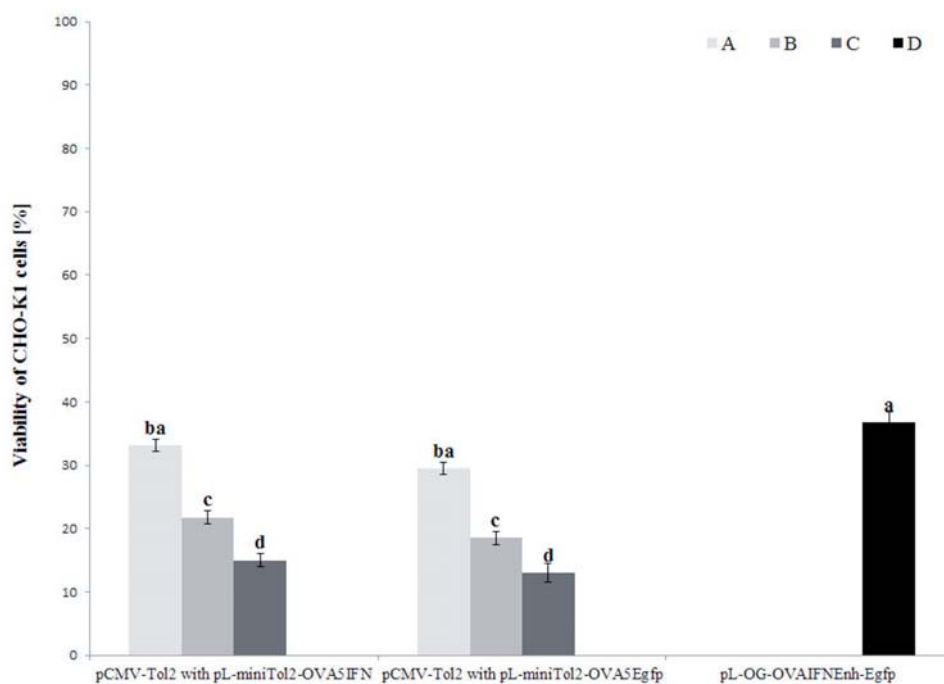


Fig. 3. Flow cytometric analysis of transfected CHO-K1 cells. Transfection efficiency of cells after lipofection with transposon vectors and different protocols (A, B or C) or pL-OG-OVAIFNEnh-Egfp and protocol D. Lipofections were performed at a cell density of  $5 \times 10^5$  cells/ml. Letters a-d indicate significant differences at  $p < 0.05$ .

#### Effectiveness of PGC transfection assessed by FACS analysis

The viability of freshly isolated and purified PGCs was high ( $98.9 \pm 1\%$ ) (Fig. 4). These cells were transfected with the most effective lipofection conditions identified in CHO-K1 cells. The percentage of live cells was used as a measure of *in vitro* transgene expression efficiency. We analyzed transfection efficiency by FACS analysis after the cells had been cultured with G418 selection. We found significant ( $p < 0.05$ ) differences in cell viability between cells transfected at a density of  $1 \times 10^5$  cells/ml and cells transfected at a density of  $5 \times 10^5$  cells/ml (Fig. 5). Similarly, in CHO-K1 cells transfected with transposon vectors via protocol A and pL-OG-OVAIFNEnh-Egfp via protocol D, the percentage of viable cells did not differ significantly (Fig. 5). However, among transposon vectors, the highest percentage of transfected cells was  $67 \pm 0.5\%$ ; this percentage was achieved by transfecting the cells with pCMV-Tol2 and pL-miniTol2-OVA5IFN at a density of  $5 \times 10^5$  cells/ml. The most unfavorable transfection conditions for PGCs were a density of  $1 \times 10^5$  cells/ml with pCMV-Tol2 and pL-miniTol2-OVA5Egfp ( $56.8 \pm 1.8\%$ ) (Fig. 5).

#### Discussion

PiggyBac and Tol2 transposons enable the introduction of exogenous DNA into host chicken chromosomes without silencing and increase the frequency of transgene integration in chicken PGCs, thereby greatly enhancing the efficient production of transgenic chickens (MACDONALD *et al.* 2012; PARK & HAN 2012). The efficiency of transgenic chicken generation also depends on PGC transfection efficiency, which in turn depends on the method used to introduce transposon vectors into PGCs. Previous studies have shown that transposons can be introduced with electroporation (SATO *et al.* 2007; LIU *et al.* 2013) or nucleofection (NAITO 2015). Nucleofection is a useful tool to transfect cells, but it requires specialized equipment and accessories, making it expensive and limiting its availability in the laboratory. The best alternative to electroporation, which achieves higher gene transfer efficiency, viability of manipulated embryos and hatching rates, is a combination of transposons and lipofection (TYACK *et al.* 2013).

In our study, we used lipofection as a transfection method to modify cells in combination with Tol2 transposon vectors carrying gene expression

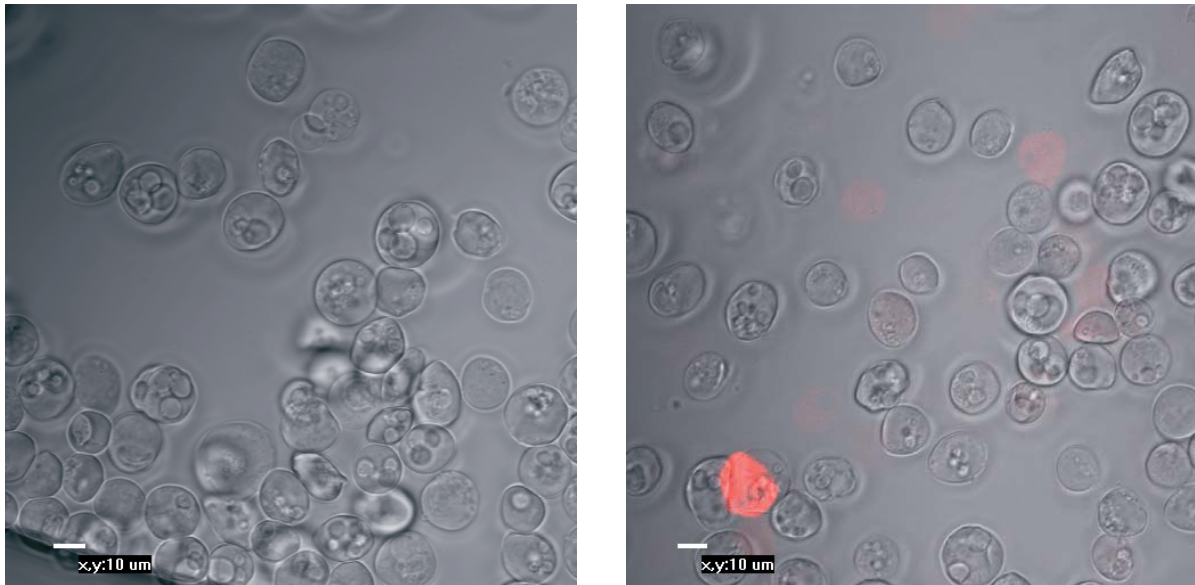


Fig. 4. Freshly isolated PGCs after staining with PI. Left – bright field, right – after excitation of PI fluorescence.

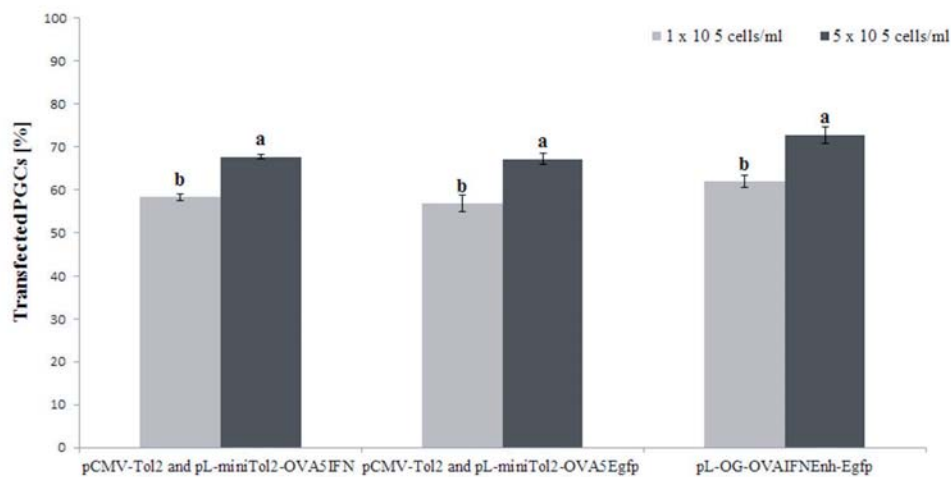


Fig. 5. Analysis of PGC transfection efficiency by FACS. Percentage of transfected cells after lipofection with transposon vectors and protocol A or pL-OG-OVAIFNenh-Egfp and protocol D. Lipofections were performed at a cell density of  $1 \times 10^5$  cells/ml or  $5 \times 10^5$  cells/ml. Letters a-b indicate significant differences at  $p < 0.05$ .

cassettes, insulators and enhancer elements. Our research focused on two areas. First, we optimized lipofection conditions using commercially available CHO-K1 cells. CHO-K1 cells are a good model for evaluating the effects of different factors, such as cell density, lipofection conditions and plasmid constructs, on transfection efficiency; thus, we reduced the number of chicken embryos used as PGC donors. Second, we transfected purified PGCs with the most effective lipofection protocol identified for CHO-K1 cells.

Lipofection in combination with transposons has been used to transfect both PGCs and CHO-K1 cells (BALASUBRAMANIAN *et al.* 2011; MATASCI *et al.* 2011; MACDONALD *et al.* 2012; PARK & HAN 2012; GLOVER *et al.* 2013; LI *et al.* 2013; LIU *et al.* 2013; RAJENDRA *et al.* 2016). In these studies, transposon vectors were combined with different transfection reagents, such as Lipofectamine 2000, 1,2-dimyristyloxy-propyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE) and polyethylenimine (PEI). This is the first report using X-treme Gene HP to transfect cells with transposon vectors.

We transfected CHO-K1 cells under various conditions, cell densities and vector constructs. After the cells had been cultured with G418 selection, we examined the percentage of live cells by flow cytometry. Successfully transfected cells, unlike nontransfected cells, produced the enzyme that digests G418, thereby allowing cell survival. Regardless of the protocols and vectors used to modify the cells, significantly higher ( $p < 0.05$ ) transfection efficiencies were obtained for transfections at a density  $1 \times 10^5$  cells/ml than for transfections at a density of  $5 \times 10^5$  cells/ml. Figs 2-3 show that the viability of cells transfected with nontransposon vectors via protocol D differed significantly ( $p < 0.05$ ) from that of cells transfected with transposon vectors via protocols B and C. Furthermore, there were no significant ( $p < 0.05$ ) differences in transfection efficiency between transposon vectors via method A (from  $60.0 \pm 1$  to  $62.0 \pm 1\%$ ) and pL-OG-OVAIFNEnh-Egfp via method D ( $63.4 \pm 1.5\%$ ). This finding suggests that our transposon vectors can be successfully applied to transfect PGCs via method A.

As reported in previous studies (OISHI 2010; MATÁS *et al.* 2011; CHOJNACKA-PUCHTA *et al.* 2015), the uptake of foreign DNA by PGCs and thereby transfection efficiency can be increased by Percoll purification. Because PGCs have a lower proliferation rate than CHO-K1 cells (VAN DE LAVOIR *et al.* 2006; KONG *et al.* 2018) and require suspension culture conditions, purified cells were seeded at two densities:  $1 \times 10^5$  cells/ml and  $5 \times 10^5$  cells/ml in 24-well plates. We transfected purified PGCs with transposon vectors via method A or nontransposon vectors via method D. After selection in G418-containing medium, cell viability was examined by flow cytometry. The most appropriate transfection conditions for introducing transposon vectors into PGCs were lipofection at a density of  $5 \times 10^5$  cells/ml with method A (Fig. 5). This procedure achieved a transfection efficiency of  $67 \pm 0.5\%$ . In a study by MACDONALD *et al.* (2012), PGCs were transfected with transposon vectors combined with lipofection (via DMRIE transfection reagent), and 45.2% of cells were transfected. We show that our optimized method is effective and can be used to transfect PGCs with transposon vectors. Modified in this way, PGCs, injected into the bloodstream of recipient embryos, may be useful for producing transgenic chickens via chimeric intermediates (KUWANA 1993; VAN DE LAVOIR *et al.* 2006; NAKAMURA *et al.* 2010; CHOJNACKA-PUCHTA *et al.* 2015; NAITO 2015).

Introduction of exogenous DNA into PGCs is also possible by *in vivo* transfection. Unlike *in vitro* transfection, this method is based on direct injection of DNA-liposome/-PEI complexes into the

subgerminal cavity of newly laid eggs or into the vasculature of developing chicken embryos. WANG *et al.* (2016) performed PGC transfections *in vivo* using different transposon vectors (PB, SB, TOL2 or ZB) combined with transfection reagent (PEI) and obtained 65-70% nonviable hatched individuals. *In vitro* transfection enables to select the most effective methods for transposon vectors before generating transgenic chickens.

In conclusion, this is the first study to optimize cell lipofection using transposon vectors. We presented an effective method for the transfection of chicken PGCs with the use of transposon vectors combined with lipofection. The method employed in this work will facilitate the production of transgenic chickens in future projects.

### Acknowledgements

This work was partially supported by the National Center for Research and Development (Grant No. PBS3/A8/30/2015).

### Author Contributions

Research concept and design: D.S, L.C.-P.; Collection and/or assembly of data: D.S, L.C.-P.; Data analysis and interpretation: D.S, L.C.-P.; Writing the article: D.S, L.C.-P.; Critical revision of the article: D.S, L.C.-P.; Final approval of article: D.S, L.C.-P.

### Conflict of Interest

The authors declare no conflict of interest.

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