《Research Note》

Expression of GFP Gene in Gonads of Chicken Embryos by Transfecting Primordial Germ Cells *in vitro* or *in vivo* using the PiggyBac Transposon Vector System

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In the present study, chicken primordial germ cells (PGCs) were transfected with GFP gene *in vitro* or *in vivo* using the piggyBac transposon vector system. PGCs cultured for 465 days were transfected *in vitro*, and GFP gene expression was observed in 25% of the treated PGCs after culturing for further 42 days. The cultured PGCs expressing GFP gene were transferred to recipient embryos and strong GFP gene expression was observed in the recipient gonads at day 18.5 of incubation. Circulating PGCs were transfected *in vivo*, and intense GFP gene expression was observed in the gonads of recipient embryos at day 18.5 of incubation. The procedure employed in the present study will contribute to successful gene transfer into chickens.

Key words: chick embryo, GFP gene, primordial germ cells, transfection, transposon

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Introduction

Production of transgenic chickens is useful for generating pharmaceutical materials in eggs, analysis of cloned gene function and genetic improvement in chickens. Primordial germ cells (PGCs) are progenitor cells of ova and spermatozoa and are one of the most appropriate cells for germline manipulation in chickens (Kuwana, 1993; Tajima, 2002, 2013; Naito, 2003, 2015). Exogenous DNA could be introduced transiently into the gonads of recipient embryos by transfecting freshly collected PGCs *in vitro* and then transferring them to recipient embryos (Naito *et al.*, 1998). Recent development of the PGC culture method enabled us to transfer exogenous DNA into chickens without using a viral vector (van de Lavoie *et al.*, 2006). The efficiency of DNA transfer into PGCs was improved by using the piggyBac or the Tol2 transposon vector system, and this system is effective for inserting exogenous DNA into the host chromosome with the aid of transposase, as well as for avoiding the silencing of transgene expression (Macdonald *et al.*, 2012; Park and Han, 2012; Glover *et al.*, 2013). Very recently, we have developed a novel method for the long-term culture of chicken PGCs isolated from embryonic blood

without using xeno-animal cells as feeder cells (Naito *et al.*, 2015). This method is especially effective for the long-term culture of female PGCs. However, DNA transfer into PGCs cultured by this method has not been attempted. On the other hand, exogenous DNA was introduced efficiently by transfecting circulating PGCs *in vivo* (Watanabe *et al.*, 1994; Naito *et al.*, 2007). The introduced DNA expressed strongly in the gonads of recipient embryos, but they gradually disappeared with ongoing embryonic development (Naito *et al.*, 2007). By using the Tol2 transposon vector system for this *in vivo* transfection, exogenous DNA was stably incorporated in the circulating PGCs, and transgenic chickens were produced (Tyack *et al.*, 2013). In the present study, we attempted to introduce GFP gene into the chicken germline by transfecting PGCs *in vitro* or *in vivo* using the piggyBac transposon vector system. PGC population used for *in vitro* transfection was cultured for long-term and almost established as a cell line.

Materials and Methods

Fertilized Eggs and Animal Care

Fertilized eggs of White Leghorn (WL) and Barred Plymouth Rock (BPR) chickens were obtained by artificial insemination. WL and BPR populations are maintained at the National Institute of Livestock and Grassland Science. All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (National Institute of Agrobiological Sciences, Animal Care Commit-

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tee), and was specifically approved for this study (H18-028-1).

PGC Culture *in vitro*

PGCs were isolated from the embryonic blood of 2.5-day incubated chicken embryos (BPR). The isolated PGCs were dispersed in the culture medium and placed on the feeder cells derived from chicken embryonic fibroblasts (WL) using a 4-well plate (176740, Nunc, Roskilde, Denmark). The PGC culture medium used was KAv-1 containing 5% fetal bovine serum and 5% chicken serum (Kuwana *et al.*, 1996), supplemented with 10 ng/mL human basic fibroblast growth factor (060-04543, Wako Pure Chemicals, Osaka, Japan), 2% chick embryo extract and 15% knockout serum replacement (10828-028, Invitrogen, Carlsbad, CA, USA) (Naito *et al.*, 2015). The chick embryo extract was prepared as follows. Five-day incubated WL embryo was homogenized with 800 μ L KAv-1 medium, then centrifuged and supernatants were filtered. The cultured PGCs proliferated were passaged every 5-7 days and cultured for more than one year. The PGC population used for *in vitro* transfection proliferated without forming cell colonies (Naito *et al.*, 2015), and the presence of female cells were not detected by PCR analysis (Clinton *et al.*, 2001).

Preparation of Recipient Embryos

Freshly laid WL fertilized eggs at stage X (Eyal-Giladi and Kochav, 1976) were broken, the albumen capsule removed, the yolk was then transferred to a small host eggshell, and the egg reconstituted (System II, Perry, 1988; Naito *et al.*, 1990). The reconstituted eggs were incubated for 2.5 days until the embryo reached stages 14-16 in a forced air incubator (P-008B, Showa Furanki, Saitama, Japan).

Transfection of Cultured PGCs *in vitro* and Cell Transfer

The cultured PGCs were recovered and washed with Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS (-)) (28-103-05 FN, Dainippon Sumitomo Pharma, Osaka, Japan). The transfection of PGCs was performed using a new electroporation-based technique known as "nucleofection" and the piggyBac transposon vector system (System Biosciences, Mountain View, CA, USA). The collected PGCs were dispersed in a 100 μ L nucleofector solution (solution V, Amaxa GmBH, Köln, Germany) containing 5 μ g of PB513B-ACT plasmid (PB513B-1 plasmid modified with GFP gene under the control of chicken β -actin gene promoter) and 5 μ g of PB200PA-1 plasmid. The transfection was accomplished using the nucleofection program A-033. The recovered

PGCs were further cultured on freshly prepared feeder cells to reduce the proportion of PGCs expressing GFP gene transiently. The cultured PGCs were recovered and dispersed in fresh culture medium. The cell suspension was placed on a plastic dish and GFP gene expression was observed under a fluorescent microscope (DMIRE2, Leica Microsystems, Tokyo, Japan). Two hundred cultured PGCs expressing the GFP gene were picked up under the fluorescent microscope and transferred to the bloodstream of recipient embryos (Naito *et al.*, 1994). The manipulated embryos were transferred to large host eggshells and incubated for a further 16 days (System III, Perry, 1988; Naito *et al.*, 1990).

Transfection of Circulating PGCs *in vivo*

In vivo transfection of circulating PGCs was performed by lipofection. DNA-liposome complex was prepared as follows. Fifteen microliters of a cationic lipid (11668-027, Lipofectamine2000, Invitrogen, Carlsbad, CA, USA) solution was first diluted with 15 μ L Opti-MEM I reduced-serum medium (31985-062, Invitrogen, Carlsbad, CA, USA) and incubated for 5 min at room temperature (25°C). Two and a half micrograms of PB513B-ACT plasmid and 2.5 μ g of PB200PA-1 plasmid diluted with 5 μ L of Opti-MEM I reduced-serum medium were added, mixed gently, and incubated for 20 min at room temperature. The prepared transfection medium (DNA-liposome complex) was injected into the bloodstream of recipient embryos at a volume of 0.5 μ L (62.5 ng DNA). The manipulated embryos were transferred to large host eggshells and incubated for a further 16 days (System III, Perry, 1988; Naito *et al.*, 1990).

Analysis of GFP Gene Expression in Gonads of Recipient Embryos

Embryos incubated for up to day 18.5 following transfection of PGCs *in vitro* or *in vivo* were removed from the yolk, and then the gonads were exposed and isolated from the embryos. Expression of the GFP gene in the gonads was detected under a fluorescent microscope (M205FA, Leica Microsystems, Tokyo, Japan).

Results

Viability of Manipulated Embryos

Viabilities of the manipulated embryos are shown in Table 1. When transfected and cultured PGCs were transferred to the recipient embryos, half (50.0%, 15/30) of the manipulated embryos survived at day 18.5 of incubation. On the other hand, when DNA-liposome complex was injected into

Table 1. Viabilities of manipulated embryos *in vitro* or *in vivo*

Transfection of PGCs	No. of embryos cultured	Number (%) of embryos manipulated at day 2.5	Number (%) of embryos surviving at day 18.5	Number (%) of embryos with GFP-positive gonads	Sex ratio of embryos with GFP-positive gonads
<i>in vitro</i>	36	30 (83.3)*	15 (41.7)*	3 (20.0)**	♂2 : ♀1
<i>in vivo</i>	54	49 (90.7)*	37 (68.5)*	21 (56.8)**	♂11 : ♀10

* Percentage of embryos surviving among cultured embryos.

** Percentage of embryos with GFP-positive gonads among analyzed embryos at day 18.5 of incubation.

Fig. 1. Expression of GFP gene in cultured PGCs and in recipient gonads. PGCs cultured for 465 days were transfected *in vitro* with GFP gene using the piggyBac transposon vector system and then cultured for further 42 days (A: bright light, B: fluorescent light). Cultured PGCs expressing GFP gene were transferred to recipient embryos and observed GFP gene expression in the gonads of recipient embryos at day 18.5 of incubation (C and D: male, E and F: female; The gonad was rotated slightly in D). Bars indicate 20 μ m (A and B) and 0.5 mm (C, D, E and F).

the recipient embryos, three quarters (75.5%, 37/49) of the manipulated embryos survived at day 18.5 of incubation.

Expression of GFP Gene in Cultured PGCs Transfected in vitro and in Gonads of Recipient Embryos

PGCs cultured for 465 days were transfected with GFP gene *in vitro* by nucleofection and further cultured for 42 days. Then, GFP gene expression was observed in 25% (201/804) of the cultured PGCs (Fig. 1A and 1B). Cultured PGCs expressing GFP gene were transferred to the recipient embryos and analyzed the GFP gene expression in the gonads of recipient embryos at day 18.5 of incubation. The GFP gene expression in the gonads was detected in 20% (3/15) of embryos analyzed (Table 1), and strong GFP gene expression was observed in limited areas of the recipient gonads of males (Fig. 1C and 1D) and female (Fig. 1E and 1F).

Expression of GFP Gene in Gonads of Recipient Embryos Transfected in vivo

Circulating PGCs in the bloodstream were transfected with GFP gene *in vivo* by lipofection, and 24 hours later many GFP gene expressing cells were observed in the blood circulation. GFP gene expression in the gonads was detected in 56.8% (21/37) of embryos analyzed at day 18.5 of incubation (Table 1). Although the GFP gene expression tended to be observed in limited areas of the recipient gonads, intense expression of the GFP gene was observed both in males (Fig. 2A and 2B) and females (Fig. 2C and 2D).

Discussion

The present study shows that the GFP gene was strongly expressed in the gonads of 18.5-day incubated recipient embryos by transfecting PGCs *in vitro* or *in vivo* using the piggyBac transposon vector system. The piggyBac and the Tol2 transposon vector systems are very useful for introducing exogenous DNA into host chromosome of chicken cells (Macdonald *et al.*, 2012; Glover *et al.*, 2013). The integration frequency of exogenous DNA in chicken PGCs is usually low (Naito *et al.*, 1988, 2007; van de Lavoie *et al.*, 2006), but by using the transposon vector system it was greatly enhanced and efficient production of transgenic chickens became possible (Macdonald *et al.*, 2012; Park and Han, 2012).

Concerning PGC transfection *in vitro*, freshly collected PGCs were transfected with *lacZ* gene by lipofection, and then transferred to recipient embryos (Naito *et al.*, 1998). The introduced *lacZ* gene expressed in the gonads of recipient embryos efficiently, but gradually disappeared during embryonic development. In the present study, PGCs cultured by the newly developed method (Naito *et al.*, 2015) were transfected with the GFP gene by nucleofection using the piggyBac transposon vector system. The transfected PGCs were further cultured and then transferred to the recipient embryos. Strong GFP gene expression was observed in the gonads of recipient embryos, but the GFP gene expression was limited in areas. The transfected PGCs were cultured for more than one year and as a result the migratory

Fig. 2. **Expression of GFP gene in recipient gonads.** Circulating PGCs were transfected *in vivo* with GFP gene using the piggyBac transposon vector system. The GFP gene expression was observed in the gonads of recipient embryos at day 18.5 of incubation (A and B: male, C and D: female). Bars indicate 0.5 mm.

ability of the manipulated PGCs seemed to decline. When cultured PGCs are transfected *in vitro*, the PGC culture period should probably be as short as possible in order to maintain the migratory ability to recipient gonads and also maintain differentiation potency to functional gametes of cultured PGCs (Naito *et al.*, 2015).

On the other hand, circulating PGCs were efficiently transfected *in vivo* with *lacZ* gene or GFP gene by lipofection (Watanabe *et al.*, 1994; Naito *et al.*, 2007), but the introduced *lacZ* gene or GFP gene disappeared gradually from the gonads during embryonic development. In the present study, circulating PGCs were transfected *in vivo* using the piggyBac transposon vector system and intense expression of the GFP gene was observed in the gonads of 18.5-day incubated recipient embryos. Tyack *et al.* (2013) produced

transgenic chickens by *in vivo* transfection of circulating PGCs using the Tol2 transposon vector system. This *in vivo* transfection method for circulating PGCs is easy compared with that *in vitro* transfection method due to not needing to culture PGCs, although hatched chicks must be analyzed to determine whether they have transgenes.

In the present study, the GFP gene was introduced into PGCs using the piggyBac transposon vector system and the GFP gene expression continued long term in the recipient gonads. It is, therefore, expected that the introduced GFP gene was stably incorporated in the germline of recipient embryos. The procedure employed in the present study will contribute to producing transgenic chickens.

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