

# Evidence of *in vivo* transfection of circulating primordial germ cells in chickens

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Primordial germ cells (PGCs) are progenitor cells of ova and spermatozoa. They appear in the centre of the area pellucida at stage X, and circulate in the bloodstream before migrating to the germinal ridges. In order to introduce exogenous DNA into the germline of chickens, *in vivo* transfection of circulating PGCs was attempted. DNA-liposome complex was injected into the bloodstream of embryos at stages 14-15, and the manipulated embryos were cultured up to 20.5 days of incubation. GFP gene expression was analysed in the gonads of developing embryos. When circular plasmid was introduced, GFP gene was efficiently expressed in the gonads of embryos, especially up to 10.5 days of incubation. Thereafter, the GFP gene expression decreased gradually, but GFP gene expression was still observed in the gonads of embryos at 20.5 days of incubation. When linearised plasmid was introduced, GFP gene expression was not observed in the gonads of embryos at 20.5 days of incubation except for two (0.45%) embryos. In these two embryos, strong GFP gene expression was observed in a limited area of the left ovary and the left testis. PCR analysis of the gonads of manipulated embryos showed that GFP gene introduced into the PGCs was gradually lost, and most of it by the hatching stage. Nevertheless, GFP gene was still detectable at a very low frequency in some of the gonads of embryos at 20.5 days of incubation. These results suggest that it is possible to introduce exogenous DNA into gonadal germ cells by transfecting circulating PGCs *in vivo*.

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**Keywords:** GFP gene; gonadal germ cell; gonad; primordial germ cell; transfection

## Introduction

The production of transgenic chickens provides numerous applications in the field of animal biotechnology such as producing pharmaceutical materials in eggs as well as studies on the function of cloned genes. The introduction of exogenous DNA into chickens still poses difficulties mainly due to their unique reproductive characteristics and the yolk-laden structure of the ovum (Naito, 2003ab; Sang, 2004). For practical applications of avian transgenic technology, the non-viral method is preferable for safety reasons. The only non-viral method that has successfully produced transgenic chickens to date is microinjection of DNA into the germinal disc of the fertilised ovum (Love *et al.*, 1994; Sherman *et al.*, 1998). By this method, however, only a limited number of ova can be manipulated because only one ovum can be obtained from each hen.

Primordial germ cells (PGCs) are the progenitor cells of ova and spermatozoa. PGCs in chickens are mainly located in the central disc of the area pellucida at stage X (Eyal-Giladi and Kochav, 1976; Ginsburg, 1994; Naito *et al.*, 2001a), and move to the germinal crescent region at stage 4 (Hamburger and Hamilton, 1951). They then enter into the developing blood vessels and circulate temporarily throughout the circulatory system, finally migrating to the germinal ridges, i.e., the future gonads (Kuwana, 1993; Naito, 2003c). The introduction of exogenous DNA into PGCs has been attempted at various developmental stages, e.g., PGCs or their precursors isolated from stage X blastoderm

(Carsience *et al.*, 1993), PGCs isolated from embryonic blood (Naito *et al.*, 1998), or PGCs isolated from embryonic gonads (Hong *et al.*, 1998) have been transfected *in vitro* and then transferred to the recipient embryos. The introduced DNA was expressed in those embryos, but most of it was present episomally and disappeared during embryonic development. The integration of exogenous DNA into the host chromosomes of PGCs is expected to occur in a small proportion of the *in vitro* manipulated PGC population, but so far no PGCs have been obtained in which exogenous DNA was integrated into the chromosomes, probably due to the limited number of PGCs that could be manipulated *in vitro*. Although the culture of PGCs *in vitro* has been successful, the proliferation rate is still very low (Naito *et al.*, 2001b; Han *et al.*, 2002; Park *et al.*, 2003).

*In vivo* manipulation of PGCs has the advantage of introducing exogenous DNA into germ cells. When PGCs are transfected *in vivo*, all PGCs in the embryo can be manipulated and are able to proliferate rapidly after entering the gonads. Watanabe *et al.* (1994) attempted to transfect PGCs *in vivo* by lipofection. In their experiment, liposome-DNA complexes were injected into the bloodstream of 2.5-day incubated embryos. The introduced DNA (*lacZ* gene) was expressed in the gonads of developing embryos after 2-3 days of incubation following the manipulation, and the number of *lacZ*-positive PGCs was 0.2-2.1 per embryo. Although the transfection efficiency of PGCs *in vivo* was thus very low, the introduction of exogenous DNA into PGCs *in vivo* by lipofection was shown to be possible. Recent developments in lipofection technology, however, are expected to enhance the *in vivo* transfection efficiency of PGCs circulating in the bloodstream.

The present study was performed to improve the transfection efficiency of the above technique using green fluorescent protein (GFP) gene as a marker, enabling us to achieve a highly efficient transfection of PGCs *in vivo*.

## Materials and methods

### *Fertilised eggs and animal care*

Fertilised eggs of Barred Plymouth Rock chickens were obtained by artificial insemination from the genetic stock 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 Committee).

### *Transfection of PGCs in vivo and embryo culture*

Transfection of PGCs was carried out by lipofection using cationic lipids (LA2000). Five microliters of LA2000 solution was first diluted with Opti-MEM I and incubated for 5 min at room temperature (25°C). Two micrograms of plasmid DNA (pbAEGFP; GFP gene under the control of chicken  $\beta$ -actin gene promoter) diluted with Opti-MEM I was added, mixed gently, and incubated for 20 min at room temperature.

Freshly laid and unincubated fertilised eggs (stage X) were broken, and the contents put in glass vessels. The thick albumen capsule was removed from the yolk, and the embryo (yolk) was transferred to a small host eggshell, filled with thin albumen, covered with cling film, and secured with plastic rings and elastic bands (Perry, 1988; Naito *et al.*, 1990). The reconstituted eggs were cultured at 38°C, relative humidity 50-60%, for about 53 hrs in a forced-air incubator. When the embryos reached to stages 13-14, the prepared transfection medium (DNA-liposome complexes) was injected into the dorsal aorta of each embryo at a volume of 0.5  $\mu$ l (50 ng DNA). The control medium was also injected into the bloodstream in the same manner. The manipulated embryos were then transferred to large host eggshells and incubated for up to 20.5 days (Perry, 1988; Naito *et al.*, 1990).

### *Detection of GFP gene expression in gonads of embryos*

Manipulated and cultured embryos were removed from the yolk, washed with PBS(-). The gonads were then exposed, and the GFP gene expression was observed under a fluorescent microscope (MZFL-III, Leica Microsystems).

### *Detection of GFP gene in gonads of embryos*

Gonads were removed from the embryos which developed to 20.5 days of incubation and washed with PBS(-). DNA was extracted from the gonads using a DNA extraction kit (SepaGene, Sanko Junyaku) according to the manufacturer's instructions. The extracted DNA was dissolved in TE buffer.

PCR analysis was carried out to detect the presence of the GFP gene. The PCR reaction was performed using a programmable thermal controller (Model 9700, Perkin Elmer). The primers for detecting the GFP gene were as follows; 5'-TTC AAG TCC GCC ATG CCC GAA-3', 5'-ATG GGG GTG TTC TGC TGG TAG-3' (Sano *et al.*, 2003). After an initial denaturation step at 94°C for 5 min, 40 cycles of amplification were performed; DNA was denatured at 94°C for 30 sec, annealed at 60°C for 30 sec, and extended at 72°C for 30 sec. The reactions were then incubated at 72°C for 5 min. For internal control, a sequence of the endogenous glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene was amplified using the following primers; 5'-CAG ATC AGT TTC TAT CAG C-3', 5'-TGT GAC TTC AAT GGT GAC A-3' (Love *et al.*, 1994). After an initial denaturation step at 94°C for 5 min, 40 cycles of amplification were performed; DNA was denatured at 94°C for 30 sec, annealed at 55°C for 30 sec, and extended at 72°C for 30 sec. The reactions were then incubated at 72°C for 5 min. The PCR products of each reaction were separated on a 2% agarose gel, and the bands were visualised under UV light after ethidium bromide staining.

## Results

### *Viabilities of the manipulated embryos*

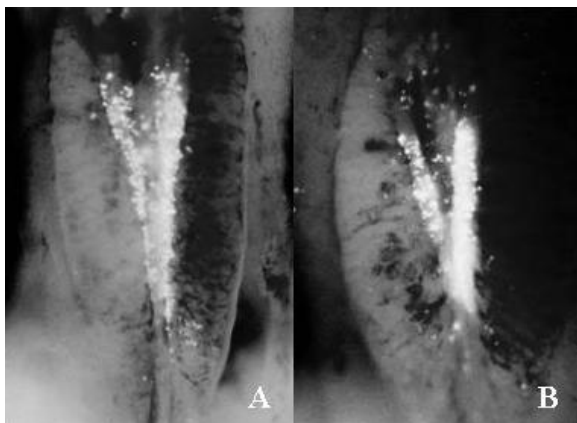
Viabilities of the manipulated embryos at 20.5-days of incubation which were injected with DNA-liposome complexes were shown in Table 1. When circular form DNA was used for lipofection, viability of the embryos was 44.6% (183/410), whereas when linearised DNA was used, viability was 69.7% (442/634).

### *Expression of the GFP gene in gonads of developing embryos*

GFP gene expression in the gonads was observed only when circular form plasmid DNA was transfected; that expression in the gonads of manipulated embryos was shown in Figure 1. A strong GFP gene expression was detected in the gonads of embryos up to 10.5 days of incubation. During this period, that strong GFP gene expression was observed throughout the gonads. After that, GFP gene expression gradually decreased as the incubation continued, but it was still detected in the gonads of 20.5-day incubated embryos just before hatching, although it was only faintly expressed. Green fluorescence was not observed at any developmental stage in the embryos injected with the control medium (lacking LF2000 or plasmid DNA).

### *Expression and presence of GFP gene in gonads of 20.5-day incubated embryos*

The manipulated embryos that developed to 20.5 days of incubation were examined for the expression and presence of the GFP gene in the gonads. That expression was observed in 86.3% (158/183) of embryos examined only when circular form plasmid DNA was transfected (Table 1), whereas its presence in the gonads was detected in only one embryo (0.55%, 1/158) when the circular form was transfected (Table 1). The GFP gene was detected in the gonads of 4.30% (19/442) of embryos analysed when linearised plasmid DNA was transfected.



**Figure 1.** Expression of GFP gene in the gonads of developing chicken embryos at 6.5 days (A) and 7.5 days (B) of incubation. Circulating primordial germ cells (st. 14) were transfected *in vivo* by lipofection at 2.5 days of incubation, and the manipulated embryos were cultured in host eggshells. GFP gene expression was observed throughout the gonads.

**Table 1. Presence and expression of GFP gene in gonads of 20.5 day-incubated embryos transfected *in vivo* by lipofection at 2.5 days of incubation**

Plasmid (pbAEGFP)	Number of embryos treated	Number (%) of embryos surviving at 20.5 days of incubation	Number (%) of embryos expressing GFP gene in gonads of 20.5 days of incubation	Number (%) of GFP-positive gonads by PCR at 20.5 days of incubation
Circular	410	183 (44.6)	158 (86.3)	1 (0.55)
Linear	634	442 (69.7)	2 (0.45)	19 (4.30)

## Discussion

Avian species show some unique characteristics of PGC migration such as circulating in the bloodstream before colonising the gonads. The present study demonstrates that circulating PGCs can be efficiently transfected *in vivo* by lipofection and, as a result, the introduced GFP gene was strongly expressed throughout the gonads when circular form plasmid DNA was used for transfection. This strong GFP gene expression persisted up to 10.5 days of incubation, i.e., 8 days after the manipulation. Thereafter, GFP gene expression decreased gradually, but was still detectable at 20.5 days of incubation just before hatching. At this stage, GFP was observed in 86.3% of embryos examined, but the presence of the GFP gene in the gonads was detected in only one (0.55%). These results suggest that the GFP gene was efficiently introduced into the PGCs circulating in the bloodstream by *in vivo* lipofection, and that the transfected PGCs colonised in the gonads and strongly expressed the GFP gene. Although this strong GFP gene expression persisted during the first 8 days after manipulation, the GFP gene was gradually lost, and most of it had disappeared by the hatching stage.

The number of PGCs circulating in the bloodstream reaches a maximum at stages 14-15 and starts to migrate to the germinal ridges at stages 15-16, although there are egg-to-egg variations in the number of PGCs present at the same developmental stage (Kuwana, 1993; Tajima *et al.*, 1999; Zhao *et al.*, 2003). Transfection of PGCs circulating in the bloodstream *in vivo* can, therefore, be carried out by injecting DNA-liposome complexes into the bloodstream at stage 14, as suggested by Watanabe *et al.* (1994). Since DNA-liposome complexes are stable for several hrs even in the presence of serum, most of the PGCs were transfected efficiently during their circulation in the bloodstream, therefore, a strong GFP gene expression throughout the gonads could be achieved by manipulating embryos at the appropriate developmental stage.

PGCs proliferate rapidly after entering the gonads and, during such a rapid proliferation phase, exogenous DNA (GFP gene) would have a chance to enter the chromosomes of PGCs or germ cells. When the linearised form of plasmid DNA was used for transfection, GFP gene was detected in the gonads of 4.3% of embryos examined at 20.5 days of incubation. These results suggest that the integration of the GFP gene into the chromosomes of PGCs or germ cells could be expected using the current *in vivo* lipofection procedure, although the frequency is very low.

The viability of embryos at 20.5 days of incubation was higher when the linearised form of plasmid DNA was used for transfection of PGCs compared with that when the circular form was used. Excessive expression of the GFP gene seems to be harmful to developing embryos, although such GFP gene expression was mainly observed in the gonads.

The analysis of same-sex and mixed-sex germline chimaeric chickens has shown that PGCs differentiate to ova in the ovary and to spermatozoa in the testes irrespective of their genetic sex (Kagami *et al.*, 1995, 1997; Naito *et al.*, 1999, 2001a). Many genes involved in the sexual differentiation of embryos have been identified, and these genes express transiently at the appropriate period of embryonic development (Shimada, 2002; Smith and Sinclair, 2004). Although genes expressing in PGCs at the stage of sexual differentiation are largely unknown, exogenous genes can be expressed strongly and transiently using the present procedure of *in vivo* lipofection. The present procedure will, therefore, be useful in studying the functions of genes involved in the differentiation of PGCs.

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