Gene Transfer into Circulating Primordial Germ Cells of Quail Embryos

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Abstract: During early stages in avian embryogenesis primordial germ cells (PGCs) show a unique migration pathway toward the gonadal anlage through the circulation. In the present study, liposomes consisting of plasmid DNA (pMiwZ; containing lacZ as a reporter) and Lipofectin were injected into the marginal veins of quail embryos during the stages PGCs were circulating in the blood vessels. The lacZ expression was then histochemically detected in the gonads at later embryonic stages, indicating the expression of the injected DNA in PGCs.

Key words: lacZ, lipofection, pMiwZ, primordial germ cells, quail

Introduction

Avian primordial germ cells (PGCs) show a unique migration pathway toward gonadal anlage [9]. They are derived from epiblastic cells that migrate from the central region of the area pellucida to the germinal crescent in the hypoblast at the anterior border of the area pellucida [5, 7]. At stage 10 [8] when the blood vessels form in the germinal crescent, the PGCs enter the vessels and are carried by the circulation to the region where hind gut is forming [6]. They then exit from the circulation, become associated with the mesentery at stages 15–18 [1], and finally migrate into the gonadal anlage by stages 20–24 [18].

A number of attempts have been made to produce transgenic birds for example by introducing the gene into a fertilized single-cell ovum [10–13], infusing the

blastoderm cells transfected by the foreign gene into the host embryo at the blastoderm stage [3, 14] or by using a retrovirus as a vector [2, 17]. These methods, however, require improvement in the gene transmission to the germline for success or require the handling of hazardous retroviruses.

When the production of transgenic offspring is the target, direct gene incorporation into the genome of PGCs will be a shorter way. Recently, Watanabe *et al.* [18] injected liposomes consisting of reporter plasmid DNA and N-[1-(2, 3-Dioleoyloxy) propyl]-N, N, N-trimethylammoniummethylsulfate (DOTAP) into the marginal veins (*sinus terminalis*) of chick embryos at stages 10–15 and transferred the exogenous DNA into PGCs circulating in the blood vessels. Chickens have been widely used for the study of avian transgenesis, but the quail may serve as better experimental animal

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for the transgenesis because of its small body size, fast growth rate and short generation interval.

In the present study, liposomes consisting of plasmid DNA (pMiwZ, containing *lacZ* as a reporter [15]) and Lipofectin were injected into the marginal veins of quail embryos at stages 12–15. The *lacZ* expression was then histochemically detected in PGCs settled in the gonads at later developmental stages, indicating the incorporation and the expression of the introduced DNA in PGCs.

Materials and Methods

Animals: Fertile quail eggs of the wild-type plumage strain maintained in our laboratory were used [4]. Eggs were collected daily and kept at 12°C for not more than 3 days. The eggs were then incubated in a commercial incubator at 37.5°C and 70% relative humidity with tilting to a 90 degree angle at 30 min intervals until the embryos reached stages 12–15.

Preparation of micropipettes: The micropipettes were made of siliconized glass capillary tubing (G-1, Narishige, Tokyo) drawn out with a micropipette puller (PB-7, Narishige) and the tips were beveled with the aid of a pipette grinder (EG-4, Narishige) at 20 degrees to an outer diameter of approximately 30 μm .

Gene introduction: The plasmid construct pMiwZ was used for gene introduction. The construct contains *Escherichia coli* β -galactosidase gene (*lacZ*) under the control of Rous sarcoma virus (RSV) enhancer and tandem promoters of RSV and chicken β -actin, and was prepared as a circular form. 15.5 μg of pMiwZ (1 $\mu\text{g}/\mu\text{l}$), 10 μl of cationic liposomes (Lipofectin, BRL, Gaithersburg, MD) and 14.5 μl of distilled water were mixed together, and incubated for 15 min at room temperature. A 2 μl aliquot was then injected into the marginal vein of each embryo at stages 12–15 (43–46 hr of incubation) with a micropipette under the dissection microscope.

Embryo culture: The treated embryos were cultured *in vitro* according to System Q3 described by Ono *et al.* [13]. Briefly, the embryos with egg yolk and albumen were transferred into the bed shells prepared from the sharp end halves of small chicken eggs. The bed shells were sealed with cling films with chicken thin albumen as a glue. The embryos were cultured at 37.5°C and 70% relative humidity with tilting to a 30

degree angle at 30 min intervals.

Detection of gene expression: The stage of development of treated embryos cultured *in vitro* was roughly estimated under the dissecting microscope without killing them. They were removed from the yolk at stages 24–25, 28–29 and 31–32 which corresponds to 4, 5 and 6 days after incubation including *in vitro* culture, respectively. They were then washed in Ca^{2+} and Mg^{2+} free Dulbecco's phosphate-buffered saline (PBS (-)) and fixed in 1% glutaraldehyde in PBS (-) for 15 min. After washing three times with PBS (-) the specimens were stained by incubation for 1 hr at 37°C in PBS (-) containing 0.05% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, Wako, Tokyo), 1mM magnesium chloride, 0.1% Triton X-100, 3mM potassium ferrocyanide and 3mM potassium ferricyanide [11]. The stained embryos were observed under the dissection microscope. Embryonic gonads positive for bacterial β -galactosidase activity were dehydrated in ethanol series, embedded in paraffin, serially sectioned at 8 μm and counterstained with hematoxylin and eosin.

Results

The *lacZ* was expressed in the PGCs of embryonic gonads at stages 12–15 as detected by X-gal staining (Table 1). When *lacZ* expression was observed at stages 24–25, all the treated embryos were surviving (18/18) and 8 of them (44.4%) exhibited the gene expression in gonads (Fig. 1A). The *lacZ* positive blue cells in the gonads were identified as PGCs on paraffin sections by virtue of their size, shape and large nuclei (Fig. 1B). With the histochemical observation of serial sections of gonads, the population of *lacZ* positive PGCs in the pair of gonads of each embryo was 1, 1, 3, 4, 5, 7, 14 and 16, respectively (Table 1). At stages 28–29, 16 out of 18 treated embryos were surviving and 2 out of 16 surviving embryos (12.5%) exhibited the gene expression in gonads. The population of the positive PGCs in them was 2 and 4, respectively. By contrast, 20 out of 22 treated embryos were surviving at stages 31–32 but none of them showed any positive populations of PGCs in gonads.

Discussion

The liposome-mediated DNA transfer into avian

Table 1. *LacZ* expression in PGCs of quail embryonic gonads after introduction of liposomes consisting of pMiwZ and Lipofectin

Stages of embryos observed	No. of embryos treated ¹⁾	No. of surviving embryos (%)	No. (%) ²⁾ of embryos showing <i>lacZ</i> expression in gonad ³⁾	No. of <i>lacZ</i> positive PGCs counted in each embryo showing <i>lacZ</i> expression in gonad ⁴⁾
24–25	18	18 (100.0%)	8 (44.4%)	1, 1, 3, 4, 5, 7, 14, 16
28–29	18	16 (88.9%)	2 (12.5%)	2, 4
31–32	22	20 (90.9%)	0 (0.0%)	

¹⁾A 2 μ l aliquot of liposomes containing 0.775 μ g pMiwZ and 0.5 μ l Lipofectin was injected into the marginal vein of the quail embryos at stages 12–15. ²⁾% of embryos surviving. ³⁾*LacZ* expression was detected histochemically with X-gal staining and observed under the dissection microscope. ⁴⁾Embryonic gonads with *lacZ* positive cells were serially sectioned and numbers of PGCs were counted under the microscope.

Fig. 1. Detection of *lacZ* expression in the gonadal region of a transgenic quail embryo. Embryos were injected with liposomes consisting pMiwZ and Lipofectin between stages 12–15 and stained with X-gal for bacterial β -gal activity, as described in Materials and Methods. A: ventral view of an embryo at stage 24. Blue cells represent the site of gene expression and arrows indicate that in the gonad (G) on the median surface of the mesonephros (M). B: a section (8 μ m) of the gonad of an embryo at stage 23 counterstained with hematoxylin and eosin. Arrows indicate the PGCs positive for *lacZ* expression in the gonad (G). scale bar=20 μ m.

PGCs was first demonstrated by Watanabe *et al.* [18] with chick embryos. They observed up to 23 positive PGCs at stages 24–27 (2–3 days after the injection) with pAcZ driven by chicken β -actin promoter and DOTAP. When pRSVZ driven by RSV promoter was substituted for pAcZ, up to 3 positive PGCs were ob-

served in the gonads at stages 24–27. In the present study for *in vivo* transfection of quail PGCs we used pMiwZ driven by RSV and chicken β -actin tandem promoters, and Lipofectin. We then observed up to 16 positive PGCs in quail embryonic gonads at stages 24–25. These differences in the expression of *LacZ* in

gonadal PGCs may be due to differences in the promoters of DNA used and/or the species of experimental animals (chicken and quail). By contrast, when the quail embryos were observed at stages 28–29, fewer embryos showed *lacZ* expression in the gonads, and, furthermore, no embryos at stages 31–32 showed the gene expression in PGCs in the gonads. Decreased gene expression as a function of embryonic development may indicate that the gene is not integrated into the host genome but it is a transient expression. The gonads of chick embryos at stages 22–27 contain hundreds to over one thousand PGCs [16]. It should therefore be noted that the gene expression observed here was limited to subpopulations of PGCs in the embryonic gonad, indicating the need for further improvement in the method used in genome integration, including a search for more effective promoters, cationic liposomes and other substances.

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