Liposome-Mediated Electric Gene Delivery into Fetal and Adult Gonads

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Liposome을 매개로 한 태아 및 웅성 생식선으로의 전기적 유전자 도입

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ABSTRACT

Gene delivery is one of the keen interests in animal industry as well as research on gene functions. Some of the *in vivo* gene delivery techniques have been successively used in various tissues for the gene therapy and transgenesis. Despite intensive efforts, it still remains to overcome problems of limited local and regional administration and low transgene expression. To improve the efficiency of gene delivery, a new procedure was tested. We injected exogenous DNA containing LacZ into the female or male gonads and then pulsed electric field. Electroporated gonads showed positive X-gal staining in many seminiferous tubules of the porcine fetal gonads. Exogenously introduced LacZ genes were also expressed in female porcine gonad. In addition, we demonstrated efficient gene delivery in gonad of adult mouse. Furthermore, we succeed to generate genetically modified germline cells showing GFP and positive X-gal signals. The results suggest that the newly developed gene delivery is an effective way of *in vivo* transfection in mammals. The developed gene delivery procedure should be useful in producing transgenic animals when combined with primary cell culture and nuclear transplantation.

(Key words : Gene delivery, Electroporation, Gonads)

I. INTRODUCTION

Gene delivery is one of the keen interests in animal industry as well as research on gene function *in vivo*. Some of the developed *in vivo* gene delivery methods have been successively used in various tissues including cancerous, germline and other target tissues for the gene therapy and transgenesis (Verma and Somia, 1997). Despite many efforts, some of the techniques employ highly-trained person and expensive equipments to deliver genes into a target tissue as in testicular gene delivery (Momose et al., 1999). Two major transfection systems have been being used to achieve varying results, namely, viral systems and non-viral systems. Retrovirus, adenovirus and adeno-associated virus are some of the viral systems are being used. The efficiency of the gene transfer by viral systems is high, however, harmful antigenicity or risks of acquiring novel infectious particles result in causing side-effects that lead to insertional mutagenesis (Kootstra and Verma, 2003; Liu and Huang, 2002). In contrast, non-viral systems may be safer and easier for obtaining target gene to use in gene delivery (Hara et al., 1999). The only drawback in the non-viral systems is low efficiency of gene transfer (Clark et al., 1999). Therefore,

* This work was supported by grant No. R11-2002-100-00000-0 from ERC program of the Korea Science & Engineering Foundation.
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many attempts have been made to increase the efficiency of the non-viral systems. Among them, cationic liposomes and polycations have widely been used (Matsuura et al., 2003).

Liposome-mediated delivery of plasmid DNA has recently been used in various fields of bioscience and medicine for curing disease with reduced morbidity and mortality. Several modes for gene delivery into mammals has also been applied for rescuing genetic abnormality using uterine injection or intravenous injection of naked DNA into fetus, yolk sac vessel and some tissues of adult mouse such as liver, brain, muscle and tails (Herweijer and Wolff,2003; Inoue and Krumlauf, 2001; Kikuchi et al., 2000). To overcome low and variable levels of gene expression, delivering brief electric field impulses to cells or tissue were introduced successfully even in embryonic tissues (Eide et al., 2000).

In the present study, we tried to improve the efficiency of gene delivery combining liposome and electric field impulse in gonadal tissues or their primary cells of porcine fetus and adult mouse. The results showed that the newly-developed gene delivery is an effective way of transfection in gonadal tissues. The gene delivery should be useful in producing transgenic animals when combined with primary cell culture and nuclear transplantation.

Ⅱ. MATERIALS AND METHODS

1. Preparation of plasmid DNA and DNA/ liposome complexes

A dual reporter gene encoding the green fluorescent protein (GFP) fused with beta-galactosidase was amplified in *E. coli* DH5 (GibcoBRL, Grand Island, NY, USA) and purified using a Qiagen Endofree Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). The DNA and liposome (Lipofectin Reagent, GibcoBRL) were mixed (1:1, v/v), stirred gently and left for 20 min at room temperature prior to injection *in vivo.*

2. Animals and *in vivo* gene transfer

Gonads were isolated from both E55 embryos of pregnant pigs showing 11cm of crown-rump length and C57Bl/6 mice at 8 weeks of age. To transfer the gene into the target tissues, liposome complexes (50 g plasmid DNA) were injected with a Hamilton hypodermic needle into the testis or the ovaries. After gene introduction, a few drops of PBS were added to the tissues prevent from being dried in an atmosphere. Tungsten microelectrodes were sharpened in potassium hydroxide (Sigma Chemical Co., St. Louis, MO, USA) and set on a micromanipulator. Electrodes were put into the sides of target tissues at a distance of 2 mm apart from the injected site. Then, pulse (20 V, 3 sec) was charged three times, which were generated by Hoefer DC Power supply (PS-3000).

Table 1. Estimation of fetal age using Crown-rump length

Approximate fetal age	Crown-rump length (cm)
20	0.9
30	2.5
40	4.8
50	8.2
60	11.9
70	15.8
80	17.6
90	19.4
100	22.6
110	23.9

(From Robinson, 1973).

3. Culture and gene transfection

Male or female gonads were washed with PBS and then trypsinized with 0.025% (v/v) trypsin and 1 mM EDTA (Sigma). Following incubation at 37℃ for 10min, the cells were dispersed into single cells and cultured in DMEM with 10% FBS and 10g/l ITS (insulin, transferrin, selenious acid), 10ng/ml hFSH, 10^{-8} M estradiol benzoate and 25 ng/ml bFGF at 37℃ in 5% CO_2. For transient transfection, pGFP-LacZ were introduced by a calcium phosphate method (Chen and Okayama, 1987). For tissue culture, gene-transferred tissues were cultured on a nitrocellulose membrane on the culture medium for 48 h.

4. X-gal staining of transfected cells or tissues

The transfected cells or tissues were briefly washed with PBS (pH 7.4) and fixed with 1% formaldehyde, 0.2% glutaraldehyde

and 0.02% Nonidet P 40 (Sigma) in PBS at room temperature for 3 h. Cells or tissues were then washed with PBS and transferred to a solution containing 1mg/ml X-gal, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide (Sigma) in PBS, and incubated at 37℃ for 30 min to allow reaction with expressed beta-galactosidase. After staining, the cells were incubated in 4 g/ml of DAPI for 15 min for nucleus staining. To discriminate nucleus or cytoplasm of transfected gonad, the X-gal-developed tissues were cut in a microtome, mounted on glass slide and dried at 65℃. The sections (20 μm) were incubated with 1 mg/ml eosin and 6 ng/ml hematoxylin for 1 and 15 min, respectively.

Ⅲ. RESULTS

1. Isolation and Characterization of Gonadal Sex from Porcine Fetuses

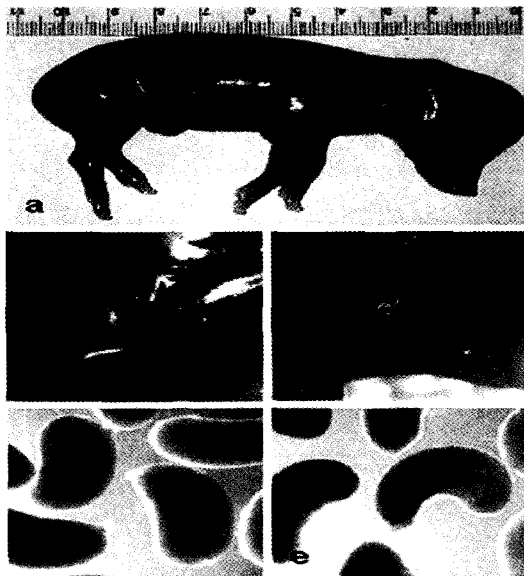

Fig. 1. Isolated male and female gonad from E55 porcine fetus. Porcine fetuses at E55 have 11cm crown-rump length (a). The sexes are classified by the appearance of the external genitalia in individual fetus. Male (b) and female (c) external genitalia and gonads were shown just before isolating primary PGCs. Sexed gonads into male or female by a morphological standard. Male gonads showed oval shape and had no turning (d) and female gonads showed sunken kidney bean-like shape (e).

It was important to differentiate sexes in early fetuses. By comparing external genitalia and subsequently dissecting out the fetal gonads, we were able to tell the exact sex of the individual fetus. This procedure allowed us to culture the gonad cells separately depending on the sexes. Fetal gonads used were about 55 days of pregnancy (E55) (Robinsin, 1973). They were about 11cm in crown-rump length (Fig. 1a). The isolated gonads were divided into two groups depending on the fetal sexes. Male gonads showed oval shape and had no turning (Fig. 1b) and female gonads had sunken kidney bean-like shape (Fig. 1c). These morphology of the gonads were verified by examining appearance of the external sexes of the fetuses.

2. Expression of Transgene Introduced into the Gonads

To examine whether we could successfully transfer transgenes into the gonads by gene transfer, we injected 50 g of DNA solution containing LacZ into the female or male gonads. After gene introduction, electric field impulses were generated three pulses at 20 V for 3 sec. Electroporated gonads were cultured and analyzed by X-gal staining to examine the efficiency of the gene expression. Many seminiferous tubules of the fetal gonads showed strong X-gal staining (Fig. 2a and c).

To examine the distribution of the expressed gene more closely, the stained tissues were cut in a microtome and stained with hematoxylin and eosin for 15 min. Positive signals of X-gal staining was detected inside of tubules and cytoplasm of cells consisting the goand (Fig. 2b and d).

Exogenously introduced LacZ genes were also expressed in female porcine gonad. In the peripheral region of the grove that will later be connected to oviduct, many round shape cells were strongly stained (Fig. 3a and c). In the sections, strong positive signals were detected not only the supporting cells (arrow) but also follicle and the oocytes (Fig. 3b and d).

We also investigate whether transgene delivery was possible in gonad of adult mouse. Testis of 8-weekold mouse was isolated and then pGFP-LacZ injected into the target sites and then electroporated at 20 V, 3 sec, three times. The transfected testis showed positive X-gal positive signals along the tubules (Fig. 5a and b). Not only the region between seminiferous tubules, but also the inside of seminiferous tubules (arrowhead) showed clear lacZ staining.

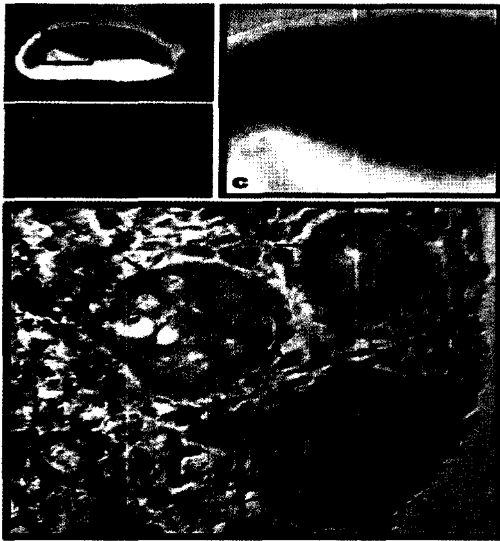

Fig. 2. Efficient transfection of LacZ gene in male goand by liposome mediated electroporation. Electroporated gonads were fixed and analyzed by X-gal staining. Many tubules of male gonad destined to develop for seminiferous tubules showed strong X-gal staining (a, c). Inside of tubules and cytoplasm of cells consisting the goand had strong positive lacZ staining (b, d).

Fig. 3. LacZ gene expression in gene transferred female gonad. Exogenously introduced LacZ genes were expressed in female porcine gonad. In the peripheral region, a number of round shape cells were strongly stained (arrow) (a, c). Strong positive signals were detected in supporting cells (arrow), follicle and the oocytes (arrowhead) in sectioned tissues (b, d).

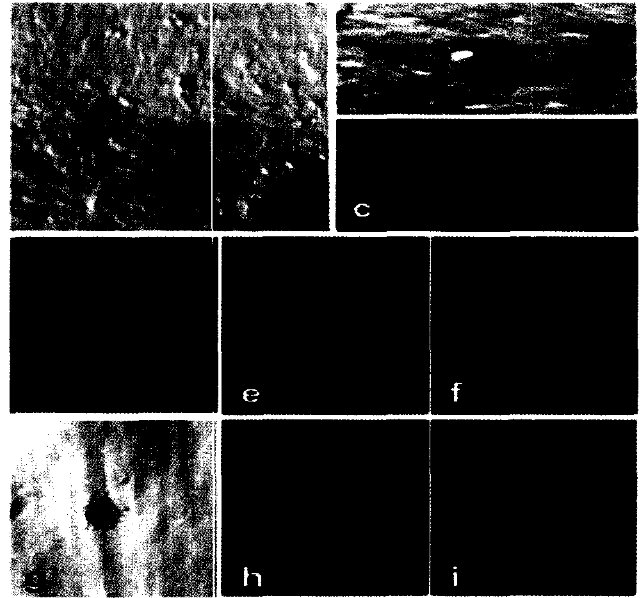

Fig. 4. Dual expression of GFP-LacZ in spermatogonic cells. Primary spermatogonic cells were transfected with pGFP-LacZ, a dual reporter gene. After 48 h, GFP peptides in the cytoplasm were analyzed by GFP-optimized filter sets without fixing step (c, e and h). After visualization of GFP, the cells were fixed and stained with X-gal. Some of the transfected cells showed positive X-gal signals (a, b, d and g) and also showed colocalized GFP expression in the cytoplasm (c, e and h).

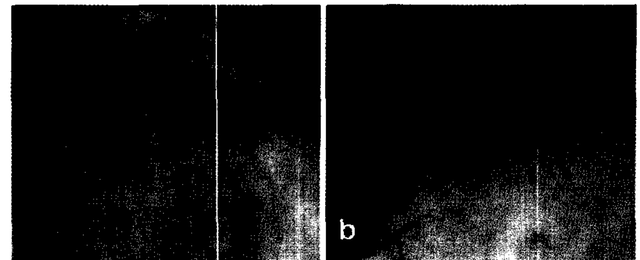

Fig. 5. Expression of LacZ gene in testis of adult mouse after gene transfer. The transfected testis showed positive X-gal positive signals along the tubules (a and b). Not only the region between seminiferous tubules, but also the inside of seminiferous tubules (arrowhead) showed clear lacZ staining.

3. *In vitro* Gene Expression in Porcine Primary Gonad Cells

To generate gene modified germline cells, we investigate whether the exogenous gene could be transfected into the

spermatogonic cells, we transfected GFP-LacZ, a dual reporter gene into the cells. After 48 h, some of the transfected cells showed positive X-gal signals (Fig. 4a, b, d and g) and also showed colocalized GFP expression in the cytoplasm (Fig. 4c, e and h).

Ⅳ. DISCUSSION

It has been recently reported that the use of DNA injection or electroporation can introduce DNA into cells of chicken embryos *in ovo* or mouse embryos *in vivo* (Muramatsu et al., 1998; Ogura et al., 2002). However there are still difficulties in an accuracy of expression at target sites in these methods. Moreover, the viability of the electroporated embryos is low due to the toxicity of the electric current on the embryos (Momose et al., 1999). To take advantage of fetal gonads for gene delivery and subsequent use for *in vitro* culture for later use of somatic nuclear transplantation. We tried to inject an exogenous gene into the gonadal tissues of fetus or adult.

Although liposome-mediated methods have been used extensively to introduce DNA into cultured cells, trials to transfer into whole embryos or tissues have often yield low expression (Dickinson et al., 2002). To increase efficiency of gene transfection using liposome, we applied electroporation additionally. In this method, electric treatment would increase binding of liposomes to cells according to previous study (Chernomordik et al., 1991). By using a dual reporter, GFP-LacZ gene that are driven by CMV, a ubiquitous promoter that is constitutively active in various types of cells, such as neuron, spermatogonic cells and CHO cells, it would be possible to select GFP-expressing cells in culture and subsequently to be cloned by single cell isolation. Such transfected-germline cell nucleus could be used in making transgenic animals by somatic cell nuclear transplantation.

In this study, we tried to establish an efficient technique for gene transfer in the gonads of fetus and adult. The results presented here showed the exogenous genes were expressed inside of male gonad seminiferous tubules and cytoplasm of cells consisting goands. In female gonad, gene was expressed in supporting cells, follicle and the oocytes. The GFP and LacZ expressions in transfected primary spermatogonic cells also suggest that spermatogonic cells could be transformed by gene transfection. The cytoplasmic expression may be the result from no signal peptides of transgene. Localization of transgene in seminiferous tubules and the oocytes suggests that the gene transfer procedure used in this study was sufficient to transfect exogenous gene into germ cells. These results provide a possibility in solving difficulties of generation of transgenic animals such as low viability, low production and high cost. In near future, improved techniques in gene delivery should be applied for the purpose of overcoming low efficiency, and application to other tissues should also be necessary. We consider that liposome-mediated gene electroporation, when combined with nuclear transplantation technique, would be a powerful addition to current gene delivery techniques in generation of transgenic animals and clinical treatment for disease.

Ⅴ. REFERENCES

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(Received 12 February 2004 / Accepted 15 March 2004)