◀Research Note▶

Effect of Electrode Size on Transfection Efficiency of Chicken Stage X Blastoderm by *in vivo* Electroporation

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In order to improve transfection efficiency of the stage X blastoderm of chickens by *in vivo* electroporation, the effect of the size of the upper and lower electrodes was examined. The plasmid DNA (pbAEGFP) was injected into the stage X blastoderm followed by applying electric pulses vertically to the blastoderm layer. The manipulated embryos were incubated using an embryo culture system, and the GFP gene expression was then examined. By decreasing the size of the upper electrode or by increasing the size of the lower electrode, the effect of the electric pulses was clearly enhanced. Although the introduced GFP gene was efficiently expressed in the embryonic and extra-embryonic tissues, the expression in the embryos was transient. No integration of the GFP gene into the chromosomes of germline cells of chickens was detected.

Key words : blastoderm, electrode, electroporation, GFP gene

Introduction

Introduction of exogenous DNA into early chicken embryos is very useful for studying the functions of genes related to embryogenesis, morphogenesis or sexual differentiation, even though they are only expressed in the embryos transiently. Recent development of *in vivo* electroporation methods have made it possible to transfect stage X blastoderm (Naito *et al.*, 2000, 2002). The introduced DNA expressed efficiently in the early stage embryos and distributed various tissues and organs including gonads (Sano *et al.*, 2003). When electroporation was carried out to the stage X blastoderm *in vivo*, a high rate of gene expression in the embryonic tissues was achieved by applying electric pulses vertically rather than horizontally to the blastoderm layer (Naito *et al.*, 2002). In the stage X blastoderm, the marginal area opaca will contribute to extra-embryonic structures, and the central area pellucida will give rise to the embryo proper and some extra-embryonic tissues (Petitte *et al.*, 1999 ; Petitte, 2002). By applying electric pulses vertically to the blastoderm layer, the exogenous DNA could be efficiently introduced into the central part of the blastoderm. As a result, a high rate of embryonic tissue expression of the exogenous DNA was achieved (Naito *et al.*, 2002).

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In the present study, the effect of the size of the upper and lower electrodes was examined to improve the transfection efficiency of the stage X blastoderm by *in vivo* electroporation when the electric pulses were applied vertically to the blastoderm layer. The possibility of transmitting the introduced DNA into the germline of chickens was also examined.

Materials and Methods

Embryo manipulation and detection of gene expression

Embryo manipulation and *in vivo* electroporation techniques were described by Naito *et al.* (2002). The procedure may be briefly described as follows. Unincubated fertilised eggs were broken, and the thick albumen capsule was removed from the yolk. Plasmid DNA (pbAEGFP, GFP gene under the control of the chicken β -actin gene promoter) was injected into the blastoderm ($0.25 \mu\text{g}/\text{egg}$). The manipulated embryo (yolk) was then transferred into a vessel in which the lower electrode was attached to the bottom, and the blastoderm was placed on top of the yolk by rotation. The upper electrode (stick type) was placed on the yolk membrane in the centre of the blastoderm. The diameter of the upper electrode was 1 or 2 mm, against 2 or 4 cm in the lower one. Electric square pulses were applied 5 times at 5–20 V for loading periods of 50 msec at one-second intervals. The manipulated embryos were cultured using an embryo culture system II (Perry, 1988 ; Naito *et al.*, 1990) for 3 days, and the GFP gene expression was detected under a fluorescent microscope (MZFL-III, Leica Microsystems, Tokyo).

Presence of introduced DNA in germline of chickens

When the manipulated embryos were cultured until hatching, the linealised plasmid DNA was used. Diameters of the two electrodes employed were 1 mm (upper) and 2 cm (lower). The manipulated embryos were cultured until hatching using embryo culture systems II and III (Perry, 1988 ; Naito *et al.*, 1990). The hatched chicks were raised until maturity. Sperm samples were collected from the males, and DNA was extracted and analysed for the presence of the GFP gene by PCR (Sano *et al.*, 2003). Fertilised eggs were obtained from the females by artificial insemination, and the GFP gene expression was analysed in the 3-day incubated embryos.

Results

Viability of embryos and expression of GFP gene

Viabilities of the manipulated embryos and expression of the GFP gene in the embryonic and extra-embryonic tissues are shown in Table 1. With the 4-cm lower electrode, embryo viability was poor and no embryo developed to stage 18 with 15-V or 20-V electric pulses. Thus, the relative size of the upper and lower electrodes affected the viability of embryos. Expression of the GFP gene was detected in the embryonic and extra-embryonic tissues (Table 1). Embryonic tissue expression of the GFP gene was observed when 10-V or higher electric pulses were applied in all cases. Using the upper electrode of 1-mm diameter, the GFP gene expression was observed at a lower voltage such as 10-V electric pulses, but not when using the 2-mm electrode.

Presence of introduced DNA in sperm or eggs of hatched chickens

Following the electroporation of stage X blastoderm *in vivo*, a total of 47 chickens were hatched and 29 chickens grew to maturity (Table 2). The introduced DNA was not detected in the sperm samples of the male chickens, and the GFP gene expression was not detected in the embryonic samples of the female chickens.

Discussion

The results show that the transfection efficiency of the stage X blastoderm by *in vivo* electroporation depends on the size of the upper and lower electrodes. By decreasing the size of the upper electrode or by increasing the size of the lower electrode, the effect of the electric pulses tended to enhanced and the GFP gene was

Table 1. Expression of GFP gene in early chicken embryos following transfection of stage X blastoderms by *in vivo* electroporation

Electrodes			Number of blastoderms treated	Number (%) of blastoderms or embryos expressing GFP gene			Number (%) of embryos developed to stage 18 (H & H)	Expression of GFP gene in stage 18 embryos Number (%)		
Upper (ϕ)	Lower (ϕ)	Voltage		Day 1	Day 2	Day 3		Embryonic and extra-embryonic tissues	Extra-embryonic tissues only	No expression
2 mm	4 cm	10 V	6	6 (100.0)	4 (66.7)	3 (50.0)	3 (50.0)	0 (0.0)	3 (100.0)	0 (0.0)
		15 V	6	6 (100.0)	5 (83.3)	3 (50.0)	0 (0.0)	—	—	—
		20 V	6	6 (100.0)	5 (83.3)	5 (83.3)	0 (0.0)	—	—	—
2 mm	2 cm	10 V	6	6 (100.0)	3 (50.0)	4 (66.7)	4 (66.7)	0 (0.0)	3 (75.0)	1 (25.0)
		15 V	6	6 (100.0)	5 (83.3)	5 (83.3)	4 (66.7)	1 (25.0)	2 (50.0)	1 (25.0)
		20 V	6	6 (100.0)	4 (66.7)	4 (66.7)	4 (66.7)	2 (50.0)	1 (25.0)	1 (25.0)
1 mm	4 cm	5 V	9	6 (66.7)	3 (33.3)	0 (0.0)	8 (88.9)	0 (0.0)	0 (0.0)	8 (100.0)
		10 V	9	8 (88.9)	8 (88.9)	6 (66.7)	6 (66.7)	4 (66.7)	1 (6.8)	1 (6.8)
1 mm	2 cm	10 V	6	6 (100.0)	5 (83.3)	4 (66.7)	4 (66.7)	2 (50.0)	1 (25.0)	1 (25.0)
		15 V	6	6 (100.0)	6 (100.0)	6 (100.0)	3 (50.0)	1 (33.3)	2 (66.7)	0 (0.0)
		20 V	6	6 (100.0)	6 (100.0)	5 (83.3)	2 (33.3)	1 (50.0)	0 (0.0)	1 (50.0)

Table 2. Development and hatching of manipulated embryos following *in vivo* electroporation

Electric pulses*	Number of embryos manipulated	Embryos developed to stage 18	Chickens			
			Hatched (%)	Matured	Male	Female
15 V	107	71 (66.4%)	30 (28.0%)	17	6	11
20 V	108	49 (45.4%)	17 (15.7%)	12	7	5
Total	215	120 (55.8%)	47 (21.9%)	29	13	16

*Diameters of electrodes were 1 mm (upper) and 2 cm (lower).

Electric square pulses were applied 5 times for loading periods of 50 msec at one-second intervals.

efficiently expressed in the embryonic tissues even in the lower voltage of the electric pulses. Since the cells that give rise to the embryo proper locate in the central part of the area pellucida of stage X blastoderm (Petitte *et al.*, 1999 ; Petitte, 2002), it is considered that the GFP gene could be introduced in the cells located in the very central part of the area pellucida by using a smaller upper electrode (e.g., 1 mm), and embryonic tissue expression of the GFP gene could be enhanced. In fact, DNA can be introduced and expressed in a targeted site of a chicken embryo by using a micro-electrode (e.g., 0.3–0.5 mm in diameter ; Momose *et al.*, 1999). Thus, the effect of the electric pulses on the chicken embryos differs with the size of the upper and lower electrodes. Therefore, optimum size electrode and optimum voltage for electric pulses should be selected depending on the purpose of the experiment.

Primordial germ cells or their precursors reportedly distributed in the central part of the area pellucida, and on the ventral surface, of the stage X blastoderm (Karagenc *et al.*, 1996 ; Kagami *et al.*, 1997 ; Naito *et al.*, 2001). The application of electric pulses to the blastoderm layer from bottom to top is considered to be suitable for transfecting the primordial germ cells or their precursors *in vivo*. The expression of the introduced GFP gene was transient, however, and could not be detected in the germline cells of the adult chickens. Therefore, *in vivo* electroporation of stage X blastoderm can not be expected to integrate the introduced DNA into the chromosomes of the germline cells.

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