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Establishment of An Efficient and Stable Transgene Expression System in Chicken Primordial Germ Cells

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Chicken primordial germ cells (cPGCs) are founder germ cells in embryonic stage of development that eventually give rise to sperms or oocytes. Currently cPGCs are only known cells enabling germline transmission in chicken and their cultivation protocols were recently established. Although genome modifications of chickens are now theoretically possible using cPGCs, there are still several hurdles to overcome to practically use cPGCs as mediators for chicken transgenesis. First, efficiency of gene delivery into cPGCs remains low with current methods. Second, there aregene silencing mechanisms against the expression of foreign genes in cPGCs. In this study, we successfully increased the efficiency of gene delivery in cPGCs by taking advantage of the TTAA-specific *piggybac* transposon system. Moreover, a pipette-type electroporator significantly enhanced transfection efficiency up to 5-fold compared withcuvette-type methods. Taken together, the technological advances in our study will provide practical benefits for the application to fulfill genetic modifications of chicken genome.

Key Words : Chicken primordial germ cells (cPGCs), Transfection, *piggybac* transposon, Transgenesis

Introduction

Historically, the chicken egg was one of the first research materials in embryology. However, compared with recent progresses in mouse genetics, chicken became less attractive as a transgenic model system owing to the absence of standardized transgenesis protocol in avian species. Chicken embryonic stem cells (cESCs) were first recognized as a cell model for transgenesis.[1] They self-renew in culture and give rise to ectodermal, mesodermal and endodermal derivatives *in vivo* and *in vitro* at similar levels to mouse embryonic stem cells (mESCs). However, cESCs were shown to lack the ability to make germline contribution in contrast to mESCs.[2] Therefore, it has been suggested that subtle differences in developmental potency between mammals and avian species require different strategies for transgenesis.

cPGCs are the embryonic progenitor cells that eventually give rise to sperms or oocytes in adult.[3] In mouse, PGCs are specified at the beginning of gastrulation around embryonic day 7.25 when Blimp1 expression is induced by secreted factors from extra embryonic tissues.[4] In avian species, maternally inherited cytoplasmic components in the germ plasm determine germ cell fate.[5] In chicken, PGCs are initially localized to the central zone of the area pellucida in stage X embryos[5] and migrate to the germinal crescent at stage 4.[6] Later, between stage 10 and 12, they move into blood vessels, begin circulation in the blood stream and finally migrate to the genital ridge where they aggregate with somatic cells to form embryonic gonads.[7-9]

Recently, an *in vitro* culture method for cPGCs was developed and these embryonic germ cells were recognized as a new cell model capable of contributing to germline transmission.[10] However, at the same time, experimental evidence also suggested that there are two major issues to overcome for possible practical uses of the cells in chicken transgenesis.

First, active transcriptional silencing mechanisms against transgene expression appear to exist in PGCs.[11] It has been postulated that active transgene silencing is required in germ cells to prevent the occurrence of genetic modifications since any mutations in the cell may be transmitted to the next generation.[12] Therefore, in order to stably express exogenous genes in germ cells, one should use strategic approaches to avoid the silencing mechanisms. To this end, HS4 insulator elements in chicken β-globin were utilized in the plasmid DNA constructsto stably express green fluorescence protein (GFP) in cPGCs. In a subsequent study, increased frequency of integration was achieved when the ϕC31 integrase was used to insert transgenes into endogenous pseudo attP sites.[13] However, transfection efficiency remained low.

Piggybac, a DNA transposon from the cabbage looper moth *Trichoplusiani*, harbors 2472 bp transposons with 13 bp inverted terminal repeats (ITRs) and a 594 amino acid transposase.[14] It was reported that *piggybac* transposons recognize ITRs and efficiently insert them into the tetranucleotide TTAA sites.[15,16] In our study, *piggybac* transposon vector system was used for transgene expression in cPGCs. The results indicated that the transfection efficiency by *piggybac* transposon vector system was about five times more efficient than ϕC31 integrase vector system in cPGCs and the transgene expression was stably maintained even in the absence of insulator sequences. Although most gene delivery systems could insert genes into the genomes of target cells at reasonable rate, the efficiency of gene delivery significantly varies depending on transfection methods. Using *piggybac* transposon system, we compared the efficiency of gene delivery by using lipofection and two types of

electroporation in cPGCs. The results indicated that the efficiency was markedly enhanced by a pipette-type electroporator.

In summary, a stable transgene expression system and an efficient transfection method have been developed for chicken transgenesis using cPGCs and these technological progresses will facilitate biological studies and their possible uses in industrial applications.

Experimental Section

Derivation and Maintenance of cPGCs. In brief, 1-5ul of blood was taken from the vasculature of stage 14-17 (H&H) embryos and seeded in single wells of 48-well plates with mitotically inactivated Buffalo rat liver (BRL-3A) cells. In a week or so, PGCs became visible while other type of cells gradually degenerated from the culture. Throughout derivation and maintenance, cPGCs were grown in BRL-conditioned KO-DMEM (Invitrogen, USA) supplemented with following materials[10]: 7.5% fetal bovine serum (Hyclone, UT, USA), 2.5% chicken serum (Sigma-Aldrich, USA), 2 mM glutamax (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1x nucleosides (Millipore MA, USA), 1x non-essential amino acids (Invitrogen), 0.1 mM β-mercapto-ethanol (Invitrogen), 1x penstrep (Invitrogen), 6 ng/mL recombinant human SCF (R&D systems, MN, USA) and 4 ng/mL human recombinant basic FGF (Invitrogen).

Isolation and Culture of cESCs. To obtain cESCs lines, The area pellucida was isolated from stage X (EG&K) White Leghorn embryos as previouslydescribed.[17] The tissues were washed twice with PBS and dispersed using a 200 mL pipette into single cell suspension. The cESCs became visible in approximately one week after seeding the blasto-dermal cells. The cells were relatively small with a large nucleus and a pronounced nucleolus and grew in single layers as individual cells. Typically, the cells were grown to 80-100% confluency before they were passaged at 1:5 ratio. To maintain optimal growth and to prevent differentiation, we passaged the cells by transferring 30-50% of the old medium into the new well. Irradiated Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (iSTO) cells were required as feeders to support the growth of cESCs. The cells were dispersed using short incubation with trypsin (0.25%) for subculture. cESC medium contains all the components of cPGC medium but SCF, basic FGF and chicken serum as previously reported.[10]

Alkaline Phosphatase Staining. Cells were collected in a microfuge tube, media aspirated and fixed with 4% para-formaldehyde in PBS for 1-2 minutes. AP staining Kit (Millipore) was applied to the fixed cells as manufacturer's instruction and the samples were then incubated in dark at room temperature for 15 minutes. Next, the cells were rinsed with PBS-0.01% Tween 20 and covered with 1x PBS to prevent drying. Images of stained cells were captured using a Carl-Zeiss Axio Observer D1 microscope and an Axio-Cam camera.

Periodic Acid-Schiff Staining. In brief, cells were collected in a microfuge tube, media aspirated and fixed with 4% paraformaldehyde in PBS for 10 minutes. Cells were then washed three times with distilled water. Periodic acid solution (Sigma-Aldrich) was applied to stain the samples. Cells were then washed three times with PBS and Schiff's reagent was incubated with the samples for 15 minutes. Next, the cells were washed three times with distilled water. Images of stained cells were captured using a Carl-Zeiss Axio Observer D1 microscope and an AxioCam camera.

RNA Extraction and RT-PCR. For standard reverse transcription polymerase chain reaction (RT-PCR) procedures, total RNA was extracted using TRIzol reagent (MRC, London, UK). 1 mg of total RNA was reverse-transcribed into cDNA by using DNaseI Amplification Grade (Invitrogen) and Superscript II RNase H-Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instruction. List of primers used are indicated in Table 1.

Transfection of 293T Cells and cPGCs. For transfection using Lipofectamine (Invitrogen), the experiments were performed with either *piggybac* transposon system vectors (PB[Act-RFP (red fluorescence protein)] *piggybac* transposon vector and Act-PBase transposase helper vector[18]) or ϕC31 integrase system vectors (CMV-RFP-attB vector with multimerized HS4 insulators and CMV-ϕC31 helper vector) as directed by the manufacturer. The DNA-Lipofectamine mixture was removed from the cells after 4-5 hours of transfection and culture medium was then added to the cells. For electroporation using a cuvette-type electroporator, a total of 5×10^6 cPGCs were resuspended in 400 μL of cPGC medium to which 20 μg of *piggybac* transposon system vectors was added in a 0.2 cm cuvette. Wave pulses (250-350V, 100 us) were given by using Multiporator (Eppendorf, Hamburg, Germany) or ECM 830 electroporator (BTX, MA, USA). For transfection using a pipette-type electroporator, a total of 5×10^5 cPGCs were resuspended in 10 μL of Neon electroporation buffer (Invitrogen) to which 2 μg of

Table 1. List of chicken primers used

Gene	Forward primer	Reverse primer
Vasa	GCTCGATATGGGTTTTGGAT	TTCTCTTGGGTTCCATTCTGC
Dazl	GCTTGCATGCTTTTCCTGCT	TGCGTCACAAAGTTAGGCA
Nanog	CCTGGCCATGCCGTCCTACGGCTC	GGTGGTCTGCAGTAGGGCTAGTGG
Oct4	CCTGCTGCGAATGTGTAAATGGCT	GCCTGAGTGAAGCCCAGCATGATG
Blimp1	ACATGGAGGATGCTGACATGACCC	CGTATCGCTGGTATAGATCTCTCC
Actin	AACACCCCAGCCATGTATGTA	TTTCATTGTGCTAGGTGCCA

piggybac transposon system vectors was added in the electroporator tip. Wave pulses (850 V, 40 ms) were given by using Neon electroporator (Invitrogen). Fluorescence was detected using a Nikon Ti-u microscope, and images were captured using a Qimaging Qicam Fast 1394.

Results and Discussion

Isolation and Derivation of cPGCs. To establish cPGC lines, blood containingmigrating cPGCs was collected from dorsal aorta of day 3 embryos (Fig. 1(a)) and seeded on a layer of irradiated BRL-3A (iBRL) (Fig. 1(b)). cPGC medium contains chicken serum, fetal bovine serum, human recombinant SCF, and basic FGF and this culture condition allowed rapid proliferation of cPGCs while other types of cells gradually degenerated from the culture. From the attempts using total 154 embryos, 33 clones of cPGCs were initially isolated but finally 18 clones were successfully maintained. Derivation efficiency was about 23% for male cPGCs but female cPGCs didn't survive long in the culture as previous reported (Table 2).[10] In this culture condition, cPGCs have been successfully cultured for more than 1 year.

Molecular Characterization of cPGCsin Culture. To characterize cultured cPGCs, we first carried out AP and PAS stainings on cPGCs (Fig. 2). cPGCs were positive for both AP and PAS stainings. These results indicate that most cells are undifferentiated and inactively proliferating state. It has been known that PGCs express genes associated with both pluripotency and germline properties.[19] To analyze characteristic gene expression profile of cPGC lines established in this study, we checked the expression of the chicken homologues of Oct4, Nanog, Vasa and Dazl. Most cPGC clones expressed germ cell specific and pluripotency marker genes as expected (Fig. 3(a)). This expression pattern was further confirmed when the pattern was compared with those of gonads from chicken embryo H-H stage 41, which contain post migratory PGCs (Fig. 3(b)). Chicken embryonic fibroblasts (CEFs) were used as a negative control. Taken together, these results demonstrated that we successfully isolated and cultured functional cPGCs.

Transgene Expression in cPGCs. Next, we attempted to deliver transgenes into cPGCs. In an earlier study, to stably express a transgene in cPGCs, Van der Lavoir and colleagues

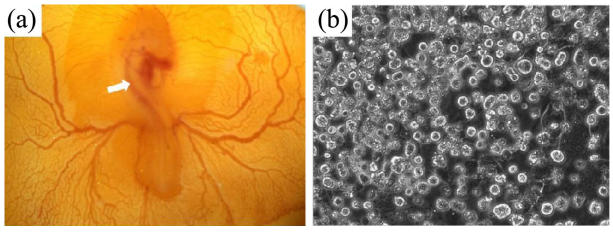

Figure 1. Morphology of H-H stage 14-17 embryo and cPGCs *in vitro*. (a) In these stages, cPGCs migrate along the blood stream from germinal crescent to gonad. Dorsal aorta is indicated by an arrow. (b) cPGCs grow floating over the feeder cells, inactivated BRL-3A by γ-irradiation. Magnification: (a) 10x, (b) 200x.

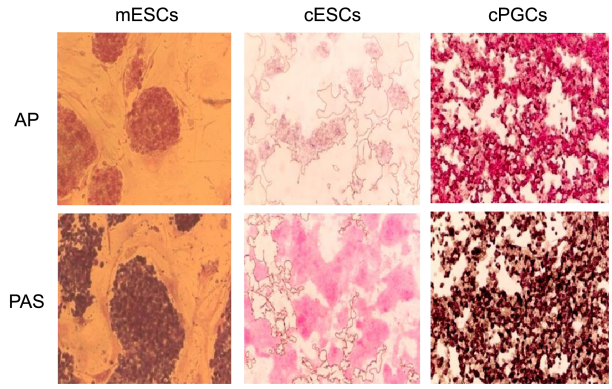

Figure 2. AP and PAS stainings of mESCs, cESCs and cPGCs. Like other pluripotent stem cells, cPGCs are positive both for AP and PAS. Magnification: mESCs 200x, cESCs, cPGCs 100x.

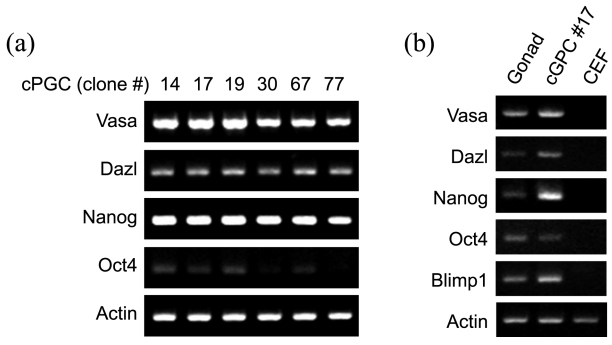

Figure 3. Gene expression profile of cPGCs. (a) Most cPGC clones exhibit a strong expression of Vasa, Dazl, Nanog and Oct4. (b) Comparing gene expression among chicken gonad from chicken embryo H-H stage 41, cPGC clone #17 and a negative control, CEFs.

Table 2. Derivation efficiency of cPGCs.cPGCs were isolated from the blood of H-H stage 14-17 White Leghorn embryos. Among 154 embryos used, 18 cPGC lines were successfully derived

		1st Exp.		2nd Exp.	
		Number of embryo	Number of derived cPGCs	Number of embryo	Number of derived cPGCs
Total (n)		108	6	46	12
Male	PGC (+)	17	6	12	12
	PGC (–)	35	0	16	0
Female	PGC (+)	4	0	0	0
	PGC (–)	52	0	18	0

Table 3. Comparison of gene delivery vector systems and transfection methods in cPGCs. The method in our study is in bold

Vector	Transfection	Promoter	Insulator
eGFP (*Nature*, 2006)[10]	Electroporation (cuvette-type)	*β*-actin	HS4
attB-eGFP+ϕc31 (*Mol. Repro. Dev.*, 2008)[13]		*β*-actin CAG	HS4
***piggybac*-RFP + transposase**	**Electroporation (pipette-type)**	**CAG**	**NONE**

used a linearized vector expressing GFP transgene flanked by multimerized HS4 insulators to avoid silencing effect.[10] In a subsequent study, Leighton *et al.* enhanced gene delivery efficiency using ϕC31 integrase vector system but still had to use HS4 insulators (Table 3).[20] In this study, we decided to use a *piggybac* transposon system. *Piggybac* transposon system has been widely used as an efficient gene delivery method in insects and mammals. Recently, it was also shown to function well for exogene expression in chicken spinal cord.[21] The expression system we used consists of *piggybac* transposon vector (PB[Act-RFP]) expressing RFP driven by β-actin promoter with a helper vector (Act-PBase) expressing transposase by β-actin promoter without using any insulator sequences. We first preformed transfection into 293T cells to compare gene delivery efficiency between ϕC31 integrase vector and *piggybac* transposon vector systems using lipofection in 293T cells. In this experiment, gene delivery efficiency of *piggybac* transgene expression system was about 40% higher than that of ϕC31 integrase vector system according to the percent of RFP positive cells (Fig. 4(a and d)). This result indicated that the frequency of transgene integration increased with *piggybac* transposon system. Next, we performed a transfection experiment for cPGCs with the same vector systems using lipofection. However, lipofection appeared to be toxic to cPGCs and thus most cells were not viable or started differentiation after the transfection (data not shown). Electroporation is commonly used for cells sensitive to traditional transfection methods such as lipofection and calcium phosphate. In fact, electroporation was the only known method to show reasonable transfection efficiency for cPGCs. Therefore, we carried out transfection for cPGCs using two different types of electroporation, a traditional cuvette-type and a noble pipette-type. The result showed that the pipette-type electroporation generated much higher gene delivery efficiency with about 5-7 fold increase in efficiency by both transgene expression systems and most cPGCs remained viable (Fig. 4(b-d)). Moreover, despite concern regarding transgene silencing effect, transfection efficiency by *piggybac* transposon vector was 5-fold higher than by ϕC31 integrasevectoreven in the absence of HS4 insulator sequences (Fig. 4(b-d)). These RFP expression was maintained about more than 3 months in transfected cPGCs, clearly indicating that transgene expression is stably maintained without being silenced (Fig. 4(e)). These results demonstrate that *piggybac*

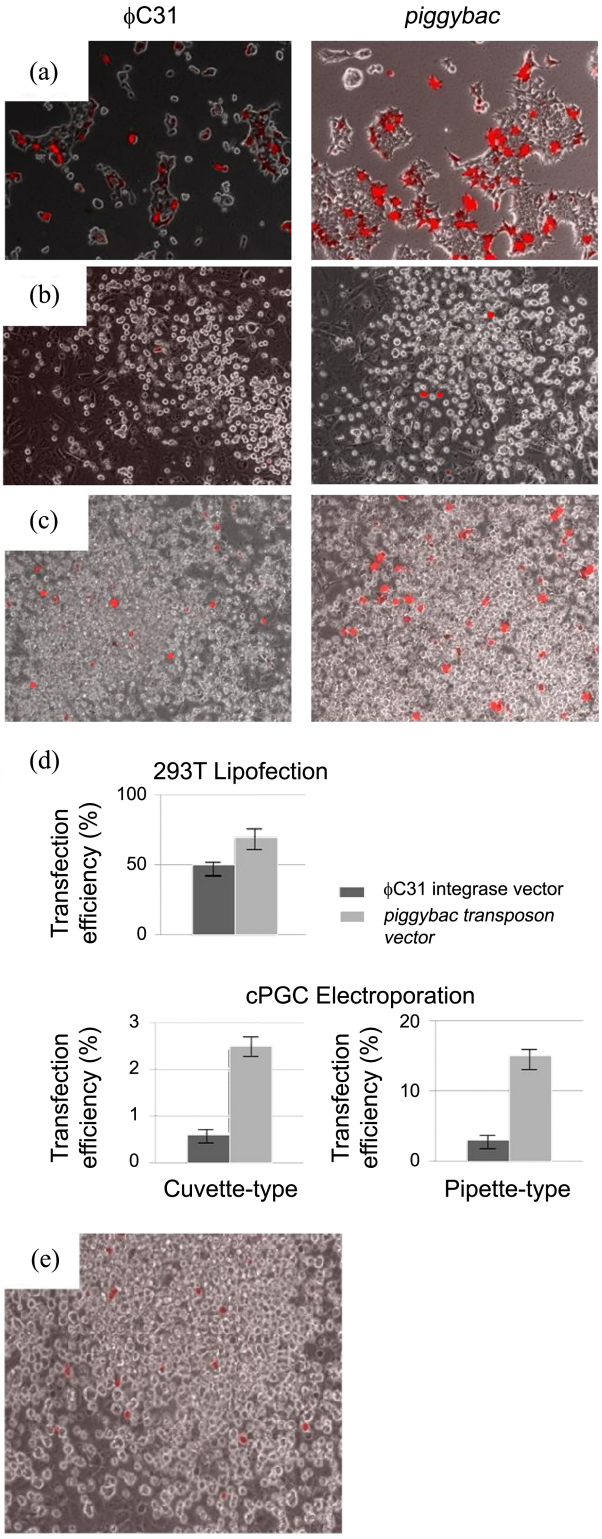

Figure 4. Transfection efficiency using different vector systems and delivery methods. Comparison of transfection efficiency between ϕC31 integrase vector and *piggybac* transposon vector systems 48 hrs postransfection (a) Images of cells expressing RFP in 293T cells by lipofection, (b) in cPGCs by a cuvette-type electroporation and (c) in cPGCs by a pipette-type electroporation. (d) Diagrammed transfection efficiency shown in (a-c). (e) A representative image of cPGCs expressing stable RFP by *piggybac* transposon vector. The RFP signal was maintained for more than 3 months. Magnification: (a), (b), (c) and (e) 100x.

transposon system with the pipette-type electroporator method efficiently delivered transgenes into the cPGCs and allowed stable expression by escaping transgene silencing mechanisms.

Conclusion

The establishment of *in vitro* cultures of avian PGCs offers a unique system for the study of early germ cell development as a comparative system of mammalian counterparts. Moreover, cPGCs are considered valuable tools for transgenic technology for industrial application as a promising bioreactor.[22] However, there are several obstacles to overcome to practically use cPGCs for transgenesis. In this study, to circumvent silencing mechanisms and to efficiently insert transgene into the genome of cPGCs, a *piggybac* transposon vector system was used. Transfection efficiency was markedly enhanced and the foreign gene was not silenced even without the use of HS4 insulators in cPGCs. Moreover, transfection using a noble pipette-type electroporator was significantly more efficient with more viable cells compared to traditional cuvette-type electroporation and a cationic lipid-mediated transfection. In summary, this study will provide unprecedented opportunities that chicken genome can be efficiently modified to accomplish germline transmission.

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