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Short Communication

DsRed gene expression by doxycycline in porcine fibroblasts and cloned embryos using transposon

SuJin Kim[1], JoonHo Moon[1], BegoRoibas da Torre[1], Islam M. Saadeldin[1], JungTaek Kang[1], JiYei Choi[1], SolJi Park[1], Byeong-Chun Lee[1] and Goo Jang[1,2]*

[1]Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Research Institute of Veterinary Science, Seoul National University, Seoul 151-742, Republic of Korea.
[2]Emergence Center for Food-Medicine Personalized Therapy System, Advanced Institutes of Convergence Technology, Seoul National University, Gyeonggi-do, Korea

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To develop a transgenic pig, introduction of foreign genes into fibroblasts is required. In this study, *Piggybac* transposition was used to produce tetracycline dependent gene expressing cloned embryos. Red fluorescence proteins (DsRed) combined with tetracycline promoter flanked transposon sequences were transfected into fetal fibroblasts, and the transfected cells were used as the donor for somatic cell nuclear transfer. Induction of DsRed expression was successfully controlled by doxycycline treatment in donor fibroblasts and early stage embryos. In conclusion, this study suggested that *Piggybac* transposition could deliver genes into cells or embryos for developing transgenic pig.

Key words: Miniature pigs, transfection, *Piggybac*, somatic cell nuclear transfer (SCNT), RFP.

INTRODUCTION

Pigs are one of the valuable animal models for human biomedical research, particularly, xeno-transplantation, because they have similar characteristics in the size of their organs and physiology (Aigner et al., 2010b; Ekser et al., 2011; 2012). Hence, for xeno-transplantation, transgenesis in pigs is considered as a prerequisite process for reducing or removing the immune rejection. Since the first transgenic pigs via microinjection were born (Hammer et al., 1985), several transgenic pigs have been produced by somatic cell nuclear transfer (SCNT). It involves a somatic donor cell injected into an enucleated oocyte, and then cloned embryos transferred into surrogate mothers (Aigner et al., 2010b). Many cloned transgenic pigs were derived from plasmid vector or viral infection system for knockout or over-expression of the target gene (Aigner et al., 2010a, b; Klymiuk et al., 2010). Since constitutive expressions of the foreign gene often make transgenic animals lethal, it is preferred that the target genes are expressed in a temporarily and spatially controlled manner.

To circumvent these problems, methods for inducible transgenic systems have been developed, including tetracycline inducible system that has been extensively used mostly in rodent animal models (Zhu et al., 2002; Sun et al., 2007).

Recently, a gene delivery system, *Piggybac* (*PB*) transposition was successfully applied to produce transgenic mice (Ding et al., 2005; Nakanishi et al., 2010). In those studies, *PB* transposition delivered genes into transgenic mice efficiently.

*Corresponding author. E-mail: snujang@snu.ac.kr. Tel: 82-2-880-1280. Fax: 82-2-873-1269.

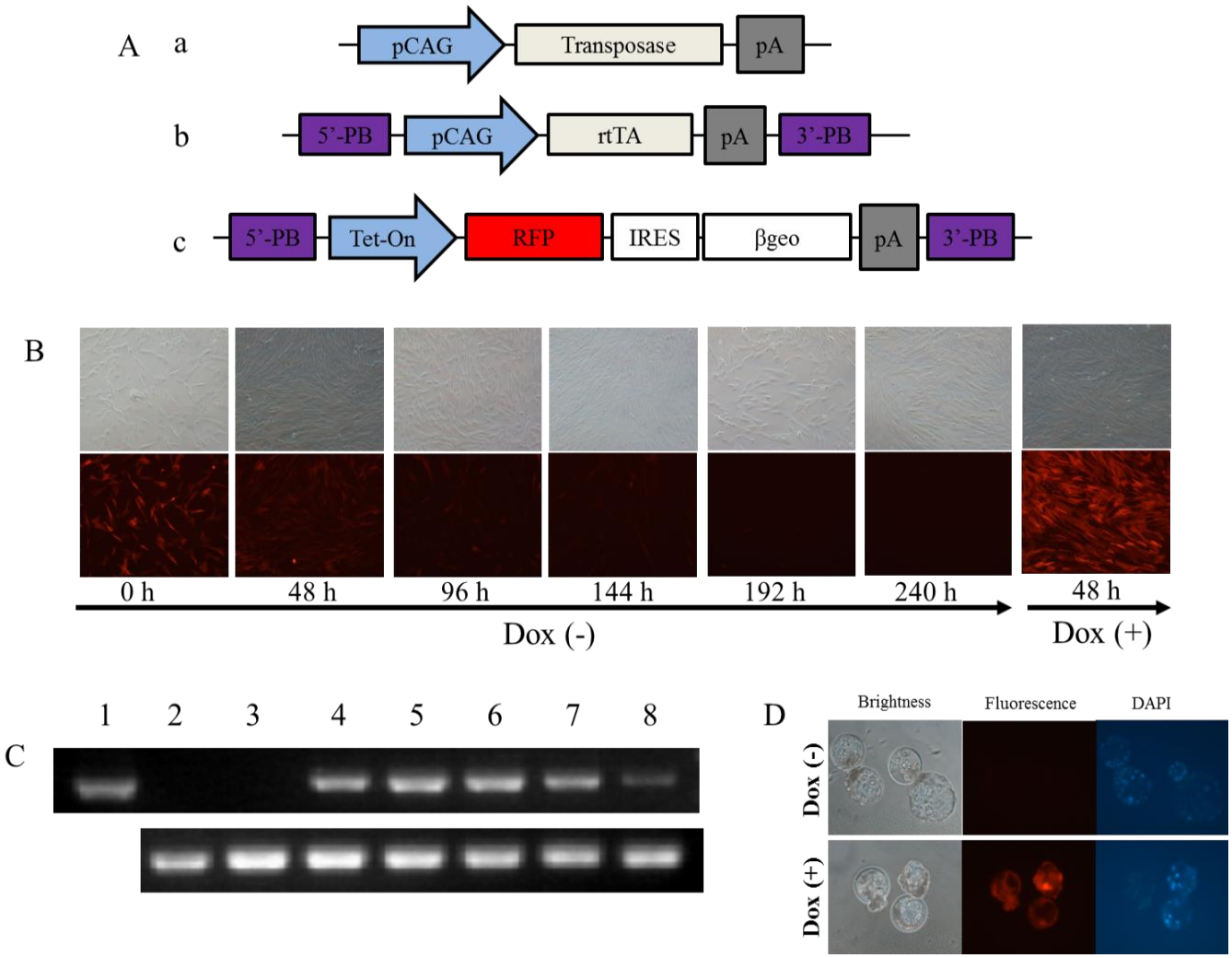

Figure 1. DNA map for inducible DsRed protein in somatic cells or embryos (A). Three plasmid DNA (A-a, -b and -c) transfected into fetal fibroblast and then treated with neomycin for removing non-transfected cells. After treating neomycin for 2 weeks, DsRed proteins in transfected cells were regulated with or without doxycycline (B, upper panel: under brightness, lower panel: under fluorescence). Expression level of mRNA of DsRed using RT-PCR was decreased without doxycycline (C, Lane 1: positive control, plasmid DNA, 2: non-transfected cells, 3: without doxycycline before turn on, 4: with doxycycline after 48 h, 5: with doxycycline after 96 h, 6: without doxycycline after 48 h, 7: without doxycycline after 96 h, 8: without doxycycline after 144 h, Upper panel is for RFP expression, band size (435 bps), lower panel is for GAPDH (252 bps). Transfected cells were reprogrammed in enucleated oocytes and developed into blastocysts. Blastocysts after addition of doxycycline expressed RFP in doxycycline addition group (D).

Accordingly, this study investigated the feasibility of tetracycline inducible gene expression using *PB* transposition to produce transfected cell line and transgenic embryos.

MATERIALS AND METHODS

Basic *PB* transposition system was provided by Addgene (Plasmid #20909, http://www.addgene.org). DsRed was amplified using PCR primer containing gateway sequence; forward: ggggacaagtttgtacaaaaaagcaggcttcACCATGGATAGCACTGAGAAC GTCAT, reverse: ggggaccactttgtacaagaaagctgggtcCTACTGGAACAGGTGGTGGC. DsRed PCR amplicons through BP and LR recombination (Invitrogen, Gaithersburg, USA) were inserted into the expression destination vector, PB-TET with a tetracycline inducible promoter from Addgene. Three plasmid genes (0.5 µg PB-TET-DsRed, 0.5 µg PB-CA-rtTA, from Addgene, plasmid #20910 and 1 µg Transposase) were transfected using Fugene HD (Promega, Wisconsin, USA). Fetal fibroblasts from a male miniature fetus were used for the transfection. Transfections protocol was based on manufacturer's instruction. Briefly, once growing cells were 50-60% confluent, transfection was carried out. Serum-free DMEM (Invitrogen) containing a ratio of one to three (DNA: transfection reagent) was added as a culture medium. For establishing a stable cell line, 800 µg/mL neomycin (G418, Gibco, Invitrogen) and 2 µg/mL doxycycline (Sigma-Aldrich,St Louis MO, USA) during two weeks were used after transfection for 48 h. After DsRed expressing cells were established, the cells were cultured for more 10 days without doxycycline in order to observe disappearance of DsRed protein under the

fluorescent microscope.

Thereafter, the DsRed expressing cells were used for SCNT (Park et al., 2010; Cho et al., 2011). Briefly, immature oocytes were cultured in TCM 199 medium supplemented with 10 ng/mL EGF, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 µg/mL insulin, 1% (v/v) Pen-Strep (Invitrogen), 0.5 µg/mL follicle stimulating hormone, 0.5 µg/mL luteinizing hormone and 10% porcine follicular fluid at 39°C in a humidified atmosphere of 5% CO_2 in air; this was done at first with gonadotropin-releasing hormone for 22 h and then without it for another 22 h. After the maturation of the oocytes, genetic materials of oocytes were removed under the nuclear staining and a DsRed expressing cell was micro-injected into enucleated oocytes. The reconstructed oocytes were fused and electrically activated as follows; the fusion medium was 0.26 M mannitol solution containing 0.5 mM Hepes, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$. Oocytes were activated with a single DC pulse of 1.5 kV/cm for 60 µs, utilizing a BTX electro-cell Manipulator 2001 (BTX, Inc., San Diego, USA). The activated embryos were cultured in PZM5 medium (IFP0410P, Funakoshi, Tokyo, Japan) for seven days.

RESULTS AND DISCUSSION

To make a stable DsRed expressing cell line, it took two weeks from initial transfection. Expression on or off of DsRed mRNA or protein using *PB* transposition in porcine fibroblasts worked well in the presence or absence of the doxycycline (Figure 1). With the transfected cells, 123 oocytes for SCNT were used. Developmental ratios of two-cell and blastocyst stage from an activated reconstructed oocytes were 58.5% (n = 72) and 11.4% (n = 14), respectively. When early blastocysts were cultured for two more days with doxycycline, the blastocysts expressed ubiquitously DsRed in all embryonic cells (Figure 1).

Technically, for delivering a foreign gene, the cDNA from the cells or tissues was cloned, sequenced and inserted into expression vector with circular form. Before the DNA was transfected with several transfection protocols including electroporation, the DNA was linearized more efficiently for transferring the gene into the host genome. However, without linearization, circular form, *PB*-DNAs are transposed through “cut-and-paste” mechanism, whereby transposase catalyzes the excision of the transposon from one chromosomal site and reinserted elsewhere in the genome. Recently, it was shown that *PB* efficiently transposes in human (Owens et al., 2012) and mouse cell lines. That could readily mediate the introduction of foreign genes (up to 14 kb) in the mouse germ line (Ding et al., 2005). While viral infection and simple plasmid transfections have been applied to generate cloned transgenic pigs (Hyun et al., 2003; Cho et al., 2011), the data from this study, transposon using *PB* could be an alternative way to produce transgenic pig models.

In summary, these results demonstrated that *PB* system combined with inducible gene expression by doxycycline can successfully deliver foreign genes (DsRed) into porcine fibroblasts and embryos.

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