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Short communication

Production of porcine cloned embryos derived from cells conditionally expressing an exogenous gene using Cre-*loxP*

JoonHo Moon[2], *SuJin Kim*[2], *HeeJung Park*[2], *JungTaek Kang*[2], *SolJi Park*[2], *OkJae Koo*[2], *Begona Roibas da Torre*[2], *Islam M. Saadeldin*[2], *ByeongChun Lee*[2] *and Goo Jang*[1]
Department of Theriogenology and Biotechnology, College of Veterinary Medicine and the Research Institute for Veterinary Science, Seoul National University, Seoul 151-742, Republic of Korea

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Summary

It is increasingly evident that conditional gene expression in pigs is necessary to make transgenic models. In this study, we investigated conditional expression in porcine fetal fibroblasts using Cre-*loxP* recombination, a system that has had limited application in large animals to date. Transformed fibroblasts were reprogrammed in enucleated oocytes to support further early embryonic development. Fetal fibroblasts from miniature pigs were used for transfection with a plasmid that contained a red fluorescent protein marker (pCALNL-DsRed) and a floxed neomycin-resistance gene. Cells were selected with 750 μg/ml neomycin for 2 weeks following transfection but did not express DsRed after visualization under a fluorescence microscope. Expression was achieved only after transient transfection with plasmid DNA that expressed the Cre recombinase enzyme. The cells that expressed DsRed were used for somatic cell nuclear transfer (SCNT). A total of 121 oocytes were used for SCNT and 76 cloned embryos (62.8%) were seen to have cleaved. Six blastocysts developed after SCNT and expressed DsRed. Deletion of the floxed neomycin-resistance gene was confirmed by reverse transcription polymerase chain reaction (RT-PCR) in cloned blastocysts. This study demonstrated that Cre-*loxP* recombination can be conducted successfully in miniature pig fibroblasts and that the sequentially transformed cells can develop to the pre-implantation embryo stage via SCNT.

Keywords: Cre-*loxP*, Porcine fibroblast, Somatic cell nuclear transfer, Transfection

Introduction

Cyclic recombinase (Cre)-*loxP* isolated from the bacteriophage P1 is one of the well known site-specific recombination systems. *loxP* is 34 bp in length, which includes an 8-bp asymmetric core region enclosed by two 13-bp inverted repeat regions (Anastassiadis *et al.*, 2009). The 38 kD Cre protein specifically recognizes *loxP* sites to cause excision, insertion, inversion and translocation (Branda & Dymecki, 2004; Nagy, 2000; Nagy *et al.*, 2009). Cre-*loxP* recombination was originally described in prokaryotes, but this system has been developed to delete *loxP*-flanked chromosomal DNA sequences at high efficiency in eukaryotes (Sauer, 1987). This system has been applied mainly in small animals such as mice (Brault *et al.*, 2007), and to date its application in large animals has been very limited.

Pigs are popular as human disease model animals because humans and pigs have many genetic similarities (Li *et al.*, 2009) and their organ sizes are similar to that of humans. This factor, and the fewer ethical considerations related to organ extraction, make this approach the best animal

[1]All correspondence to: Goo Jang. Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul, Republic of Korea, 151-742. Tel: +82 2 880 1280, Fax: +82 2 873 1269. e-mail: snujang@snu.ac.kr
[2]Department of Theriogenology and Biotechnology, College of Veterinary Medicine and the Research Institute for Veterinary Science, Seoul National University, Seoul 151-742, Republic of Korea.

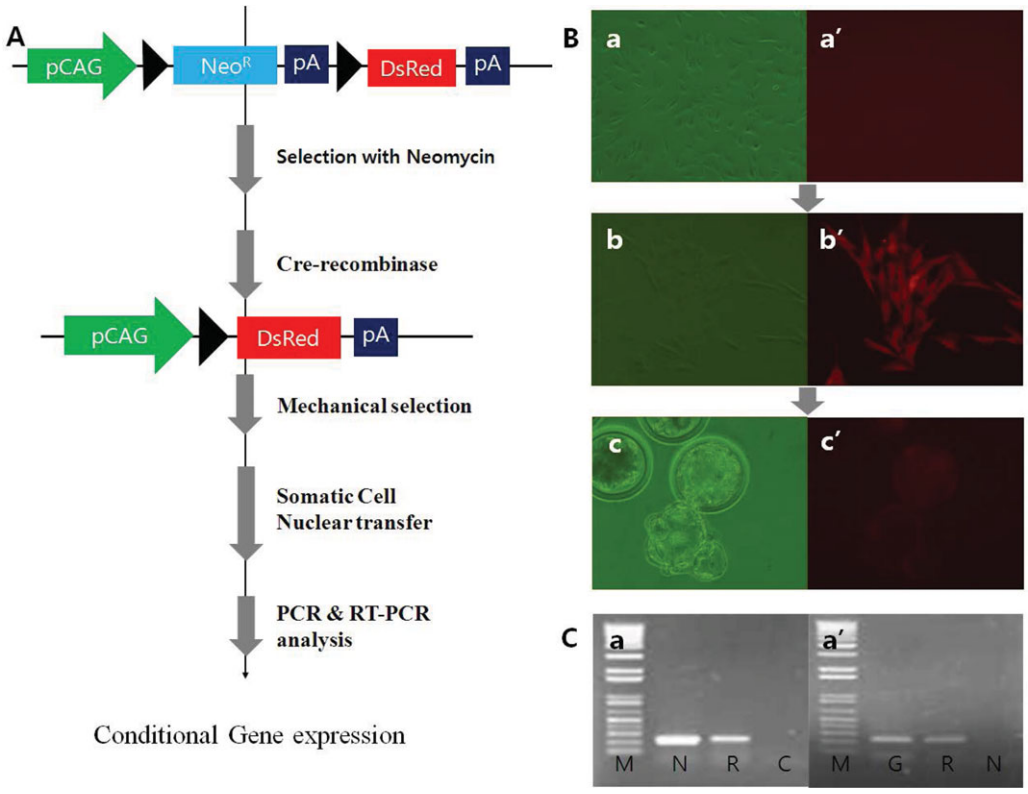

Figure 1 (*A*) Flow chart of experimental design. (*B*) Transfected donor fibroblasts and embryo. Cells (*B-a*) were selected with neomycin for around 2 weeks and did not express the red fluorescent protein (RFP) (*B-a′*). After transient Cre transfection (*B-b*), the cells expressed RFP due to deletion of the floxed neomycin-resistance gene and polyA sequences (*B-b′*). After mechanical selection, RFP-expressing cells (*B-b′*) developed into cloned blastocysts (*B-c*) and expressed RFP (*B-c′*). (*C*) Polymerase chain reaction (PCR), reverse transcription (RT)-PCR analysis. Gene insertion into cells and deletion in a blastocyst were confirmed by PCR (*C-a*) and RT-PCR (*C-a′*), respectively. M, DNA ladder; N, neomycin resistance gene (235 bp); R, DsRed (250 bp); G, glutaraldehyde phosphate 3-dehydrogenase (GAPDH) (252 bp); C, control. (See online for a colour version of this figure.)

option for xeno-transplantation (Ahn *et al.*, 2004). Removal or insertion of specific genes is required to alleviate immunological reaction and to establish xeno-transplantation. However, the process of removal or insertion of some genes may cause early embryonic lethality (Li *et al.*, 2009). The application of Cre-*loxP* recombination to these early embryonic lethal genes enables the production of live offspring (Li *et al.*, 2009). Furthermore, Cre-*loxP* recombination enables verification of specific gene functions. The aim of this study was to determine if the Cre-*loxP* system is applicable in porcine fibroblasts and if sequentially transfected cells can be used as donor cells for somatic cell nuclear transfer (SCNT).

Materials and methods

Cell culture, transfection and selection

Fetal fibroblasts from a miniature pig were used for transfection and as donor cells. The DNA, floxed neomycin-resistance gene that included a red fluorescent protein (RFP) plasmid system, designated pCALNL-DsRed (from Addgene, http://www.addgene.org), was transfected into the fibroblasts with Fugene HD (Roche, Germany). One day before transfection, the fibroblasts were plated at a density of 5×10^4 cells/ml in a 35-mm culture dish and cultured overnight to achieve 50–70% confluency. Two days after transfection 750 μg/ml neomycin (G418, Invitrogen, USA) was added, transfected cells were then cultured for about 2 weeks to isolate stable cell lines. Further transfection with transient Cre was performed in order to remove floxed genes by Cre recombination. Following Cre expression, RFP-expressing cells were isolated mechanically and cultured for use in SCNT.

Somatic cell nuclear transfer (SCNT)

RFP-expressing cells were subjected to SCNT, which was carried out in accordance with the protocol established previously in our laboratory (Koo *et al.*, 2008). In brief, *in vitro* matured (IVM) pig oocytes

were enucleated using an aspiration pipette, then microinjected with a transfected donor cell, fused by electrical stimulation and activated. The activated embryos that resulted were cultured for 7 days. Cleavage and blastocyst stages were observed on days 2 and 7 after culture, respectively. Deletion of the neomycin-resistance gene was confirmed in cloned blastocysts by reverse transcription polymerase chain reaction (RT-PCR).

Results and Discussion

Colonized pCALNL-DsRed cells were observed after 2 weeks of neomycin selection, and the cells were then cultured further. RFP could not be seen (Fig. 1) in these cells under ultraviolet light exposure. However, RFP was detected under ultraviolet light after transfection with the transiently expressed Cre gene (Fig. 1). Out of a total of 121 SCNT embryos (SCNT Replication number; 4) that had RFP-expressing cells, 76 embryos (62.8%) were observed to have cleaved and six (5.0%) had developed to the blastocyst stage. Developed blastocysts were confirmed to express RFP by fluorescence microscopy (Fig. 1). In addition, excision of the neomycin-resistance gene in genomic DNA of cloned blastocysts was confirmed by RT-PCR (Fig. 1).

In this experiment we found that, following insertion and excision of the floxed target gene, donor fibroblasts were reprogrammed in enucleated oocytes and developed into pre-implantation stage embryos. The need to employ two sequential transfection steps for gene insertion and then excision, made it more difficult for the donor fibroblasts to produce the embryonic cell numbers and, therefore, the number of resulting SCNT blastocysts was lower compared with our previous studies (Koo *et al.*, 2008). To overcome this issue *in vitro*, it would be necessary, therefore, to make immortalized cell lines. One approach would be to use the telomerase catalytic subunit TERT (Garcia-Escudero *et al.*, 2010) and test the Cre-*loxP* system on various target genes *in vitro*. In addition, the gene of a floxed target and Cre could be transfected into female or male cells, as determined by PCR or karyotyping before SCNT, and cloned offspring could be bred for conditioned gene deletion.

In conclusion, we demonstrated that Cre-*loxP* recombination was a successful procedure in pig fibroblasts and that successfully transformed cells could develop into blastocysts. Retention of the transformed traits was also confirmed. In the future, the use of a floxed endogenous gene for Cre-*loxP* recombination could be applied for the production of conditional gene knock-out pigs for xeno-transplantation.

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