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Production of homozygous *klotho* knockout porcine embryos cloned from genome-edited porcine fibroblasts

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ABSTRACT

Even though klotho deficiency in mice exhibits multiple aging-like phenotypes, studies using large animal models such as pigs, which have many similarities to humans, have been limited due to the absence of cell lines or animal models. The objective of this study was to generate homozygous *klotho* knockout porcine cell lines and cloned embryos. A CRISPR sgRNA specific for the *klotho* gene was designed and sgRNA (targeting exon 3 of *klotho*) and Cas9 RNPs were transfected into porcine fibroblasts. The transfected fibroblasts were then used for single cell colony formation and 9 single cell‑derived colonies were established. In a T7 endonuclease I mutation assay, 5 colonies (#3, #4, #5, #7 and #9) were confirmed as mutated. These 5 colonies were subsequently analyzed by deep sequencing for determination of homozygous mutated colonies and 4 (#3, #4, #5 and #9) from 5 colonies contained homozygous modifications. Somatic cell nuclear transfer was performed to generate homozygous *klotho* knockout cloned embryos by using one homozygous mutation colony (#9); the cleavage and blastocyst formation rates were 72.0% and 8.3%, respectively. Two cloned embryos derived from a homozygous *klotho* knockout cell line (#9) were subjected to deep sequencing and they showed the same mutation pattern as the donor cell line. In conclusion, we produced homozygous *klotho* knockout porcine embryos cloned from genome-edited porcine fibroblasts.

(Key word: *Klotho*; Knockout; CRISP/Cas9; Somatic cell nuclear transfer; Porcine embryo)

Introduction

Pigs are useful as animal models for human disease because these two species have many genetic similarities (Li *et al.*, 2009), and the anatomy and physiology of pigs are similar to those of humans (Prather *et al.*, 2003).

Recent advances in genome engineering tools, including Zinc-Finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs) and the Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas9) system, have made it possible to produce specific gene knockout animals. In particular, the high targeting efficiency and mutation rate of the CRISPR/Cas9 system has led to enhanced ability to perform genome editing in pigs (Zhou *et al.*, 2015).

The klotho deficient mice display multiple aging-like phenotypes similar to human premature-aging syndromes with extremely shortened life-span (Kuro-o *et al.*, 1997). Homozygous *klotho* knockout mice ($KL^{-/-}$) develop normally up to 3 weeks. Afterwards, they began to manifest multiple aging-like phenotypes such as growth retardation, infertility, arteriosclerosis and osteoporosis. Eventually, loss of klotho function results in premature death at 2 months of age.

Although studies have been conducted recently to identify the functions of the *klotho* gene in mice, there is growing evidence that animal models other than rodents are needed for biomedical applications (Plews *et al.*, 2012; Harding *et al.*,

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2013) due to inappropriate mimicking of some human genetic diseases (Walters and Prather, 2013). Therefore, the aim of this study was to generate *klotho* knockout porcine cloned embryos *via* somatic cell nuclear transfer (SCNT) in order to produce a more suitable animal model for elucidating the role of this gene in aging and disease.

Materials and Methods

Primary culture of porcine fetal fibroblasts

Female fibroblasts from one porcine fetus were isolated and cultured. The euthanized fetus was dissected into three parts: head, body and tail. The fetal body parts were washed three times in phosphate-buffered saline (PBS) and then chopped into small pieces in a 60 mm dish with trypsin. Trypsinized tissues were then incubated for 30 min at 37 °C. Well-dissociated tissues were centrifuged at 1,500 g for 2 min. The supernatant was discarded, and the pellet was resuspended with PBS and then centrifuged at 1,500 g for 2 min. These procedures were repeated twice. Finally, the supernatant was discarded, and the pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS; Gibco), 1% Penicillin/Streptomycin (P/S; Gibco), 1% nonessential amino acids (NEAA; Gibco), and 100 mmol/l β-mercaptoethanol (β-ME), by inverting the tube several times. The cells resuspended in this medium were held at room temperature (~25 °C) for 5 min, and then the suspension was transferred into a cell culture dish for ~10 days with the culture medium changed every 2 - 3 days. These primary cells were cultured, expanded and finally frozen at −196 °C for further use. The cell cultures were maintained in DMEM with 15% FBS, 1% P/S, 1% NEAA and 100 mmol/l β-ME.

Generation of *klotho* knockout cell lines

CRISPR/Cas9 nucleases for porcine *klotho* gene exon 3 were designed and synthesized as described (Kim *et al.*, 2014). Cas9/sgRNA ribonucleoproteins (RNPs) were transfected into porcine fibroblasts *via* Neon (Life technologies). Transfected cells were subjected to limiting dilution in a 48-well plate and a monoclonal cell population was selected and propagated. Genomic DNA from each isolated clone was analyzed by a T7 endonuclease I (T7E1) assay and deep sequencing on the *klotho* gene target locus to identify and characterize the *klotho* knockout alleles.

T7 endonuclease I assay

Genomic DNA was extracted using Exgene ™ cell SV (GeneAll Biotech., Seoul, Korea) according to the manufacturer's instructions. PCR amplicons including CRISPR/Cas9 target sites were generated using the primers listed in Table 1. The T7E1 analysis was done as described previously (Kim *et al.*, 2009). In brief, the PCR amplicons were denatured at 95 °C and annealed to form heteroduplex DNA, which was treated with 5 units of T7 endonuclease 1 (ToolGen Inc., Seoul) for 20 min at 37 °C and then analyzed by 2% agarose gel electrophoresis.

Deep sequencing

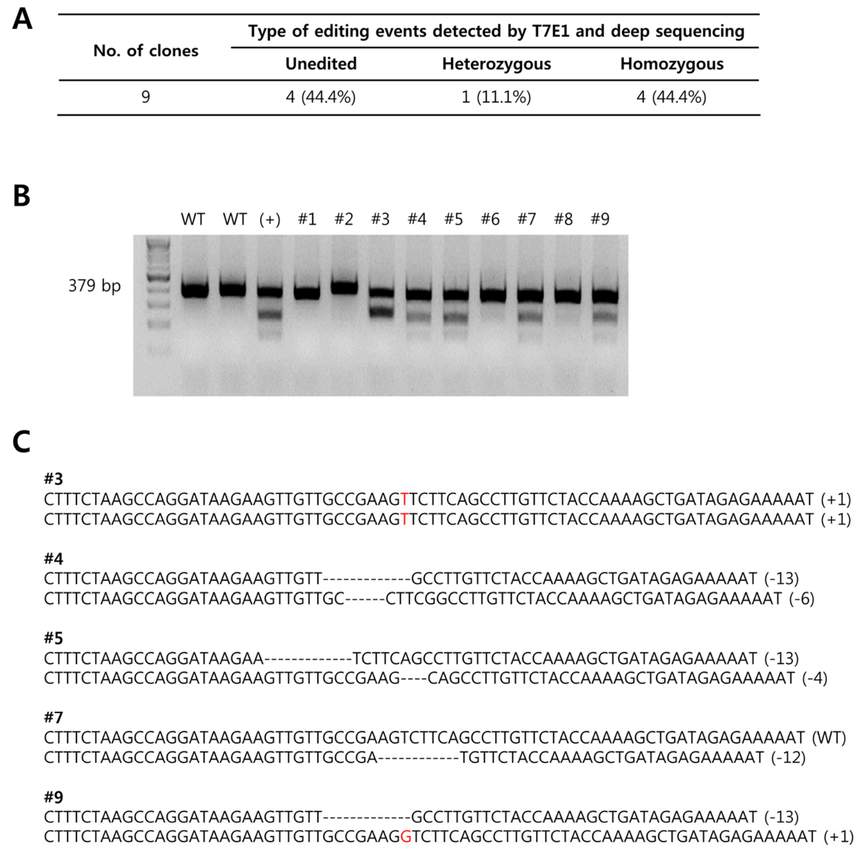
The on-target regions were amplified from genomic DNA and used for library construction. Equal amounts of the PCR amplicons were subjected to paired-end read sequencing using Illumina MiSeq (v2, 300-cycle). Insertions or deletions located around the CRISPR/Cas9 cleavage site (3 bp upstream of the PAM) were considered to be the mutations induced by CRISPR/Cas9.

Somatic cell nuclear transfer

One of the homozygous *klotho* knockout cell lines was subjected to SCNT, which was performed as described in a previous report (Lee *et al.*, 2016). In brief, *in vitro* matured (IVM) pig oocytes were enucleated by aspirating the first polar body and the adjacent cytoplasm containing chromosomes with an aspiration pipette. Then, using a fine pipette, a trypsinized

Table. 1. Details of primers used for T7E1 assay and deep sequencing.

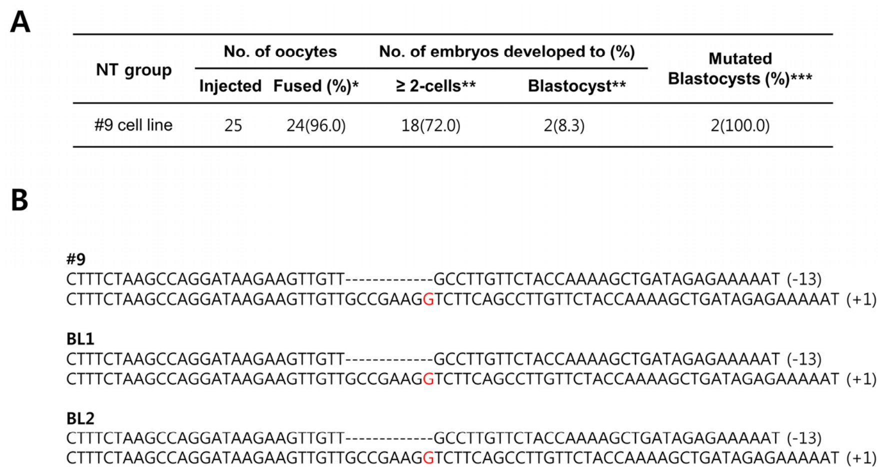
Target	Use	Primer sequences (5'-3')	Product size (bp)
Klotho	T7E1	F: CCTCAAGTAGTAAAACCCTC R: GGTTTTGTCAGCTGACTTAC	379
	Deep sequencing	F: CTTGCTCTTGTCCTCTTTCC R: CAACAATTCCCCAAGCAAAG	282



A

No. of clones	Type of editing events detected by T7E1 and deep sequencing		
	Unedited	Heterozygous	Homozygous
9	4 (44.4%)	1 (11.1%)	4 (44.4%)

Fig. 1. Generation of *klotho* gene knockout cells and their analysis. A: Summary of *klotho* knockout results in porcine fibroblasts. B: T7 endonuclease I (T7E1) assay: the T7E1 assay was conducted using genomic DNA from nine single cell-derived colonies. (WT; wild type, (+); positive control) C: Deep sequencing to determine mutations in the *klotho*-targeted region. Deep sequencing was conducted using genomic DNA from five mutation-confirmed colonies by T7E1 (#3, #4, #5, #7 and #9). Multiple deletions or insertions are depicted using parentheses.



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NT group	No. of oocytes		No. of embryos developed to (%)		Mutated Blastocysts (%)***
	Injected	Fused (%)*	≥ 2-cells**	Blastocyst**	
#9 cell line	25	24(96.0)	18(72.0)	2(8.3)	2(100.0)

Fig. 2. Production of homozygous *klotho* knockout porcine cloned embryos. A: Summary of SCNT results using homozygous *klotho* knockout porcine fibroblasts (#9) (*; Percentage of the number of injected oocytes, **; Percentage of the number of fused oocytes, ***; Percentage of the number of blastocysts derived from the #9 cell line). B: Deep sequencing to determine the same mutation pattern between cell line (#9) and SCNT blastocysts derived from this cell line.

klotho knockout cell, with a smooth cell surface, was transferred into the perivitelline space of an enucleated oocyte. These couplets were electrically fused with a single DC pulse of 200 V/mm for 30 µs using an electro cell fusion generator (LF101; Nepa Gene Co., Japan). Then, 30 min after fusion, fused couplets were activated with a single DC pulse of 1.5 kV/cm for 60 µs using a BTX Electro-Cell Manipulator 2001 (BTX, Inc., San Diego, CA, USA). The resulting activated embryos were cultured in Porcine Zygote Medium-5 (PZM-5; Funakoshi Corporation, Tokyo, Japan) medium for 7 days. Cleavage and blastocyst formation rates were observed on days 2 and 7 after culture, respectively. Mutation of the *klotho* gene was confirmed in cloned blastocysts by deep sequencing.

Results and Discussion

Klotho knockout in porcine fibroblasts *via* delivery of CRISPR/Cas9 RNPs

After transfection of Cas9/sgRNA RNPs, transfected porcine fibroblasts were cultured in a 100 mm dish and grown into colonies; 9 single cell‑derived colonies were established. In a T7E1 mutation assay, we found 5 colonies (#3, #4, #5, #7 and #9) that were mutated (Fig. 1). These 5 colonies were subjected to deep sequencing for determination of homozygous mutated colonies and 4 (#3, #4, #5 and #9) of 5 colonies contained homozygous modifications (Fig. 1). In colony #3, 1 bp insertions were observed; in # 4, 13 and 6 bp deletions were observed; in #5, 13 and 4 bp deletions were observed; in #9, a 13bp deletion and 1 bp insertion were observed (Fig. 1).

SCNT using a homozygous *klotho* knockout cell line

One homozygous mutated colony (#9) with morphologically good cells was finally selected and used for SCNT. The cleavage and blastocyst formation rates were 72.0% and 8.3%, respectively (Fig. 2). Two cloned embryos derived from the homozygous *klotho* knockout cell line (#9) were subjected to deep sequencing and they showed the same mutation pattern as the donor cell line (Fig. 2).

In conclusion, we established several *klotho* knockout cell lines that may be useful cell sources for SCNT procedures to generate *klotho* knockout pigs. *Klotho* knockout pigs may exhibit a reduced life span with multiple aging-like phenotypes and thus will be a good model for studying aging and its consequences in humans. However, further studies are required to generate *klotho* knockout pigs.

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