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Knockout of exogenous EGFP gene in porcine somatic cells using zinc-finger nucleases

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ABSTRACT

Zinc-finger nucleases (ZFNs) are expected as a powerful tool for generating gene knockouts in laboratory and domestic animals. Currently, it is unclear whether this technology can be utilized for knocking-out genes in pigs. Here, we investigated whether knockout (KO) events in which ZFNs recognize and cleave a target sequence occur in porcine primary cultured somatic cells that harbor the exogenous enhanced green fluorescent protein (EGFP) gene. ZFN-encoding mRNA designed to target the EGFP gene was introduced by electroporation into the cell. Using the Surveyor nuclease assay and flow cytometric analysis, we confirmed ZFN-induced cleavage of the target sequence and the disappearance of EGFP fluorescence expression in ZFN-treated cells. In addition, sequence analysis revealed that ZFN-induced mutations such as base substitution, deletion, or insertion were generated in the ZFN cleavage site of EGFP-expression negative cells that were cloned from ZFN-treated cells, thereby showing it was possible to disrupt (i.e., knock out) the function of the EGFP gene in porcine somatic cells. To our knowledge, this study provides the first evidence that the ZFN-KO system can be applied to pigs. These findings may open a new avenue to the creation of gene KO pigs using ZFN-treated cells and somatic cell nuclear transfer.

1. Introduction

Gene knockout (KO) is an effective tool for analyzing gene function and generating model animals that recapitulate genetic disorders [1]. However, the currently available techniques for targeting a specific gene of interest for knockout by homologous recombination are highly laborious and time-consuming, and thus, somewhat inefficient [2].

In mammals, the use of gene knockout technology has been largely restricted to the laboratory mouse [3]. This reflects the fact that the efficiency of gene targeting is still extremely low in species from which embryonic stem (ES) cell lines have not been established, with the exception of mice [4–6]. In pigs, for example, to the best of our knowledge, the only genes that have been successfully knocked-out are α1,3-galactosyltransferase (Gal-T) [7,8] and cystic fibrosis transmembrane conductance regulator (CFTR) [9].

With their physiological and anatomical similarities to humans, pigs have attracted attention as a large experimental animal with the capability of providing valuable information that could be easily extrapolated to humans [10,11], and the possibility of acting as a donor for xenotransplantation [12]. Pig models for diseases such as diabetes [13] and Alzheimer's disease [14] might be expected to contribute to advances in our understanding and treatments for these intractable diseases. Therefore, the development of effective gene modification techniques in pigs, particularly efficient gene KO techniques, is very important in biomedical research [15,16].

Recently, it was shown that KO rats could be produced rapidly and efficiently by microinjection of DNA construct expressing zinc-finger nuclease (ZFN) or ZFN-encoding mRNA into rat embryos [17,18]. Although ZFNs are expected to open up a new means of generating gene knockouts in laboratory animals, it is currently unclear whether this methodology will be feasible for modifying or knocking-out genes in pigs. Because the creation of pig clones by somatic cell nuclear transfer (SCNT) is highly reproducible [19,20], it is important to determine whether gene KO using ZFNs is possible in primary cultures of pig somatic cells. In other words, generating pigs with knockouts of specific genes would become more realistic and practical if the ZFN approach performs efficiently in porcine somatic cells. The aim of the present study was to determine whether ZFNs could recognize and cleave a target sequence in an exogenous enhanced green fluorescent protein (EGFP) gene in porcine primary

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cultured somatic cells and, thereby, knock out the gene. Our study provides the first evidence that the ZFN-KO system can be applied to pigs.

2. Materials and methods

2.1. Chemicals

Unless otherwise specified, all reagents, including EGFP-targeted ZFN mRNA, were purchased from SIGMA-Aldrich. One of the two ZFNs used in this experiment had 6 zinc-finger proteins recognizing 18 bases, the other had 5 zinc-finger proteins recognizing 15 bases (Fig. 1); these ZFNs have been confirmed to specifically recognize and cleave the EGFP gene sequence in EGFP-transgenic rats [17].

2.2. Preparation of cells and culture conditions

A primary culture of pig fetal fibroblast cells (EGFP-transgenic fetal fibroblasts) that had been confirmed to contain approximately 10 copies of the transgene pCX-EGFP [21] was used (data not shown). The fibroblast cells and their derivatives were seeded on type I collagen-coated dishes or plates (IWAKI) and cultured in MEMα (Invitrogen), supplemented with 15% FBS (CBB), 0.1 mM MEM Non-Essential Amino Acids Solution (Invitrogen), 1 × ITS-X supplement (Invitrogen), and 1 × Antibiotic-Antimycotic (Invitrogen) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Transfer of ZFN-encoding mRNA

The EGFP-transgenic fetal fibroblasts were cultured to 70–90% confluence, then washed twice with D-PBS(-) (Invitrogen) and treated with 0.05% trypsin-EDTA (Invitrogen) to isolate and collect the cells. The cells (1×10^6) were suspended in 100 μl R buffer (supplied as part of the Neon Transfection System, Invitrogen), given either 2 μg/5 μl of ZFN-expressing mRNA solution (ZFN-treated cells) or 5 μl of RNase free water (control cells), and electroporated under the following conditions: pulse voltage, 1100 V; pulse width, 30 ms; and pulse number, 1 (program #6). Following electroporation, the cells were cultured in the medium described above without antibiotics for 24 h and then in the medium with antibiotics. In this study, mRNA transfer experiment was replicated three times.

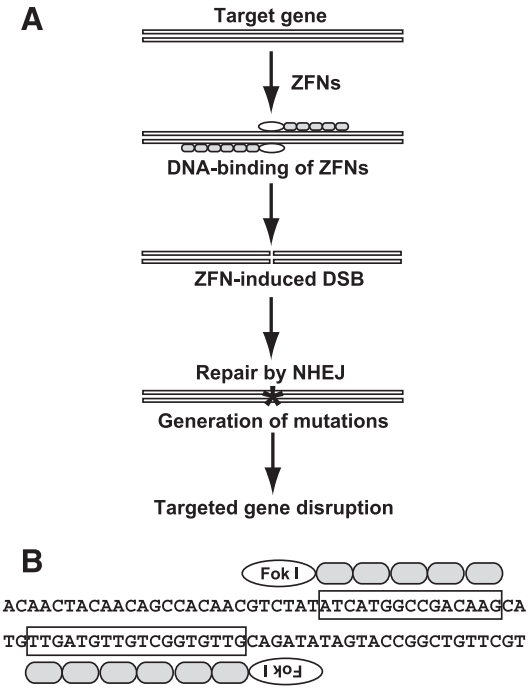

Fig. 1. ZFN-mediated gene disruption. (A) A pair of ZFNs binds to the target gene in a sequence-specific manner. Dimerized Fok I nucleases cleave DNA, producing double-strand breaks (DSBs). When the DSBs are repaired by nonhomologous end-joining (NHEJ), gene mutation often occurs (*), causing targeted gene disruption [33]. (B) EGFP-targeted ZFNs used in this experiment. The ZFN target sequences are boxed. Each ZFN consists of a finger protein that recognizes the DNA sequence (gray ellipses) and the non-specific cleavage domain of Fok I, a restriction enzyme that cleaves the DNA sequence (white ellipses). One finger protein recognizes three bases.

2.4. Surveyor nuclease assay (mutation detection assay)

ZFN-induced mutations were detected using the SURVEYOR Mutation Detection Kit (Transgenomic) in accordance with the manufacturer's protocol. The assay detects nonhomologous end-joining (NHEJ)-mediated imperfect repair of ZFN-induced double-strand breaks (DSBs) by digesting heteroduplexes consisting of wild-type and mutant DNA with Surveyor nuclease, a mismatch DNA-specific cleavage enzyme (Fig. 2A) [22]. Briefly, 24 h after electroporation, genomic DNA was extracted from the ZFN-treated and control cells using Nucleospin (MACHEREY-NAGEL). The genomic DNA was used as a template, and a region recognized and cleaved by ZFNs was amplified by PCR. The PCR was performed using PrimeSTAR HS DNA polymerase (Takara), a high-fidelity enzyme, under the following conditions: 95 °C for 1 min, followed by 95 °C for 30 s, 68 °C for 30 s, and 72 °C for 1 min for 30 cycles. The sequences of the PCR primers used are as follows: 5′-AAGAATTCGCCACCATGGTGAGCAAG and 5′-GACCATGTGATCGCGCTTCTCGT. The ZFN-treated cell-derived (Z) and the control cell-derived (C) amplicons were obtained by PCR and, along with a mixture of both (Z/C), were heat denatured, allowed to rehybridize, and then digested by the Surveyor nuclease. The digested samples were subjected to polyacrylamide gel electrophoresis to confirm ZFN-induced mutations (Fig. 2B). This assay was performed in all three mRNA transfer experiments.

2.5. Flow cytometric analysis

The green fluorescence intensity (FL1) in cells was measured using FACSCalibur cytometer (Becton Dickinson) equipped with a 488 nm argon laser. Briefly, 1×10^5 cells were harvested at 7 days after electroporation and then resuspended in 1 ml D-PBS(-) supplemented with 5% FBS. Cell debris and aggregates were gated out of the analysis using bivariate, forward/side scatter (FSC/SSC) parameters. Gated 1×10^4 events per sample were acquired, and the values were calculated as a percentage of the cell population using CELLQuest software (Becton Dickinson). EGFP-expression positive and negative cells were discriminated using the EGFP fluorescence intensity 10^1 as a standard.

2.6. Cloning of EGFP-expression negative cells

Cells lacking EGFP expression were cloned from ZFN-treated cells that had been confirmed by the Surveyor nuclease assay to have ZFN-induced mutations. The presence/absence of EGFP expression in the cells was determined by fluorescence microscopy (Nikon). Cell cloning was performed at 7 days after the transfer of ZFN-encoding mRNA in accordance with a protocol recommended by the manufacturer (SIGMA-Aldrich). Briefly, the ZFN-treated cells were cultured for 7 days after mRNA transfer, then seeded onto five 96-well plates at a concentration of 5 cells/well, and further cultured. Twelve days after seeding, cultures containing cells that lacked EGFP expression were continued, while those containing mixed colonies of cells that showed or lacked EGFP expression were excluded from the analysis. The cloned cells that lacked EGFP expression were allowed to proliferate in the 6-well plates until confluence and then subjected to DNA analysis.