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

Masahito Watanabe [a,b], Kazuhiro Umeyama [b,d], Hitomi Matsunari [b], Shuko Takayanagi [a,b], Erika Haruyama [b], Kazuaki Nakano [b], Tsukasa Fujiwara [b], Yuka Ikezawa [b], Hiromitsu Nakauchi [a,c], Hiroshi Nagashima [a,b,d,*]

[a] Japan Science and Technology Agency (JST), ERATO, Nakauchi Stem Cell and Organ Regeneration Project, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[b] Department of Life Sciences, School of Agriculture, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan
[c] Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, Tokyo University, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[d] International Cluster for Bio-Resource Research, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan

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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

* Corresponding author at: Department of Life Sciences, School of Agriculture, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan. Fax: +81 44 934 7824.
E-mail address: hnagas@isc.meiji.ac.jp (H. Nagashima).

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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.

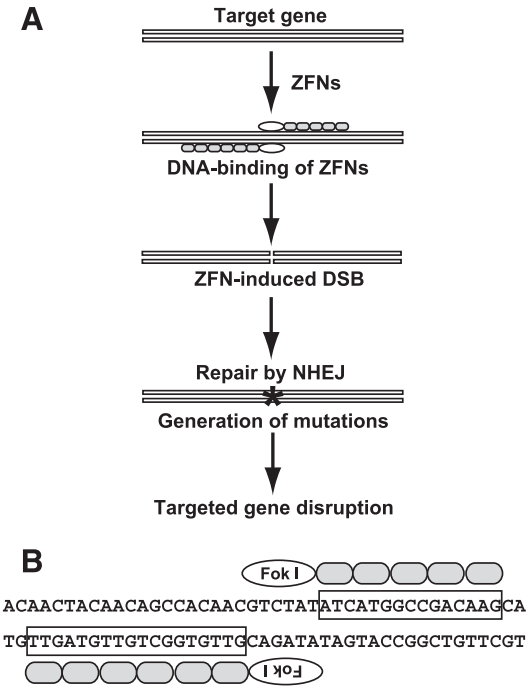

Fig. 1. ZFN-mediated gene disruption. (A) A pair of ZFNs binds to the target gene in a sequence-specific manner. Dimerized Fok I nucleases cleave DNA, producing double-strand breaks (DSBs). When the DSBs are repaired by nonhomologous end-joining (NHEJ), gene mutation often occurs (*), causing targeted 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.

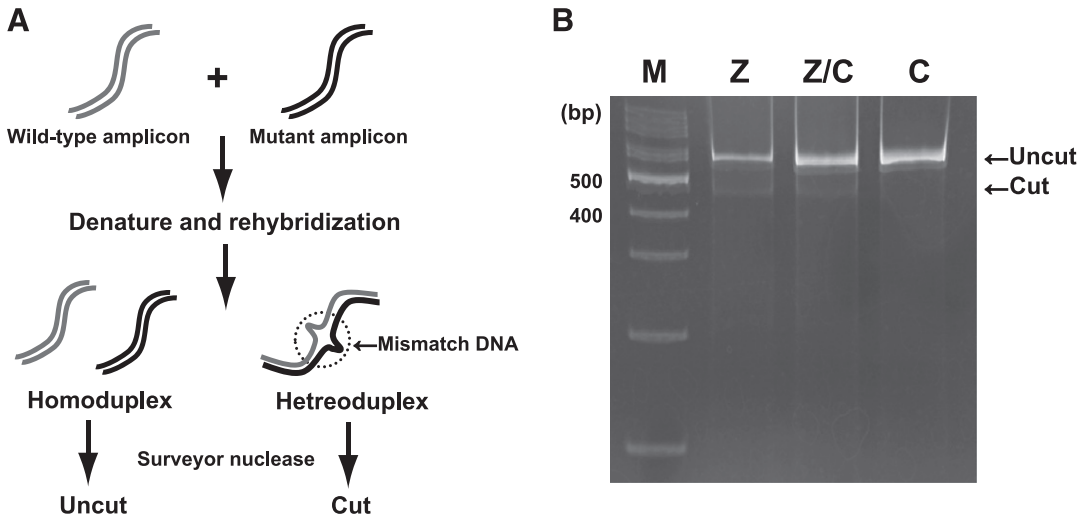

Fig. 2. Confirmation of ZFN-induced mutations by the Surveyor nuclease assay. (A) Schematic overview of the Surveyor nuclease assay. Genomic DNA was purified from ZFN-treated cells or control cells. The PCR amplicons encompassing the site of ZFN recognition were denatured and rehybridized. If the amplicons contain both wild-type and mutated DNA sequences, heteroduplexes would be formed. The Surveyor nuclease recognizes and cuts heteroduplexes but not homoduplexes. (B) Amplicons from ZFN-treated cells (Z), control cells (C), and a mixture of ZFN-treated and control cells (Z/C). In Z and Z/C, a band cut by the Surveyor nuclease was detected at an expected size (near 470 bp), indicating the presence of mismatch DNA. In C, the band was not detected.

2.7. Analysis of ZFN-induced mutations by DNA sequencing

Genomic DNA was extracted from cells lacking EGFP expression (cloned from ZFN-treated cells) and used as a template for PCR to amplify the region recognized and cleaved by the ZFNs (see above). The amplification products were subcloned into pCR4Blunt-TOPO plasmid vector (Invitrogen). Plasmids were extracted from the resultant *Escherichia coli* colonies (at least from 10 colonies per cell clone) for DNA sequencing. Sequencing was performed using the BigDye Terminator Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

3. Results

3.1. Transfer of ZFN-encoding mRNA

The day after electroporation, there were slightly more dead ZFN-treated cells than dead control cells. No unusual morphological changes were observed in ZFN-treated cells, which adhered to the culture dishes similarly to the control cells. However, the day after the first passage following the electroporation, there were obviously more dead ZFN-treated cells than dead control cells. Similar observations were made in all three mRNA transfer experiments.

3.2. Surveyor nuclease assay (mutation detection assay)

A Surveyor nuclease assay was performed on cells the day after electroporation. It was expected, depending on the type of mutation induced by ZFN treatment, that if mutations had occurred in the EGFP target sequence, fragments of approximately 470 bp would be produced by digestion with the Surveyor nuclease. In agreement with this expectation, a band of approximately 470 bp was detected in the ZFN-treated cell-derived amplicon (Z) and the mixture of amplicons (Z/C) from ZFN-treated and control cells (Fig. 2B). By contrast, no bands were detected near 470 bp in the amplicon from control cells (C). These results show that mismatch DNA was present in the ZFN-treated cell-derived amplicon, confirming the presence of mutations induced by ZFN treatment. This experiment confirms that ZFNs can induce cleavage and mutation in a target sequence. Identical results were obtained in all three mRNA transfer experiments.

3.3. Flow cytometric analysis

Following the confirmation by the Surveyor nuclease assay of induced mutations in the ZFN-treated cells, EGFP expression in the ZFN-treated and control cells was analyzed by flow cytometry. The proportion of control cells lacking EGFP expression was 5.74%, compared to 20.54% of ZFN-treated cells (Fig. 3). Thus the rate of cells not expressing EGFP rose considerably after ZFN treatment. We also found that the intensity of EGFP fluorescence in ZFN-treated cells decreased (i.e., the peak shifted to the left) compared to control cells. These results showed ZFNs could recognize and cleave an EGFP sequence in porcine cells, thereby quenching or decreasing EGFP expression. Furthermore, EGFP expression profiles were similar in control cells and EGFP-transgenic fetal fibroblasts not treated by electroporation, confirming that electroporation did not affect the intensity of EGFP fluorescence (data not shown).

3.4. Cloning of EGFP-expression negative cells and DNA sequencing

Following the confirmation by the Surveyor nuclease assay and flow cytometric analysis of ZFN-induced cleavage of the target sequence and the disappearance of EGFP fluorescence expression, we derived clones from cells lacking EGFP expression after ZFN treatment. ZFN-treated cells were seeded onto five 96-well plates at a concentration of 5 cells/well, and 1–2 colonies formed in the majority of wells. Twelve days after seeding, 78 of 480 wells (16.2%) developed colonies that only contained EGFP-expression negative cells. After further time in culture, the ZFN-target region of these cells was sequenced and various mutations were identified in seven of the clones (Fig. 4), as follows: 15 bp deletion in clones 1-A7 and 1-B4 (Y146F and loss of five amino acids, NSHNV), 1 bp substitution in clones 1-H2 and 5-C4 (V151G), 1 bp substitution + 1 bp deletion (clone 3-E7), 2 bp deletion (clone 3-F10), and 4 bp insertion (clone 1-C10). This analysis confirmed that genetic mutations were induced in the target sequence region, that is, the target sequence in the cloned cells that lacked EGFP expression had been knocked out by the ZFN treatment.

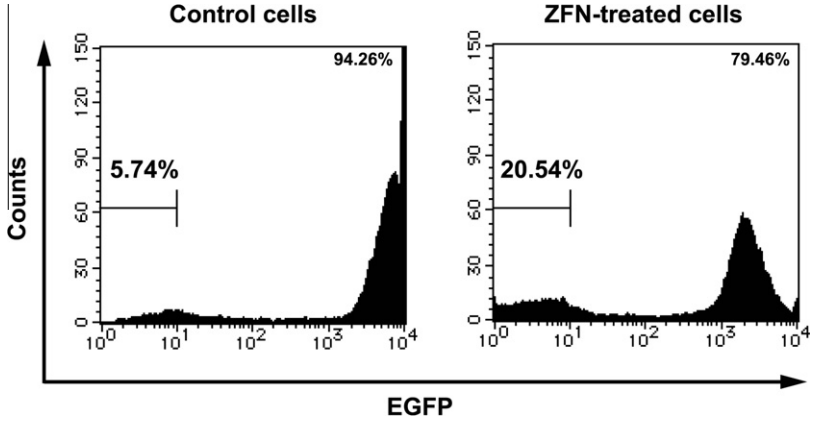

Fig. 3. Analysis for EGFP fluorescence intensity by flow cytometry. The *X*- and *Y*-axes in the histogram indicate EGFP fluorescence intensity and cell counts, respectively. In the ZFN-treated cells (right), the number of cells lacking EGFP expression (10^0–10^1) clearly increased (20.54%), and the intensity of EGFP fluorescence also decreased compared to the control cells (left). A small number of cells lacking EGFP expression were detected in the control (left).

4. Discussion

To our knowledge, this study is the first to show ZFN-induced knockout of an exogenous gene integrated into the genome of porcine primary cultured somatic cells. The only other mammalian species in which this approach has been successful is the rat: Geurts et al. knocked out an exogenous EGFP gene integrated into the rat genome using ZFNs [17], while Mashimo et al. subsequently showed that it was possible to knock out an endogenous rat gene (IL2RG) [18]. These reports in rats, in combination with our finding of exogenous EGFP gene knock out in primary cultures of porcine cells, suggest that it is likely that it will also be possible to knock out endogenous genes in pigs. The production of cloned pigs by SCNT is now possible with high reproducibility [19,20]. Therefore, the development of efficient techniques for establishing KO cells using ZFNs increases the possibility that pigs with knock outs of specific genes might be generated using SCNT with ZFN-treated cells. Furthermore, the establishment of KO cells using ZFNs does not require a drug selection step, unlike traditional methods, for example using homologous recombination. Drug selection might compromise viability of the cells, but ZFN-based methods can avoid any such problem and is therefore one of best advantages for creating KO pigs by SCNT.

Two methods are currently available for expressing ZFNs: ZFN-expression constructs (plasmids) or ZFN-encoding mRNA [17,23,24]. While further study is necessary to determine which of these methods is more suitable for modifying genes using ZFNs, there are distinct differences between these approaches. The former generally uses circular plasmids, giving rise to the possibility of the permanent expression of ZFNs as a result of integration of the plasmid into the host genome. Such integration might increase the risk of off-target effects. Moreover, there is also the possibility of ZFN-expression constructs being integrated into endogenous genes, inducing undesirable insertional mutations. Alternatively, with the ZFN-encoding mRNA method the problems associated with insertion into the host genome are avoided. To reduce the rate of additional and undesirable mutations due to off-target effects, it is desirable that ZFN expression be short-term [25]. In the present study, we chose to exploit the above-mentioned advantages of mRNA transfer for future application with SCNT in pigs. Here, ZFN-encoding mRNA that targeted the EGFP gene was introduced by electroporation into EGFP-transgenic fetal fibroblasts. The Surveyor nuclease assay detected a band of the size expected of DNA mutated at the ZFN target site, thereby, providing proof of targeted gene mutations (Fig. 2B). This result was replicated in three independent experiments. The gene-modification efficiency of ZFNs has been reported to be as high as 10–20% [17,23,26], and there are even studies that report an efficiency exceeding 20% [18,27]. Flow cytometric analysis in this study showed that cells lacking EGFP expression increased by approximately 15% after ZFN treatment (Fig. 3),

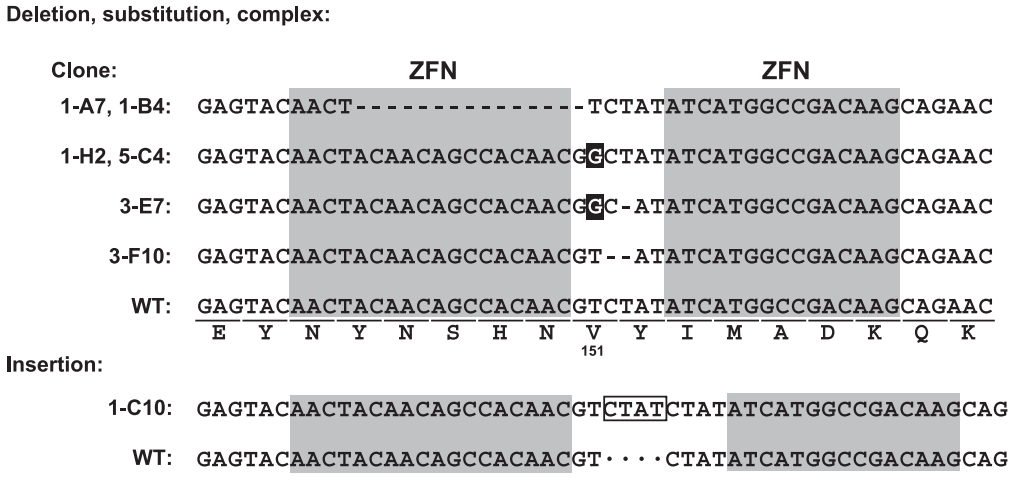

Fig. 4. ZFN-induced mutations in clones of cells lacking EGFP expression. The gray boxes indicate the sites recognized by the ZFNs. WT indicates the wild-type (non-mutated) EGFP sequence. Substituted bases and inserted bases are indicated by black and white boxes, respectively. Deleted bases are indicated by "–". The lines under the WT sequence indicate codons, and the alphabetical letters and the number under the lines indicate the corresponding amino acids and position, respectively.

and DNA analysis confirmed that the disappearance of the EGFP expression phenotype was due to the KO of the target gene. These data therefore indicate that in primary cultures of porcine cells, gene KO efficiency equivalent to those previously reported was obtained.

In addition, this study characterized the mutations induced by the ZFNs (Fig. 4). The types of mutation recovered were consistent with those already reported, i.e., deletions, substitutions, insertions, and more complex mutations [24,28,29]. EGFP is a fluorescent protein consisting of 239 amino acids [30]. A 15 bp deletion that caused a single amino acid substitution (Y146F) and the loss of five amino acids (NSHNV, amino acids 147–151) was observed in clones 1-A7 and 1-B4. Thus, these clones may possibly produce an abnormal EGFP consisting of 234 amino acids. In clones 1-H2 and 5-C4, the substitution of amino acid 151 (V151G) was observed. The valine positioned at position 151 of EGFP is important for EGFP fluorescence and is located close to the tyrosine at position 67 at the center of a chromophore [31]. Therefore, we suggest that the amino acid substitution (V151G) in clones 1-H2 and 5-C4 hinders the formation of a chromophore necessary for EGFP fluorescence expression. It is probable that the 1 bp substitution + 1 bp deletion observed in clone 3-E7 created a premature stop codon due to a frameshift and might have resulted in the production of a truncated EGFP consisting of 163 amino acids. Also, in the 2 bp deletion observed in clone 3-F10 and the 4 bp insertion observed in clone 1-C10, the original stop codon was lost due to a frameshift, possibly resulting in the incorrect amino acids at and after amino acid position 153. Indeed, it was not clear whether the abnormal locations of stop codons due to the mutations observed in clones 3-E7, 3-F10, and 1-C10 produced immature and abnormal EGFPs. However, it is possible that nonsense mediated decay (NMD), a cellular mechanism of mRNA surveillance, could rapidly degrade abnormal EGFP mRNAs and decrease the expression of any abnormal proteins [32]. Based on these results, we suggest that in the cell clones lacking EGFP expression after ZFN treatment, the ZFN-induced mutations disrupted (knocked out) the functions of the EGFP.

In the present study, we made use of EGFP-transgenic fetal fibroblasts that have approximately 10 copies (i.e., approximately 10 ZFN target sequences) of the transgene pCX-EGFP (data not shown). Flow cytometric analysis confirmed a decrease in the fluorescence intensity of EGFP in the ZFN-treated cells, probably as a consequence of damage at the target sites. We suggest that disruption of gene functions (i.e., KO) occurred as a result of cleavage or mutation at some of the multiple ZFN targets, thereby decreasing the EGFP fluorescence intensity. This suggestion is supported by the fact that DNA sequencing analysis of cells lacking EGFP expression showed the presence of both mutant and wild-type (i.e., non-mutated) EGFP sequences in 6 (1-A7, 3-E7, 5-C4, 1-B4, 3-F10, and 1-C10) of the seven cell clones in which gene mutations were induced by ZFN treatment.

In conclusion, this study has confirmed that ZFNs can recognize and cleave the target EGFP sequences in porcine primary cultured somatic cells, thereby showing that it is possible to disrupt (knock out) the EGFP gene. Using ZFNs, gene KO could be performed in a relatively short time with minimum effort (i.e., transfer of ZFN-expression mRNA). If more specific, safer ZFNs are developed in future, then ZFN techniques will become very useful and powerful for the creation of KO pigs.

Acknowledgments

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