Targeted Mutagenesis of Beta-Lactoglobulin Gene in Caprine Fetal Fibroblasts by Context-Dependent Assembly Zinc-Finger Nucleases

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Abstract

Targeted mutagenesis by zinc-finger nucleases (ZFNs) can be used to generate knock-out mammalian cell lines with high efficiency. A number of different methods have been developed for the design and assembly of gene-specific ZFNs, making them easily accessible to researchers. In this study, we used ZFNs assembled through the CoDA (context-dependent assembly) platforms to generate mutant caprine fetal fibroblasts cells for the BLG gene. ZFN plasmid was introduced into the caprine fetal fibroblasts cell by electroporation. ZFN-induced cleavage of the target sequence was confirmed by the Surveyor nuclease assay analysis. Sequence analysis revealed that ZFN-induced mutations such as base insertion, deletion, or substitution were generated in the ZFN cleavage site of BLG. The simplicity and efficacy of CoDA will enable broad application of ZFN technology. This technique could be used with homologous arm, which may target foreign genes into the BLG locus at higher efficiency.

Keywords

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1. Introduction

Zinc finger nucleases (ZFNs) are artificial, hybrid restriction enzymes created by covalently linking a DNA-binding zinc finger (Zif) domain (composed of three to six finger-arrays) to the non-specific DNA cleavage domain (or simply FN) of the *Flavobacterium okeanokoites* bacterial restriction endonuclease-Fok I. ZFNs have recently become a powerful tool for genome modification since they are able to generate site-specific double-strand breaks (DSBs) [1]. Repair of DSBs provides the molecular basis for gene disruption, gene correction, or gene addition during ZFN-mediated genome editing [2]. Specifically, a given DSB may be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways [1]. HDR utilizes a homologous donor sequence as template for the conservative repair of the DNA break. Provision of a suitably designed donor DNA molecule can, therefore, specify gene addition or gene correction at the ZFN-induced DSB [3]-[5]. NHEJ, on the other hand, catalyzes the rejoining of the two DNA ends, a process that can result in the deletion or insertion of nucleotides at the repair junction. DNA repair via NHEJ is, therefore, mutagenic [6]-[9]. To date, ZFN-induced DNA repair via HDR or NHEJ has been utilized to target modifications to the genomes of numerous species [10].

There are some options for designing and assembling ZFN for specific genes. Ready to use expression plasmids can be purchased from commercial sources (e.g. ZFNs from Sigma-Aldrich) or assembled in the laboratory using publicly available resources, such as OPEN, CoDA, and modular-assembly for ZFNs. Selection assays for identifying zinc fingers that bind specific targets are laborious and challenging for non-specialist laboratories [10]. An alternative method, known as modular assembly, combines pre-selected zinc-finger modules into arrays. These ZFNs are relatively easy to generate but have low success rates [11], although significant progress has recently been made. Proprietary methods have also been used to generate ZFNs that are effective in organisms [5]-[9] [12], but these nucleases must be purchased and are expensive. While assembling ZFNs through OPEN and modular assembly are technically challenging and labor-intensive, the CoDA approach is straightforward and requires two straight-forward steps. The first is a computational analysis of the target gene to identify potential ZFP binding sites, and the second involves the synthesis and cloning of the ZFP coding sequences into appropriate expression vectors containing the heterodimeric Fok1 nuclease domain. These procedures can be completed in 1 to 2 weeks without the need for labor-intensive selection which can be moved directly into cells for testing as ZFNs. Therefore, in our study, we used the CoDA approach for designing ZFNs. *β*-lactoglobulin (BLG) is a major ruminant milk protein and is an ideal locus in mammary gland bioreactors for producing complex bioactive proteins. Yu *et al.* [13] had successfully applied ZFNs to knockout BLG in cattle with high effciency. In this study, we describe methods to analyze caprine BLG gene mutations in cultured cells introduced by expression of ZFNs.

2. Materials and Methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA), unless otherwise indicated.

2.2. Design and Assembly of CoDA ZFNs

ZFN plasmids were designed to bind and cleave intron 3 of the caprine *β*-lactoglobulin gene (Z33881.1), using the ZiFIT CoDA Targeter (http://zifit.partners.org/ZiFiT) [8] [14] [15]. The full binding site of the ZFN pair used is 4761-ACCCCCCTTCCCTTGCTGGGCAGTGT-4786, where the uppercase letters are the ZFN binding sites and the lowercase letters are where the double-strand break was created. DNA fragments encoding zinc-finger arrays (**Table 1**) were synthesized (shanghaishenggong Inc, Shanghai, China) and cloned into FokI EL/KK heterodimeric expression vectors by BamHI/XbaI (pMLM290 and pMLM292). Each ZFN contained three zinc fingers. Plasmids were obtained from the non-profit plasmid repository Addgene.

2.3. Preparation of Cells and Culture Conditions

Fetal fibroblasts were obtained from 35-day-old fetuses that were produced by mating nanjiang yellow goats. The head, viscera and bones of each fetus were removed and washed three times using Dulbecco's phosphate buffered saline (D-PBS). The washed tissue was finely chopped into fragments smaller than 1 mm in size, and

Table 1. Zinc fingers used in ZFN (CoDA source)

Finger	Helix	Triplet	Reference Number
Left F1	RKHHLGR	GGG	C12-211
Left F2	RREHLVR	GGG	C12-211
Left F3	QDGNLGR	GAA	C23-212
Right F1	RSHILTN	GTG	C12-103
Right F2	QSTTLKR	GCA	C12-103
Right F3	RSDHLSL	TGG	C23-106

the tissue fragments were placed in 35-mm culture dishes (Falcon, BD, USA) and incubated for 30 min in a humidified incubator at 38.5°C with 5% CO_2. After confirming that the tissue fragments adhered to the culture dish, a high-glucose Dulbecco's modified Eagle medium (HyClone, Beijing, China) containing 10% FCS (HyClone, Beijing, China) was gently added. After 2 - 3 days, the tissue fragments were removed, and the cells adhering to the bottom of the culture dish were cultured. After two to three passages, the cells were stored frozen based on conventional methods.

2.4. ZFNs Plasmid Transfection

For ZFNs transfection, the cells were seeded at 3×10^5 cells per well in 24-well dishes. The next day, cells were transfected with 15 µg of each ZFN expression plasmid (in pairs) by electrotransfection. For the mutation analysis, 24 hours post-transfection, cells were divided into two groups. One group was the mixed cells (10^6) and the total DNA was extracted for PCR and sequenced. The other group (10^4) was to form single colonies by limited dilution (1 cell per well on average). The fetal fibroblasts were cultured to 70% - 90% confluency (after 10 - 14 d culture), then washed twice with D-PBS (-) and treated with 0.05% trypsin-EDTA to isolate and collect the cells. In this study, ZFNs plasmid transfer experiment was repeated at least three times.

2.5. Surveyor Nuclease Assay (Mutation Detection Assay)

ZFN-induced mutations were detected using the SURVEYOR Mutation Detection Kit (Transgenomic, Omaha, USA) based on the manufacturer's protocol. The assay detects non-homologous end-joining (NHEJ)-mediated imperfect repair of ZFN-induced double-strand breaks (DSBs) by digesting hetero duplexes consisting of wild-type and mutant DNA with Surveyor nuclease, a mismatch DNA-specific cleavage enzyme [13]. Briefly, 24 h after electroporation, genomic DNA was extracted from the ZFN-treated and control cells using a DNA extraction kit (Shanghai Ruifeng Agro-tech, Shanghai, China). 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, Dalian, China), a high-fidelity enzyme, under the following conditions: 95°C for 1 min, followed by 95°C for 30 s, 65°C for 30 s, and 72°C for 1 min for 30 cycles. The sequences of the PCR primers used are as follows: 5'-TCGTCCTAAATCCG AGATGAGAAAG-3' and 5'-CTTCCTCTTCCCTGCTGC CACTGCC-3'. The cell-derived and the control cell-derived amplicons were obtained by PCR and, along with a mixture of both, 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. This assay was performed in all three ZFNs transfection experiments.

2.6. Analysis of ZFN-Induced Mutations by DNA Sequencing

Genomic DNA was extracted from single clone cells (cloned from ZFN-treated cells) and mixed cells, and used as a template for PCR to amplify the region recognized and cleaved by the ZFNs. The amplification products from mixed cells were cloned into pGEM-T Easy Vector (Promega, Madison, USA). Plasmids were extracted from the resultant *E. coli* colonies for DNA sequencing.

3. Results and Discussion

Traditionally, creating a gene knockout animal is solely dependent on the availability of embryonic stem cell lines that have only been established in mouse and rat [1]. The low efficiency of gene targeting in cultured somatic cells is the main barrier for gene targeting in large animals. Recently, zinc-finger nuclease (ZFN) technology has provided powerful tools for editing genomes of any animals [10]. The success of ZFN technology for gene targeting in many organisms (e.g. insects, amphibians, plants, nematodes), and several mammals (e.g. humans) encouraged us to establish a high-efficiency gene-targeting platform in large animals such as goats. The CoDA method for designing a pair of ZFNs in this report provide a rapid, highly effective, and publicly available platform for engineering zinc-finger arrays [7] [14]-[16]. To mutate the BLG gene (Z33881.1) in goat, we designed one pair of ZFNs that targets the caprine BLG gene using the CoDA method. When the coding sequence of BLG was analyzed by the ZiFIT CoDA Targeter with parameter of 5 - 6 spacers, the targeted sequence of all ZFNs were located in the introns. Since our later aim was to obtained transgenic cell, which foreign gene were knocked in at BLG locus by homologous recombination co-transfection with ZFNs, the ZFNs for intron 3 but not for exon were choosed and used for gene knocked-in. To test the activity of the pair of ZFNs, we evaluated each for its ability to bind to its cognate DNA target site using a well-established bacterial two-hybrid (B2H) reporter assay (data not show). The two ZFAs for the intron 3 target sites functioned effectively as ZFNs and were further subjected for testing in caprine fetal fibroblast cells. Fetal fibroblasts were transfected with the ZFN plasmid. A surveyor nuclease assay was performed on mixed 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 BLG target sequence, fragments of approximately 378 bp and 157 bp would be produced by digestion using the Surveyor nuclease. In agreement with this expectation, two bands of approximately 378 bp and 157 bp were detected in the ZFN-treated cell-derived amplicon (**Figure 1**). By contrast, no bands were detected near 535 bp in the amplicon from control cells. 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 confirmed that ZFNs with CoDA method can induce cleavage and mutation in the target sequences. CEL I assay revealed 3.14% ZFN activity (**Figure 1**), indicating 3.14% mutated alleles in the pooled cell population. Similar results were obtained in all of the three plasmid transfer experiments.

Functional genomics, the use of gene knock-out or knock-in techniques, is severely limited by methods for rapid targeting and disrupting of a gene of interest. Early approaches to somatic cell gene disruption used genome-wide non-targeted methods, including ionizing radiation and chemical-induced mutagenesis, whereas more recent methods used targeted homologous recombination (HR). However, the >1000-fold lower frequency of the targeted HR event relative to random integration in most mammalian cell lines (beyond mouse ES cells) can necessitate screening thousands of clones and take several months to identify a biallelic targeted gene knockout. Strategies including positive and negative marker selection and promoter-trap can considerably boost efficiencies, although these approaches present their own technical challenges and are not always successful in achieving high efficiency targeting [3]. Although advances with adeno-associated viral delivery strategies continue to improve knockout efficiency, the frequency is still very low and the time required to achieve biallelic
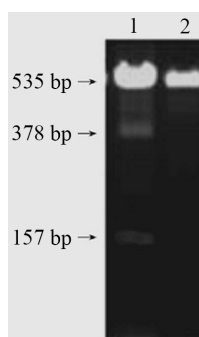
Figure 1. Confirmation of ZFN-induced mutations by the Surveyor nuclease assay. 1. Amplicons from ZFN-treated cells, a 535-bp PCR product was cleaved into two fragments: 378 bp and 157 bp; 2. Control cells.

gene knockout remains a barrier to its routine adoption [10].

The use of ZFN can greatly facilitate the mutation or integration of a transgene at a precise genomic locus [2]. In order to select mutated cells, 264 isolates cloning were analyzed via direct sequencing of PCR products of the genomic target locus. The presence of double peaks after the targeting site in the sequencing curves clearly distinguishes mutants from non-targeted cells (**Figure 2**). Various mutations were identified in nine of the 264 clones (98 from fibroblast cells and 166 from *E. coli* clones; 3.4% efficiency) (**Figure 3**), as follows: 5 bp deletion in clones 1-A4 and 1-C4, 2 bp substitution in clones 1-H5 and 4-C6, 1 bp substitution + 2 bp deletion (clone 7-E2), 2 bp deletion (clone 3-F10), and 3 bp insertion (clone 3-C9). Two were found to be homozygous mutations, with the remaining seven clones being heterozygous. All mutations were centered on the ZFN cleavage site, and were consistent with intron 3. The types of mutation recovered were consistent with those already reported, such as deletions, substitutions and insertions [8] [9] [13] [17]-[20]. To test whether ZFN plasmid randomly integrated into the goat genome, PCR was performed to examine ZFN plasmid integration by amplifying CMV-FokI catalytic domain from all mutant clones. The desired sequence was not found in nine clones that were screened (data not shown). For further proof-of-concept that the introduction of foreign genes in the BLG locus with ZFNs for nuclear donor cells to produce transgenic goats, different lengths of homologous arms would be prepared for cotransfection.

ACCCCCCTTCCCTTGCTGGGCAGTGT
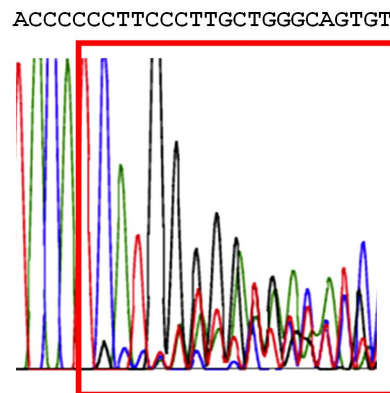
Figure 2. DNA sequencing revealed the mutation at ZFN targeted region. To detect mutations at ZFN targeted region in a single-cell colony, the PCR products of a single-cell colony were directly sequenced. If the colony is a mutant clone, the peak after the ZFNs cutting site should be double peaks (red) box.

Insertion

Clone:

3-C9	4761-ACCCCCCTTCCC**ACG**TTGCTGGGCAGTGT-4786
WT	4761-ACCCCCCTTCCC...TTGCTGGGCAGTGT-4786

Deletions, substitutions, complex:

1-A4, 1-C4	4761-ACCCCC-----CTTGCTGGGCAGTGT-4786
3-F10	4761-ACCCCCCTTCCCTTGC--GGCAGTGT-4786
7-E2	4761-ACC--CCTT**T**CCTTGCTGGGCAGTGT-4786
1-H5	4761-ACCCCCCTTCC**AG**TGCTGGGCAGTGT-4786
4-C6	4761-ACCCCCCTTCCTT**AT**TGGGCAGTGT-4786
WT	4761-ACCCCCCTTCCCTTGCTGGGCAGTGT-4786

Figure 3. ZFN-induced mutations in clones of cells. WT indicates the wild-type (non-mutated) Sequence; Substituted bases and inserted bases are indicated by black; Deleted bases are indicated by "–".

4. Conclusion

This study demonstrates that CoDA provides an effective alternative method for using publicly available reagents to engineer ZFNs. Therefore, this strategy could be designed against an endogenous genomic locus with high efficiency. Creation of goats with targeted gene disruption or knock-in has been hampered by the inefficiencies of relying on homologous recombination to create a single disrupted allele. The results presented here suggest that ZFNs are promising approach for specific gene editing in goat and can be used in combination with an appropriately structured donor DNA for targeted transgene integration into the BLG locus for expression of foreign proteins.

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Approved

Our study is approved by ethics committee of Yangzhou university.

Conflict

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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