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Expression, purification and characterization of zinc-finger nuclease to knockout the goat beta-lactoglobulin gene

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ABSTRACT

Engineered zinc-finger nucleases (ZFNs) have been widely used for precise genome editing. ZFNs can induce DNA double-strand breaks at specific genomic locations and drive the introduction of an insertion or deletion of base pairs at the targeted region, consequently resulting in a loss-of-function mutation. In this study, we investigated the cloning, expression and purification of ZFN fusion proteins targeting the goat beta-lactoglobulin (BLG) gene and detected the cleavage activities of these ZFN proteins in vitro and in cells, respectively. The results showed that the pET-BLG-LFN and pET-BLG-RFN prokaryotic expression plasmids can be constructed correctly and expressed efficiently in *Escherichia coli* BL21 (DE3) cells to produce the 6× His-tagged ZFN proteins that can be purified by Ni-IDA-Sefinose Column. The predetermined sequence of BLG can be recognized and excised both in vitro and in goat fibroblasts by the purified ZFN fusion proteins, which demonstrated that the purified ZFN fusion proteins can be used as gene modification tools to knock out the BLG gene. Furthermore, these results lay the foundation for eliminating allergen BLG from goat milk and improving the quality of goat milk products.

Introduction

Goat milk is the primary source of nutrition for humans second only to cow milk. However, some infants are allergic to goat milk products in the first year of life [1]. Beta lactoglobulin (BLG),[1] which is a major whey protein of ruminant milk but absent in human milk [2], is considered an allergen for infants [3–5]. The milk allergen has been reduced through several technologies, including heat treatment, fermentation, hydrolyzed protein desensitization, glycation, and RNAi [6,7]. However, these approaches cannot solve this problem thoroughly. By contrast, Gene targeting can work as a direct and complete method to reduce and even eliminate the allergen BLG from goat milk without any change in the other milk components. Traditional gene targeting technology is inefficient and time consuming. The appearance and development of engineered chimeric nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9, significantly improve the efficiency of gene targeting [8].

ZFNs, the efficient site-specific gene modification tools, are created by fusing both the recognition motif Cys_2His_2 zinc fingers and the cleavage domain type IIS restriction enzyme FokI [9–11]. The FokI cleavage domain must dimerize to cut DNA, so a monomer does not work at single binding site. It needs to construct two sets of Cys_2His_2 zinc fingers directed to neighboring sequences and joined each to a monomeric cleavage domain [12,13]. Only both the combined left and right ZFNs can induce site-specific double-strand breaks (DSBs) [8] and trigger cellular repair mechanisms involving either error-prone non-homologous end joining (NHEJ) [14,15] or homology-directed repair (HDR) [16]. Therefore, BLG knockout with ZFNs is an appropriate technology to solve the problem of milk allergy.

Although ZFNs have been successfully applied for gene disruption or addition in mouse [17,18], porcine [15] and human cells [19,20] by traditional transfection methods and mRNA delivery systems, they are limited by high random insertion and off-target cleavage [21]. These limitations and defects can be avoided by

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[1] *Abbreviations used:* DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; LB, Luria-Bertani; His, histidine; IPTG, isopropyl-β-D-1-thiogalactopyranoside; SDS–PAGE, sodium dodecyl-sulfate–polyacrylamide gel electrophoresis; ZFNs, Zinc-finger nucleases; DSBs, DNA double-strand breaks; BLG, beta lactoglobulin; NLS, nuclear localization signal; FokI, type IIS restriction enzyme; *E. coli* BL21 (DE3), *Escherichia coli* BL21 (DE3); NHEJ, non-homologous end joining; HDR, homology-directed repair; mRNA, messenger ribonucleic acid; RNAi, RNA interference; TALEN, transcription activator-like effector nuclease; CRISPR/Cas9, clustered regulatory interspaced short palindromic repeat/Cas 9; DTT, DL-Dithiothreitol; PMSF, phenyl-methylsulfonyl-fluoride; DMEM, Dulbecco's Modified Eagle Media; SCNT, somatic cell nuclear transfer.

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the method of direct delivery of ZFNs as proteins. Compared with plasmid delivery systems, direct delivery of site-specific nuclease proteins has more advantages, including no risk of insertional mutagenesis, reduced toxicity and few off-target cleavage events [22]. Previous studies showed that direct delivery of ZFNs or TALENs proteins yielded highly efficient gene knockout in various mammalian cell types [22,23].

In this research, we purified ZFN proteins targeting the goat BLG gene from *Escherichia coli* by using Ni-IDA-Sefinose column and assayed their cutting efficiency and specificity in vitro and in cells. Finally we investigated the feasibility of using direct protein delivery of ZFNs to knock out the BLG gene.

Materials and methods

Construction of the expression vector

Two prokaryotic expression constructs pET-BLG-L/RFN were generated to express a pair of ZFNs targeting goat BLG gene (GenBank: Z33881). The left (L) and right (R) zinc fingers that were flanked by N-terminal nuclear localization signal (NLS) [24] and C-terminal specialized FokI cleavage domain were PCR amplified from their respective templates pZFN3 and pZFN4 with the primers 5′EcoRI-NLS and 3′FoKI-XhoI listed in Table 1. The respective templates pZFN3 and pZFN4 and their sequences were engineered and customized by the CompoZr ZFN Operations Group at Sigma–Aldrich Biotechnology (Fig. 1). The ZFNs used in this study are available through Sigma–Aldrich (St. Louis, MO; lot number: 01051223MN). PCR products were digested with EcoRI and XhoI (TaKaRa, Dalian, China), then recovered from agarose gel by the DNA purification kit (Tiangen, Beijing, China) and finally cloned into the EcoRI and XhoI restriction sites of the pET-32a (+) expression vector (Novagen, Madison, USA) to generate the plasmids pET-BLG-LFN and pET-BLG-RFN. Sequencing analysis (Sangon, shanghai, China) was conducted to verify the construction of pET-BLG-L/RFN (Fig. S1).

Table 1
Primer sequences used in this study.

Primer name	Sequences (5′–3′)
5′EcoRI-NLS	**CCG**GAATTCCCCAAGAAGAAGAGGAAGGT
3′FoKI-XhoI	AACAGATGGCTGGCAACTAG
BLG5-F	GCATTCCCACTGCTCCTG
BLG3-R	**CC**ATCGATTTCAGCTCCTCCACGTACACT
SNA-F	TCGTAGAGGAAGCCACCCCG
SNA-R	CACACGTGGCAACATTTGGATCTT

Notes: Letters underlined means restriction sites, bolded means protective base. *F* means sense primer, while *R* means anti-sense primer.

Expression of ZFN fusion proteins

The expression of ZFN fusion proteins was performed according to the method described by Thomas Gaj and Rodriguez [25,26] with some modifications. The ZFN prokaryotic expression plasmids pET-BLG-L/RFN were transformed into BL21 (DE3) respectively and the transformed BL21 (DE3) were incubated at 37 °C for 12–16 h to form single colonies. One of the grown single colonies was chosen to grow overnight in 5 mL LB medium containing 100 μg/mL ampicillin at 37 °C. 100 μL of the overnight inoculant cultures continuously grown in 50 mL LB media supplemented with 100 μM $ZnCl_2$, 100 μg/mL ampicillin, 100 μg/mL chloramphenicol and 0.2% glucose until the OD600 reached 0.8. In order to confirm optimum conditions of the ZFN proteins expression, induction

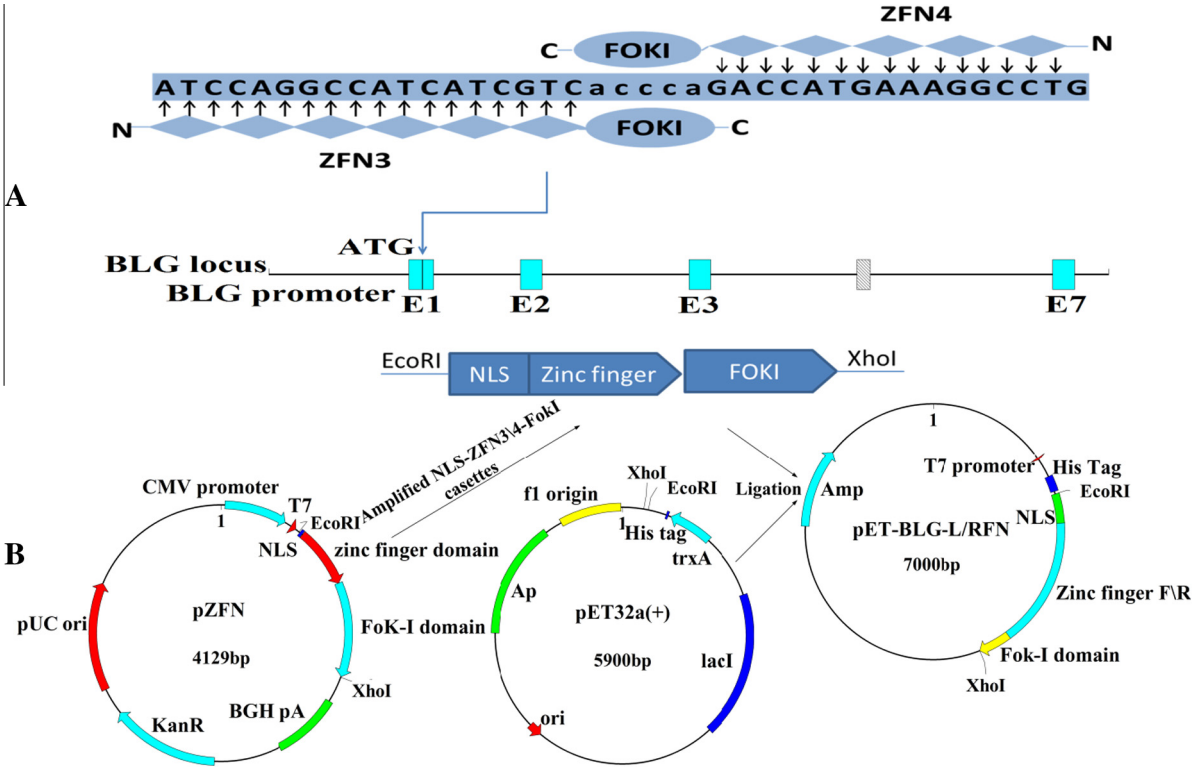

Fig. 1. Schematic overview depicting the binding sites of the left and right ZFNs and the construction of the prokaryotic expression vectors pET-BLG-L/RFN. (A) Cartoons of one pair of ZFNs binding at specific genomic sites. The binding sites of the left and right ZFNs are located in the Exon 1 of BLG gene. The DNA sequence of the primary binding site for each ZFN is uppercase; the cut sites cleaved by dimerization of the FokI nuclease domains are lowercase. (B) The flow chart of pET-BLG-L/RFN construction. Clone the NLS-ZFN3/4-FoKI cassettes by PCR with their respective mammalian expression vectors as templates. Then, doubly digested the amplicon with EcoRI and XhoI and inserted it into the prokaryotic expression vector pET 32a (+) which was cut with the same endonucleases.