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Production of microhomologous-mediated site-specific integrated *LacS* gene cow using TALENs

Xiaohu Su [a], Shenyuan Wang [b], Guanghua Su [c], Zhong Zheng [c], Jiaqi Zhang [d], Yunlong Ma [b], Zongzheng Liu [e], Huanmin Zhou [b], Yanru Zhang [b, **], Li Zhang [c, *]

[a] *Key Laboratory of Gene Engineering of the Ministry of Education, Guangzhou Key Laboratory of Healthy Aging Research and State Key Laboratory of Biocontrol, SYSU-BCM JointResearch Center, School of Life Sciences, Sun Yat-sen University, Guangzhou, 510275, PR China*
[b] *Key Laboratory of Biological Manufacturing of Inner Mongolia Autonomous Region, College of Life Sciences, Inner Mongolia Agricultural University, Hohhot, Inner Mongolia Autonomous Region, 010018, PR China*
[c] *The State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock, Inner Mongolia University, Hohhot, Inner Mongolia Autonomous Region, 010018, PR China*
[d] *State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China*
[e] *Qingdao Animal Husbandry and Veterinary Research Institution, Qingdao, ShanDong, 266100, PR China*

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ABSTRACT

Gene editing tools (Zinc-Finger Nucleases, ZFN; Transcription Activator-Like Effector Nucleases, TALEN; and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas)9, CRISPR-Cas9) provide us with a powerful means of performing genetic engineering procedures. A combinational approach that utilizes both somatic cell nuclear transfer (SCNT) and somatic cell gene editing facilitates the generation of genetically engineered animals. However, the associated research has utilized markers and/or selected genes, which constitute a potential threat to biosafety. Microhomologous-mediated end-joining (MMEJ) has showed the utilization of micro-homologous arms (5–25 bp) can mediate exogenous gene insertion. Dairy milk is a major source of nutrition worldwide. However, most people are not capable of optimally utilizing the nutrition in milk because of lactose intolerance. *Sulfolobus solfataricus* β-glycosidase (LacS) is a lactase derived from the extreme thermophilic archaeon *Sulfolobus solfataricus*. Our finally aim was to site-specific integrated *LacS* gene into cow's genome through TALEN-mediated MMEJ and produce low-lactose cow. Firstly, we constructed TALENs vectors which target to the cow's β-casein locus and *LacS* gene expression vector which contain TALEN reorganization sequence and micro-homologous arms. Then we co-transfected these vectors into fetal derived skin fibroblasts and cultured as monoclone. Positive cell clones were screened using 3′ junction PCR amplification and sequencing analysis. The positive cells were used as donors for SCNT and embryo transfer (ET). Lastly, we detected the genotype through PCR of blood genomic DNA. This resulted in a *LacS* knock-in rate of 0.8% in TALEN-treated cattle fetal fibroblasts. The blastocyst rate of SCNT embryo was 27%. The 3 months pregnancy rate was 20%. Finally, we obtained 1 newborn cow (5%) and verified its genotype. We obtained 1 site-specific marker-free *LacS* transgenic cow. It provides a basis to solve lactose intolerance by gene engineering breeding. This study also provides us with a new strategy to facilitate gene knock-ins in livestock using techniques that exhibit improved biosafety and intuitive methodologies.

1. Introduction

Livestock such as cattle, sheep, and pigs have been domesticated for thousands of years. Traditionally, cross breeding has represented the predominant method to improve performance in livestock. However, following the development of gene editing technologies, molecular breeding strategies now offer a realistic

* Corresponding author.
** Corresponding author.
E-mail addresses: yanru1964@163.com (Y. Zhang), zhanglinmg@aliyun.com (L. Zhang).

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approach to optimize livestock performance. Compared with traditional breeding approaches, molecular breeding can result in a significant reduction in the time required to generate the desired outcomes. TALEN is a programmable nuclease that contains a *Fok*I nuclease domain and a DNA-binding domain known as "transcription activator-like effector"; the latter is derived from plant pathogenic *Xanthomonas spp* [1]. TALEN facilitates the induction of a site-specific double-strand break (DSB) and results in the occurrence of gene mutations when the DSBs are repaired erroneously by non-homologous end-joining (NHEJ). This phenomenon permits the incorporation of indels (insertions and/or deletions) into the host genome [2,3]. Furthermore, DSB occurrence facilitates the improved integration of exogenous donor DNA into the genome [4], thereby promoting improved transgenic efficiency. Human induced pluripotent stem cell (iPSC) and neural stem cell (NSC) lines have already been transgenically modified using TALENs [5]. Furthermore, livestock gene editing studies have allowed scientists to generate gene knock-out and knock-in cattle using TALENs [1,6,7].

Dairy milk, especially cows' milk, is a major source of nutrition worldwide, supplying essential proteins, energy, vitamins, trace elements, and other nutritional elements. However, most people are not capable of optimally utilizing the nutrition in milk because of lactose intolerance. Lactose intolerance due to primary adult hypolactasia is a frequent condition in the world. Its prevalence differs among ethnic groups, ranging from 2% in Northern Europeans to almost 100% in Asians [8]. In Brazil, the reported prevalence varies from 45%–85%, and Oriental and Afro-descendants are more affected than other ethnic groups [9]. This condition results in a variety of symptoms, such as abdominal pain, flatulence, bloating, borborygmus and osmotic diarrhea, caused by the breakdown of non-digested lactose by the gut microflora [10]. The management of lactose intolerance is basically through dietary restriction of dairy products. In addition, intolerance symptoms can be alleviated with the intake of lactase enzyme supplements together with lactose-containing foods. However, these effects are temporary and depend on lactase preparation and dosage [11].

Sulfolobus solfataricus β-glycosidase is a lactase derived from the extreme thermophilic archaeon *Sulfolobus solfataricus*. It is a thermostable tetrameric protein with a molecular mass of 240 kDa, which is stable in the presence of detergents and has a maximal activity at temperatures greater than 95 °C [12]. Recombinant hyperthermostable β-glycosidase from archaeon *Sulfolobus solfataricus* presented continuous conversion of lactose in different media, including whey and milk [13]. The objective of this study was to integrate the *LacS* gene into the bovine genome using TALENs; a technique that facilitates MMEJ-dependent site-specific knock-in. This method does not require the use of selectable markers or vector backbones, thereby relatively improving biosafety. The promoter (associated with the target gene) is the β-casein promoter, which is activated during lactation and specifically expresses in bovine mammary glands. The obtained milk can hydrolyze lactose achieved by simple heating maybe. This strategy permits the safe and economical production of milk for lactose intolerant patients.

2. Material and methods

2.1. Experimental design

Firstly, we constructed TALEN vector and *LacS* gene vector. Then we transfected them into bovine fetal fibroblasts and cultured as monoclone. After PCR screening, the positive monoclone was used as donor to SCNT. Then the blastocysts were transferred to synchronized recipient heifers. At last, the newborn cow was genotyped by PCR.

All chemicals and culture media used in this study were of cell culture grade and obtained from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise indicated. The plastics that were used were obtained from Nunc (Roskilde, Denmark). PCR related reagents were purchased from TAKARA, and the genomic DNA kit was purchased from TIANGEN Biotech Co. LTD.

2.2. Ethics statement

The study was approved by the animal research ethics board of Inner Mongolia Agricultural University (China), and the return of the survey was considered consensual with respect to participation. Bovine ovaries from slaughtered mature cows were collected from North Asia Muslim Abattoir (Hohhot, China). A Holstein fetus was used to obtain nucleus donor cell cultures and Simmental cows were used as recipient animals (Fengzhen Yongmin livestock Co., Ltd., P. R. China).

2.3. Bovine fetal fibroblast culture

The bovine fetal fibroblast culture was performed as detailed in Bressan et al. [14].

Holstein cattle that were two months into pregnancy were used during this study. Briefly, after the fetus was removed by cesarean section, the skin tissues were cut up and plated into 25 cm^2cell culture bottles. Four hours after the tissues were cultured using standard conditions (37 °C, 5% CO_2, and saturated humidity), 5 ml of DMEM/F12 medium was supplemented with 15% (vol/vol) FBS and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively) were added. Upon reaching 90% confluence, the cells were digested with 0.25% trypsin/EDTA and subcultured and/or frozen. The ratio used for subculture was 1:4. The frozen medium contained DMEM/F12: FBS: DMSO = 7:2:1 (vol).

2.4. TALENs and donor DNA constructs

TALENs plasmid constructs were generated according to the Viewsolid™ Tale construction kit (VK006-02) manufacturer's protocol [15]. The *LacS* gene and the β-casein promoter donor DNA were generated in our laboratory. Briefly, the donor DNA primers were annealed to the TALENs recognition sequence resulting in the generation of 8-bp micro-homology arms at the 5′-end. This construct was subsequently ligated to the pMD19-T clone vector. The sequence of TALENs recognition region and *LacS* gene primers was showed in Table 1.

Table 1
Primers and TANEL design sites.

Primer name	Primer sequence
P1	GCCCTTGCAAGAGAGGTAAAT
P2	GACACAGCTAAACCTAGCACT
P3	GGAGAAATCACAAGAGGCAATG
P4	TCACGGGTGGCACAGAGT
LacS f	TCTGAGTCTAGTGCTTTCTGTACCATTATAGTATCTGT ACCATCAATCCCTACAATACATGACCCAGATTC
LacS r	TAGGGATTGATGGTACAGATACTATAATGGTACAG AAAGCACTAGACTCAGATGATGAGTTTGGACAAACC
	TALEN target sequence
Tale1	TCATCCACTATCTCAG tagtatcctatggg ACCACAAGTCTGAGTCT
Tale2	CTGAGTCTAGTGCTTTC tatagtattgtaccat CTGTACCATCAATCCCT

*The capital letter were TALEN reorganization region. The small letter were TALEN spacer region.