Production of hornless dairy cattle from genome-edited blastocysts

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Abstract. Cattle of polled phenotype is convenient for breeders, as it decreases the risk of animals being hurt and ensures safety of workers. We developed the system for editing cattle genome using CRISPR/Cas9 which will allow production of animals with polled phenotype genetically based on any cattle breed without changing its main phenotypic traits.

1 Introduction

Cattle of polled phenotype is convenient for breeders, as it decreases the risk of animals being hurt and ensures safety of workers. It was shown that several existing in nature autosomal dominant genetic variants can result in polled phenotype. Replacement of a sequence of 10bp with duplicated adjacent sequence of 212bp in non-coding region of chromosome 1 was discovered in Alpine and Scottish cattle [1]. In 2013, Tan et al, using TALEN approach, edited genome of bovine fibroblasts in vitro and reproduced this genotype [2]. After that, transgene animals with the corresponding phenotype were created using nuclear transfer from fibroblasts to oocytes [3]. Later, these animals naturally produced offspring which inherited the phenotype [4]. However, transferring of the polled phenotype to the genetic basis of other cattle breeds might take a lot of time and result in disappearance of other traits important for the breed. In this work we propose a method of editing cattle genome using CRISPR/Cas9 for obtaining genetic variants of polled cattle. This method allows producing of polled animals genetically based on any cattle breed without changing any of the phenotypic traits of the breed [5].

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2 Materials and methods

Guide RNA cloning was performed in the px330 vector (Addgene, <https://www.addgene.org/crispr/zhang/>) in accordance with the manufacturer's instructions. The vector was cut using BbsI-HF restrictase (New England Biolabs), dephosphorylated using FastAP enzyme (Thermofisher Scientific), then purified in agarose gel and then isolated using the Monarch DNA Gel Extraction Kit. Oligonucleotides containing a sequence of guide RNAs were synthesized by Eurogen.

5' – CACCGNNNNNNNNNNNNNNNNNNNNNN – 3'
3' – CNNNNNNNNNNNNNNNNNNNNNCAAA – 5'

Scheme of oligonucleotide synthesis for cloning guide RNA. Added nucleotides to complement the sticky ends formed when cutting the px330 vector with BbsI-HF restrictase [6, 7].

The oligonucleotides were phosphorylated using the PNK kinase enzyme (Thermofisher Scientific), annealed with each other, and ligated with the previously prepared vector and using the T4 DNA ligase enzyme (Thermofisher Scientific). The ligase mixture was transformed into competent cells of the XL1blue strain (Eurogene). The colonies were screened using Isogen PCR kits. Two colonies each containing sgRNA were grown in the night culture and isolated using the Monarch Plasmid Miniprep Kit. Sequencing of the obtained plasmids was performed using the U6-forv primer by Eurogen. The sequencing analysis was performed using SnapGene software.

The research is executed at the expense of a grant of the Russian scientific fund (project No. 19-76-10022).

3 Results

Polled phenotype will be achieved by replacement on Bos Taurus chromosome 1 of a sequence of 10bp with duplicated adjacent sequence of 212bp. Guided Cas9 will cleave genomic DNA upstream and downstream of 10bp sequence. A plasmid containing sequence corresponding to the polled phenotype with mutations destroying gRNA's PAM-sites will be used for homology directed repair (HDR) (Fig 1.) [9-11].

On-line tool <http://crispor.tefor.net/> was used for gRNA selection. We also had regard to distance from the 10bp sequence (Table 1.).

Fig. 1.Diagram of making polled allele into dairy cattle by genome editing.

Table 1.PotentialsgRNAs for introducingpolledalleleintodairycattle by genomeediting.

#guidel d	targetSeq	mit Spec Score	cfds pec Score	Offt arget count	Off-targets for 0-1-2-3-4 mismatches + next to PAM	note
	5' region					
215forw	<u>AGAAGGCGGCACTATCTTGA*TG</u> <u>G</u>	84	87	96	0-0-1-2-93 0-0-0-0-1	
183rev	TGCCGCCTTCTTGAAGTCCC*AGG	83	88	97	0-0-0-9-88 0-0-0-1-2	
140forw	TTCTGACTTTCTAATATTCG*AGG	82	87	147	0-0-2-12- 133 0-0-0-0-0	*
157forw	TCGAGGAATGCTTAGAAGTG*TGG	78	87	98	0-0-1-11- 86 0-0-1-2-2	
160rev	CACAAAGGACTATTTTCTAC*CGG	73	85	166	0-0-2-17- 147 0-0-1-2-2	*
186forw	GAAAATAGTCCTTTGTGCCT*GGG	72	84	162	0-0-4-16- 142 0-0-1-1-4	*
161forw	GGAATGCTTAGAAGTGTGGC*CGG	71	82	176	0-0-4-18- 154 0-0-2-1-2	
	3' region					
187forw	ACATGACTCACGATACATTC*TGG	95	97	46	0-0-0-1-45 0-0-0-0-0	*
188forw	CATGACTCACGATACATTCT*GGG	91	94	56	0-0-0-2-54 0-0-0-0-0	*
300rev	<u>GTGGGAAATCCCCACCCGCT*GG</u> <u>G</u>	87	93	77	0-0-1-2-74 0-0-0-0-2	
370forw	ATGGGGATATTAGTCAATGT*TGG	86	94	87	0-0-0-10- 77 0-0-0-0-2	
56forw	GTCTATCCCAAAAGTGTGGG*AGG	85	92	80	0-0-1-4-75 0-0-1-1-3	

*- inefficient. These gRNA contain a motif proven to lower the cleavage efficiency in CRISPR/Cas9 system [8]

We discovered 2 suitable sequences for gRNAs construction in the 5'-region of 10 bp sequence with nearly equal efficiency scores. However one of them had 1 potential off-target with as few as 3 mismatches located at the end of the sequence opposite the PAM-site. So we chose another one: AGAAGGCGGCACTATCTTGA*TTG, which, moreover, lies closer to the target sequence.

In 3'-region two sequences with highest MIT and CFD scores turned out to be unsuitable due to TT-motif next to PAM-site. This motif is reported to reduce Cas9 cleavage efficiency [8]. 3rd and 5th suggested sequences had equally high efficiency scores. 3rd had less off-target sequence whereas 5th lied ~250 bp closer to the target sequence. Finally we stopped our choice on the third one with fewer off-targets and with off-targets with more mismatches: GTGGGAAATCCCCACCCGCT*GGG

For chosen gRNA cloning we synthesized following oligos:

Sg_poll1_forv 5'-caccgGTGGGAAATCCCCACCCGCT-3';

Sg_poll1_rev 5'-aacAGCGGGTGGGGATTTCCCACc-3';

Sg_poll2_forv 5'-caccgAGAAGGCGGCACTATCTTGA-3';

Sg_poll2_rev 5'-aacTCAAGATAGTGCCGCCTTCTc-3'.

A

B

Fig.2.Sequence analysis. A. px330+sg_poll1 B. px330+sg_poll2

4 Conclusion

We developed the system for editing cattle genome using CRISPR/Cas9 which will allow production of animals with polled phenotype genetically based on any cattle breed without changing its main phenotypic traits.

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