Modifications of the beta-lactoglobulin gene in bovine and goats for correction of milk composition using CRISPR/Cas9 technology

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Abstract

New methods of genome editing open up promising opportunities for animal husbandry, including for correcting the composition of milk to improve its consumer qualities, as well as for obtaining recombinant biologically active proteins with milk. Beta-lactoglobulin (BLG) is the main serum protein in almost all mammals, except rodents and primates. Cow's milk is a complete food product containing proteins, fats, carbohydrates and minerals. But it often causes allergic reactions that seriously affect the absorption and use of nutrients in dairy products: the main allergen is β-lactoglobulin. Regulatory regions of the BLG gene in sheep, goats, and cattle are used as part of genetic constructs. The article provides a brief overview of successful experiments in the world practice on editing the beta-lactoglobulin gene in cattle and goats using genomic editing methods, in particular the CRISPR / Cas9 technology. The article reports on the creation of a ready-made set of crispr / Cas9 components in plasmid form for microinjection into the pronucleus of bovine zygotes in order to knock out the BLG gene and replace it with the gene of a biologically active target protein, as well as a well-thought-out strategy for analyzing its possible modifications.

A plasmid containing 5'-and 3' - arms of homology to the bovine BLG gene was created as a replacement DNA matrix. A fragment containing a biologically active protein gene can be embedded in the resulting plasmid at the site for EagI restriction enzyme at the junction of the arms of homology. The resulting DNA matrix is designed for site-specific integration by homologous recombination into the BLG gene using CRISPR/Cas9 technology. To select sites for double-stranded cuts (DSC), a polymorphic variant of the bull BLG gene was determined, whose sperm was used for fertilization of cow eggs in vitro. A strategy for making DSC in the BLG gene was developed and four pX330 plasmids encoding the CRISPR/Cas9 components were obtained. The pBLGcmvEGFP plasmid containing homology arms to the BLG gene and the green fluorescent protein gene under the cytomegalovirus promoter is intended for site-specific integration by homologous recombination into the BLG gene to evaluate the effectiveness of site-specific operation of the CRISPR/Cas9 system components under in vitro conditions.

Key words: genetic engineering; genetic constructs; homologous recombination; beta-lactoglobulin; cattle; CRISPR/Cas9.

Introduction

The search for and development of effective solutions to food security and health problems are inextricably linked to research in the field of genetic engineering. The possibility of high-precision molecular genetic manipulations in the near future should accelerate the development of therapeutic biomaterials for the prevention and treatment of human diseases; modifications to the genome of farm animals are necessary to increase the manufacture and production of human food with the desired properties (Tan et al., 2012; Bishop and Van Eenennaam, 2020).

Historically, the application of genetic engineering in animal husbandry has been a difficult task. To obtain genetically modified (GM) animals, the vast majority of studies used a very labor-intensive method of somatic cell nuclear transferring (SCNT). In the last decade significant advances have been made in the development of genome editing tools: ZFN (zinc-finger nucleases), TALEN (transcription activator-like effector nucleases) and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9). These tools are based on the generation of a double-stranded DNA break (DCR), followed by one of two repair pathways - non-homologous end joining (NHEJ) or homology directed repair (HDR). These tools are based on generating a double-stranded cut DNA followed by one of two repair pathways - non-homologous end joining (NHEJ) or homologous directed repair (HDR). Compared to traditional approaches, these tools significantly reduce the time and effort required to create a GM animal. Another advantage of using new genome editing tools is the ability to use direct injection (microinjection, MI) in developing embryos to induce target mutations, which avoids the disadvantages of SCNT. New genome editing technologies have significantly improved the efficiency of creating large GM animals that produce small offspring, for both biomedical and agricultural purposes (Larkina et al., 2018). Genomic editing technologies, especially variants of CRISPR technology, can make a real breakthrough to increase livestock production, in terms of increasing efficiency, reducing the impact of agriculture on the environment, enhancing pest control, and preserving animal health. The time of genome editing has already arrived, which requires a serious assessment of potential opportunities and threats, benefits and risks, ethical and scientific views, and legal regulation (Menchaca et al., 2020).

The processes associated with editing the genome of livestock are still time-consuming and currently ineffective. These are a number of stages and poorly predictable and reproducible biological variables: gamete collection and maturation, administration of editing reagents, cloning and transfer of embryos to synchronized surrogate mothers. Each stage has its own limitations. Microinjections of embryos often lead to mosaic offspring, and subsequent breeding to produce heterozygous genetically modified offspring is a long and expensive process, especially for large farm animals. However, new methods of genome editing offer promising opportunities for animal husbandry. Genome editing tools can be used both to clarify the function of genes and to identify the cause-and-effect relationships of the main productive indicators, and to accurately introduce useful genetic modification in livestock breeding programs with specified characteristics. Genetic modification may include the correction of genetic defects, the inactivation of undesirable genes, and the transfer of useful alleles of genes between breeds of farm animals. Gene editing can be a useful tool in the evolution of animal breeding. (Bishop, Van Eenennaam, 2020).

Cattle breeding is conducted in three directions – meat, mixed dairy-meat and dairy in order to obtain products of the desired category. The use of endonuclease technologies using the HDR mechanism is an effective method of changing the composition of milk by replacing the genes of endogenous milk proteins in farm animals in order to improve the consumer qualities of milk, using the animal's mammary gland as a bioreactor for the production of heterologous proteins (Shepelev et al., 2018). You can exclude any protein from the composition of milk, replace it with a protein of pharmacological or diagnostic purpose, and modify milk proteins in order to increase their nutritional value and digestibility, for processing into different dairy products or obtaining biologically active peptides from them as vaccines (Whitelaw et al., 2016).

Cow's milk is a complete food product containing proteins, fats, carbohydrates and minerals. But it often causes allergic reactions that seriously affect the absorption and use of nutrients in dairy products. An effective method for reducing the allergic reaction caused by milk has not yet been developed. The main milk allergen is the whey protein β-lactoglobulin (BLG). To reduce its allergenicity, various methods are used, but as a result of such processing of milk, the structure and functions of other milk proteins are disrupted, which significantly affects the nutritional value of milk. Knockout of the BLG gene using gene editing technology completely solves a problem of great importance for the creation of hypoallergenic dairy products. The BLG gene is a promising target both for its knockout in order to obtain hypoallergenic milk, and for replacing it with a heterologous protein gene when using endonuclease editors; precise embedding of the transgene in a gene structure containing homology arms to BLG will ensure its expression under the control of full-size endogenous regulatory sequences.

The concentration of β-lactoglobulin in cattle milk is on average 4.6 mg / ml. Regulatory regions of the BLG gene of sheep, goats, and cattle have long been used as part of gene constructions (GC) in the production of transgenic animals by the method of classical transgenesis (Trubitsina et al., 2018). For example, the expression of human alpha-1-antitrypsin in the milk of transgenic sheep reached 5 mg / ml when using the sheep BLG gene promoter as part of the gene design (Wright et al., 1991).

There are known works on directed modifications of the BLG gene in cattle using ZFN and TALEN technologies, but not using the CRISPR/Cas9 system (table 1). In the first GM cloned cow (SCNT) obtained in 2011 using the ZFN editing method, a biallel modification of the BLG gene was obtained with a small deletion without

reading frame failure and without the formation of functional knockout alleles (Yu et al., 2011). Later, the same method was used to obtain a cow that does not contain β-LH in milk (Sun et al., 2018).

In experiments on cattle zygotes, site-specific mutations of the BLG gene were shown to be highly effective using the ZFN method (30-80% of embryos). For precise modification, single-stranded DNA matrices with homology arms flanking the integrable sequence were used. Analysis of microinjected embryos showed the effectiveness of site-specific modifications up to 33% for ZFN and 46% for TALEN blastocysts. Sequencing showed that the modification of the target BLG allele can reach 100%; this means that by using the method of genomic editing with microinjection of cattle zygotes, it is possible to obtain a non-mosaic animal with pre-developed biallel modifications already in the F0 generation (Wei et al., 2015). In 2018, zygote microinjection using the TALEN editor produced a live bull and cow with a BLG gene knockout due to the purposeful deletion of nine nucleotides at the very beginning of BLG protein synthesis and the generated stop codon, which caused the gene knockout (Wei et al., 2018).

Using TALEN technology applied to fetal fibroblasts, a cattle transgenic by human serum albumin (HSA) with a knockout of the BLG gene was obtained. The concentration of HSA synthesized instead of endogenous β-lactoglobulin in the milk of mono-allelic cows reached 2.3 g / l, the protein had the correct folding. The HSA content in the milk of homozygous cows was significantly higher – 3.3-3.5 g/l (Luo et al., 2016). Goats were also obtained not only with the knockout of the BLG gene, but also with the genes of human milk proteins – lactoferrin integrated into its locus (Cui et al., 2015; Song et al., 2016) and alpha-lactalbumin (Zhu et al., 2016).

There have been no reports of receiving cattle with a modified BLG gene using the CRISPR/Cas9 system. BLG-knockout goats were created in 2017 by microinjecting zygotes with CRISPR/Cas9 components (Zhou et al., 2017).

The aim of this work was to create a gene construction containing a green fluorescent protein (EGFP) gene under a cytomegalovirus (CMV) promoter for integration by a homologous recombination mechanism into the bovine BLG locus during common microinjection with site-specific components of the CRISPR/Cas9 system. Precise embedding (substitution) of a transgen having relatively short (up to 1 kbp) homology arms to the BLG gene will ensure its operation under the control of full-size endogenous regulatory sequences. To achieve this goal, it was necessary to select the sequences of guide RNAs and develop a system of PCR analysis of possible variants of changes made for a specific allelic variant of BLG for its modifications (knockout of the gene by local indels or major deletion).

Materials and methods

All works were performed on the basis of All-Russian Research Institute of Physiology, Biochemistry and Animal Nutrition in the framework of the 2019 state task on the topic 0445-2019-0030. Sequence of the bovine β-lactoglobulin gene (Bos taurus) was taken from the GenBank database, entry X14710. Genomic DNA was extracted from the semen of a black-and-white bull named “Moroz” (Bykovo breeding farm) using the phenol-chloroform method. The selection of primers, construction of recombinant DNA, and restriction analysis were performed in the Vector NTI program.

We used enzymes and reagents: FastAP Thermosensitive Alkaline Phosphatase, EagI restrictases (analog − BseX31, SibEnzyme), BglII, BamHI, BsmBI with corresponding buffers. For intermediate cloning of PCR products, components of the InsTAclone PCR Cloning Kit (Thermo Scientific) were used. During PCR, a mixture of dNTP (2 mM) (Fermentas), Taq polymerase (5 u/mcl), 10×Taq buffer (with 25 mM $MgCl_2$), and Pfu-DNA polymerase (5 u/ mcl) (Silex) was used. The time and temperature parameters of the PCR were selected depending on the structure of the primers. The sequences and descriptions of the primers used to create the gene constructions are shown in table 2. PCR was performed on a DNA amplifier "Tertsik" ("DNA Technology LLC", Moscow).

The transformation of competent E. coli Dh5a and TG1 cells was performed using the method and reagents of the Transform-Aid Bacterial Transformation Kit. The transformed cells were seeded on a Luria-Bertani medium containing 100 micrograms / ml of ampicillin (Am+) and 1.5% agarose. The grown clones were reseed and isolated DNA for PCR analysis. A suitable clone was accumulated, and plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit with our modifications (Koloskova, Ezerskii 2019) and the classic method of alkaline lysis. The quality and quantity of DNA were evaluated visually in UV light after electrophoresis in agarose gel. DNA from agarose gel was isolated using the Gene JET Gel Extraction Kit. The concentration of plasmid DNA was determined after restriction (x1), by sequential aliquot dilution, by electrophoresis in agarose gel with ethidium bromide. As a standard, a linearized plasmid of known concentration was taken in similar dilutions. The size of the DNA fragments was estimated using the DNA Ladder Mix (Fermentas) as the standard.

RNA guides were selected using on-line CHOP CHOP programs <http://chopchop.cbu.uib.no/>, CRISPR direct <http://crispr.dbcls.jp/>, CRISPOR V. 4. 8. <http://crispor.tefor.net/>. A pX330-U6-Chimeric_BB-Cb-hSpCas9 (Addgene plasmid # 42230) plasmid was used to obtain the CRISPR/Cas9 components (Cong et al., 2013). Sequences of oligonucleotides and their description, sequences of primers for evaluating deletions of various sizes and and integration of the gene structure by homologous recombination, and their description are given in table 3. All primers and oligonucleotides, plasmid DNA sequences were ordered from “Syntol” company <http://www.syntol.ru>.

Table 1. Modification of the beta-lactoglobulin gene in cattle and goats resulting in edited offspring

Species	Cell type	Method	Modification / mechanism (DNA matrix)	Editor	Reference
Cattle	FFB, SCNT	NF	KO/ NHEJ	ZFN	Yu et al., 2011
	FFB, SCNT	NF	KO/ NHEJ	ZFN	Sun et al., 2018
	Zygotes	CPI	KO/ HDR (ssODN)	TALEN	Wei et al., 2018
Goats	FFB, SCNT	EP	KI, KO/ HDR (human lactoferrin gene at the locus of BLG)	TALEN	Cui et al., 2015
	FFB, SCNT	EP	KI, KO/ HDR (human lactoferrin gene at the locus of BLG)	TALEN	Song et al., 2016
	EFB, FFB, SCNT	EP	KI, KO/ HDR (human alpha lactalbumin gene at the locus of BLG)	TALEN	Zhu et al., 2016
	Zygotes	CPI	KO/ NHEJ	CRISPR/Cas9	Zhou et al., 2017

FFB - fetal fibroblasts; EFB – ear fibroblasts; SCNT - somatic cell nuclear transfer; NF – nucleofection; EP – electroporation; CPI– cytoplasmic injection; NHEJ - non-homologous end joining; HDR - homology directed repair; ssODN – single-stranded oligonucleotide; KO – knockout; KI (knock – in) - gene introduction; ZFN,TALEN, CRISPR/Cas9 - endonuclease methods of gene editing.

Table 2. Primers used to create the gene construction

Name	5'-3' sequence	Introduced site of the restriction
S5-F	gagctcgacctgaaccccat	-
S5-R	cctccaggggtaccaggaaa	-
S3-F	ggacgacgaggccctggagaaatt	-
S3-R	gctctccgcatcatgtggccaa	-
BLG51	gctctaga aaccccagggcccaaggata	XbaI
BLG52	aaagtcgaccggccg ccacctggggagggaccttgagct	SalI, EagI
BLG31	tacggccg agcagtgccacatctaggtgag	Eag I
BLG32	gaagtcgacagatct tgccgactccctacctggtt	SalI-BglII

Table 3. Oligonucleotides for creating CRISPR/Cas9 system components and primers for PCR analysis of modifications

Name	5'-3' sequence	Appointment
bg51f	caccggagatgtcgctggccgcca	pX330-51b
bg51r	aaactggcggccagcgacatctcc	
bg52f	caccgacttggtactccttggcca	pX330-52b
bg52r	aaactggccaaggagtaccaagtc	
bg31f	caccgatcctgacgctcccccgcca	
bg31r	aaactggcgggggagcgtcaggatc	pX330-31b
bg32f	caccggggagcgtcaggatgggac	pX330-32b
bg32r	aaacgtcccatcctgacgctcccc	
T5L	tccaaatgttgccacgtgtg	
T5R	ataagcagccttgggtggac	
T3L	ggccctggagaaattcgaca	
T3R	gaacccctaccccatgtgtg	
HA5F	cttggcctcgaatggaagaa	5'utr before 5'HA, for HDR
HA5R	tggaaagtccctattggcgt	5' end cmv for HDR
HA3F	ttgtctgagtaggtgtcatt	3' end bGH polyA for HDR
HA3R	gactgaacactccaagttaa	3'utr after 3'HA, for HDR

Table 4. Determination of the BLG gene polymorphism of the bull "Moroz"

	According to the Gladyr method	With our primers
Primers	primer1/ primer2	BLG51/S5-R
PCR amplificate, bp	1248	1195
PvuII for A-allele, bp	774, 474	743, 452
PvuII for B-allele, bp	774, 297, 177	155, 297, 743

Results and Discussion

Creating a pBLGcmvEGFP plasmid. Genotyping of the bovine beta-lactoglobulin (BLG) gene.

The BLG gene of cattle is located on the 10th chromosome, contains 4 exons and encodes a protein of 127 amino acids, of which 19 is a signal peptide. The scheme for selecting homology shoulders and fragments for sequencing is shown in figure 1.

The effectiveness of the CRISPR/Cas9 components depends on the quality of the sgRNA target sequences of possible target gene polymorphism in the region of potential double-stranded cuts. This is the ovaries of cattle selected in meat processing plants from cows of different breeds, but in experiments in vitro fertilization of eggs, as a rule, is

produced by the sperm of a single bull. PCR amplifications with genomic DNA of the bull "Moroz " 5' and 3' regions of the BLG gene (about 1.5 kbp each), made using pairs of S5-F/ S5-R and S3-F/ S3-R primers, were cloned into the pTZ57R/T plasmid vector in order to obtain DNA fragments for sequencing (Fig.1).

The classic method of genotyping the BLG gene in cattle is using the Medrano technique (Medrano and Aguilar-Cordova, 1990), which allows finding polymorphism in the 4th exon. Using the Gladyr technique (Gladyr, 2001), the region of the 1st exon and intron is analyzed, which allows us to determine the BLG gene polymorphism from the DNA of the bull "Moroz" using one of the pairs of our primers and PvuII restrictase (table 3).

Using a pair of BLG51/S5-R primers, PCR amplification was obtained, which after treatment with PvuII restriction endonuclease showed the formation of allele-specific fragments corresponding to the A variant (table 4). The sequence of cloned sequences of the BLG gene of the black-and-white bull "frost" showed an A-allele, while the records published in the GenBank database X14710 and Z48305 (B. taurus gene for beta-lactoglobulin variant B) correspond to the B-allele.

Creating a pTZHABLG matrix plasmid, pBLGcmvEGFP plasmid

Sequences for XbaI, EagI, and Eag I, BglII restrictases were introduced was introduced in the primers for PCR amplification of 5' and 3' arms homology (5'HA и 3'HA), respectively (table 2). The 5' HA fragment contained the 1st exon of the BLG gene with the signal peptide sequence, the 1st intron. PCR-amplification products were cloned into the pTZ57R/T vector with obtaining the intermediate plasmids pTZ5'HABLG and pTZ3'HABLG.

From the pTZ5'HABLG plasmid by XbaI and EagI restrictases, cut out the fragment-insert XbaI_5'HABLG_EagI and after cleaning cloned it into the prepared XbaI_pTZ3'HABLG_EagI acceptor plasmid. After ligation of the fragments, a pTZHABLG plasmid was obtained, which has a unique site for EagI restriction at the junction of the arms homology, which can be used for cloning the target protein's DNA (Fig.2, 3).

The genes of colored fluorescent proteins are often used as reporters in creating genetic constructs: protein expression in the case of integration of a transgene into the genome can serve as an indicator of transgenesis (Kato et al., 1999). It was assumed that the cmvEGFP sequence embedded in the genetic construction between the 5' and 3' arms of homology to the BLG gene, in the case of HDR using CRISPR/Cas9 technology, would provide in vitro expression of a green fluorescent protein, which would be an effective marker for evaluating the HDR event. From a previously created plasmid, pBLgLfcmvEGFP (Ezerskii & Shevchenko, 2008), a fragment of cmvEGFP-bGHpolyA was cut and purified by NotI restriction with the formation of EagI sticky ends. The insertion was cloned into a prepared pTZHABLG plasmid in the site-sequence of EagI. A pBLGcmvEGFP plasmid was obtained, which can be used as a DNA matrix in circular or linearized form (after treatment with restriction enzymes EcoRI and BglII) (Fig. 2).

Selecting target sites in the BLM gene of cattle, obtaining plasmids pX330-b51, pX330-b52, pX330-b31 and pX330-b32 encoding Cas9 and sgRNAs to the corresponding sites of the BLG gene.

For knockout by local indels or by deletion of a large fragment of the BLG gene, sgRNA sequences were selected using online programs and a PCR analysis system for possible gene modifications was developed. Target sequences for gRNAs for double-stranded cross-section in the 5'region of the BLG gene were selected in the region bounded by the BLG52 / S5-R primers: 120 nucleotides were taken for analysis after the 5' homology arm. This fragment is completely part of the 2nd exon. Target sequences in the 3'region of the BLG gene were selected in the region bounded by S3-F / BLG31 primers: 120 nucleotides were taken at the end of the 5th intron –the beginning of the 6th exon.

Oligonucleotides for sgRNAs production were processed according to the Protocol described in the article by Menzorov et al. (2016) with some modifications and cloned into the pX330 plasmid (tables 5, 6).

Cloning Protocol

1. Oligonucleotides (table. 2) diluted with water (deionized water, with a resistivity greater than 18 MOm * cm) to a concentration of 100 pM/µl.
2. To obtain a short double-stranded cut insert with sticky ends, the mixture was prepared in test tubes for PCR, incubated at 37^{o}C for 40-50 minutes, kept at 95^{0}C for 5 minutes and slowly cooled to room temperature.
3. For the reaction of restriction-ligation mixtures were prepared whose composition is given in table 6.
4. The reaction of the restriction-ligation was carried out in the PCR-thermocycler under the following conditions: 1) 37^{o}C (restriction) - 5 minutes; 2) 20^{o}C (ligation) – 5 minutes: a total of 38 cycles.
5. E. coli Dh5a cells transformed by ligase mixture were seeded on solid medium LB-Am+. Grown clones were re-seeded for PCR analysis, DNA from transformed clones was isolated using a lysing mixture with proteinase K. Compositions of PCR mixtures for screening pX330-based plasmids are shown in table 7. PCR amplification conditions: denaturation of 94^{o}C - 30 sec (in the first cycle – 2 min.), annealing of primers 62 ^{o}C – 30 sec., elongation of 72^{o}C – 1 min. (in the last cycle-3 min); a total of 27 cycles.
6. A suitable clone was developed, plasmid DNA was isolated, and plasmid concentrations were determined after its linearization by XbaI restrictase.

As a result, plasmids encoding Cas9 and sgRNAs to the corresponding sites of the BLG gene were obtained.

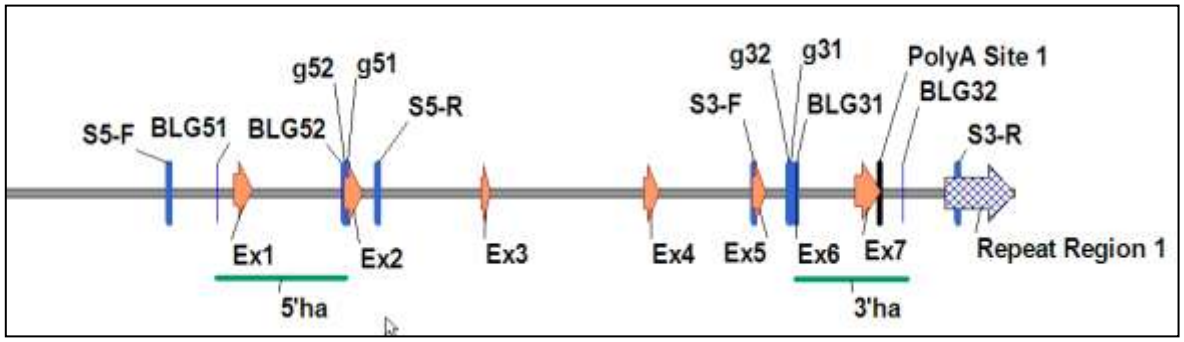

Fig. 1. Scheme for selecting homology arms of the BLG gene in cattle to create a DNA matrix for homologous recombination. The 5' - homology arm (5'ha) measuring 929 bp includes the 1st exon and 8 bp of the second exon. The 3'ha with a size of 805 bp contains a fragment of the 6th exon and the 7th exon. The binding sites of the primers used, localization of proto-spacer sequences (g52, etc.) are indicated.

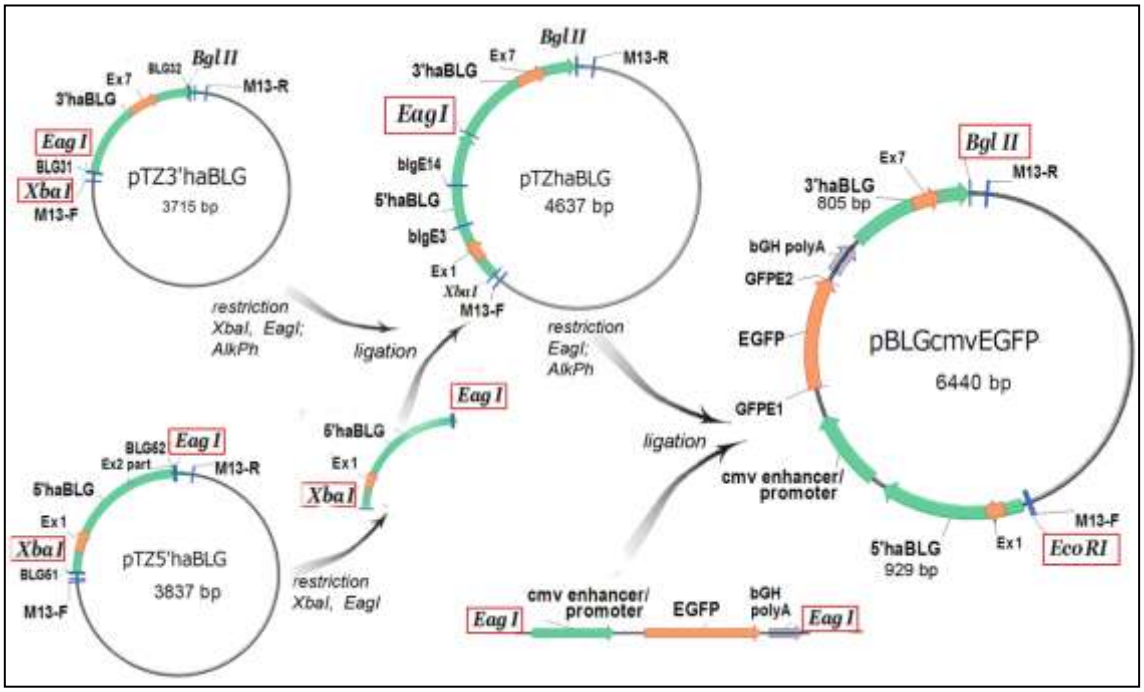

Fig. 2. Scheme for obtaining pTZHABLG and pBLGcmvEGFP plasmids containing 5' and 3' homology arms to the BLG gene. The main sites of restriction (within the framework) and binding of the primers used are indicated.

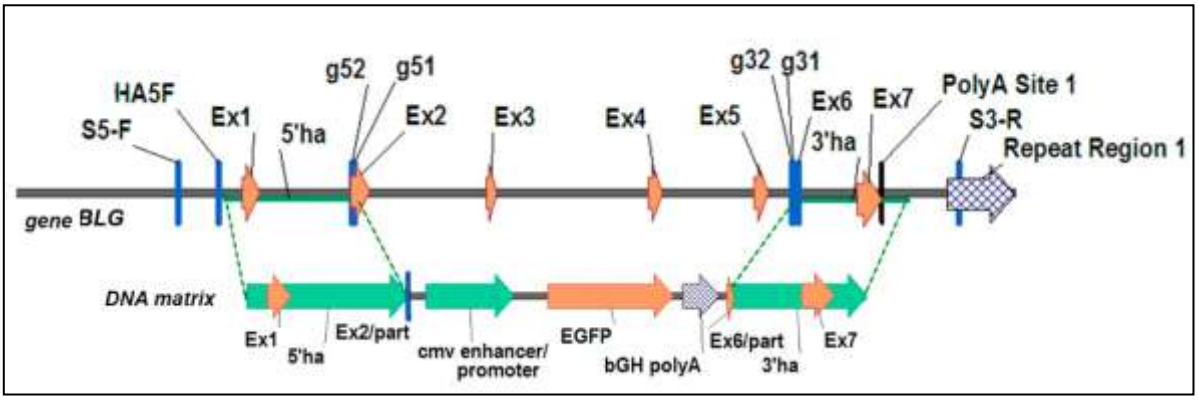

Fig. 3. Scheme of homologous recombination of the BLGcmvEGFP gene construction with the BLG gene as a result of the CRISPR/Cas9 operation. Shown are: g51, g52, g31, g32 targets for gRNAs; 5'ha and 3'ha homology arms; structural elements of the gene and construction; primers.

Table 5. Composition of mixtures for the formation of double-chain inserts with sticky ends

	Mixture for the formation of an insert, µl			
Component, (conc.).	**bg51**	**bg52**	**bg31**	**bg32**
Water	13	13	13	13
bg51f (100 pM/µl)	2	-	-	-
bg51r (100 pM/µl)	2	-	-	-
bg52f (100 pM/µl)	-	2	-	-
bg52r (100 pM/µl)	-	2	-	-
bg31f (100 pM/µl)	-	-	2	-
bg31r (100 pM/µl)	-	-	2	-
bg32f (100 pM/µl)	-	-	-	2
bg32r (100 pM/µl)	-	-	-	2
T4PNK (5 U/ µl)	1	1	1	1
10x T4 DNA ligase buffer	2	2	2	2
Mineral oil	20	20	20	20
In total:	40	40	40	40

Table 6. Composition of ligase mixtures for producing pX330-based plasmids

	Mixture for the formation of plasmids, µl			
Component, (conc.).	**pX330-b51**	**pX330-b52**	**pX330-b31**	**pX330-b32**
Water	9	9	9	9
5x T4 DNA ligase buffer	4	4	4	4
BSA* (10 mg / ml)	1,5	1,5	1,5	1,5
pX330 (150 ng/ µl)	2	2	2	2
BbsI (10 un / µl)	1	1	1	1
T4 DNA ligase	1	1	1	1
bg51	1,5	-	-	-
bg52	-	1,5	-	-
bg31	-	-	1,5	-
bg32	-	-	-	1,5
Mineral oil	20	20	20	20
In total	40	40	40	40

* BSA – bovine serum albumin

Table 7. Composition of PCR mixtures for screening based on pX330 plasmids (per test tube).

	PCR-mixture for plasmid analysis, µl			
Component, (conc.).	**pX330-b51**	**pX330-b52**	**pX330-b31**	**pX330-b32**
Taq buf ×10	2	2	2	2
dNTP ×10 (2 мМ)	1,8	1,8	1,8	1,8
ENH-R (3 pM / µl)	1	1	1	1
bg51f (3 pM / µl)	1	-	-	-
bg52f (3 pM / µl)	-	1	-	-
bg31f (3 pM / µl)	-	-	1	-
bg32f (3 pM / µl)	-	-	-	1
Water	12,9	12,9	12,9	12,9
DNA from the corr.clone.	1	1	1	1
TaqPol (5 un / µl)	0,3	0,3	0,3	0,3
Mineral oil	20	20	20	20
In total:	40	40	40	40

Table 8. PCR analysis of BLG gene modifications obtained using CRISPR/Cas9 system components and HDR matrix

A pair of primers	Amplification product size, bp		Detection
	Intact animal	Genetically modified animal	
T5L / T5R	490	< 490 (ΔG51/G52)	Small deletions, PAAG
T3L /T3R	397	< 397 (ΔG31/G33)	
T5L / T3R	3598	≈ 320 for ΔG51(G52)/G31(G32)	
HA5F/ HA3R	3621	≈ 350 for ΔG51(G52)/G31(G32)	
HA5F /HA5R	-	1179 (5'HA for HDR)	Large deletions, AG
HA3F/ HA3R	-	972 (3'HA for HDR)	

Δ - deletion, PAAG – polyacrylamide gel; agarose gel

Analysis of BLG gene modifications

The pBLGcmvEGFP plasmid in circular or linear form (by cutting out the gene structure by EcoRI and BglII restrictases) can be used as a DNA matrix for homologous recombination with the BLG gene using CRISPR/Cas9 components (Fig. 3).

As a result of embedding the gene construction the locus of the BLG gene will be modified. To confirm the fact of homologous recombination, HA5F /HA5R and HA3F/ HA3R pairs were selected (tables 3,6).

Another variant of possible genetic modifications resulting from the operation of the created components of the CRISPR / Cas9 system is double-stranded cut repair by the NHEJ mechanism. Options for detecting possible small and large deletions are presented in table 8.

Conclusions.

The method of somatic cell nuclei transfer is still the main one in obtaining gene-edited large agricultural animals, that produce few offspring, although the method of microinjection of zygotes is considered simpler and more reliable, especially in combination with CRISPR/Cas9 technology. However, we have not found any such work on obtaining genetically modified cattle. We created a recombinant pTZhaBLG plasmid containing 5' and 3' homology arms (929 bp and 805 bp in size, accordingly) to the BLG gene of cattle, which can be used to clone the DNA of a target biologically active protein using the EagI site, with the prospect of obtaining a transgenic cattle that produces recombinant proteins with milk instead of BLG, using the CRISPR/Cas9 technology. Plasmids encoding Cas 9 endonuclease and sgRNAs to the corresponding sites of the BLG gene were obtained. A system for evaluating potential genetic modifications of the gene was developed using PCR and analysis of its products in agarose and polyacrylamide gels.

In addition, pBLGcmvEGFP plasmid was created containing a fragment of cmvEGFP-bGHpolyA (a reporter gene of a green fluorescent protein under a cytomegalovirus promoter) flanked by homology arms to BLG gene sequences to evaluate the effectiveness of transgen integration under in vitro culture.

Genome editing technologies will improve and create new breeds of farm animals in order to obtain both full-fledged food products with new qualities, and to obtain a source of biologically active proteins for pharmacological purposes from milk. The use of CRISPR / Cas9 technology in scientific and practical research can significantly accelerate the production of such animals. Our work provides a ready-made set of CRISPR/Cas9 components in plasmid form for microinjection into the zygote pronucleus of cattle in order to knock out the BLG gene, as well as a ready-made strategy for analyzing its possible modifications.

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Authors' contributions

KE developed a research program, conducted a search and analysis of literature, compiled a manuscript, worked with specialized computer programs, including on-line and participated in the implementation of the molecular genetic block of work. EV carried out a complex of molecular genetic studies and research, analyzed literature data, worked with specialized computer programs, including on-line, and participated in the writing of the manuscript. All authors approved the final version of the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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