

The growth and reproduction performance of TALEN-mediated β -lactoglobulin-knockout bucks

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Abstract With the technological development of several engineered endonucleases (EENs), such as zinc-finger nucleases, transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9, gene targeting by homologous recombination has been efficiently improved to generate site-specifically genetically modified livestock. However, few studies have been done to investigate the health and fertility of these animals. The purpose of the present study is to investigate if gene targeting events and a recloning procedure would affect the production traits of EEN-mediated gene targeted bucks. TALEN-mediated β -lactoglobulin (*BLG*) gene mono-allelic knockout ($BLG^{+/-}$) goats and bi-allelic knockout ($BLG^{-/-}$) buck produced by using sequential gene targeting combined with recloning in fibroblasts from $BLG^{+/-}$

buck were used to evaluate their health and fertility. Birth weight and postnatal growth of $BLG^{+/-}$ bucks were similar to the wild-type goats. None of the parameters for both fresh and frozen-thawed semen quality were significantly different in $BLG^{+/-}$ or $BLG^{-/-}$ bucks compared to their corresponding comparators. In vitro fertilization (IVF) test revealed that the proportion of IVF oocytes developing to the blastocyst stage was identical among $BLG^{+/-}$, $BLG^{-/-}$ and wild-type bucks. Conception rates of artificial insemination were respectively 42.3, 38.0 and 42.6 % for frozen-thawed semen from the $BLG^{+/-}$, $BLG^{-/-}$ and wild-type bucks. In addition, germline transmission of the targeted *BLG* modification was in accordance with Mendelian rules. These results demonstrated that the analyzed growth and reproductive traits were not impacted by targeting *BLG* gene and recloning, implicating the potential for dairy goat breeding of $BLG^{+/-}$ and $BLG^{-/-}$ bucks.

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Abbreviations

EEN	Engineered endonuclease
TALEN	Transcription activator-like effector nucleases
BLG	β -lactoglobulin
SCNT	Somatic cell transfer
IVF	In vitro fertilization

AI Artificial insemination
 GE Genetically engineering

Introduction

Traditional breeding and selection had been thought the only approach to shape the genetic makeup of livestock to promote production for increasing human demands for food and other products. In the past 30 years, the transgenic and cloning technology have become an alternative approach to make genetic manipulations in livestock for various application in agriculture and biomedicine (Garas et al. 2015). However, to successfully apply the transgenic technology in the animal production, an intense concern must be prompted about the health and welfare of these genetically engineering (GE) animals (Jagadeesan and Bin Salem 2015).

In the past decades, the GE livestock have been commonly generated by either microinjection or transfection of expression cassettes into the animal genome, in which the transgenes insertion were essentially random (Laible et al. 2015a). The random integration possibly results in unpredictable expression of transgenes, insertional mutagenesis and unwanted pathological side effects (Clark et al. 1997; Dobie et al. 1996; Fattori et al. 1994; Gao et al. 2007; Lewis et al. 1993; Milot et al. 1996; Pedram et al. 2006; Ramirez et al. 2001). Although gene targeting by homologous recombination (HR) could overcome these shortcomings, the lower frequency of HR in somatic cells hampers its application in GE livestock. Since the first gene-targeted sheep in 2000 were born, only few studies demonstrated gene targeting in somatic cells followed by embryonic cloning to produce GE livestock (Hauschild et al. 2011; Laible and Alonso-Gonzalez 2009). The advent of engineered endonucleases (EENs), including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9, has hold much promise for site-specifically and precise genomic modification in livestock. These endonucleases can efficiently cleave DNA at preferred genomic regions and induced double-strand breaks (DSBs), which are repaired by either non homologous end joining

(NHEJ) or HR (Gaj et al. 2013). Using EENs mediated NHEJ or HR, a lot of GE livestock, such as cattle, pig, goat, and sheep, were generated to enhanced certain traits, improve ability to defense disease, and produce models of human disease (Bosch et al. 2015; Carlson et al. 2013; Laible et al. 2015b; Lillico et al. 2016; Tu et al. 2015). However, few studies was focused on the welfare and health of these animals.

β -Lactoglobulin (BLG), the major whey protein of the milk from goats and other ruminants, is considered to be a dominant milk allergen (Sharma et al. 2001). Removal of BLG by chemical or physical methods is costly, and the treated milk may have been changed in quality. Genetic engineering technology can be used to solve this problem radically. In the previous study, we have generated $BLG^{+/-}$ goats by using TALEN-mediated gene targeting in goat fetal fibroblasts and somatic cell transfer (SCNT) (Cui et al. 2015). The sequential targeting manipulation was performed on the somatic cells from $BLG^{+/-}$ goats to produce the $BLG^{-/-}$ goats by SCNT. The level BLG was lower in the milk secreted by $BLG^{+/-}$ females, and the BLG was lack in the milk secreted by $BLG^{-/-}$ females. In the present study, the growth and fertility of the $BLG^{+/-}$ and $BLG^{-/-}$ were evaluated to determine whether BLG gene targeted events impacted the production and reproductive fitness of these animals.

Materials and methods

Animals

BLG -knockout goats used in the present study were reported previously (Cui et al. 2015). Briefly, a 20 bp deletion of the BLG gene and a 1935 bp neomycin resistance (*neo*) gene were introduced into the cognate chromosomal location in $BLG^{+/-}$ goats by using precisely genetic modification technology, and a 80-bp fragment containing the ATG start codon of the goat BLG gene was replaced with the 3153 bp puromycin (*puro*) selection maker in $BLG^{-/-}$ goats. Three $BLG^{+/-}$ bucks born in 2013 and one $BLG^{-/-}$ buck born in 2014 were detected in this study. Wild type bucks with the same genetic background and age were selected as control.

Growth trial

The kids were fostered according to the standard procedure of our dairy goat facility. The animals were observed daily for clinical changes. Body weight was recorded weekly after birth. Prior to weaning, alfalfa hay was introduced to supplement the goats' nutritional needs. At the age of 4 months, the goats were weaned. The birth weights, weaning weights of the *BLG*-knockout and control bucks were recorded and compared over a 4-month interval.

Semen collection, freezing and thawing

Fresh semen was acquired from each *BLG*-targeted and control buck of 18 months old. The collection was carried out at the end of September and continued through early November, using an artificial vagina and estrous females as mounts for the bucks. Semen samples for analysis were collected three times with an interval of at least 3 days.

To prepare frozen sperm, the fresh semen was diluted (1:5, V/V) in TFC solution (Tris 375 mM, fructose 27 mM, citric acid 124 mM, streptomycin sulfate 1 g/l, and penicillin 0.06 g/l, pH 7.0) and centrifuged at room temperature for 10 min at 800 g to remove the seminal plasma. The remain is diluted in TFC-egg yolk-glycerol diluent (TFC solution supply with egg yolk 20 % and glycerol 6.0 % by volume, pH 7.0) at 37 °C to 400×10^6 sperm/ml. Then the samples were loaded into 0.25 ml straws, sealed, and cooled to 5 °C over 2 h, frozen in liquid nitrogen vapor for 7 min and subsequently plunged into the liquid nitrogen for storage. After 1 week of freezing, the straws were thawed in water bath at 37 °C for 30 s.

Semen evaluation

The characteristics of fresh and post-thaw semen were evaluated, including ejaculate volume, sperm concentration, motility, morphology and acrosome integrity. After collection and determination of volume of ejaculate, a portion of the semen was extended 1:100 with PBS supplemented with 5 % FBS (v/v). Sperm concentration was determined using a hemocytometer. Viability and percentage of morphological normal spermatozoa were measured using sperm viability stain kit and Diff-Quick staining kit respectively (Bred life science, Shenzhen, China), and the percentage of

acrosome integrity was assessed using Giemsa stain (Solarbio, Beijing, China). The sperm motility was examined under a phase contrast microscope (Olympus, Tokyo, Japan).

Seminal plasma parameters, including fructose, Zn^{2+} , neutral α -glucose glycosidase and acid phosphatase were detected using corresponding commercial kit (Bred life science, Shenzhen, China).

To evaluate the post-thaw semen quality, the thawed sample was diluted tenfold and incubated at 37 °C for 1 h. Then the sperm parameters, including viability, motile spermatozoa, acrosome integrity, plasma membrane integrity were measured as described above.

Sperm capacitation and IVF

The motile spermatozoa of fresh semen was selected by the swim-up method (Parrish et al. 1986). Seventy microliter of each ejaculate was added into tubes bottom containing 2 ml modified Defined Medium (mDM) (Younis et al. 1991). After 1 h of incubation at 38.5 °C, the upper layer was removed to new tubes and centrifuged for 5 min at 200 g. The pellet was resuspended in mDM, and then the resuspension was mixed with equal volume of mDM with 100 $\mu\text{g/ml}$ heparin. The mixture was incubated for 45 min at 38.5 °C, 5 % CO_2 and saturation humidity for the spermatozoa capacitation (Izquierdo et al. 2002).

The motile spermatozoa of the frozen-thawed semen was obtained by discontinuous Percoll gradient (Parrish et al. 1986) centrifuged and resuspended with mDM. The capacitation process was performed as described above.

Ovaries were collected at a local abattoir, and the collection of cumulus oocyte complexes (COC) and in vitro maturation of oocytes were performed as described previously (Liu et al. 2011; Zhang et al. 2008). After maturation, a total of around 30 mature oocytes were transferred into 100- μl drops of in vitro fertilization (IVF) medium, and co-cultured with sperm in a final concentration of 4×10^6 fresh or frozen motile sperm/ml for 24 h at 38.5 °C and in an incubator with 5 % CO_2 in humidified air. Then the presumptive zygotes were cultured in G-SeriesTM Culture Media G1 and G2 (Vitrolife AB, Gothenburg, Sweden), within the 72 h in G1 and another 96 h in droplet of G2 covered with mineral oil. Embryos were cultured for 7 days to evaluate the in vitro developmental rate.

Artificial insemination

Adult females with proven fertility were selected according to their age (2–5 years old), good body condition, medical and reproductive history. Estrus synchronization treatment was performed by insertion of intravaginal progestogens sponge as previously described (Dorado et al. 2007). On day 9 after sponge insertion, the goats were injected with cloprostenol. On day 11, the sponge was taken away and the estrous goats were detected using a teaser buck. The goats were presented individually to the male and estrous was defined and recorded.

The cryopreserved sperm of one $BLG^{+/-}$ buck (MK1) and the $BLG^{-/-}$ buck (BK1) and 3 control bucks were used for artificial insemination. The sperm was thawed for 30 s in a 37 °C water bath just before insemination. Each of the estrous goats were inseminated intracervically twice using the sperm from the same individual. Transrectal ultrasonography was done 30 days after artificial insemination to detect pregnancy.

Statistical analysis

The data were analyzed using statistical software (SPSS 22.0) by a one-way ANOVA for independent samples. A value of $P < 0.05$ was considered to be statistically significant.

Results

Birth weight and postnatal growth

The birth weights and weaning weights of the BLG -targeted bucks and controls were shown in Table 1. The mean birth weight of the $BLG^{+/-}$ bucks was consistent with that of the wild-type goats, and no significant difference was detected in the weaning weight and average daily gain (ADG) among these groups. The body weights and ADG of the $BLG^{-/-}$ buck were within the normal range. In addition, the BLG -knockout goats were immunized according to conventionally immunized program, and no anomalies was observed through the process of growth from birth to weaning.

Semen quality

The fresh semen of BLG -knockout bucks was analyzed for ejaculate volume, concentration, viability, morphology, motility, acrosome integrity, and plasma membrane integrity. There were no significant differences in all the examined parameters between BLG -knockout bucks and their corresponding controls. There was also no significant differences for age between $BLG^{+/-}$ and $BLG^{-/-}$ bucks (Table 2).

Table 3 presented the analysis of seminal plasma of the fresh semen. The parameters of fructose, Zn^{2+} , α -glucose glycosidase and acid phosphatase of semen from the $BLG^{+/-}$ bucks were similar to that of the controls. Despite the parameters of fructose and neutral α -glucose glycosidase of semen from $BLG^{-/-}$ buck were a little lower than that of $BLG^{+/-}$ bucks (25.72 ± 0.67 vs. 26.88 ± 1.17 ; 0.26 ± 0.02 vs. 0.29 ± 0.03), they were in conformity with the tendency of the corresponding controls of the present year (25.95 ± 1.93 vs. 26.01 ± 1.88 ; 0.25 ± 0.05 vs. 0.26 ± 0.06).

The parameters of the percentage of viability, motility, acrosome integrity and plasma membrane integrity for frozen-thawed semen were showed in Table 4. The $BLG^{-/-}$ buck had advantage of viability compare to $BLG^{+/-}$ buck and controls, and MK2 presented better motility, acrosome integrity and plasma membrane integrity. But analysis of the value revealed that there were no significant differences among these animals in all the examined parameters.

Fertility of BLG -knockout Bucks *in vitro*

The IVF test reflected fertilizing capacity of the fresh and frozen-thawed sperm from BLG -targeted bucks and a wild type buck of proven fertility (Table 5). Fresh sperm and frozen-thawed sperm possessed identical *in vitro* developmental rates from the 2-cell embryo to the blastocyst stage were normal.

The AI test of the semen from the BLG -knockout Bucks

The frozen-thawed semen of the MK1, BK1 and controls in the same estrus synchronization period was used for AI. A summary of the reproductive status of inseminated doe was represented in Table 6. The pregnancy rate of the doe was similar between the

Table 1 Growth parameters of the *BLG*-knockout and control animals

	<i>BLG</i> ^{+/-} bucks			Mean ± SD of the <i>BLG</i> ^{+/-} bucks	Control ^a (n = 15)	<i>BLG</i> ^{-/-} buck BK1	Control ^b (n = 15)
	MK1	MK2	MK3				
Birth weight (kg)	3.62	3.38	4.12	3.71 ± 0.38	3.70 ± 0.63	3.71	3.61 ± 0.49
Weaning weight (kg)	23.12	22.19	25.15	23.48 ± 1.51	23.67 ± 0.75	23.29	22.29 ± 1.65
Average daily gain (g)	162.5	156.75	175.25	164.83 ± 9.47	166.39 ± 10.09	163.17	155.68 ± 10.16

^a Contemporary wild type bucks of *BLG*^{+/-} bucks^b Contemporary wild type bucks of *BLG*^{-/-} buck**Table 2** Characteristics of fresh semen from *BLG*-knockout bucks and their corresponding controls

	<i>BLG</i> ^{+/-} bucks			Mean ± SD of the <i>BLG</i> ^{+/-} bucks	Control ^a (n = 6)	<i>BLG</i> ^{-/-} buck BK1	Control ^b (n = 6)
	MK1	MK2	MK3				
Ejaculate volume (ml)	1.34 ± 0.06	1.26 ± 0.05	1.21 ± 0.04	1.27 ± 0.07	1.30 ± 0.11	1.31 ± 0.10	1.26 ± 0.11
Concentration (10 ⁹ /ml)	2.43 ± 0.08	2.72 ± 0.10	2.57 ± 0.08	2.57 ± 0.14	2.61 ± 0.35	2.49 ± 0.14	2.52 ± 0.17
Viability (% live)	88.20 ± 0.78	86.63 ± 0.80	87.60 ± 0.21	87.49 ± 0.93	88.10 ± 1.63	88.10 ± 0.56	87.11 ± 1.54
Morphology (% normal)	88.40 ± 0.82	88.83 ± 0.68	89.03 ± 0.30	88.76 ± 0.62	88.10 ± 0.92	88.40 ± 0.62	87.97 ± 0.92
Motile (%)	64.37 ± 0.96	63.73 ± 0.50	65.2 ± 0.36	64.43 ± 0.86	64.30 ± 1.45	65.47 ± 0.91	64.31 ± 1.25
Acrosome integrity (%)	81.15 ± 0.75	79.52 ± 0.85	80.75 ± 0.37	80.47 ± 0.95	80.17 ± 1.03	80.69 ± 0.19	79.94 ± 1.34
Plasma membrane integrity (%)	63.20 ± 0.59	62.28 ± 0.40	64.07 ± 0.59	63.18 ± 0.91	62.14 ± 2.04	62.74 ± 0.78	62.14 ± 1.36

^a Contemporary wild type bucks of *BLG*^{+/-} bucks^b Contemporary wild type bucks of *BLG*^{-/-} buck

BLG^{+/-} group and control (42.86 and 42.59 % respectively) which were higher than that of *BLG*^{-/-} group (38.00 %). The duration of gestation for pregnant does of the three groups were within the normal range for goats, and there were no significant differences among these groups. In addition, the kidding rate of pregnant goats from *BLG*^{+/-} (1.28, 27/21), *BLG*^{-/-} (1.37, 26/19), and control groups (1.39, 32/23) were similar.

Germline transmission of the targeted *BLG* modification in the *BLG*-knockout bucks was demonstrated in Table 7. Twenty-seven offspring were derived from semen of *BLG*^{+/-} buck, 44.4 % (12/27) of which inherited the targeted allele from their father. Twenty-six offspring were derived from semen obtained from *BLG*^{-/-} buck, and all of them were detected as *BLG*^{+/-}. 42.3 % (11/26) of these offspring inherited

the modified *BLG* allele which a neomycin resistance (*neo*) gene was targeted in, and the remainders inherited another modified *BLG* allele which a puromycin (*puro*) selection cassette was targeted in.

No significant difference was observed in the birth weights of the male or female offspring derived from *BLG*^{+/-}, *BLG*^{-/-} and control bucks. In addition, the birth weights of the *BLG*-knockout offspring were not affected by the genotype they inherited.

Discussion

Genetically engineering studies using livestock, such as cattle, sheep and goats usually aim to enhance disease resistance, alter the composition of milk and meat, produce pharmaceutical proteins in mammary

Table 3 Seminal plasma parameters of *BLG*-knockout bucks and their corresponding controls

	<i>BLG</i> ^{+/-} bucks			Mean ± SD of the <i>BLG</i> ^{+/-} bucks	Control ^a (n = 6)	<i>BLG</i> ^{-/-} buck BK1	Control ^b (n = 6)
	MK1	MK2	MK3				
Fructose (μM)	26.57 ± 0.91	26.07 ± 0.22	28.01 ± 1.25	26.89 ± 1.18	26.01 ± 1.88	25.72 ± 0.67	25.95 ± 1.93
Zn (μM)	0.21 ± 0.01	0.19 ± 0.01	0.20 ± 0.02	0.20 ± 0.01	0.18 ± 0.05	0.23 ± 0.01	0.18 ± 0.03
Neutral α-glucose glycosidase (mU)	0.31 ± 0.03	0.27 ± 0.02	0.29 ± 0.02	0.29 ± 0.03	0.26 ± 0.06	0.26 ± 0.02	0.25 ± 0.05
Acid phosphatase (U)	2.18 ± 0.04	2.24 ± 0.08	2.16 ± 0.04	2.19 ± 0.06	2.19 ± 0.07	2.20 ± 0.01	2.23 ± 0.09

^a Contemporary wild type bucks of *BLG*^{+/-} bucks^b Contemporary wild type bucks of *BLG*^{-/-} buck**Table 4** Characteristics of post thawed semen of *BLG*-knockout and control bucks

	<i>BLG</i> ^{+/-} bucks			Mean ± SD of the <i>BLG</i> ^{+/-} bucks	<i>BLG</i> ^{-/-} buck BK1	Control (n = 6)
	MK1	MK2	MK3			
Viability (%)	47.76 ± 0.88	48.71 ± 1.16	47.75 ± 1.07	48.07 ± 1.02	49.34 ± 1.58	48.53 ± 1.66
Motile spermatozoa (%)	35.00 ± 1.05	37.12 ± 0.63	35.75 ± 1.32	36.14 ± 1.17	36.97 ± 0.92	35.76 ± 1.12
Acrosome integrity (%)	65.42 ± 0.55	68.13 ± 0.80	67.44 ± 1.61	67.00 ± 1.54	67.51 ± 1.13	66.06 ± 1.95
Plasma membrane integrity (%)	42.86 ± 0.79	45.21 ± 1.03	44.46 ± 0.72	44.18 ± 1.28	43.65 ± 0.91	43.01 ± 2.34

Table 5 IVF tests using semen from *BLG*-knockout and control bucks

Animals	No. oocytes used	No. (%) ≥ 2-cell embryos	No. (%) ≥ 4-cell embryos	No. (%) ≥ Morulas	No. (%) blastocyst
<i>Fresh sperm</i>					
MK1	202	128 (63.36 ± 1.58)	107 (52.97 ± 2.99)	81 (40.11 ± 1.59)	59 (29.25 ± 3.13)
BK1	198	121 (61.07 ± 2.43)	105 (52.99 ± 3.29)	78 (39.35 ± 2.85)	60 (30.21 ± 4.04)
Control	196	123 (62.73 ± 1.99)	103 (52.48 ± 2.78)	75 (38.30 ± 4.17)	58 (29.62 ± 4.12)
<i>Frozen-thawed sperm</i>					
MK1	194	121 (62.41 ± 3.91)	101 (52.14 ± 2.79)	78 (40.26 ± 3.51)	56 (28.90 ± 4.27)
BK1	200	119 (59.47 ± 3.39)	103 (51.44 ± 4.08)	77 (38.50 ± 2.43)	57 (28.47 ± 2.71)
Control	197	121 (61.39 ± 2.91)	104 (52.75 ± 3.75)	74 (37.55 ± 3.15)	58 (29.47 ± 4.59)

Seven replicate experiments were performed for per source of semen. Numbers in parentheses represent development rates (mean ± SEM %), and other numbers represent total embryo number of the seven replicates

gland, or generate models of human disease for agriculture or biomedicine application. To achieve these goals, the health and welfare of GE livestock should be considered. It has been suggested that insertional mutagenesis and transgene expression due

to the random transgenes integration, and in vitro reproduction technologies are the major potential factors which may affect the genetically engineering livestock health and welfare (Broom and Kirkden 2012; Kruip and Blokhuis 2001). However, Huber

Table 6 Artificial insemination(AI)tests using frozen-thawed semen from the *BLG*-targeted bucks and controls

Source of semen	No. females inseminated	No. pregnant females (%)	Gestation length (days)	No. kidding (%)	Ratio female/male
MK1	49	21 (42.86)	151.00 ± 1.09	27/21 (1.28)	15/12
BK1	50	19 (38.00)	150.68 ± 0.94	26/19 (1.37)	15/11
Control (<i>n</i> = 3)	54	23 (42.59)	150.78 ± 0.95	32/23 (1.39)	18/14

Table 7 Germline transmission of *BLG*-targeted bucks and birth weights of their offspring

Source of semen	<i>BLG</i> ^{+/-} (<i>neo</i>)		<i>BLG</i> ^{+/-} (<i>puro</i>)		Wild type	
	Female	Male	Female	Male	Female	Male
MK1	3.60 ± 0.49 (<i>n</i> = 7)	3.56 ± 0.50 (<i>n</i> = 5)	–	–	3.72 ± 0.54 (<i>n</i> = 8)	3.89 ± 0.57 (<i>n</i> = 7)
BK1	3.51 ± 0.61 (<i>n</i> = 6)	3.78 ± 0.35 (<i>n</i> = 5)	3.50 ± 0.45 (<i>n</i> = 9)	3.53 ± 0.52 (<i>n</i> = 6)	–	–
Controls	–	–	–	–	3.51 ± 0.48 (<i>n</i> = 18)	3.72 ± 0.49 (<i>n</i> = 14)

BLG^{+/-} (*neo*) and *BLG*^{+/-} (*puro*) were the genotype of the offspring, *neo* indicating neomycin resistance gene targeted in MK1/BK1, and *puro* indicating puromycin selection cassette targeted in BK1

et al. (2012) performed an extensive welfare assessment including reproductive performance and behavior, general health and development, social behavior, exploratory behavior and emotionality comparing GFP transgenic pigs with wildtype animals along various stages of post natal development, which suggested that the random integration of GFP gene had no effect on welfare of the genetic modified animals. Advances in transgenic technology and EENs have made precisely targeted genetic modification available, offering a more robust tool to produce GE livestock for various applications. In the present study, *BLG*-knockout bucks were compared with wildtype bucks with regard to general growth and reproductive performance. To our knowledge, this was the first time that a comprehensive assessment study was carried out in GE bucks produced by TALEN-mediated gene targeting and SCNT to assess the impact of targeted genetic modification on animal health. Especially, the *BLG*^{-/-} buck was generated by a sequential gene targeting and re-cloning process, which means that the *BLG*^{-/-} donor cells used for SCNT undergone two times of consecutive reprogramming.

To generate *BLG*-knockout goats, the TALENs-encoding mRNAs were introduced to eliminate the possible genomic integration of TALEN–DNA constructs. Additionally, the Southern blot analysis

revealed that there was no randomly integration of HDR template plasmids (Cui et al. 2015). Thus, any major impact resulting from insertional mutagenesis or off tissue transgene expression on the general welfare of these animals was not of concern. Despite targeted genome editing by EENs has provided a powerful tool for GE livestock production, the off-target cutting of EENs may leading to unexpected mutagenesis. In our study, we did not observe any abnormality in all the *BLG*-knockout newborn goats, and abnormal postnatal development or growth in either female (data not shown in this study) or male. In addition, the postnatal growth study showed that the weight gain of *BLG*^{+/-} and *BLG*^{-/-} bucks from birth to weaning were within the normal range of our dairy goat facility. These results revealed that the impact of any off-target cutting of TALENs has been negligible in these animals.

Traditional livestock breeding is a painstakingly slow process of accumulating incremental gains over a long period. By contrast, a variety of new transgenic animals with desirable production and composition changes can be produced within a single generation. When these dairy livestock with particular traits are projected onto the production scale of the dairy industry, GE sire with favorable fertility is required for developing large production herds. With the help

of cryopreservation and assisted reproductive technology, the GE genotype can be rapidly propagated on large scale at low cost. It has been confirmed that cloned or GE sire could propagate normal fertile offspring, because the possible epigenetically error could be amended by germ cells (Jackson et al. 2010; Gauthier et al. 2004; Wilmut et al. 1997; Tamashiro et al. 2002). In our study, despite the reproductive ability of *BLG*-knockout bucks were not significantly different from wildtype controls, there were some differences in seminal plasma parameters and semen parameters among the *BLG*^{+/-} and *BLG*^{-/-} bucks, and these differences may suffer from the quality diversity of the feed supply or varying environmental conditions in different ages. The frozen-thawed sperm also exhibited favorable viability and acrosome integrity. In IVF test, it gave the similar response to make the oocyte fertilized and the blastocyst rate as frozen-thawed sperm from corresponding controls. In artificial insemination test, the cryopreserved sperm of MK1 and BK1 showed similar conception rate compared to controls, and the gestation period and the birth weights of the offspring were normal. Detection of the *BLG* gene status of the offspring revealed the stable germline transmission of the targeted genetic modification. Overall, the *BLG*-knockout bucks exhibited normal fertility both in vitro and in vivo, indicating their potential for dairy goat breeding.

Recently reports indicate that it is possible to produce recloned animals with somatic cell nuclei derived from previously cloned animals (Cho et al. 2007; Kubota et al. 2004; Kurome et al. 2008; Wakayama et al. 2013; Yin et al. 2008). As it is difficult to obtain homozygous targeted cells in one clonal selection process, the sequential gene targeting in somatic cells from the heterozygous targeted animals is an alternative to produce homozygous targeted genetic modified animals. In the present study, *BLG*^{-/-} goats generated by sequential gene targeting combined with recloning showed normal health and fertility, suggesting that this strategy may be a useful approach to generate the homozygous or multiple site-specific genetic modification in livestock.

In summary, our results demonstrated the normal health growth and fertility of TALEN-mediated gene knockout goats, indicating that the reproduction indices and weight development in these goats were not compromised by the gene targeting events and the

recloning. Our results would be invaluable for the knowledge required to make science-based regulatory decisions regarding the use of site-specific genetic modified livestock.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

- Bosch P et al (2015) Exogenous enzymes upgrade transgenesis and genetic engineering of farm animals. *Cell Mol Life Sci* 72:1907–1929. doi:[10.1007/s00018-015-1842-1](https://doi.org/10.1007/s00018-015-1842-1)
- Broom D, Kirkden R (2012) Welfare of genetically modified and cloned animals used for food. A report for Compassion in World Farming, Godalming. <http://www.ciwf.org.uk>
- Carlson DF, Tan W, Hackett PB, Fahrenkrug SC (2013) Editing livestock genomes with site-specific nucleases. *Reprod Fertil Dev* 26:74–82. doi:[10.1071/RD13260](https://doi.org/10.1071/RD13260)
- Cho SK et al (2007) Serial cloning of pigs by somatic cell nuclear transfer: restoration of phenotypic normality during serial cloning. *Dev Dyn* 236:3369–3382. doi:[10.1002/dvdy.21308](https://doi.org/10.1002/dvdy.21308)
- Clark AJ, Harold G, Yull FE (1997) Mammalian cDNA and prokaryotic reporter sequences silence adjacent transgenes in transgenic mice. *Nucleic Acids Res* 25:1009–1014
- Cui C et al (2015) Gene targeting by TALEN-induced homologous recombination in goats directs production of beta-lactoglobulin-free, high-human lactoferrin milk. *Sci Rep* 5:10482. doi:[10.1038/srep10482](https://doi.org/10.1038/srep10482)
- Dobie KW et al (1996) Variegated transgene expression in mouse mammary gland is determined by the transgene integration locus. *Proc Natl Acad Sci USA* 93:6659–6664
- Dorado J, Rodriguez I, Hidalgo M (2007) Cryopreservation of goat spermatozoa: comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. *Theriogenology* 68:168–177. doi:[10.1016/j.theriogenology.2007.04.048](https://doi.org/10.1016/j.theriogenology.2007.04.048)
- Fattori E, Della Rocca C, Costa P, Giorgio M, Dente B, Pozzi L, Ciliberto G (1994) Development of progressive kidney damage and myeloma kidney in interleukin-6 transgenic mice. *Blood* 83:2570–2579
- Gaj T, Gersbach CA, Barbas CF (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 31:397–405. doi:[10.1016/j.tibtech.2013.04.004](https://doi.org/10.1016/j.tibtech.2013.04.004)
- Gao Q et al (2007) Telomeric transgenes are silenced in adult mouse tissues and embryo fibroblasts but are expressed in embryonic stem cells. *Stem Cells* 25:3085–3092. doi:[10.1634/stemcells.2007-0478](https://doi.org/10.1634/stemcells.2007-0478)

- Garas LC, Murray JD, Maga EA (2015) Genetically engineered livestock: ethical use for food and medical models. *Annu Rev Anim Biosci* 3:559–575. doi:[10.1146/annurev-animal-022114-110739](https://doi.org/10.1146/annurev-animal-022114-110739)
- Gauthier M, Pierson J, Drolet M, Bhatia B, Baldassarre H, Keefer CL (2004) Sexual maturation and fertility of male Nigerian Dwarf goat (*Capra hircus*) clones produced by somatic cell nuclear transfer. *Cloning Stem Cells* 3:151–155
- Hauschild J et al (2011) Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. *Proc Natl Acad Sci USA* 108:12013–12017
- Huber RC et al (2012) Welfare assessment in transgenic pigs expressing green fluorescent protein (GFP). *Transgenic Res* 21:773–784. doi:[10.1007/s11248-011-9571-1](https://doi.org/10.1007/s11248-011-9571-1)
- Izquierdo D, Villamediana P, Lopez-Bejar M, Paramio MT (2002) Effect of in vitro and in vivo culture on embryo development from prepubertal goat IVM–IVF oocytes. *Theriogenology* 57:1431–1441. doi:[10.1016/S0093-691x\(02\)00647-7](https://doi.org/10.1016/S0093-691x(02)00647-7)
- Jackson KA, Berg JM, Murray JD, Maga EA (2010) Evaluating the fitness of human lysozyme transgenic dairy goats: growth and reproductive traits. *Transgenic Res* 19:977–986. doi:[10.1007/s11248-010-9371-z](https://doi.org/10.1007/s11248-010-9371-z)
- Jagadeesan P, Bin Salem S (2015) Transgenic and cloned animals in the food chain—are we prepared to tackle it? *J Sci Food Agric* 95:2779–2782. doi:[10.1002/jsfa.7205](https://doi.org/10.1002/jsfa.7205)
- Kruip TA, Blokhuis H (2001) Transgenesis may affect farm animal welfare: a case for systematic risk assessment. *J Anim Sci* 79:1763–1779
- Kubota C, Tian XC, Yang X (2004) Serial bull cloning by somatic cell nuclear transfer. *Nat Biotechnol* 22:693–694. doi:[10.1038/nbt975](https://doi.org/10.1038/nbt975)
- Kurome M et al (2008) Production efficiency and telomere length of the cloned pigs following serial somatic cell nuclear transfer. *J Reprod Dev* 54:254–258
- Laible G, Alonso-Gonzalez L (2009) Gene targeting from laboratory to livestock: current status and emerging concepts. *Biotechnol J* 4:1278–1292. doi:[10.1002/biot.200900006](https://doi.org/10.1002/biot.200900006)
- Laible G, Wei J, Wagner S (2015a) Improving livestock for agriculture—technological progress from random transgenesis to precision genome editing heralds a new era. *Biotechnol J* 10:109–120
- Laible G, Wei J, Wagner S (2015b) Improving livestock for agriculture—technological progress from random transgenesis to precision genome editing heralds a new era. *Biotechnol J* 10:109–120. doi:[10.1002/biot.201400193](https://doi.org/10.1002/biot.201400193)
- Lewis DB et al (1993) Osteoporosis induced in mice by overproduction of interleukin 4. *Proc Natl Acad Sci USA* 90:11618–11622
- Lillico SG et al (2016) Mammalian interspecies substitution of immune modulatory alleles by genome editing. *Sci Rep* 6:21645. doi:[10.1038/srep21645](https://doi.org/10.1038/srep21645)
- Liu J et al (2011) Effects of interval between fusion and activation, cytochalasin B treatment, and number of transferred embryos, on cloning efficiency in goats. *Theriogenology* 76:1076–1083. doi:[10.1016/j.theriogenology.2011.05.013](https://doi.org/10.1016/j.theriogenology.2011.05.013)
- Milot E et al (1996) Heterochromatin effects on the frequency and duration of LCR-mediated gene transcription. *Cell* 87:105–114
- Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL (1986) Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology* 25:591–600
- Pedram M, Sprung CN, Gao Q, Lo AW, Reynolds GE, Murnane JP (2006) Telomere position effect and silencing of transgenes near telomeres in the mouse. *Mol Cell Biol* 26:1865–1878. doi:[10.1128/MCB.26.5.1865-1878.2006](https://doi.org/10.1128/MCB.26.5.1865-1878.2006)
- Ramirez A et al (2001) Sequence and chromosomal context effects on variegated expression of keratin 5/lacZ constructs in stratified epithelia of transgenic mice. *Genetics* 158:341–350
- Sharma S, Kumar P, Betzel C, Singh TP (2001) Structure and function of proteins involved in milk allergies. *J Chromatogr B* 756:183–187. doi:[10.1016/S0378-4347\(01\)00107-4](https://doi.org/10.1016/S0378-4347(01)00107-4)
- Tamashiro K et al (2002) Cloned mice have an obese phenotype not transmitted to their offspring. *Nat Med* 8:262–267. doi:[10.1038/Nm0302-262](https://doi.org/10.1038/Nm0302-262)
- Tu Z, Yang W, Yan S, Guo X, Li X-J (2015) CRISPR/Cas9: a powerful genetic engineering tool for establishing large animal models of neurodegenerative diseases. *Mol Neurodegener* 10:1–8
- Wakayama S et al (2013) Successful serial recloning in the mouse over multiple generations. *Cell Stem Cell* 12:293–297. doi:[10.1016/j.stem.2013.01.005](https://doi.org/10.1016/j.stem.2013.01.005)
- Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* 385:810–813
- Yin XJ et al (2008) Production of second-generation cloned cats by somatic cell nuclear transfer. *Theriogenology* 69:1001–1006. doi:[10.1016/j.theriogenology.2008.01.017](https://doi.org/10.1016/j.theriogenology.2008.01.017)
- Younis A, Zuelke K, Harper K, Oliveira M, Brackett B (1991) In vitro fertilization of goat oocytes. *Biol Reprod* 44:1177–1182
- Zhang YL et al (2008) Phytohemagglutinin improves efficiency of electrofusing mammary gland epithelial cells into oocytes in goats. *Theriogenology* 69:1165–1171. doi:[10.1016/j.theriogenology.2007.10.028](https://doi.org/10.1016/j.theriogenology.2007.10.028)