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Identification and Generation of Transgenic Mice and Goats with *Capra hircus SCD1* Gene

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BL, CZ and QZ designed the study. KJ, MFA and CC performed research and analyzed data. KJ and XS wrote the paper. JS provided guideline on the manuscript.

Key words
SCD1, Testicular injection, Transgenic goat, Fatty acid, Generation

ABSTRACT

Goat (*Capra hircus*) stearoyl-CoA desaturase 1 (SCD1) plays a crucial role in fatty acid metabolism including milk and muscular fatty acid. This study investigated the expression of *SCD1* gene in goat and examined its inheritability and expression in transgenic SCD1 mice and goats. Our results suggested the possibility of generating transgenic goats by sperm-mediated gene transfer (SMGT). The expression of *SCD1* gene and fatty acid metabolism genes in goat mammary gland tissues and muscular tissues was aligned with GEO database, and tested by qRT-PCR. F_1 transgenic mice were mated and then the target genes and protein expression level in F_2 transgenic mice, as well as fat content in F_1 and F_2 transgenic mice muscle were examined. Successful transgenic goats generation was identified by testicular injection. The results showed a developmental time specific *SCD1* expression in the goat mammary gland tissue and muscular tissue. The rate of positive expression of exogenic gene and exogenic protein in F2 transgenic mice was 30% and 6.67%, respectively, Moreover, muscular fat content in the positive group was significantly higher than the control group both in F1 and F2 ($P > 0.05$). The rate of positive expression of exogenic gene and exogenic protein was 10.29% and 8.82% in the transgenic goat by testicular injection. Results of the present study showed that *SCD1* gene plays a crucial function in goat fatty acid metabolism, and that *SCD1* can regulate fat synthesis in *SCD1*-transferred mice and be inherited stably. Also, *SCD1*-transferred goat could be generated through testicular injection, suggesting a practical approach for transgenic livestock breeding.

INTRODUCTION

Stearoyl-CoA desaturase-1 (SCD1) is an endoplasmic reticulum enzyme that catalyzes the rate-limiting step towards the formation of monounsaturated fatty acids (MUFAs), specifically oleate and palmitoleate from stearoyl-CoA and palmitoyl-CoA, a key enzyme in fatty acid metabolism (Sampath *et al*., 2007; Paton *et al*., 2009). Chevon meat is a nutritious and healthy diet for human because it contain a variety of unsaturated fatty acids (UFA)which could significantly reduce the serum lipids levels in patients with hyperlipidemia (Ryzhenkov *et al*., 1995). Coincidently, *SCD1* transgenic goat can produce meet that satisfies with such patients' requirement through changing the composition of fatty acid and regulating *SCD1* expression.

SCD1 gene has been proven involved with the synthesis of fatty acids and fat in mouse and human (Hammond *et al*., 2005; Ntambi *et al*., 2002; Cohen *et al*., 2007; Maeda, 2007). In goat, SCD1 is a rate-limiting enzyme that regulates UFA synthesis *in vivo* (Wang *et al*., 2014), and its knockout can decrease the levesl of triglyceride (TG), cholesteryl ester (CE), wax ester (WE), diacylglycerol (DAG), lipidoses, palmitic acid (PA) and oleic acid (OA) in adipose tissue, while that of stearic acid increases (Flowers *et al*., 2012). SCD1 is also found to be a key enzyme participating into the synthesis of conjugated linoleic acid (CLA) in ruminant meat. It plays

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a significant role in some physiological processes such as bone mineralization, lipid metabolism and immune regulation (Lee *et al*., 1998). Furthermore, *SCD1* is the main candidate gene that may improve the quality of goat milk and meat because it can regulate the ratio of saturated fatty acids (SFA) and UFA in milk and lipidosis in meat (Brooks *et al*., 2011).

Transgenic technology is a powerful tool in animal improvement and function research, and it has been successfully used in an array of animals. However, only a few studies have ever addressed the molecular mechanism underlying unsaturated fatty acids and fatty acid metabolism in goats. This has hindered the breeding of quality mutton and milk. We previously investigated the distribution of *SCD1* gene in goat tissues and cells, successfully generated *SCD1* gene-transferred mice by testicle injection (Zuo *et al*., 2018). In this study, the heredity of *SCD1* gene in the transgenic mice was examined, and transgenic goats with *SCD1* gene were generated by testicle injection to test its application for livestock breeding in terms of fatty acid metabolism.

MATERIALS AND METHODS

Ethics statement

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Yangzhou University Animal Experiments Ethics Committee (permit number: SYXK [Su] IACUC 2012-0029). All experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China. Goats were provided by Dongshan Town Animal and Veterinary Station in Soochow City, Jiangsu Province, China, and Male ICR mice were purchased from the Comparative Medicine Centre of Yangzhou University.

Materials

The test goats were provided by Dongshan Town Animal and Veterinary Station in Soochow City, Jiangsu Province, China. The recombinant plasmid pEGFP-C1-SCD1 and transgenic mice (F1) were from our laboratory.

Polyethylene imine (PEI), protease K and trypsase were obtained from Sigma (USA). The First Strand cDNA Synthesis Kit, Primer STAR MaxDNA Polymerase and DL5000DNAMarker were purchased from TaKaRa (Dalian, China). SYBR Green Master Mix Kit was obtained from Vazyme Biotech (Nanjing, China). Tryptone, yeast powder, NaCl, agar powder were obtained from Solarbio Science and Technology Co., Ltd (Beijing, China).

GEO database and homology as well as structure analysis of goat SCD1

The expression data was downloaded from GEO Public Database (https://www.ncbi.nlm.nih.gov/geo/). The expression of mammary gland in different lactation period data ID: GSE87089 and the expression of Muscle Tissue in Different Growth Stages data ID: GSE85014. The homology of SCD1 was analyzed with MEGE6.0 software. The topography of goat SCD1 was modeled by SWISS-MODEL, and then compared with that of the x-ray of mice SCD1.

qRT-PCR

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen). cDNA was reverse transcribed from 1 μg total RNA using the First Strand cDNA Synthesis Kit following manufacturer's instructions. qRT-PCR was performed using SuperRealPreMix Plus (SYBR Green). Primers used for qRT-PCR analyses are listed in Table I. All experiments were performed in triplicates.

Generation and identification of F_2 transgenic mice

The male and female mice of F_1 transgenic generation of mice (Zuo *et al*., 2018) were mated after sexual maturation. Tail tissues of the F2 generation were collected to test gene expression and protein levels. Primers used for qPCR assay are as follows.

F-GFP: 5′-CCTGAAGTTCATCTGCACCA-3′
R-GFP: 5′-TCATGCCTCTTTCTCGTAAG-3′
F-GFP-SCD1:5′-CCTGAAGTTCATCTGCACCA- 3′
R-GFP-SCD1:5′-TCAGCCACTCTTGCAGCTTT- 3′

Western blot was used to test the expression of EGFP in F_2 offspring as previously suggested (Zuo *et al*., 2018).

Determination of muscular fat content in transgenic mice

The positive and negative controls of F_1 and F_2 were killed by cervical dislocation and fat content was measured in crureus and longissimus dorsi by Soxhlet extractor method as previously described (Zuo *et al*., 2018).

Generation and identification of transgenic goats

The recombinant plasmid pEGFP-C1-SCD1 was encapsulated by polyethylene imine (PEI) (μL) and DNA (μg) at a ratio of 3: 1. Both testicles of male goats were injected with 3mg of encapsulated recombinant plasmid pEGFP-C1-SCD1. Seven days after first injection, the males were injected again to strengthen the effects. Forty days after second injection, these goats were mated with female goats, and the breeding ratio of male to female was 1: 3. Testicles of the control group were not injected. DNA and protein were extracted from the lambs, and the target gene and protein were detected as described above.

Statistical analysis

The data are presented as the mean with SEM. Differences in the expression of specific markers were evaluated using Student's t-test (SPSS22.0, IBM). The results of the different treatments were considered statistically significant at $P < 0.05$ and were highly significant at $P < 0.01$.

RESULT

Expression of SCD1 *and fatty acid metabolism genes in goats*

In mammalian, SCD1 is the main enzyme responsible for the conversion of saturated fatty acyl-CoAs, stearoyl-CoA (18:0) and palmitoyl-CoA (16:0), to their respective mono-unsaturated fats acyl-CoAs, oleyl-CoA (18:1) and palmitoleyl-CoA (16:1) (Fig. 1A) (Gutierrezjuarez *et al*., 2006). FASN, PPARG and ACACA regulate the *de novo* fat acid synthesis, DAGT1, DAGT2, LIPE and PNPLA2 regulate triacylglycerol synthesis and degradation, and CD36, ACOX1 and CPTB1 regulate the uptake, intracellular transport and oxidation of fatty acids (Yao *et al*., 2017). The results of protein-protein interaction (PPI) analysis showed that *SCD1* gene has probably regulated fatty acid metabolism through its interaction with these key genes mentioned in Figure 1B.

To investigate the expression of *SCD1* gene in goat, development-specific expression of this gene in mammary gland and muscular tissue was downloaded from the GEO database. The results showed that *SCD1* gene was expressed lower in adult's dorsal longissimus and dried mammary gland. In contrast, it was higher in juvenile dorsal longissimus and lactation-period mammary gland. Moreover, the downstream expression pattern of fatty acid metabolism gene suggested its regulation by SCD1. The results indicated a critical role of *SCD1* gene in fatty acid metabolism in goat muscle and mammary gland (Fig. 1C).

Xuhuai goat SCD1 was highly conserved and highly expressed in juvenile dorsal longissimus and lactation-period mammary gland tissue

Phylogenetic analysis indicated that *SCD1* gene sequence is highly conserved across species, suggesting that SCD1 has a sequence synonymous mutation (Fig. 2A). Furthermore, protein sequence and structure analysis showed that goat SCD1 had a highly similarity to mice SCD1 when aligned with mice SCD1 X-ray structure (Fig. 2B). To investigate the distribution of SCD1 in goat, we evaluated its expression patterns in different tissue samples of goat with qRT-PCR. The results showed that although SCD1 was widely expressed in different tissues, its expression in mammary gland was the highest (Fig. 2C). In particular, the expression of SCD1 during the lactation period was significantly higher than that in the mammary gland, suggesting a critical role of SCD1 in goat's milk production. However, SCD1 expression decreased gradually in longissimus dorsi with goat development, which was consistent with the result of RNA-seq. Our results suggested a critical role of SCD1 in goat fatty acid metabolism (Fig. 2D).

Generation of transgenic mice and stable inheritance

To explore the function of goat *SCD1* gene, transgenic mice carrying this gene was generated by testicular injection of the pEGFP-SCD1 vector. The plasmids were linearized and injected into mice testes to produce F_1 transgenic mice. The resulting mice were identified and mated to produce F_2 transgenic mice (Fig. 3A). A total of 60 healthy offspring of F_1 transgenic mice were obtained. *SCD1* gene and its expression in tail tissue were assayed by PCR and western blot. The results showed that the positive rate of gene expression and protein expression in the F_1 transgenic mice was 18.33% and 6.67%, respectively (Fig. 3D). Thirty healthy offspring of F_2 transgenic mice were mated with the positive F_1 transgenic mice, and the results showed that the positive rate of gene expression and protein expression in the F1 transgenic mice was 30% and 26.67% (Fig. 3B, 3C and 3D). The detection of muscular fat content in the crureus and longissimus dorsi of F_1 and F_2 transgenic mice showed that muscular fat contents in positive individuals in two kinds of muscle were significantly increased compare with negative individual (Fig. 3E, 3F).

Identification and generation of transgenic goats

To produce transgenic goats, the testis injected healthy males were mated with estrus female goats. A total of 68 healthy offspring of the transgenic goats was produced by testicular injection method (Fig. 4A, 4B). Tail tissue samples were collected to identify the expression of transgenic gene in the transgenic goat by PCR. The normal lambs were negative controls and pEGFP-SCD1 plasmid positive controls. Seven positive individuals were generated and the positive rate of gene expression was 10.29% (Fig. 4C, 4E). Protein expression of the positive individual was identified by Western the results showed that we got 6 positive individuals and the positive rate of gene expression was 8.82% with the protein of normal lamb was negative control (Fig. 4D, 4E).

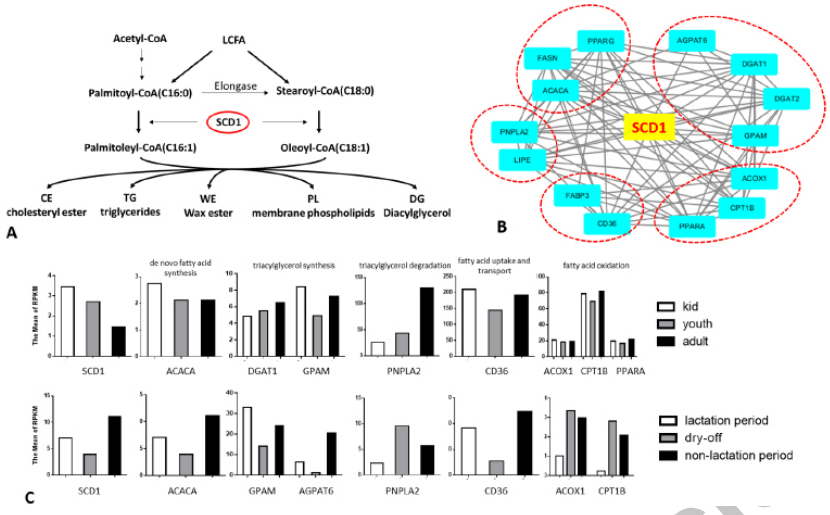

Fig. 1. *SCD1* and fat acid metabolism genes expression in goat. (A) Schematic diagram of SCD1 role in lipid synthesis. CE, cholesteryl esters; TG, triglycerides; WE, wax esters; PL, phospholipids; DG, diacylglycerol. (B) Effects of SCD1 on lipid accumulation and expression of genes related to lipid metabolism. (C) Variation of genes in lipid metabolism in mammary gland and muscular tissue at different developmental stages including *de novo* fat acid synthesis (FASN, ACACA, and PPARG), triacylglycerol synthesis (DGAT1, DGAT2, GPAM, AGPAT6, and LPIN1), triacylglycerol degradation (PNPLA2 and LIPE), fat acid uptake and intracellular transport (FABP3 and CD36), and fat acid oxidation (PPARA, ACOX1, and CPT1B) by RNA-seq.

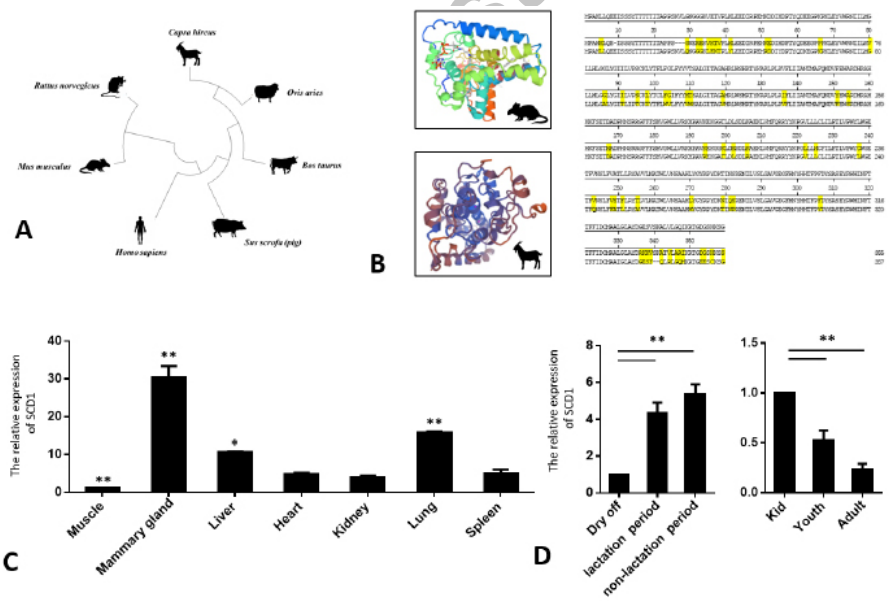

Fig. 2. Xuhuai goat *SCD1* was highly conserved and highly expressed in juvenile dorsal longissimus and lactating mammary gland tissue. (A) Phylogenetic relationships of SCD1. Cloned gene sequence was submitted to GenBank with numbers: *Homo sapiens*, *Ovis aris, Capra hircus, Mus musculus, Rattus norvegicus, Suss crofa,* and *Bos taurus*. Phylogenetic relationship of these sequences was estimated by neighbor-joining algorithm using PAM distances. Branch length is proportional to amount of evolution assumed to occur in each lineage. Numbers indicate the percentage of bootstrap replications that support each node. (B) Alignment of goat and mouse SCD1 protein structure, the different amino acid is yellow line. (C) SCD1 is more highly expressed in GOAT mammary gland, liver and lung, but low expressed in muscle. Error bars represent mean value ± SEM. (* $p < 0.05$, ** $p < 0.01$). (D) Expression of *SCD1* increased significantly during lactation compared with the dry period in mammary gland and lamb during adult period in muscle. Quantitative PCR data were normalized to the dry period. Error bars represent mean value ± SEM. (* $p < 0.05$, ** $p < 0.01$).

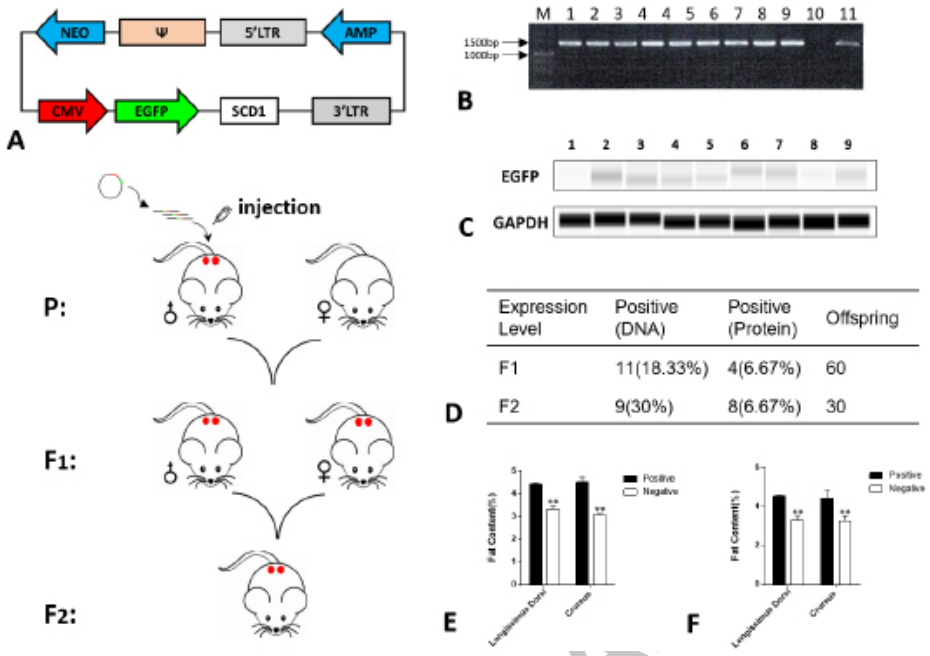

Fig. 3. Establishment of transgenic mice and stable inheritance. (A) Protocol for transgenic mice generation. The healthy adult male mice were chosen for testicle injection, and after 5 days one treated male and three healthy non-pregnant adult female mice were put in one cage. The resulting positive F1 males were mated with female mice after sexual maturity to produce F2 generation. (B) DNA PCR assay of F1 generation tail tissue. M: DL5000bp marker; 1-9, experimental group; 10: negative control; 11: positive control. (C). Western blot assay results of F1 generation. 1-9: the experimental group. (D). Number of F1 and F2 with *SCD1* expression in transgenic mice. (E). Expression of SCD1 increased significantly in longissimus dorsi and crureus of F1 transgenic mice. Error bars represent mean value ± SEM. (* $p < 0.05$, ** $p < 0.01$).(F). Expression of SCD1 increased significantly in Longissimus dorsi and Crureus of F2 transgenic mice. Error bars represent mean value ± SEM. (* $p < 0.05$, ** $p < 0.01$).

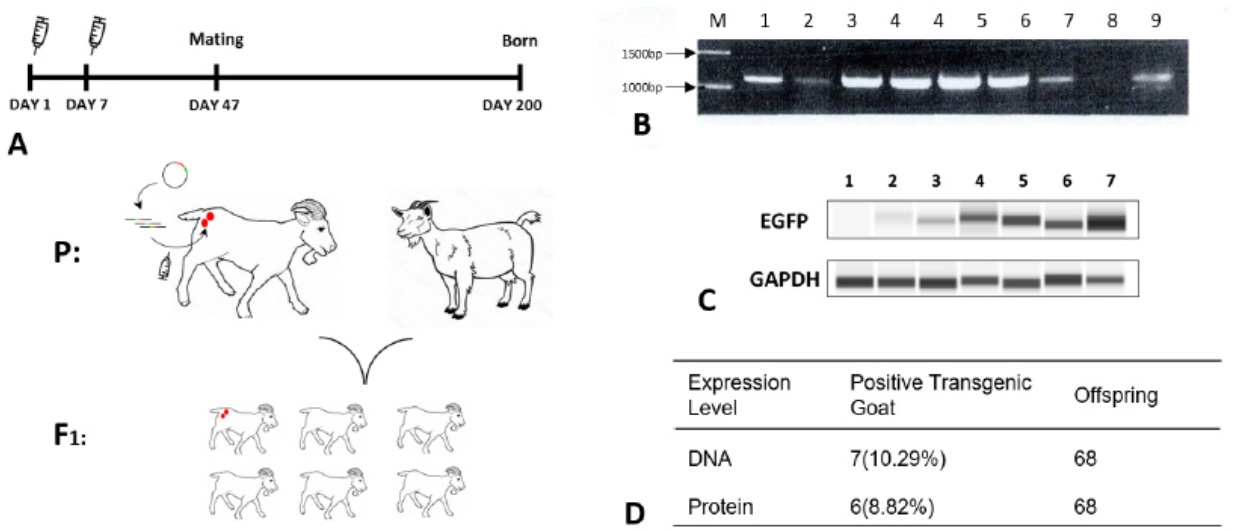

Fig. 4. Identication and generation of transgenic goat. (A) Protocol for transgenic goat production. The healthy adult male goat were chosen for testicle injection twice, and after 47 days, one treated male and three healthy non-pregnant adult female goat were mated to produce F1 generation. (B) DNA PCR assay of tail tissue in F1 generation. M, DL5000 bp marker; 1-7, experimental postive group; 8, negative control; 9, positive control. (C) Western blot assay results of F1 generation. 1-7, the experimental group. (D) Number of transgenic F1 goat with SCD1 expression.

Table I. Primers used for RT-PCR and qRT-PCR analyses.

Gene name	Primer (5'-3')	Gene ID
SCD1	F: CCATCGCCTGTGGAGTCAC R: GTCGGATAAATCTAGCGTAGCA	GU947654
ACACA	F: CTCCAACCTCAACCACTACGG R:GGGGAATCACAGAAGCAGCC	JN236219.1
DGAT1	F: CCACTGGGACCTGAGGTGTC R: GCATCACCACACACCAATTCA	DQ380249.1
GPAM	F: GCAGGTTTATCCAGTATGGCATT R: GGACTGATATCTTCCTGATCATCTTG	AY515690
AGPAT6	F: AAGCAAGTTGCCCATCCTCA R: AAACTGTGGCTCCAATTTCGA	JI861797.1
PNPLA2	F: GGAGCTTATCCAGGCCAATG R: GCGGGCAGATGTCACTCT	GQ918145
CD36	F: GTACAGATGCAGCCTCATTTCC R: TGGACCTGCAAATATCAGAGGA	X91503
ACOX1	F: CGAGTTCATTCTCAACAGTCCT R: GCATCTTCAAGTAGCCATTATCC	NM_001035289.2
CPT1B	F: AAGGACCTCTACGCCAACACG R: TTTGCGGTGGACGATGGAG	FJ415874.1
PPARA	F: TACTCTCGGCAGACTTCCTAC R: CCTCCTCACATCTGTCATACAC	HM600811.1
GAPDH	F: CAAAGTGGACATCGTTGCCA R: TGGAAGATGGTGATGGCCTT	XM_005680968.3

DISCUSSION

Numerous researches have suggested SCD1 is closely associated with fat acid synthesis and fat content in human (Heinemann *et al*., 2003; Miyazaki *et al*., 2003). In dairy cow and goat, the polymorphism of SCD1 was associated with altered milk fatty acid composition and milk (Mcmanaman *et al*., 2007; Schennink *et al*., 2008). In this study, the expression pattern of *SCD1* and its downstream genes in the mammary gland (dry-off, lactation and non-lactation) and muscular tissue (cubs, juveniles and adults) was analyzed and identified by public GEO database; qRT-PCR was used to identify the heredity of transgenic mice. We also generated and identified the transgenic goat.

Testicular injection is a widely applied method to generate transgenic animals (Houdebine, 2002; Qin *et al*., 2016). Lavitrano *et al*. (1989) incubated the plasmid with capacitated sperm to obtain mice offspring carrying the plasmid. Yonezawa *et al*. (2001) injected Liposome-encapsulated plasmids into rat testes to generate transgenic rats. Farre *et al*. (1999) successfully obtained transgenic pigs by transfected testis *in vivo*. Reh *et al*. (2004) found that the ratio of monounsaturated and conjugated linoleic acid in milk of rat SCD1-transfered goat was significantly higher than control. In a previous study we successfully generated transgenic mice with goat SCD1 gene by testicular injection (Zuo *et al*., 2018). In the present study, the heredity and fat content in transgenic mice were identified and analyzed. The results showed that transgenic goat SCD1-transferred mice could be stably inherited and fat content in the crureus and longissimus dorsi was significantly improved. This study provides a model and reference for the integration and expression of exogenic genes in xenogeneic animals. We successfully generated SCD1 gene-transferred goats by testicle injection with a positive rate being 8.82%, indicating that testicular injection is a potential method for the generation of transgenic animals. Moreover, this method neither requires precise instruments such as embryonic injection device, nor is the safety risk assessment of lentivirus transfection necessary. In addition, this method is simple and easy to operate, suggesting a great application prospect.

In this study, it was found that the expression level of SCD1 gene is higher in juvenile dorsal longissimus and lactating mammary gland tissue, indicating its crucial role in goat mammary gland and musular lipogenesis. SCD1 regulates fat acid synthesis through its interaction with numerous downstream genes; Shi *et al*. (2013) found that SCD1 interacts with AGPAT6, ACACA, CD36, FASN, DGAT1, FABP3 and PPARG to regulate fatty acid

synthesis and fat content in mammary gland tissues; Lin *et al*. (2013) proved that SCD1 regulates fat synthesis in conjunction with ACOX1, CPT1B, LIPE, PNPLA2 and PPARA in mammary epithelial cells; Bionaz and Loor (2007, 2008) demonstrated that SCD1-mediated fat acid synthesis in mammary gland cells is involved with the expression of DGAT2 and GPAM. Meanwhile, this study showed that the expression of fat acid metabolism genes is also contingent upon the development specific expression of *SCD1*. Previous study showed that rat *SCD1* gene transfer into goat has affected the fat content in goat milk (Zidi *et al*., 2010). The present study shows that the muscular fat content in mice has been significantly increased after goat *SCD1* gene transfer, indicating that SCD1 has been expressed in mice and functioned to regulate fat synthesis across species. Structure analysis of mice and goat *SCD1* genes also showed that *SCD1* gene in both species has similar sequence and structure. It probably indicated that the functional regulation of *SCD1* gene was generalized across different species. As a result, malfunction of SCD1 could cause some diseases such as diabetes, atherosclerosis, cancer and obesity. Our study also provides a reference for further study of the function of *SCD1* gene (Brown *et al*., 2008).

In conclusion, this study investigated the role of *SCD1* gene in goat fat acid metabolism and demonstrated that goat *SCD1* gene-transferred mice could regulate fat synthesis and be inherited stably. The transgenic goat was successfully generated by testicular injection, which provides a potential approach for the production of transgenic livestock.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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