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Scd1 mammary-specific vector constructed and overexpressed in goat fibroblast cells resulting in an increase of palmitoleic acid and oleic acid

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ABSTRACT

Stearoyl-CoA desaturase-1 (*Scd1*) is a rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids. Overexpression of *Scd1* in transgenic animals would modify the nutritional value of ruminant-derived foods by increasing the monounsaturated fatty acid (MUFA) and decreasing the saturated fatty acid (SFA) content. The aim of this study was to develop an effective *Scd1* vector that is specifically expressed in dairy goat mammary glands. We successfully amplified the goat full length *Scd1* cDNA and evaluated its activity in goat ear skin-derived fibroblast cells (GEFCs) by lipid analysis. In addition, we constructed a mammary gland-specific expression vector and confirmed efficient expression of *Scd1* in goat mammary epithelial cells (GMECs) by qRT-PCR and Western blot analysis. Fatty acid analysis showed that *Scd1*-overexpression resulted in an increase in levels of palmitoleic acid (16:1n-7) and oleic acid (18:1n-9), from 1.73 ± 0.02% to 2.54 ± 0.02% and from 27.25 ± 0.13% to 30.37 ± 0.04%, respectively (both $p < 0.01$) and the ratio of MUFA to SFA was increased. This work lays a foundation for the generation of *Scd1* transgenic goats.

1. Introduction

Stearoyl-CoA desaturase-1, a membrane-bound protein of the endoplasmic reticulum that is encoded by the *Scd1* gene, has recently been identified as one of the major enzymes in the control of lipid metabolism [1,2]. SCD1 is a Δ-9 desaturase that introduces a cis-double bond at the Δ-9 position of carbon-14 through carbon-19 fatty acyl-CoAs to yield the corresponding monounsaturated fatty acids (MUFAs) [3]. SCD1 is also responsible for the conversion of *trans*-11 C18:1 to *cis*9, *trans*11-conjugated linoleic acid (CLA), a major isomer of CLA in ruminant-derived foods which are significant sources of CLA in the human diet [4].

Increasing evidence indicates that excessive intake of saturated fatty acids (SFAs) leads to cardiovascular disease, insulin resistance and obesity [5–7]. MUFAs and two major isomers of CLA (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) have a range of positive health effects including reducing body fat and decreasing the risk of chronic diseases such as cardiovascular diseases and cancers [8–11].

Milk and milk products provide high-quality protein and other macro- and micronutrients but contribute significantly to total SFAs in the human diet [12,13]. The nutritional value of milk can be modified by alteration of the diet management and transgenic approaches. The fatty acid content in foods can be modified by gene transfer or increasing the expression of target genes *in vivo* [14]. Many studies have focused on increasing the synthesis of n-3 PUFA (polyunsaturated fatty acid) and reducing the *n*-6/*n*-3 ratio via transgenic technologies [15]. To our knowledge, there is only one study on increasing MUFA levels in ruminant [16].

To modify the nutritional value of milk by increasing the MUFA content, we aimed to express *Scd1* exclusively in transgenic mammary glands of dairy goats. Firstly, we amplified the goat full length *Scd1* cDNA and demonstrated that the expressed SCD1 protein possessed Δ-9 desaturase activity for the conversion of C16:0 to C16:1n7 and C18:0 to C18:1n9. Secondly, mammary gland-specific expression vector of *Scd1* was constructed and its function was confirmed in GMECs. The ultimate goal of this study in the future is that, MUFA-rich milk can be produced from the transgenic goats with *Scd1*. In sum, it lays a foundation for the study on the molecular mechanisms of this gene in lipid metabolism or *Scd1*-related signal pathways in the mammary gland, as well as the application in animal transgenesis experiment.

Abbreviations: Scd1, Stearoyl-CoA desaturase-1; GEFCs, goat ear skin-derived fibroblast cells; GMECs, goat mammary epithelial cells; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; CLA, conjugated linoleic acid.

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2. Materials and methods

2.1. Construction and assessment of the goat Scd1 eukaryotic expression vector

The full length cDNA fragment encoding *Scd1* (GeneBank No. AF325499) was amplified from saanen goat lactating mammary gland tissue. The primers F1/R1 used for the synthesis of *Scd1* cDNA, were capped with *Xho*I sites in the 5′ terminal end to facilitate the subcloning of pIRES-EGFP. The synthesized fragment was inserted into the pMD-19T vector (Takara, China), followed by sequencing. The *Scd1* gene was isolated from pMD-SCD1 and inserted into the vector pIRES-EGFP (Clontech, USA) by *Xho*I site, followed by enzymatic digestion and partial DNA sequencing. The plasmid obtained was named pSCD1-EGFP. A schematic representation of this vector was shown in Fig. 1A and primers used for the plasmid construction were listed in Table 1.

2.2. Construction of the goat Scd1 mammary-specific expression vector

The *Scd1* cDNA was released by *Xho*I enzyme (Takara) and inserted into the same *Xho*I site of pBC1 (Invitrogen, USA). The obtained vector was named pBC1-SCD1. Loxp-Neo-IRES-EGFP was amplified from pNeo-IRES$_2$-EGFP vector [17] and inserted into

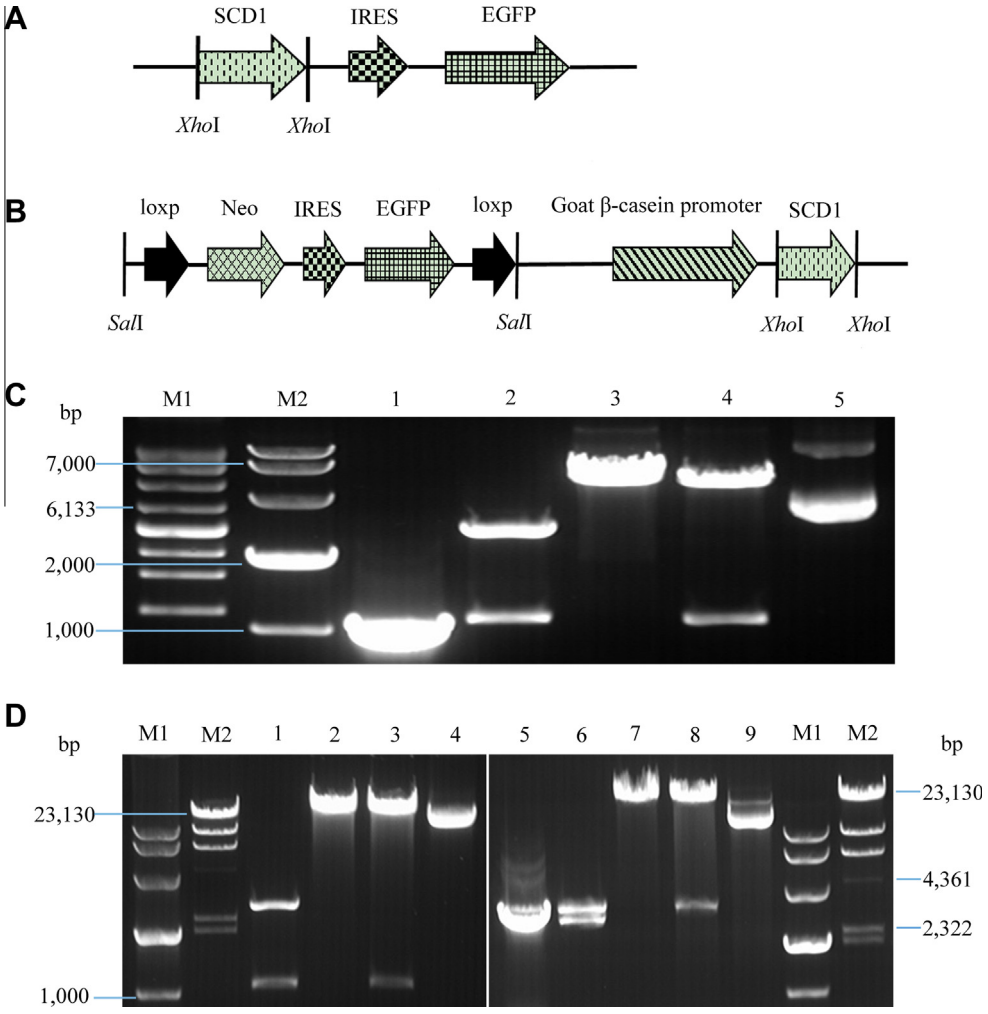

Fig. 1. Construction and assessment of pSCD1-EGFP and pBC1-SCD1-LNIE. (A) The synthesized *Scd1* was cloned into pIERS-EGFP (Clontech) which has been digested by *Xho*I to generate the pSCD1-EGFP vector. (B) DNA construct for *Scd1* mammary gland-specific vector with a goat β-casein promoter and dual selectable marker genes Neo and EGFP. (C) Restriction enzymatic digestion of pSCD1-EGFP. Lane1: *Scd1* fragment (1080 bp); Lane 2–4: pMD-SCD1, pIERS-EGFP and pSCD1-EGFP digested by *Xho*I; Lane 5: pSCD1-EGFP plasmid, M1: Supercoiled DNA Ladder Marker, M2: DL10000 DNA marker. (D) Restriction enzymatic digestion of pBC1-SCD1-LNIE. Lane 1–3: pMD-SCD1, pBC1 and pBC1-SCD1 digested by *Xho*I; Lane 4: pBC1-SCD1 plasmid; Lane 5: Loxp-Neo-IRES-EGFP fragment (3114 bp); Lane 6–8: pMD-LNIE, pBC1-SCD1 and pBC1-SCD1-LNIE digested by *SalI;* Lane 9: pBC1-SCD1-LNIE plasmid. M1: DL10000 DNA marker, M2: λ-*Hind* III digest DNA marker.

Table 1
Primers for the plasmid construction.

Gene name and DNA fragment	Primer sequences	Size of PCR product (bp)	Annealing temperature (°C)
Scd1	F1: CCGCTCGAGATGCCGGCCCACTTGCTGC R1: CCGCTCGAGTCAGCCACTCTTGCAGCTTTCCTCT	1080	60
Loxp-Neo-IRES-EGFP	F2: GGCGGGTCGACATAACTTCGTATAGCATACATTATACGAAGTTATTAGTTATTAATAGTAATCAATTACGG R2: GTAATGTCGACATAACTTCGTATAGCATACATTATACGAAGTTATTTGGACAAACCACAACTAGAATG	3114	65

Table 2
Primer sequences and conditions used for real-time PCR analysis.

Genes and reference sequences (Genbank No.)	Primer sequences	Size of PCR product (bp)	Annealing temperature (°C)
Scd1(AF325499)	F: GGCACATCAACTTTACCACATTCTT R: TTTCCTCTCCAGTTCTTTTCATCC	120	60
Gapdh (NM001034034)	F: CGACTTCAACAGCGACACTCAC R: CCCTGTTGCTGTAGCCGAATTC	119	60

the pMD-19T vector (Takara, China), followed by sequencing. The primers F2/R2 used for the synthesis of the marker genes, contained *Sal*I sites and loxp sites in the same orientation in the 5′ terminal end, *Sal*I sites were to facilitate the subcloning of pBC1-SCD1 vector and two loxp sites were used for the deletion of marker genes (Neo and EGFP). The obtained vector was named pMD-LNIE. The Loxp-Neo-IRES-EGFP fragment was released by *Sal*I enzyme (Takara) and cloned into the same *Sal*I site of pBC1-SCD1. The final vector was named as pBC1-SCD1-LNIE. The plasmids were confirmed by enzymatic digestion and partial DNA sequencing. The schematic diagram of this vector was shown in Fig. 1B and primers used for the plasmid construction were listed in Table 1.

2.3. Cell culture and transfection

Goat ear skin-derived fibroblast cells (GEFCs) were transfected with pSCD1-EGFP and goat mammary epithelial cells (GMECs) were transfected with pBC1-SCD1-LNIE plasmid by the Lipofectamine™ LTX and PLUS reagent (Invitrogen, USA) according to the manufacturer's protocol. The GEFCs were cultured in fresh growth medium 6 h after transfection, the GMECs were cultured in inductive medium (DMEM-F12 + 10% FBS + 5 μg/ml insulin + 5 μg/ml hydrocortisone + 10 ng/ml EGF + 5 μg/ml Prolactin). After incubation for 48 h, the confluent cells were harvested for lipid analysis, real-time PCR or Western blot analysis.

2.4. Lipid analysis

As previously described, the confluent cells were collected for fatty acid analysis after 48 h incubated [18,19]. Fatty acid methyl esters (FAME) were quantified using a GC-2010 Plus gas chromatograph (Shimadzu, Japan) with a CP-Sil 88 WCOT fused silica column (100 m × 0.25 mm i.d. × 0.2 μm film thickness). Peaks were identified by comparison with fatty acid methyl esters standards (Sigma) and peak areas of various analyzed fatty acids were used to calculated relative contents of fatty acids.

2.5. Real-time PCR analysis

Total RNA were isolated from GMECs and T-GMECs (transfected GMEC) using PureLink® RNA Mini Kit (Invitrogen, USA) according to manufacturer's protocol. Real-time PCR was performed on an ABI 7300 Real-Time PCR System (Applied BioSystems Carlsbad, CA) and detected by SYBR Green Master in a reaction volume of 20 μl. The Genbank accession numbers and primer sequences used for amplification of the target genes were presented in Table 2. Comparative quantification of *Scd1* mRNA was done by the $^{\triangle\triangle}Ct$ method [20].

2.6. Western blotting

Protein were isolated from GMECs and T-GMECs using RIPA Lysis Buffer (Beyotime, China). Western blotting was conducted using a standard protocol. A primary rabbit anti-SCD1 antibody (1:500, Santa, CA) and HRP-conjugated GAPDH (1:500, Abcam, USA) (loading control) were used in the Western blot analysis. Band intensities were estimated by densitometry and corrected by the respective GAPDH band intensities [21].

2.7. Statistical analysis

All experiments were repeated at least three times. Results were expressed as mean ± standard error (SEM). Statistically significant differences were performed using the SPSS software (version 18.0). Differences of $p < 0.05$ were considered to be significant.

3. Results

3.1. Construction and assessment of plasmids by restriction digestion

The full length *Scd1* cDNA (1080 bp) was amplified from goat cDNA and the cloned sequence was 100% homologous with the *Scd1* sequence available in GenBank. The Loxp-Neo-IRES-EGFP fragment was also amplified and the cloned sequence was 100% homologous with the template. The pSCD1-EGFP plasmid and the mammary gland-specific plasmid pBC1-SCD1-LNIE was successfully confirmed by PCR, restriction enzyme digestion and sequencing. As predicted, *Xho*I cleaved pSCD1-EGFP into 5.3-kb and 1.1-kb fragments (Fig. 1C) and cleaved pBC1-SCD1 into 21-kb and 1.1-kb fragments (Fig. 1D). *Sal*I cleaved pBC1-SCD1-LNIE into 22-kb and 3.1-kb fragments (Fig. 1D).

3.2. Expression of the Scd1 gene in goat ear skin-derived fibroblast cells

A eukaryotic expression vector for goat *Scd1*, pSCD1-EGFP, was successfully constructed and introduced into GEFCs to evaluate the activity of *Scd1 in vitro*. Microscopic inspection of cultures under UV light 24 h post-transfection confirmed expression of EGFP and indicated that *Scd1* was successfully co-expressed in the cells. Real-time PCR analysis confirmed that the relative expression level of *Scd1* was 16.5-fold higher than that in control cells.

GEFCs transfected with pSCD1-EGFP or pIRES-EGFP were subjected to fatty acid analysis. Consistent with the qRT-PCR results, gas chromatography data showed that overexpression of *Scd1* resulted in changes in fatty acid composition (Fig. 3 and Table 3). Levels of palmitoleic acid (16:1n-7) and oleic acid (18:1n-9) were markedly increased in the transfected cells compared with the control (2.54 ± 0.02% vs. 1.73 ± 0.02% and 30.37 ± 0.04% vs. 27.25 ± 0.13% respectively, $p < 0.01$). There was no significant increase in *cis*9, *trans*11-CLA ($p > 0.05$), but the concentration of *trans*10, *cis*12-CLA (0.42 ± 0.01% vs. 0.24 ± 0.01%) was greatly increased ($p < 0.01$). The amount of total monounsaturated fatty acids was significantly increased compared with the control (43.10 ± 0.01% vs. 40.31 ± 0.52%, $p < 0.01$). In contrast, the amount of total saturated fatty acids was significantly lower than in the control (29.41 ± 0.33% vs. 31.28 ± 0.45%, $p < 0.05$). In addition, compared with the control, the conversion ratio of C16:1n-7/C16:0 in the transfectants increased from 0.10 to 0.14 and the ratio of C18:1n-9/C18:0 increased from 2.09 to 2.70, both of which were significant changes ($p < 0.01$).

Table 3
Partial fatty acid composition in GEFCs (goat ear skin-derived fibroblast cells) transfected with pIRES-EGFP or pSCD1-EGFP.

Fatty Acids	GEFCs	
	Control	Transfected
C16:0	17.98 ± 0.37	17.84 ± 0.25
C18:0	13.01 ± 0.19	11.26 ± 0.08**
C16:1n-7	1.73 ± 0.02	2.54 ± 0.02**
C18:1n-9	27.25 ± 0.13	30.37 ± 0.04**
c9, t11 CLA	0.09 ± 0.02	0.11 ± 0.00
t10, c12CLA	0.24 ± 0.01	0.42 ± 0.01**
C16:1n-7/ C16:0	0.10 ± 0.00	0.14 ± 0.00**
C18:1n9/C18:0	2.09 ± 0.04	2.70 ± 0.02**
MUFA	40.31 ± 0.52	43.10 ± 0.01**
SFA	31.28 ± 0.45	29.41 ± 0.33*

Fatty acids composition is presented as a percentage of the total cellular lipids from the control cells and *Scd1* cells. Each value represented the mean ± SEM. Significant differences between the control and *Scd1* cells were marked (*$p < 0.05$; **$p < 0.01$). Total monounsaturated fatty acids are given by C16:1, C18:1, C20:1. Total saturated fatty acids are given by C16:0 , C18:0, C20:0.

3.3. Induced expression of Scd1 in goat mammary epithelial cells

GMECs were transiently transfected with pBC1-SCD1-LNIE and the expression of *Scd1* was analyzed by real-time PCR and Western blotting analysis. Inspection of cultures under UV light 24 h after transfection revealed that the plasmid was successfully introduced into GMECs (Figs. 2E–H). QRT-PCR analysis showed a 14.1-fold increase in expression of *Scd1* in T-GMECs (Fig. 4A). In Western blot analysis, lysates of both GMECs and T-GMECs reacted positively with anti-SCD1 antibody. GAPDH was used as a loading control and protein expression was normalized to GADPH expression (Fig. 4B). Quantification of signal intensity using IPP 6.0 software indicated a 7-fold increase in SCD1 protein expression in T-GMECs compared with cells transfected with the pBC1-LINE control plasmid (Fig. 4C).

4. Discussion

With increasing awareness of the benefits of lower consumption of SFAs, many studies have focused on increasing the unsaturated fatty acid (UFA) content of our food. In 2004, transgenic goats carrying the rat *Scd1* gene were successfully produced, and which showed a significant increase of UFAs and *cis*9, *trans*11-CLA in milk [16]. Expression of human *Scd1* in 293 cells *in vitro* resulted in an increase in levels of CLA and $n-7$ fatty acids [22]. In the present study, we found that *Scd1* overexpression significantly up-regulated MUFA and *trans*10, *cis*12-CLA in GEFCs. However, the level of *cis*9, *trans*11-CLA was not significantly increased, possibly due to a lack of additional *trans*11-C18:1 in the culture media. The level of C18:0, the substrate of C18:1, was decreased whereas that of C16:0, the substrate of C16:1, did not significantly decrease. This difference may stem from their different biosynthesis pathways: approximately half of the cellular C16:0 can be synthesized *de novo* whereas all C18:0 must be provided [3]. The unsaturated/saturated FA ratios in milk are usually used to estimate the desaturate

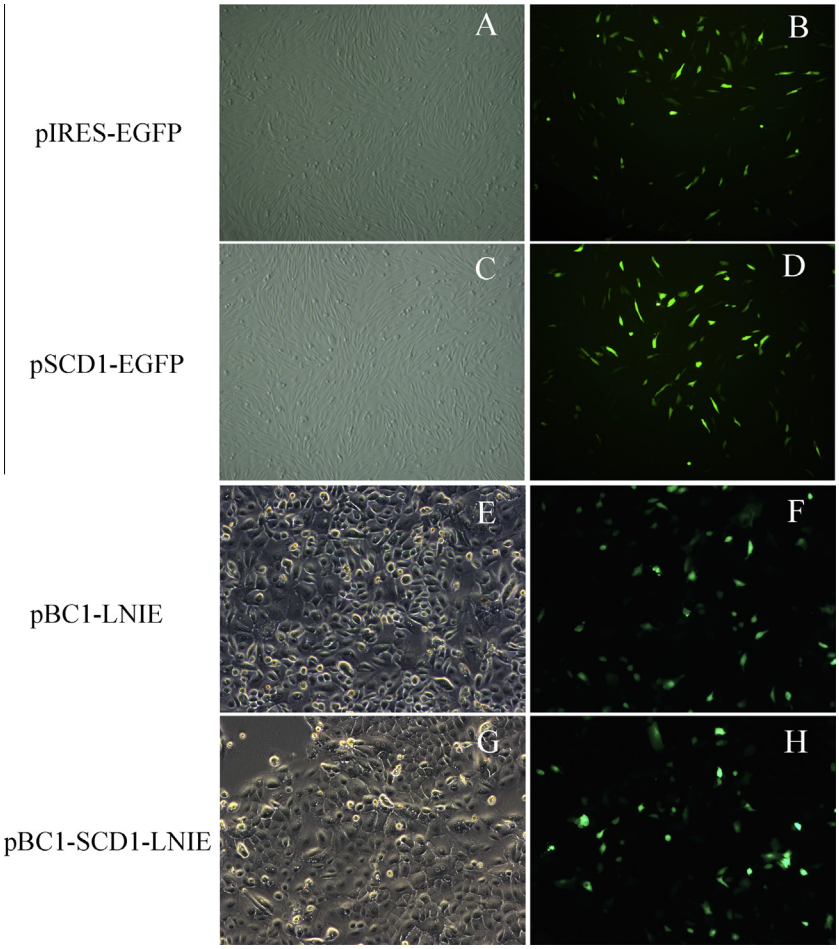

Fig. 2. GEFCs transfected with pSCD1-EGFP and GMECs transfected with pBC1-SCD1-LNIE plasmid. EGFP fluorescence was detected at 24 h post transfection. A, C, E, G (×100) under UV light; B, D, F, H (×100) under bright field. A, B transfected by pIRES-EGFP; C, D transfected by pSCD1-EGFP; E, F transfected by pBC1-LNIE; G, H transfected by pBC1-SCD1-LNIE.

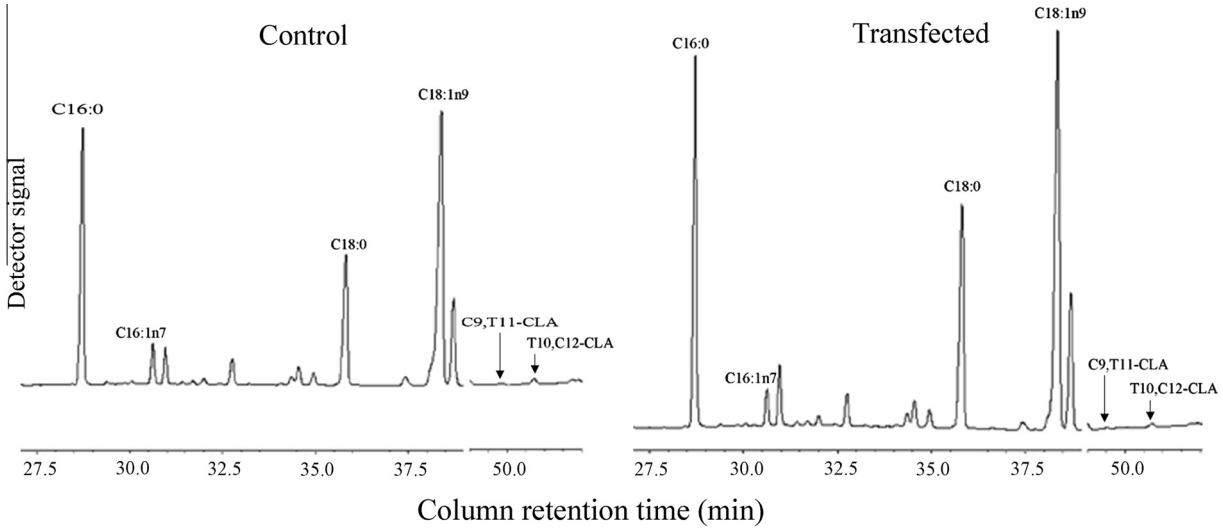

Fig. 3. Partial gas chromatogram traces showing fatty acid profiles of total cellular lipids extracted from the GEFCs transfected with pIRES-EGFP or pSCD1-EGFP. The lipid profiles revealed that the level of C16:1n-7, C18:1n-9 and t10, c12-CLA were significantly increased and the level of C18:0 was markedly decreased in cells transfected by pSCD1-EGFP, while the level of C16:0 and c9, t11 CLA did not have a significant change compared with the control cells (**represent $p < 0.01$).

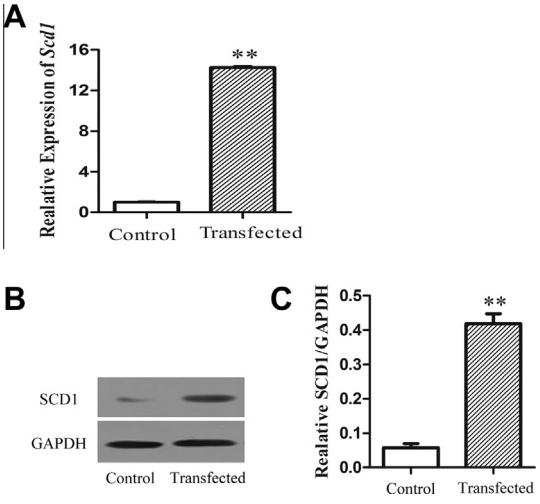

Fig. 4. Induced expression of *Scd1* in GMECs. GMECs were transfected with pBC1-LNIE or pBC1-SCD1-LNIE cultured in induced medium for 48 h. (A) *Scd1* mRNA levels were determined by real-time quantitative PCR in triplicate. The expression data were normalized to the amount of cellular GAPDH. (B) Whole cell lysates were harvested and Western-blotting was performed to detect SCD1 expression. GAPDH served as an internal control. (C) Quantification of relative SCD1 protein level by gradation analyses. IPP 6.0 software was applied to analyze the gradation of each band and the relative expression level was normalized to the expression amount of GAPDH (**$p < 0.01$).

activity of *Scd1*. In this study, ratios of both 16:1 to 16:0 and 18:1 to 18:0 were increased, but the increase in the 16:1/16:0 ratio was slightly less than the increase in the 18:1/18:0 ratio. In previous studies on mammary lipid synthesis, it demonstrated that only a small proportion of C16:0 was desaturated to C16:1 [23,24] and C18:0 was the primary substrate for *Scd1* in the mammary gland [3,23].

Transgenic gene expression requires a stable highly-expressed promoter and regulatory elements. In our current study, the pBC1 plasmid was used as the vector backbone and carried the 5′ flanking regulation region and 3′ untranslated region of goat β-casein. The goat β-casein promoter allows expression of the gene of interest exclusively in lactating mammary glands. The insulator derived from the chicken β-globin gene suppressed the position effect and blocked the action of cis-acting regulatory elements on transgene expression [25].

Selectable marker genes are generally required for the preparations of donor cells. Once the genes of interest have been transferred into the genome of donor cells, the marker genes become unnecessary and undesirable. Moreover, marker genes raise public concerns on biology and food safety. Thus, it is reasonable to excise marker genes after they fulfills their function. In the mammary-specific vector, two loxp sites with the same orientation were amplified on either side of the selectable marker genes (Neo and EGFP) to excise the marker genes from the genome [26].

For transgenic livestock production, assessing the function of the expression vector *in vitro* can significantly reduce the probability of the foreign gene being silenced in offspring [27]. Because the *Scd1* gene was cloned from goat cDNA, we evaluated the efficiency of the mammary-specific vector by measuring the relative expression level of *Scd1* in GMECs. The data demonstrated that the *Scd1* transcript was overexpressed in T-GMECs 14.1-fold and the SCD1 protein was overexpressed in T-GMEC cell lysates 7-fold relative to the control. These results indicated that the *Scd1* mammary-specific vector was successfully expressed in GMECs.

For the purpose of production of *Scd1* transgenic goats, the goat *Scd1* mammary-specific vector was constructed, which homologously expressed in coordination with the endogenous *Scd1*. *Scd1* functions as a desaturase in GMECs without introducing other new proteins, which reduces the risk of transgenic food. To our knowledge, this is the first report on construction of goat *Scd1* mammary-specific vector to increase MUFA content in milk. The role of *Scd1* as a lipogenic enzyme in the synthesis of monounsaturated fatty acids is well known. Abnomal expression of *Scd1* has been reported in some diseases, including obesity and cancers [28]. In our study, we confirmed overexpression of *Scd1* did not cause synthesis of new fatty acids, but it is still unknown whether overexpression of *Scd1* can cause lipid metabolism disorders and metabolic diseases or not.

In conclusion, we successfully amplified goat *Scd1* cDNA and demonstrated that its expression could change the fatty acid composition in GEFCs. We further showed that a mammary-specific vector could be used to effectively express *Scd1* in GMECs. Therefore, this work lays a foundation for both the production of transgenic dairy goats and study of the role of *Scd1* in lipid metabolism in the mammary gland.

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