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# Effect of *QSOX1* on cattle carcass traits as well as apoptosis and triglyceride production in bovine fetal fibroblasts and mammary epithelial cells

**Xiaochuan LIU[1)], Yuwei YANG[1)], Ping JIANG[1)], Xiaohui LI[1)], Yanliang GE[1)], Yang CAO[2)], Zhihui ZHAO[3)], Xibi FANG[1)]* and Xianzhong YU[1,4)]***

[1)]College of Animal Science, Jilin University, Xi An Road 5333, Changchun, Jilin 130062, P.R. China
[2)]Branch of Animal Husbandry, Jilin Academy of Agricultural Sciences, Changchun 130033, P.R. China
[3)]Agricultural College, Guangdong Ocean University, Zhanjiang 524088, P.R. China
[4)]Department of Biological Sciences, 132 Long Hall, Clemson University, Clemson, SC 29634, U.S.A.

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**ABSTRACT.** QSOX1 (quiescin-sulfhydryl oxidase 1) is involved in various processes, including apoptosis and the development of breast diseases. Here, we investigated the effect of *QSOX1* on the meat quality of Simmental cattle by analyzing the correlation between *QSOX1* single nucleotide polymorphisms (SNPs), I2 204 C>T and I2 378 C>T, and certain meat quality traits. The effects of *QSOX1* on triglyceride synthesis and cell apoptosis were further validated by gene silencing or overexpression in bovine fetal fibroblasts and mammary epithelial cells. The results showed that I2 204 C>T and I2 378 C>T had significant correlations with loin thickness, hind hoof weight, fat coverage, liver weight, heart weight, marbling and back fat thickness ($P<0.05$). *QSOX1* overexpression also increased triglyceride production and suppressed apoptosis. In summary, *QSOX1* is an important factor for meat quality, lipid metabolism, and cell apoptosis, indicating that *QSOX1* could be used as a biomarker to assist in breeding cattle with superior meat.

**KEY WORDS:** apoptosis, carcass traits, *QSOX1*, SNPs, triglyceride

With the rapid advancement of molecular genetics, high quality offspring with excellent meat qualities are being chosen using gene marker-assisted selection (MAS) programs including higher slaughter rates, proper ratio of fat, and better testicular development [10, 18, 19].

Quiescin-sulfhydryl oxidase 1 (QSOX1) is an important enzyme for the formation of disulfide bonds, which are critical for protein folding and stability [15, 16]. *QSOX1* is located on chromosome 16 in cattle; has 13 exons; is homologous in humans, mice, rats, chickens, and seven other species; and encodes a protein containing 567 amino acid residues. The amino acid residues 41 to 153 and 407 to 502, respectively, encode the domains of thioredoxin and ERV1 [6, 13], members of two long-standing gene families. Recently, *QSOX1* was found to be involved in various biological processes including viral replication, embryonic development, inflammation, and cancer. The structural integrity of the soluble dimeric prion protein was shown to be significantly correlated with *QSOX1*, and the protein can therefore be obtained in bacteria by co-expression of human *QSOX* [1]. Overexpression of *QSOX1* ensures the complete development of the placenta and normal embryos [14]. The overexpression of *QSOX1* in alveolar type II cells indicates that *QSOX1* plays important roles in the prevention of inflammation and fibrogenesis in lung inflammatory diseases [17]. *QSOX1* is overexpressed in diverse tumor types and hence, might be involved in promoting the growth and invasion of tumor cells and altering the composition of the extracellular matrix [11]. It was suggested that *QSOX1* could be a biomarker for identifying individuals with higher risk for certain types of cancer, including neural tumors, pancreatic cancer, and breast cancer [2, 8, 12]. Site-directed mutagenesis suggested that the C449-C452 motif is essential for the activity of *QSOX1*; the C70-C73 motif is fundamental in the transfer of electrons from thiol-containing substrates, including the reduced proteins DTT (DL-Dithiothreitol) and GSH (glutathione), to the C449-C452 motif; and the C509-C512 motif is not involved in electron transfer during disulphide formation [20]. *QSOX1* is significantly differentially expressed between high- and low-fat dairy cows, suggesting that it might be correlated with meat quality [7].

Therefore, *QSOX1* might be a new factor that regulates meat quality traits, apoptosis, and triglyceride production. In the present study, the association of *QSOX1* I2 204 C>T and I2 378 C>T with cattle carcass traits was analyzed. We then transfected

*Correspondence to: Fang, X.: fangxibi@jlu.edu.cn,Yu, X.: xyu@clemson.edu

an overexpression vector and a silencing vector of *QSOX1* into bovine fetal fibroblasts (BFFs) and mammary epithelial cells (MECs) and verified their effects on cell apoptosis and triglyceride production.

## MATERIALS AND METHODS

### *Ethics statement*

This study involved 350 Chinese Simmental steers (28 months old) from cattle farm Inner Mongolian Baolongshan. These cattle were randomly selected from the offspring of a Simmental population comprising approximately 2,000 cows and 25 bulls. Blood samples (10 m*l* each) were collected with anticoagulant (acid citrate dextrose, ACD) from the jugular vein and stored at −70°C. Animal experiments were performed in strict accordance with the guidance for the care and use of laboratory animals by the Jilin University Animal Care and Use Committee (Permit number: SYXK (Ji) 2008-0010/0011). Carcass and meat qualities were measured according to the instructions of the Chinese Academy of Agricultural Sciences Meat Laboratory.

**Table 1.** SNP primers and qPCR detection primers

| Symbol | Primer Sequence (5′–3′) |
|---|---|
| *QSOX1-SNP-F-Primer* | GCCTTGAACCTGAGTGTCT |
| *QSOX1-SNP-R-Primer* | CATCACCAACCCTCTTCC |
| *QSOX1-qPCR-F-Primer* | AAGTCCTCCAAGCCATCCG |
| *QSOX1-qPCR-R-Primer* | CGAGCGTTGACCTTGTTGTG |
| *GAPDH-qPCR -F-Primer* | GTTTGTGATGGGCGTGAAC |
| *GAPDH-qPCR -R-Primer* | ATGGACCTGGGTCATGAGT |

### *DNA extraction and PCR amplification*

Genomic DNA was extracted from 1 m*l* cattle blood, using the DNA extraction kit (Tiangen, Beijing, China). The purity of DNA was evaluated using agarose gel electrophoresis. The second intron fragment of *QSOX1* was amplified from genomic DNA, using primers designed with primer premier 5.0 software according to the published sequence on the NCBI website (Gene ID: 522986) (Table 1). The primers were synthesized by the Shanghai Biological Company. The PCR conditions for *QSOX1* amplification were an initial denaturing step at 95°C for 5 min, followed by 30 cycles of 95°C for 5 sec, 60°C for 30 sec and 72°C for 45 sec, with a final extension step at 72°C for 10 min.

### *SNP detection and genotyping*

Two polymorphisms of *QSOX1* were identified by sequencing and confirmed by PCR-RFLP, using restriction enzymes (*BsaHI* for I2 204 C>T digestion and *AvaI* for I2 378 C>T identification) in the second intron.

### *Vector construction*

For gene knockdown, BFFs and MECs were transfected with shRNA vectors of *Neo-shNC*, *Neo-QSOX1-bos-374*, *Neo-QSOX1-bos-1667*, *Neo-QSOX1-bos-1057* and *Neo-QSOX1-bos-737* purchased from the Zimmer Biotechnology Company. For gene overexpression, *PBI-CMV3* and *PBI-CMV3-QSOX1* were transfected into BFFs and MECs, using FuGENE® HD Transfection Reagent (Promega, Madison, WI, U.S.A.).

### *Cell lines and cell transfection*

Bovine MECs and BFFs were obtained from the laboratory of animal genetics at Jilin University. Twenty-four hours before transfection, the BFFs and MECs were plated at a concentration of approximately $1 \times 10^6$/well into six-well culture plates with DMEM/F12 (GIBCO, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum (FBS; PAA, Pasching, Austria) and 1% penicillin-streptomycin. To investigate the regulation role of *QSOX1* on apoptosis and triglyceride production in cells, 150 $\mu l$ Opti-MEM serum-free medium (GIBCO) was mixed with 5 $\mu l$ of Lipofectamine TM 2000 (Invitrogen, Carlsbad, CA, U.S.A.) and 1.25 $\mu l$ (of 20 $\mu$mol concentrations) each of the silencing vector *Neo-QSOX1-bos-1667*, overexpression vector *PBI-CMV3-QSOX1*, and negative control *Neo-shNC*, *PBI-CMV3*. Then, cells were incubated in Opti-MEM serum-free medium at room temperature for 30 min, after which the transfection mixture was added to each well. The medium was replaced with regular cell culture medium after 3–5 hr. After 48 hr of transfection, the cell morphology and expression level of green fluorescent protein were observed under a fluorescence microscope (NikonTE2000, Tokyo, Japan) to determine the transfection efficiency. The total RNA and protein were obtained for expression analyzes.

### *Quantitative real-time polymerase chain reaction (qPCR)*

After 48 hr of transfection, total RNA was extracted from the cultured cells and cDNA was synthesized using a cDNA synthesis kit according to the manufacturer's protocol (TaKaRa Biotechnology, Dalian, China). PCR amplification was performed in 20 $\mu l$ reaction volumes under the following conditions: an initial denaturing step at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec (Table 1). Different samples were adjusted to the same concentration, and each sample was repeated three times. The data were analyzed using SPSS 19.0 software, and the fold change of expression was calculated using the $2^{-\Delta\Delta CT}$ method according to the following formula:

$\Delta\Delta Ct=[Ct\ (positive)-Ct\ (reference)]-[Ct\ (control)-Ct\ (reference)]$, where $2^{-\Delta\Delta Ct}$ refers to the relative expression ratio.

### *Western blot analysis*

After 48 hr of transfection, total protein was extracted using the RIPA buffer (Boster, Wuhan, China) following the manufacturer's instructions. The BCA Protein Assay Kit (Boster, Wuhan, China) was used for protein quantification; the

concentrations in different groups were adjusted to the same concentration, and each sample was repeated three times. The western blot analysis was performed according to previously published protocols, using *anti-QSOX1* antibody diluted as suggested by the manufacturer (Abcam, Shanghai, China) [3].

### *Cell apoptosis analysis by flow cytometery*

After 48 hr of transfection, the MECs and BFFs transfected with *Neo-QSOX1-bos-1667*, *PBI-CMV3-QSOX1*, and *Neo-shNC*, *PBI-CMV3* were harvested and washed twice with PBS. Then, three blank samples were used to to adjust the differences between the groups, using the Apoptosis Detection Kit for reference (KeyGEN BioTECH, Nanjing, China). Then, the cells were processed using the Apoptosis Detection Kit according to the instructions and analyzed by flow cytometry (BD Biosciences, San Jose, CA, U.S.A.) to verify the effects of *QSOX1* on cell apoptosis.

### *Triglyceride detection*

After 48 hr of transfection, triglycerides were detected using the total cell lysate from each of the transfected cells, following the manufacturer's instructions (Applygen, Beijing, China) and using a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The cellular content of TG was adjusted based on the quantity of protein, and each group was repeated three times. The calculation of TG was based on the following formula: TG content ($\mu$mol/g)=triglyceride concentration ($\mu$mol/*l*)/ total protein concentration (mg/m*l*).

### *Statistical analysis*

SPSS 19.0 was used to analyze the association between the genotypes of *QSOX1* (I2 204 C>T and I2 378 C>T) and the slaughter and meat quality traits of Simmental cattle. The fixed model was referring to previous research in our laboratory [10]. The expression levels of QSOX1 mRNA and protein and the effects of *QSOX1* on apoptosis and triglyceride production were analyzed using GraphPad Software with a two-tailed *t*-test (unpaired *t*-test) between two groups. The means and standard deviation of triplicates were calculated, and then the *P* values of the groups were determined for the differential expression analysis. Statistically significant differences are defined as $P<0.05$.

## RESULTS

### *Association of QSOX1 I2 204 C>T and I2 378 C>T with cattle carcass traits*

The PCR products of the *QSOX1* second intron were subjected to RFLP analysis. Two polymorphisms were found in the PCR products (Fig. 1). Associations of *QSOX1* polymorphisms with the carcass trait data were analyzed using one-way ANOVA with SPSS 19.0. The results showed that I2 204 C>T was significantly associated with meat quality traits, including tare weight, hind hoof weight, kidney fat weight, marbling, loin thickness, fat coverage, back fat thickness, liver weight, and heart weight ($P<0.05$). The tare weight, hind hoof weight, and marbling in genotype CC were higher than those in CT and TT; however, the kidney fat weight, fat coverage, and back fat thickness were higher in genotype TT, suggesting that TT might be the dominant genotype in lipid metabolism (Table 2).

Analysis showed that I2 378 C>T was also significantly correlated with carcass traits such as slaughter rate, hind hoof weight, kidney fat weight, genital fat, loin thickness, fat coverage, loin eye area, liver weight, and heart weight. In addition, kidney fat weight, loin eye area, and heart weight were higher in genotype CC, indicating that CC could potentially be used to select better meat qualities (Table 3).

### *mRNA expressions of QSOX1 in MECs and BFFs*

Given that the cell morphology and GFP expression in the MECs and BFFs of the two transfection groups were similar to those in the negative control at 24 hr post transfection (Figs. 2 and 3), the transfected cells could be used for the subsequent experiments. The mRNA expression of *QSOX1* in the MECs and BFFs transfected with *Neo-QSOX1-bos-1667* was significantly lower than that in the cells with the negative control vector ($P<0.05$, Fig. 4A and 4B). Therefore, *Neo-QSOX1-bos-1667* with the target sequence *GCAACATCGTCCTAGACTTTC* resulted in the most efficient interference and was chosen as the interference vector in subsequent experiments (Fig. 4).

Finally, the MECs and BFFs transfected with the overexpression vectors *PBI-CMV3* and *PBI-CMV3-QSOX1* had significantly higher mRNA expression levels than those transfected with the control vector ($P<0.01$, Fig. 4C and 4D).

### *Relative QSOX1 levels in MECs and BFFs*

The results of western blot showed that cells transfected with *PBI-CMV3-QSOX1* had the highest protein levels and those with *Neo-QSOX1-bos-1667* had the lowest protein levels when compared with those in the control group ($P<0.05$, Fig. 5A and 5B).

### *Triglycerides in MECs and BFFs*

Relative triglyceride levels in MECs and BFFs transfected with *PBI-CMV3*, *PBI-CMV3-QSOX1*, *Neo-shNC*, and *Neo-QSOX1-bos-1667* were analyzed. The results showed that the relative content of triglycerides was extremely significantly increased in MECs and BFFs transfected with *PBI-CMV3-QSOX1* ($P<0.01$, Fig. 6A and 6B); however, there were no significant changes in the triglyceride levels of BFFs transfected with *Neo-QSOX1-bos-1667* ($P>0.05$, Fig. 6A).

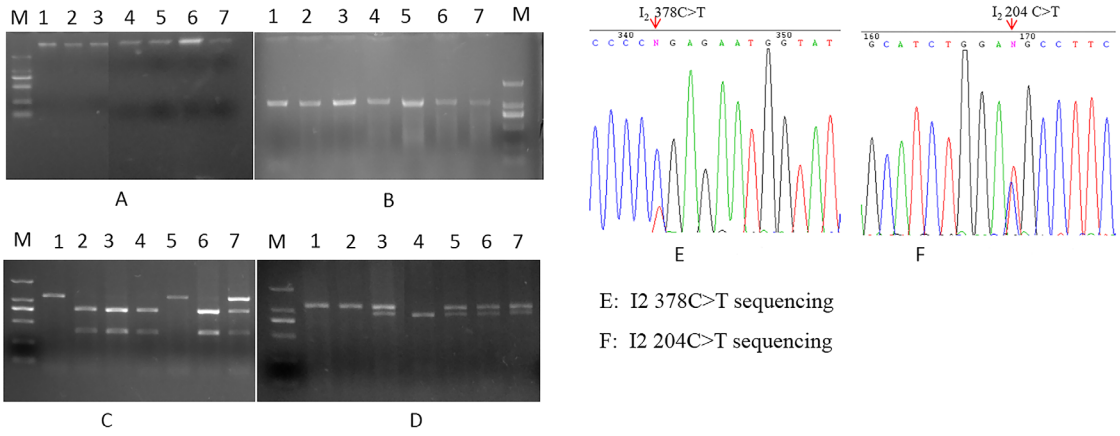



**Fig. 1.** I2 204C>T and I2 378C>T SNPs of *QSOX1*. SNPs of I2 204C>T and I2 378C>T were detected using restriction enzyme digestion and sequencing.

**Table 2.** Association between SNP I2-204 of *QSOX1* and certain cattle carcass traits

| I2 204C > T genotype | Number of individuals | Tare weight (kg) | Hind hoof weight (kg) | Kidney fat weight (kg) | Marbling ($cm^2$) | Loin thickness (cm) | Fat coverage (%) | Back fat thickness (cm) | Liver weight (kg) | Heart weight (kg) |
|---|---|---|---|---|---|---|---|---|---|---|
| CC | 165 | 43.7796 ± 6.68483$^{a)}$ | 3.6622 ± 1.07676$^{a)}$ | 4.0952 ± 2.66823$^{a)}$ | 5.5104 ± 0.63237$^{a)}$ | 6.7052 ± 0.97175$^{a)}$ | 44.1354 ± 21.93657$^{a)}$ | 0.8117 ± 0.58281$^{a)}$ | 5.6619 ± 1.13863$^{a)}$ | 1.7466 ± 0.326$^{a)}$ |
| CT | 117 | 41.9694 ± 5.92707$^{b)}$ | 3.4674 ± 1.01616$^{b)}$ | 4.7811 ± 2.703$^{b)}$ | 5.3791 ± 0.71641$^{a,b)}$ | 6.9542 ± 0.89963$^{b)}$ | 49.1699 ± 20.56123$^{b)}$ | 0.9907 ± 0.62534$^{b)}$ | 5.967 ± 1.06934$^{b)}$ | 1.8424 ± 0.384$^{b)}$ |
| TT | 28 | 41.5489 ± 6.05859$^{b)}$ | 3.405 ± 1.00288$^{b)}$ | 4.9115 ± 2.99249$^{b)}$ | 5.3014 ± 0.71729$^{b)}$ | 6.8011 ± 0.85138$^{a,b)}$ | 49.2822 ± 21.83034$^{b)}$ | 1.0492 ± 0.6745$^{b)}$ | 5.9848 ± 1.15281$^{b)}$ | 1.8141 ± 0.325$^{ab)}$ |

Numbers with different superscripts (a, b) are significantly different ($P$<0.05).

**Table 3.** Association between SNP I2-378 of *QSOX1* and certain cattle carcass traits

| I2 378C > T genotype | Number of individuals | Slaughter rate (%) | Hind hoof weight (kg) | Kidney fat weight (kg) | Genital fat (kg) | Loin thickness (cm) | Fat coverage (%) | Loin eye area ($cm^2$) | Liver weight (kg) | Heart weight (kg) |
|---|---|---|---|---|---|---|---|---|---|---|
| CC | 79 | 52.2616 ± 2.34065$^{a)}$ | 3.4803 ± 1.0666$^{a)}$ | 4.7127 ± 2.8092$^{a)}$ | 0.8704 ± 0.364$^{a)}$ | 6.8314 ± 0.9027$^{a)}$ | 47.743 ± 20.47842$^{a)}$ | 80.0157 ± 12.482$^{a)}$ | 5.9148 ± 1.09353$^{a)}$ | 1.8315 ± 0.37183$^{a)}$ |
| CT | 182 | 52.1162 ± 2.21288$^{a,b)}$ | 3.4694 ± 0.9794$^{a)}$ | 4.6801 ± 2.7354$^{a)}$ | 0.9487 ± 0.34243$^{b)}$ | 6.9255 ± 0.917$^{a)}$ | 48.7955 ± 21.5405$^{a)}$ | 78.9223 ± 13.443$^{a,b)}$ | 5.9272 ± 1.09847$^{a}$ | 1.8019 ± 0.32039$^{a)}$ |
| TT | 89 | 51.322 ± 3.01939$^{b)}$ | 3.7953 ± 1.0365$^{b)}$ | 3.897 ± 2.92718$^{b)}$ | 0.895 ± 0.31726$^{a,b)}$ | 6.5233 ± 0.8811$^{b)}$ | 43.8 ± 25.81553$^{b)}$ | 74.9117 ± 12.5183$^{b)}$ | 5.548 ± 1.32602$^{b)}$ | 1.6927 ± 0.37619$^{b)}$ |

Numbers with different superscripts (a, b) are significantly different ($P$<0.05).

### *Cell apoptosis rate*

Cells apoptosis was analyzed using flow cytometry. The higher apoptosis rate in MECs might be related to their inherent viability. Nevertheless, both BFFs and MECs with *QSOX1* overexpression had lower apoptosis rates than those with interference genes, suggesting that *QSOX1* suppresses apoptosis in both types of cells (Fig. 7).

## DISCUSSION

*QSOX1* is expressed in various cells with different functions. In pancreatic and renal cancer cell lines, the overexpression of *QSOX1* could effectively reduce tumor cell migration [11]. *QSOX1* knockdown inhibits vascular smooth muscle cell migration and proliferation and promotes the neointimal thickening of the rat carotid artery [4]. In fibroblasts and mesenchymal stem cells, *QSOX1* functions as an immune response modifier to prevent tissue inflammation and fibrosis in the lungs [17]. *QSOX1* might also be involved in the differentiation and regression of neuroblastomas through extracellular maturation and apoptosis induction [2]. It is suggested that *QSOX1* could be a potential biomarker for acute decompensated heart failure [9]. *QSOX1* also has important regulatory functions in human lung fibroblasts, guinea pig endometrial cells, and rat seminal vesicle cells [5]. In the present study, BFFs and MECs transfected with the *QSOX1* overexpression vector *PBI-CMV3-QSOX1* showed reduced apoptosis rates and increased triglyceride levels. In BFFs, the apoptosis rate in cells transfected with *PBI-CMV3-QSOX1* (8.5%) was significantly lower than that in cells transfected with *Neo-QSOX1-bos-1667* (19.32%), and *QSOX1* expression mainly affected apoptosis in late-

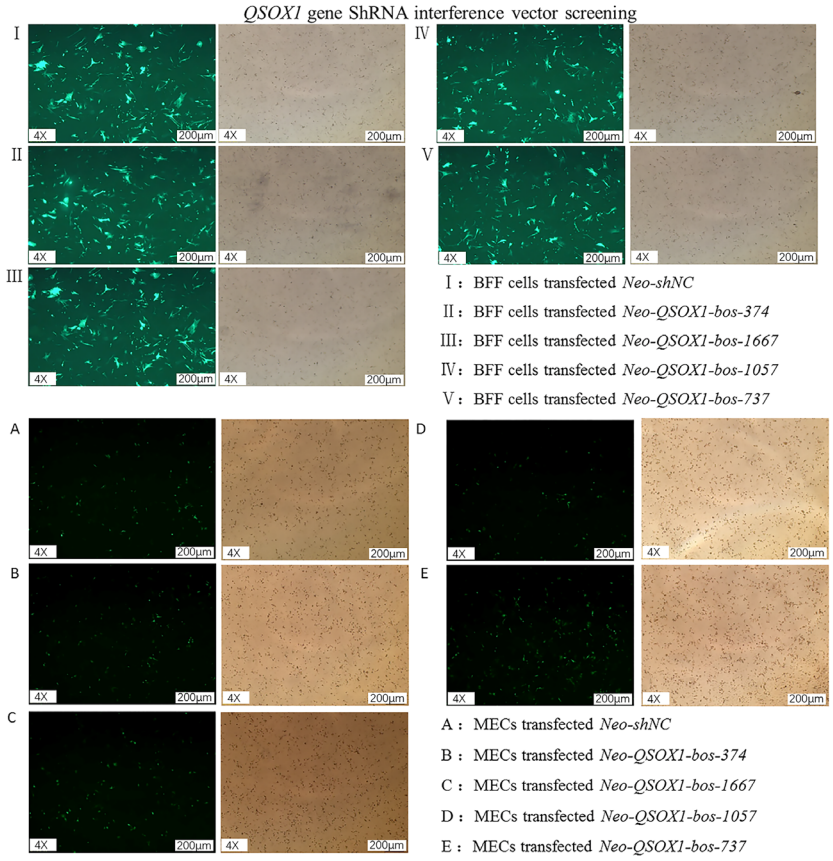



**Fig. 2.** Efficiency screening for the silencing vectors of *QSOX1*. BFFs and MECs were transfected with *Neo-shNC*, *Neo-QSOX1-bos-374*, *Neo-QSOX1-bos-1667*, *Neo-QSOX1-bos-1057* and *Neo-QSOX1-bos-737* to screen for the most effective *QSOX1* silencing vector.

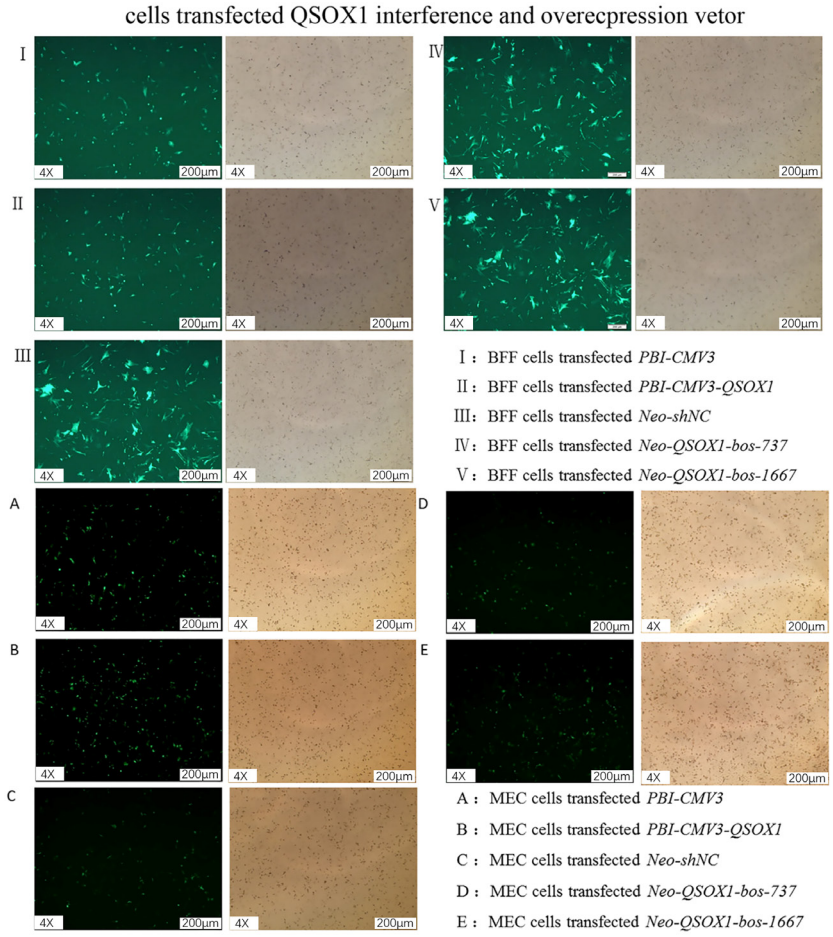



**Fig. 3.** Cells transfected with *QSOX1* silencing and overexpression vectors. BFFs and MECs were transfected with *QSOX1* silencing and overexpression vectors, including *PBI-CMV3*, *PBI-CMV3-QSOX1*, *Neo-shNC* and *Neo-QSOX1-bos-1667*.

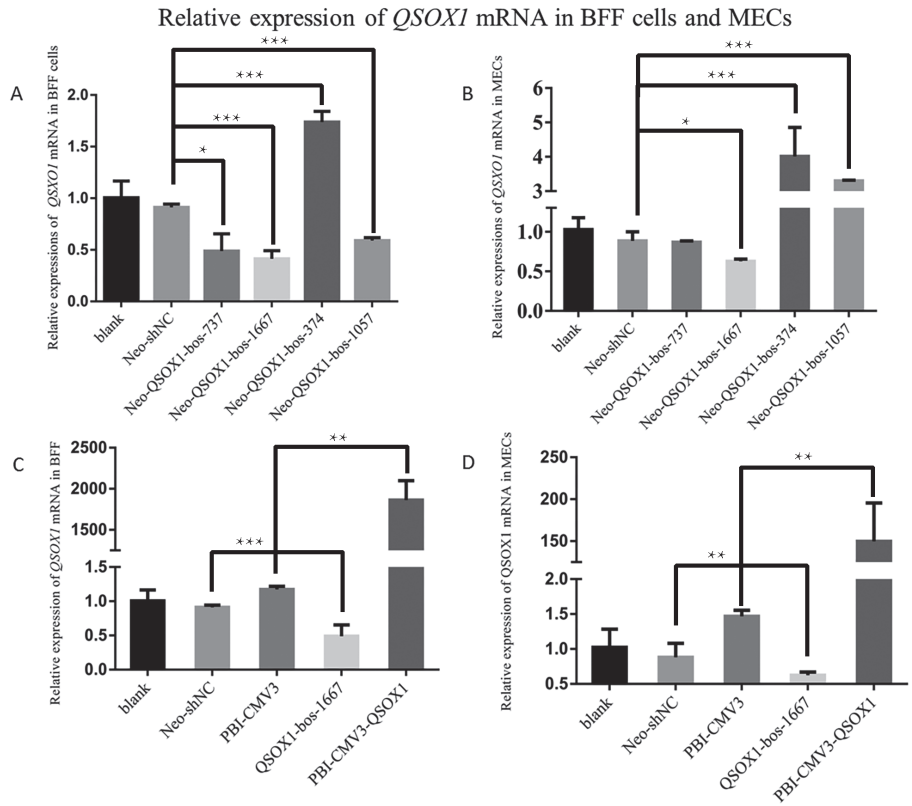



**Fig. 4.** Relative expressions of *QSOX1* mRNA in BFFs and MECs. A and B: *QSOX1* mRNA expressions in BFFs and MECs transfected with the interference vectors *Neo-shNC*, *Neo-QSOX1-bos-374*, *Neo-QSOX1-bos-1667*, *Neo-QSOX1-bos-1057* and *Neo-QSOX1-bos-737*. C and D: cells transfected with the interference vector *QSOX1-bos-1667* with control vector *Neo-shNC* and the overexpression vector *PBI-CMV3-QSOX1* with control vector *PBI-CMV3*; the test was repeated 3 times (**P*<0.05, ***P*<0.01 and ****P*<0.001).

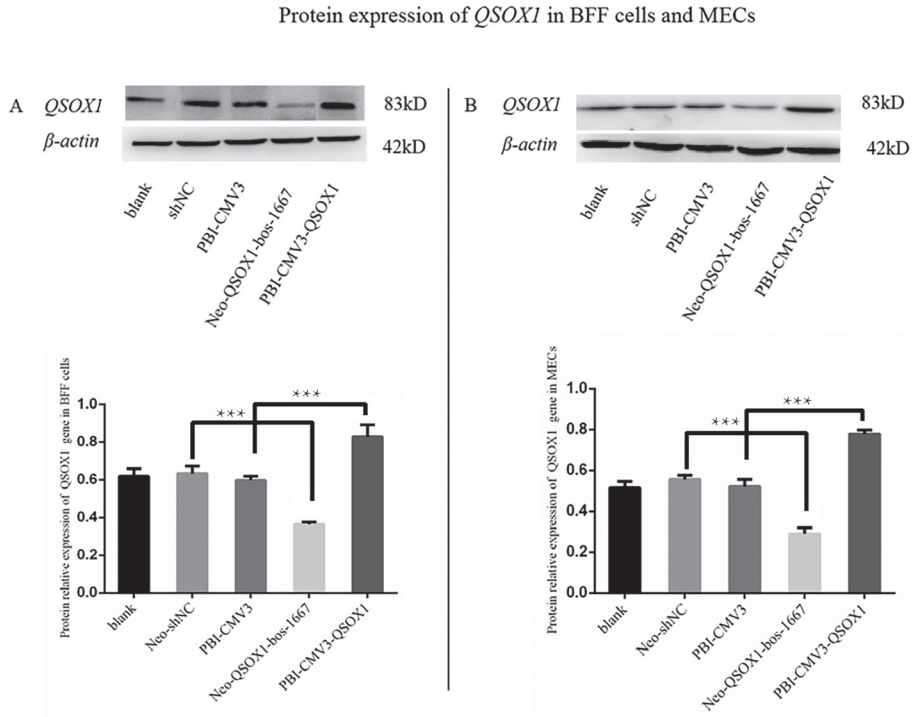



**Fig. 5.** QSOX1 levels in BFFs and MECs. A: Relative QSOX1 levels in BFFs. B: MECs transfected with *blank*, *Neo-shNC*, *PBI-CMV3*, *Neo-QSOX1-bos-1667* and *PBI-CMV3-QSOX1*; the test was repeated 3 times (****P*<0.001).

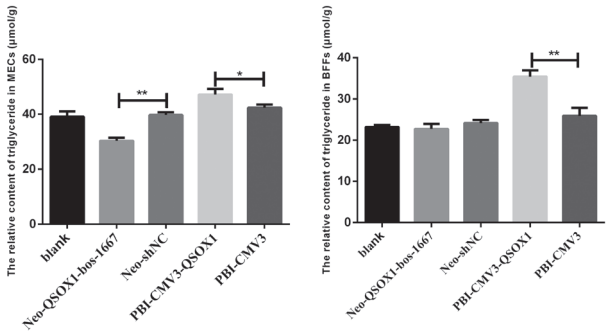
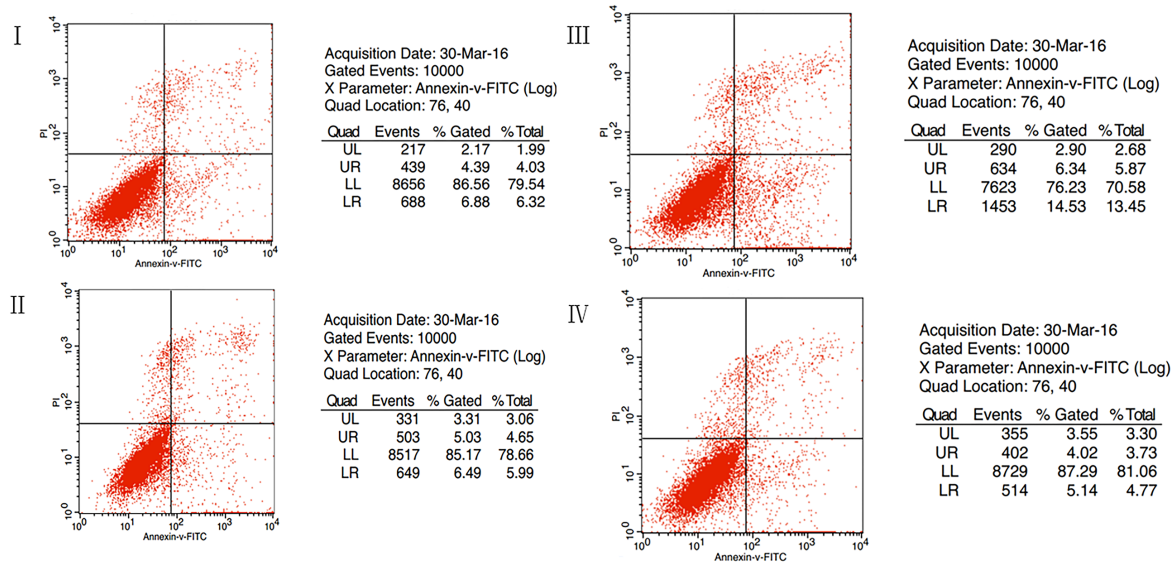
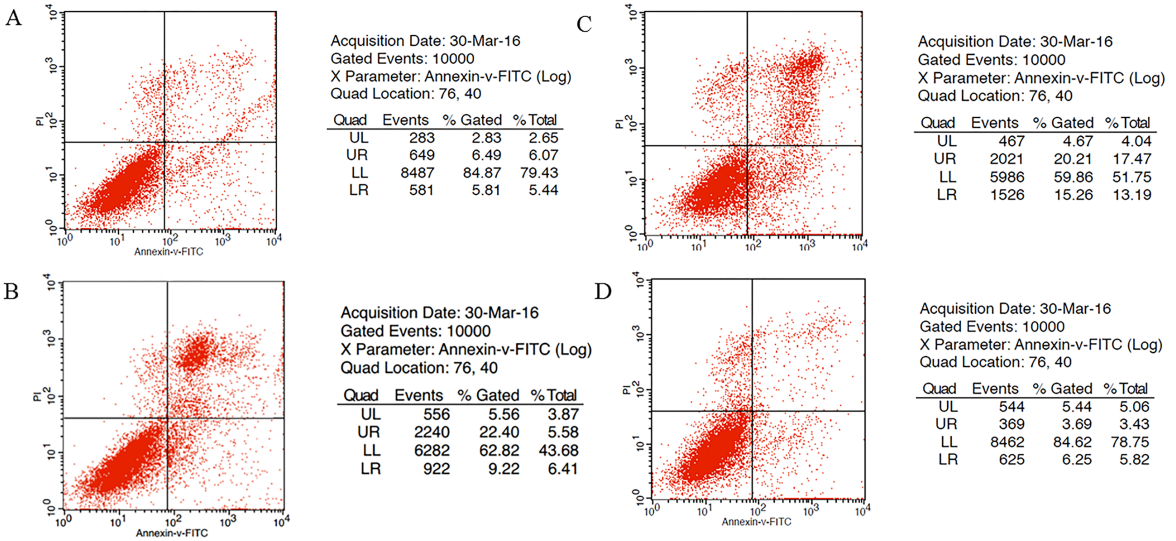



**Fig. 6.** Relative triglyceride levels in BFFs and MECs. A: BFFs transfected with *blank*, *Neo-shNC*, *PBI-CMV3*, *Neo-QSOX1-bos-1667* and *PBI-CMV3-QSOX1*. B: MECs transfected with the same vectors as in (A); the test was repeated 3 times (**P*<0.05, ***P*<0.01).




Ⅰ：BFF cells transfected Neo-shNC　　Ⅲ：BFF cells transfected Neo-QSOX1-bos-1667

Ⅱ：BFF cells transfected PBI-CMV3　　Ⅳ：BFF cells transfected PBI-CMV3-QSOX1




A：MEC cells transfected Neo-shNC　　C：MEC cells transfected Neo-QSOX1-bos-1667

B：MEC cells transfected PBI-CMV3　　D：MEC cells transfected PBI-CMV3-QSOX1

**Fig. 7.** Apoptosis rates in BFFs and MECs. Apoptosis rates of BFFs and MECs transfected with *blank*, *Neo-shNC*, *PBI-CMV3*, *Neo-QSOX1-bos-1667* and *PBI-CMV3-QSOX1* were analyzed using flow cytometry.

stage cells. In cattle MECs, the *PBI-CMV3-QSOX1* group (9.25%) had a significantly lower apoptosis rate than the *Neo-QSOX1-bos-1667* group (30.66%) did.

There was a significant correlation between SNPs of *QSOX1* and meat quality traits. Studies have shown that SNPs in introns have a great impact on gene functions. In this study, genotype CC in I2 204 C>T mainly affected the tare weight, hind hoof weight, and marbling, whereas TT in I2 204 C>T might be the dominant genotype in lipid metabolism, suggesting that TT in I2 204 C>T could be a marker for better meat qualities. Genotype CC in I2 378 C>T mainly impacted the kidney fat weight and heart weight, suggesting that CC could be a marker for visceral disorders such as cardiac hypertrophy and renal lipoma.

Based on the effects of *QSOX1* on cell apoptosis and triglyceride production in cattle MECs and BFFs and the correlation of the gene's SNPs with certain meat quality traits, we recommend that *QSOX1* be considered as a key gene for in cattle genetics and breeding. In conclusion, *QSOX1* SNPs were significantly correlated with meat quality and played an important role in lipid metabolism and cell apoptosis, suggesting that *QSOX1* could potentially be used as a biomarker in cattle breeding for better meat quality.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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