Effects of SPARCL1 on the proliferation and differentiation of sheep preadipocytes

Cheng Xiao, Hai Guo Jin, Li Chun Zhang, Jian Qiang Liu, Ming He, Hui Hai Ma, Yong Sheng Yu, and Yang Cao

Institute of Animal Biotechnology, Jilin Academy of Agricultural Sciences, Jilin, Gongzhuling, China; Institute of Animal Husbandry and Veterinary Medicine, Jilin Academy of Agricultural Sciences, Jilin, Gongzhuling, China

ABSTRACT

Important candidate genes that regulate lipid metabolism have the potential to increase the content of intramuscular fat (IMF) and improve meat quality. Secreted protein acidic and rich in cysteine like 1 (SPARCL1) is a secreted glycoprotein with important physiological functions and is involved in the proliferation and differentiation of various cells. However, the role of the SPARCL1 gene in sheep preadipocytes and its regulatory mechanism is still unclear. In this study, we explored the effect of SPARCL1 on the proliferation and differentiation of sheep preadipocytes. The results showed that the expression level of the SPARCL1 gene is higher in fat tissue than in other tissues, and the gene was significantly increased on the 6th day of preadipocyte differentiation. In the preadipocyte proliferation stage, interference of SPARCL1 gene reduced cell viability and increased cell apoptosis. In preadipocyte differentiation stage, SPARCL1 overexpression significantly inhibited lipid droplets accumulation and triglyceride content by increasing Wnt10b, Fzd8, IL6, and β-catenin and inhibiting PPARγ, C/EBPα, LPL, and IGF1 genes expression, whereas SPARCL1 deficiency significantly promoted cell differentiation by inhibiting β-catenin and increasing GSK3β, PPARγ, C/EBPα, and LPL. The results of this study suggest that SPARCL1 plays a negative role during preadipocyte differentiation and may become a novel target for regulating preadipocyte differentiation and improving IMF.

Abbreviations: IMF: Intramuscular fat; SPARCL1: Secreted protein acidic and rich in cysteine like 1; PPARγ: Peroxisome proliferator-activated receptor γ; C/EBPα: CCAAT/enhancer-binding protein-α; LPL: Lipoprotein lipase; IGF1: Insulin-like growth factor 1; Wnt10b: Wnt family member 10b; Fzd8: Frizzled class receptor 8; IL6: Interleukin 6; β-catenin: Catenin beta interacting protein; GSK3β: Glycogen synthase kinase 3 beta; LRPS/6: Low-density lipoprotein receptor-related protein 5/6

Introduction

Mutton is becoming more and more popular with consumers in China. Small Tail Han sheep is a unique breed in China, with high fecundity and strong viability, and it was widely bred in Northeast China to meet the local demand for mutton in the past [1]. However, the growth rate and meat quality of Small Tail Han sheep are not as good as foreign sheep breeds, and cannot meet the current consumer market demand, therefore it is urgent to improve its meat quality. Many factors influence meat quality, among them increasing the content of intramuscular fat (IMF) can significantly improve the taste, tenderness, colour, and quality of meat by promoting the formation and differentiation of adipocytes and the accumulation of triglycerides in lipid droplets [2,3]. Therefore, it is essential to detect new target genes that promote adipocyte differentiation.

Adipocyte formation is a complex physiological process involving a large number of genes, non-coding RNAs, growth factors, and signal pathways [4,5]. The transformation of preadipocytes into mature adipocytes requires two stages of preadipocyte proliferation and differentiation [6]. The proliferative phase of preadipocytes, although short, is a necessary process for adipocyte formation, and a variety of adverse factors can lead to apoptosis and prevent preadipocyte maturation. The peroxisome proliferator-
activated receptor gamma (PPAR_y) a transcription factor, specifically expressed in adipose tissue, plays a decisive role in preadipocyte differentiation [7]. The transcription factor CCAAT/enhancer-binding protein-alpha (C/EBPa) also plays a vital role in adipocyte differentiation [8]. PPAR_y positively regulates C/EBPa and co-initiates preadipocyte differentiation [9]. Lipoprotein lipase (LPL) is a key enzyme of fat deposition, hydrolysing triglycerides and promoting lipoprotein uptake [10]. In addition, insulin-like growth factor 1 (IGF1) also promotes adipocyte proliferation and differentiation [11]. These genes have emerged as biomarkers for detecting preadipocyte differentiation. Many signalling pathways are also involved in adipocyte differentiation. Several studies have found that the Wnt/β-catenin signalling pathway plays an important role in adipocyte formation and that the β-catenin gene has a key regulatory function as a second messenger [12]. β-catenin can affect downstream genes (PPAR_y and C/EBPa) to regulate adipocyte differentiation [13].

Secreted protein acidic and rich in cysteine like 1 (SPARCL1), a member of the SPARC family, is a glycoprotein that mediates cell-matrix interactions and is involved in many physiological processes, including cell adhesion, proliferation, differentiation, migration, and maturation [14], as well as being an important regulator of cellular metabolism. In recent years, SPARCL1 has been extensively studied in cancer and could be a potential target for cancer therapy [15], and it has recently been reported that SPARCL1 regulates adipogenesis in mice [16], but the exact mechanism is unknown and there are limited studies in sheep lipid metabolic processes. Therefore, the present study explores the potential effects of the SPARCL1 gene on the proliferation and differentiation of sheep preadipocytes and its mechanism, providing a new target for increasing IMF content and a new research direction for other fields.

Materials and methods

Experimental animal

The experimental animals were three two-month-old and three six-month-old healthy male Small Tail Han sheep from the Institute of Animal Biotechnology, Jilin Academy of Agricultural Sciences. The groin adipose tissue of two-month-old sheep was used to extract preadipocytes. Heart, muscle, small intestine, stomach, liver, duodenum, and fat tissues of six-month-age male sheep were isolated for qPCR validation. Animal experiments were performed following animal use protocols approved by the Committee for the Ethics of Animal Experiments (AWEC2017A01, 9 March 2017).

Preadipocyte isolation, culture, and differentiation

The adipose tissue was washed with PBS containing 1% penicillin/streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA), and the connective tissue and blood clots in the adipose tissue were removed using sterile tweezers. The pure tissue was cut into mm³ pieces with surgical scissors, and the blocks were digested collagenase II(Sigma-Aldrich) in a water bath at 37°C for 1 h, mixing every 15 minutes, and the undigested tissue and miscellaneous cells were filtered out using 200 mesh(75 μm) and 400 mesh filters (38 μm), and then the supernatant was removed by centrifuging at 1500 rpm for 15 min to obtain preadipocyte. Next, the preadipocytes were cultured using a complete medium containing DMEM-F12 (Sigma-Aldrich), 10% foetal bovine serum (Gemini Bio-Products, Woodland, CA, USA), and 1% penicillin/streptomycin solution in 60-mm Petri dishes (Corning, Corning, NY, USA) in a 37 °C and 5% CO₂ incubator, and change the culture medium every 48 h. When the cells overgrew the petri dish, some preadipocytes were digested with trypsin (Sigma-Aldrich) to subculture, and the remaining preadipocytes were induced by exogenous inducer I (10 mg/mL insulin (Sigma-Aldrich), 1 mM dexamethasone (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) and complete medium) and inducer II solution (10 mg/mL insulin (Sigma-Aldrich) and complete medium) into mature adipocytes. The inducer I and II have respectively cultured the cells for 48 h and then exchanged a fresh complete medium to culture the cells until becoming mature adipocytes.

Oil red O staining

When preadipocytes are differentiated into mature adipocytes, the intracellular lipid droplets can be stained red by oil red O dye to verify the maturation of the adipocytes. Adipocytes were washed three times with PBS and fixed with 4% paraformaldehyde (Sangon Biotech Co., Ltd., Shanghai, China) in a closed environment for 30 min, then washed three times with PBS and stained with 0.5% oil red O (Sangon Biotech) in an incubator for 30 min. Finally, the cells were washed a time with PBS and observed and photographed under the microscope. Isopropyl alcohol can dissolve intracellular lipid droplets, and the absorbance value at 490 nm detects lipid droplet content.

Detection of triglyceride content

Intracellular triglyceride content was determined by the triglyceride detection kit (Prilax, Beijing, China),
following the supplier’s instructions. Differentiated cells were washed three times with PBS and digested with trypsin (Sigma-Aldrich) and centrifuged at 1500 rpm for 15 min. Lysis solution was then added to the adipocytes pellet at room temperature for 10 minutes, followed by heating at 70°C for 10 minutes and centrifuging at 2000 rpm for 5 minutes. The supernatants were detected triglycerides by measuring ODs at 550 nm wavelength by an enzyme-labelled instrument and calculating triglyceride content.

**SPARCL1 interface sequence design**

Based on the sheep SPARCL1 mRNA sequence available on the NCBI website (accession number: XM_004009982.3), we designed the following small interference sequences and a negative control sequence: si-1217-SPARCL1(sense: 5’-CCAAGGAGCCUUCUAACAATT-3’; antisense: 5’-UUGUUAGAAAGGCUCUUUGGT-3’), si-1937-SPARCL1 (sense: 5’-GCACUGACAUCAGACCUATT-3’; antisense: 5’-UAGGUCUGAUUGUCAGUGCTT-3’), si-2244-SPARCL1 (sense: 5’-CCUUCUCUUAAGAGACUUUTT-3’; antisense: 5’-AAAGUCUCUUAAAGAAGGTT-3’). Negative control (sense: 5’-UUUCUGCAACGUGUCACGUTT-3’; antisense: 5’-ACGUGACAGGUGCAGAGTT-3’). These sequences were synthesized by Shanghai GenePharma Gene Co. Ltd. (Shanghai, China). We verified the optimal siRNA sequence by cell transfection and qPCR for further experiments.

**Construction of SPARCL1 overexpression plasmid**

Based on the sheep SPARCL1 mRNA sequence available on the NCBI website, we designed primer sequences to clone the CDS region of the SPARCL1 gene. The primers were synthesized by Suzhou Jin Weizhi Co. Ltd. (Suzhou, China). pEX4 vector and the restriction endonucleases XhoIII and EcoRI were used to construct recombinant plasmid completed by Shanghai GenePharma Gene Co. Ltd. pEX4 vector was used as the negative control.

**Cells transfection**

When the cells grew to 70% of the culture dish, we started the transfection of plasmids or interference sequences to the cells with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) transfection reagent. Lip2000 needs to be placed at room temperature for 5 min in advance, and 100 pmol siRNA and 5 uL lip2000 were added to 200 uL Opti-MEM (Invitrogen, Carlsbad, CA, USA) forming a mixed solution and placed at room temperature for 10 min. Each 6-well plate was spiked with 200 ul of the above mixture and 1.8 ml of fresh culture medium for 6 h. After this, we replaced the mixture with the complete culture medium, then detected the transfection efficiency by qPCR at 48th h. FuGENE (Roche Applied Science, Indianapolis, IN, USA) was a transfection reagent of the overexpression vector. 5 uL FuGENE solution was added to 200 uL Opti-MEM in an Eppendorf tube and placed at room temperature for 5 min, 3 mg plasmid was added to 200 uL Opti-MEM in another Eppendorf tube and placed at room temperature for 5 min. The two solutions were mixed well and placed at room temperature for 20 min. Each 6-well plate was spiked with 200 ul of the above mixture and 1.6 ml of fresh culture medium for 6 h. After this, we replaced the mixture with a complete culture medium. The recombinant plasmid contains green fluorescent protein, so we used fluorescence microscopy to detect the efficiency of cell transfection and qPCR to detect the results of plasmid transfection at 48th h.

**Cell counting kit**

The plasmid or siRNAs were transfected to the cells in 96-well plates for 48 h, then removed the culture medium and added the 10 mL/well of Cell Counting Kit-8(CCK8, Beyotime, China) solution to each well in a 37°C incubator for 4 h. The OD value at 450 nm was read for each well and then the cell survival rate was calculated. Untransfected cells were used as a negative control and CCK8 alone was used as a low OD value.

**Apoptosis detection by flow cytometry**

After 48 hours of cell transfection, cells were digested with 0.25% trypsin (Gibco BRL, Life Technologies) without EDTA for 5 min, then collected in Eppendorf tubes and centrifuged at 300 x g and 4°C for 5 min, next were washed twice with PBS and centrifuged at 300 x g and 4°C for 5 min, after which we resuspended the cells with 100 ul of apoptosis detection kit buffer (US Everbright*Inc, Suzhou, China) with 4 uL of annexin V and 5 uL of PI working solution and incubated it in darkness at room temperature for 15 min. Finally, 400 uL PBS was added to each tube and immediately evaluated under flow cytometry. Annexin V emits spectra at 530 nm (FITC channel) and 617 nm (PI channel). Untransfected cells were used as a negative control.
**RNA extraction and qPCR assay**

We used TRIzol reagent (Thermo Fisher Scientific) to extract cellular total RNA extracts on days 2, 4, 6, 8, and 10 during cellular proliferation and differentiation, and the experimental steps are described in the instructions. The cells growing to 75% of the culture dish were on the 2nd day; The cells growing to 100% of the culture dish and added with induction solution I were on the 4th day; The cells were replaced with induction solution II on the 6th day; Replaced with fresh culture medium were on the 8th day; many cells differentiated into the mature adipocytes on the 10th day. Monitoring RNA degradation and contamination of cells and tissues using 1% agarose gels, followed by measurement of RNA concentration using a Quawell Q5000 spectrophotometer (Quawell Technology, San Jose, CA, USA). Next, we reversed the RNA to the cDNA using a reverse transcription kit (Takara, Japan). The reverse transcription system consisted of 2 μL of 5× PrimeScript RT Master Mix, 500 ng of total RNA, and RNase Free ddH2O up to 10 μL, and reaction conditions were 37 °C for 15 min, 85 °C for 5 sec, and storage at 4 °C, and then the reaction products were diluted 10 times by double-distilled water. The quantitative fluorescent PCR detects the relative expression of mRNA levels in a Roche LightCycler 480 (Roche Applied Science), and the Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH) gene was used as an internal control. The reaction system consisted of 10 μL of 2× Light Cycler 480 SYBR Green I Master, 8 μL of ddH2O, 1 μL of cDNA, and 0.5 μL of sense and antisense primers. The reaction conditions were 40 cycles of 95 °C for 5 min, 95 °C for 10 s, 60 °C for 15 s, and 72°C for 20 s, followed by 95 °C for 5 s, 65 °C for 1 min, and 40 °C for 10 s. The number of experimental repetitions was three times. The primer sequences are in Table 1.

**Western blot analysis**

We used a protein extraction kit (Solarbio, Shanghai, China) to extract total cellular protein extracts on days 2, 4, 6, 8, and 10 during cellular proliferation and differentiation. The cells were washed with PBS and treated with lysis solution for 30 min on ice and centrifuged at 12,000 rpm at 4°C for 30 min. The protein concentration was determined BCA solution, and lystate regulates the protein concentration to make the same concentration of total protein in each group. The supernatant with proteins was then boiled at 100°C for 10 minutes to denature proteins and mixed with SDS-PAGE buffer (Beiyotime, Jiangsu, China). Preparing separation and concentrated gels using the PAGE Gel Fast Preparation kit (EpiZyme, Shanghai, China). After the gel solidification, 20 μg of proteins and 5 μl of protein markers (Thermo Fisher Scientific) were injected into the gel hole at 80 volts for 20 min. Voltage was changed to 120 V for 40 min after proteins reached the separation gel. We transferred the proteins from the gel to a 0.45 mm Immobilon poly-vinylidene difluoride membrane (Millipore, Bedford, MA, USA) at 200 mA for 1 h, after which the PVDF membrane was washed with TBST buffer for 5 min and sealed with 5% skimmed milk powder blocking solution for 2 h. The membrane was then washed three times with TBST buffer for 5 minutes each. The membrane was

---

**Table 1. Primer sequences for a quantitative real-time polymerase chain reaction.**

| Gene   | Oligo  | Primer sequence | Product size | GenBank No. |
|--------|--------|-----------------|--------------|-------------|
| Sparcl | Forward | 5'-ATCCAGCATTGTCTCTCCTAC-3' | 205 bp   | XM_004009982.3 |
|        | Reverse | 5'-CTTTCAGGAAACCTGGGACT-3' |           |             |
| PPARy  | Forward | 5'-CTGTGGACCTCTTATGATGG-3' | 193 bp    | NM_001100921.1 |
|        | Reverse | 5'-AGGGCTTCACCTGTGATGG-3' |           |             |
| C/EBPa | Forward | 5'-AGGGCTTCACCTGTGATGG-3' | 126 bp    | NM_001308574.1 |
|        | Reverse | 5'-AGGGCTTCACCTGTGATGG-3' |           |             |
| GAPDH  | Forward | 5'-AGGGCTTCACCTGTGATGG-3' | 228 bp    | NM_001190930.1 |
|        | Reverse | 5'-AGGGCTTCACCTGTGATGG-3' |           |             |
| LPL    | Forward | 5'-AGGGCTTCACCTGTGATGG-3' | 189 bp    | XM_027963889.1 |
|        | Reverse | 5'-AGGGCTTCACCTGTGATGG-3' |           |             |
| IGF1   | Forward | 5'-AGGGCTTCACCTGTGATGG-3' | 249 bp    | NM_001009774.3 |
|        | Reverse | 5'-AGGGCTTCACCTGTGATGG-3' |           |             |
| Wnt10b | Forward | 5'-AGGGCTTCACCTGTGATGG-3' | 141 bp    | XM_004202070.4 |
|        | Reverse | 5'-AGGGCTTCACCTGTGATGG-3' |           |             |
| FZD 8  | Forward | 5'-AGGGCTTCACCTGTGATGG-3' | 216 bp    | XM_027976634.1 |
|        | Reverse | 5'-AGGGCTTCACCTGTGATGG-3' |           |             |
| β-catenin | Forward | 5'-AGGGCTTCACCTGTGATGG-3' | 125 bp    | XM_027972680.1 |
|         | Reverse | 5'-AGGGCTTCACCTGTGATGG-3' |           |             |
| GSK-β | Forward | 5'-AGGGCTTCACCTGTGATGG-3' | 161 bp    | XM_001297400.1 |
|         | Reverse | 5'-AGGGCTTCACCTGTGATGG-3' |           |             |
| IL6    | Forward | 5'-AGGGCTTCACCTGTGATGG-3' | 216 bp    | XM_001009392.1 |
|         | Reverse | 5'-AGGGCTTCACCTGTGATGG-3' |           |             |
incubated with antibodies and shaken overnight at 4°C. On the second day, the membrane was washed three times with TBST buffer for 5 minutes each, incubated with secondary antibodies (anti-rabbit or anti-mouse IgG-HRP, 1:5000) at room temperature for 2 h, and washed three times with TBST buffer for 5 minutes each. The chromogenic reaction of proteins used ECL hypersensitive photoluminescence solution (Pipril, Beijing, China). The protein bands data were detected using the ChemiScope 6000 Touch imaging system (Clinx Science Instruments, Shanghai, China), and were quantified using the ImageJ software. β-actin was used as an internal reference protein. The antibody dilution ratio was in Table 2.

**Statistical analysis**

All the results are expressed as means ± SEM. Comparisons between two groups using an unpaired, two-tailed Student’s t-test. Multiple group comparisons used one-way ANOVA. Data analysis was in Graph Pad Prism 6.0 software. The original image and data are in the supplementary file. The statistical significance levels were set at P < 0.05.

**Result**

**SPARCL1 gene expression is the highest in adipose tissue, with significant expression at day 6 of cell differentiation**

SPARCL1 gene expression was highest in adipose tissue (P < 0.01; Figure 1(a)). As shown in Figure 1(b), we successfully isolated, cultured, and differentiated sheep preadipocytes. Lipid droplets in mature adipocytes were stained red by Oil Red O. We examined marker genes during preadipocyte differentiation as well as target gene expression patterns. During preadipocyte differentiation, the expression level of PPARy was the highest on the 4th day (P < 0.01; Figure 1(c)); C/EBPα gene high expression occurred on the 6th day, after which expression decreased and increased (P < 0.01; Figure 1(d)); SPARCL1 gene has significantly increased on the 6th and 10th days (P < 0.01; Figure 1(e)), and the qPCR results of these genes were consistent with Western blot results Figure 1(f,g).

**Detection of transfection efficiency**

We synthesized overexpression plasmids and interference sequences to detect the effect of the SPARCL1 gene on preadipocyte proliferation and differentiation. Post-transfection fluorescence assay of the cells also indicated high transfection efficiency Figure 2(a). Forty-eight hours after plasmid transfection of the cells, we verified the efficiency by qPCR. As shown in Figure 2(b), the experimental group’s overexpression plasmid increased the SPARCL1 gene by more than 4000-fold (P < 0.01). As shown in Figure 2(c), among the three interfering sequences, siRNA1937 had the best inhibiting effect and was used for further experiments (P < 0.01).

**SPARCL1 inhibition increases apoptosis and reduces viability on sheep preadipocytes proliferation phase**

We performed CCK8 assays on the 4th day to determine the effect of SPARCL1 on the sheep preadipocytes proliferation. The results showed that interference with SPARCL1 inhibited cell proliferation and reduced cell viability (P < 0.01), but overexpression had no significant effect on cell proliferation and viability (P > 0.05; Figure 3(a)). We found by flow cytometry assay that SPARCL1 interference increased apoptosis and thus decreased cell proliferation and viability (P < 0.01), while SPARCL1 overexpression had no significant effect on apoptosis (P > 0.05; Figure 3(b-d)).

**SPARCL1 is a negative regulator of preadipocytes differentiation in sheep**

We examined intracellular triglyceride content and expression level of marker genes to assess the effect of SPARCL1 on preadipocyte differentiation. The results showed that inhibition of SPARCL1 significantly increased triglyceride content and intracellular lipid droplets on the 12th day (P < 0.01; Figure 4(a-c)) and significantly increased the expression of the adipogenic marker genes PPARy, LPL, and C/EBPα (P < 0.05; Figure 4(d-f)). In contrast, overexpression of SPARCL1 significantly reduced triglyceride content and intracellular lipid droplets on the 12th day (P < 0.01; Figure 4(a-c)) and significantly reduced PPARy, LPL, IGF1, and C/EBPα expression (P < 0.05; Figure 4(g-i)).

**Table 2. The information of antibodies used in Western blot.**

| Gene     | Description           | Dilution | Source, Number |
|----------|-----------------------|----------|----------------|
| Sparcl1  | Rabbit polyclonal antibody | 1:1000   | Boster, DZ1007 |
| PPARy    | Rabbit polyclonal antibody | 1:1000   | Bios, bs-0530 R |
| C/EBPα   | Rabbit polyclonal antibody | 1:1000   | Bios, bs-1630 R |
| β-actin  | Rabbit polyclonal antibody | 1:5000   | Bios, bs-1571 R |
| LPL      | Rabbit polyclonal antibody | 1:1500   | Bios, bs-1973 R |
| IGF1     | Rabbit polyclonal antibody | 1:1000   | Bios, bs-0227 R |
| Wnt10b   | Rabbit polyclonal antibody | 1:1000   | Bios, bs-3662 R |
| FZD8     | Rabbit polyclonal antibody | 1:1000   | Bios, bs-13,219 R |
| β-catenin| Rabbit polyclonal antibody | 1:1000   | Bios, bs-1165 R |
| GSK3β    | Rabbit polyclonal antibody | 1:1000   | Bios, bs-0028 R |
| IL6      | Rabbit polyclonal antibody | 1:1000   | Bios, bs-4587 R |
Thus, SPARCL1 negatively regulates cell differentiation and triglyceride accumulation by regulating the expression of adipogenic marker genes.

SPARCL1 may regulate the Wnt/β-catenin pathway to affect preadipocyte differentiation

Studies showed that the SPARCL1 gene may be associated with the Wnt/β-catenin pathway and has a role in cancer as well as other diseases, so we tested whether the SPARCL1 gene can regulate the Wnt/β-catenin pathway affecting preadipocyte differentiation [17,18]. We examined the pattern of changes in key genes (Wnt10b, β-catenin, LRPS/6, Fzd8, and GSK3β) in the Wnt/β-catenin pathway during adipocyte differentiation to explore its possible mechanisms. As shown in Figure 5(a), the expression trends of these genes were similar, reaching a maximum on the 6th day, after which the expression level decreased (P < 0.05). Based on an understanding of their expression patterns, we chose day 6 of cell differentiation to detect the effect of SPARCL1 on these genes. The results showed that SPARCL1 overexpression significantly increased Wnt10b, Fzd8, β-catenin, and IL6, whereas SPARCL1 interference significantly decreased β-catenin and increased GSK3β (P < 0.05; Figure 5(b-d)). Therefore, SPARCL1 may affect Wnt/β-catenin pathway genes regulating PPARγ, C/EBPα, and LPL to affect preadipocyte differentiation.
of exogenous inducer and early pre-adipocyte differentiation. However, \textit{SPARCL1} expression increased more than 20-fold on the 8th day. This implies that \textit{SPARCL1} plays a role in late cell differentiation and in lipid metabolism. \textit{PPARy}, \textit{C/EBPa} is known to be a key transcription factor for cell differentiation. \textit{PPARy} has a decisive role in the early stages of cell differentiation. When \textit{PPARy} is activated, it induces \textit{C/EBPa} and targets to promote cell differentiation [21]. Thus, \textit{PPARy} expression was significant when exogenous inducers were added. Subsequently, \textit{C/EBPa} expression was initiated. In the present study, the expression trends of \textit{PPARy} and \textit{C/EBPa} were consistent with other reports. The results indicate that the experimental data are reliable and accurate.

Preadipocytes become mature adipocytes through proliferation and differentiation [22], so we explored the effect of \textit{SPARCL1} genes on sheep preadipocytes at two different stages of proliferation and differentiation. First, we observed that \textit{SPARCL1} expression was low in cell proliferation and did not alter cell viability and apoptosis rates. when \textit{SPARCL1} expression was increased. However, some studies have shown that \textit{SPARCL1} inhibits cancer cell proliferation [23];
**Figure 3.** SPARCL1 Inhibition decreases cell survival rate and increases apoptosis. (a) Cell counting kit-8 (CCK8) shows that siRNA1937 significantly decreases cell survival rate (**P < 0.01**). (b-d) Determination of apoptosis by flow cytometry. SPARCL1 inhibition significantly increases apoptosis (*P < 0.01*); SPARCL1 overexpression do not lead to apoptosis (*P > 0.05*). Red dots represent cells. Flow cytometry chart is divided into four areas, the lower left is living cells, the lower right is early rising apoptotic cells, the top right is apoptotic cells, the top left is dead cells. The number of detected cells is 10,000.

**SPARCL1** overexpression inhibits renal cancer cell migration and invasion, which is different from our study, where overexpression of the SPARCL1 gene did not affect sheep adipocyte proliferation [24]. It is possible that the adipocytes did not produce changes due to the regulation of other genes or factors, which needs to be explored more deeply, and this is where our present study is insufficient. **SPARCL1** interference reduced cell viability and increased the rate of apoptosis, yet in other studies, **SPARCL1** inhibited cancer cellular proliferation [25]. It is possible that adipocytes have different outcomes due to the presence of proliferation and differentiation and different mechanisms than cancer cells, a part we still need to investigate further. Our results suggest that interference with **SPARCL1** may reduce cell proliferation, whereas **SPARCL1** overexpression does not affect cell proliferation.

At the stage of adipocyte differentiation, **SPARCL1** plays a negative role in preadipocyte differentiation and the results are consistent with other studies [16]. Because **PPARγ** and **C/EBPα** have a decisive effect on adipocyte differentiation, **SPARCL1** inhibition or overexpression can affect the expression of **PPARγ**, **C/EBPα**, **LPL**, and **IGF1**, thereby regulating intracellular triglyceride and lipid droplet content. What is the mechanism by which the **SPARCL1** gene regulates preadipocyte differentiation? Some studies have shown that **SPARCL1** gene promotes C2C12 cell differentiation [Y. 26], which is not contradictory to the present study, because both adipocytes and myogenic cells are derived from mesenchymal stem cells [27], and they are interconvertible, and some genes that can inhibit mesenchymal stem cells from becoming myogenic cells and promote adipocyte differentiation. Among them, **WNT/β-catenin** signalling pathway-related genes have similar functions, so we linked **SPARCL1** and **WNT/β-catenin** signalling pathways, and also some studies have shown that **SPARCL1** is associated with **WNT/β-catenin** signalling pathways [28]. **Wnt** signalling converts mesenchymal stem cells into preadipocytes but inhibits adipocyte differentiation [29]. **Wnt** signalling also improves muscle cell differentiation in different periods [D. 30].
Figure 4. SPARCL1 gene is a negative regulator of adipocyte differentiation (a and b) effect of oil red O staining to detect SPARCL1 on preadipocyte differentiation (200×; scale bar: 50 μm); SPARCL1 inhibition significantly increases promotes adipocyte lipid droplets content (**P < 0.01); SPARCL1 overexpression inhibits adipocyte lipid droplets content (**P < 0.01); (b) The figure digitized the oil red O diagram and the absorbance value at 490 nm. (c) The content of triglyceride in the negative control, overexpression and inhibition groups was determined on the 12th day of differentiation. SPARCL1 inhibition significantly increases promotes the content of adipocyte triglyceride (**P < 0.01); SPARCL1 overexpression inhibits the content of adipocyte triglyceride (**P < 0.01); (d) Inhibition and overexpression with the SPARCL1 gene affect the mRNA expression of PPARγ, C/EBPa, LPL, IGF1. (e) Inhibition and overexpression with the SPARCL1 gene affect the protein expression of PPARγ, C/EBPa, LPL, IGF1. (f) Densitometric analyses of the Western blots. The first set of data in the figure is named ‘a’. Lowercase letters different from a represent significant data differences between groups (P < 0.05), named ‘b, c, and d’ in that order, and the same lowercase letters represent non-significant data differences (P > 0.05).

Competition for Wnt signalling exists between muscle cells and adipocytes [31]. SPARCL1 has a similar function with Wnt/β-catenin signalling. Also, both SPARCL1 and Wnt are highly expressed in the cytoplasm [32], so we examined the effect of SPARCL1 on Wnt/β-catenin signalling. Wnt10b is lowly expressed in adipocyte differentiation, and overexpression of SPARCL1 significantly increases Wnt10b, which is then enriched in membranes and binds Fzd8 in the present study [33]. Wnt10b-containing polypeptide decreases the GSK3β and increases β-catenin suppresses PPARγ and C/EBPa [D. 34]. GSK3β translocates to the nucleus, phosphorylates PPARγ and C/EBPa, and promotes adipocyte differentiation [35]. In the present study, we were also able to observe that SPARCL1 overexpression decreased GSK3β, PPARγ, C/EBPa. The results are consistent with Wnt/β-catenin signalling regulating adipocyte differentiation [36,37]. Although Wnt/β-catenin-related genes peaked at day 6 during preadipocyte differentiation, and SPARCL1 gene expression started to rise at day 6, it did not affect the conclusion of this experiment, and the results just indicate that SPARCL1 is one of the factors that can affect Wnt/β-catenin-related genes. Wnt/β-catenin is affected by a variety of factors during preadipocyte differentiation. SPARCL1 can regulate Wnt/β-catenin-related genes to influence preadipocyte differentiation.
Figure 5. SPARCL1 affects the Wnt/β-catenin pathway related genes (a) GSK3β, FZD8, Wnt10b, β-catenin, IL6 expression patterns in preadipocyte differentiation; These genes expression is the highest at day 6(* * P < 0.01). (b) Inhibition and overexpression with the SPARCL1 gene affect the mRNA expression of GSK3β, FZD8, Wnt10b, β-catenin, IL6. (c) Inhibition and overexpression with the SPARCL1 gene affect the protein expression of GSK3β, FZD8, Wnt10b, β-catenin, IL6. (d) Densitometric analyses of the Western blots. The first set of data in the figure is named ‘a’. Lowercase letters different from a represent significant data differences between groups (P < 0.05), named ‘b, c, and d’ in that order, and the same lowercase letters represent non-significant data differences(P > 0.05).

The present study is the first to show the role of SPARCL1 in sheep adipocyte differentiation, and the study has practical implications. On the one hand, this study provides a new research direction to explore the regulatory mechanism of sheep preadipocyte differentiation. The newly identified regulatory genes can be used as molecular markers for screening sheep with good meat quality, and the application of genetic engineering technology can improve the meat quality of Small Tail Han sheep. On the other hand, SPARCL1 may provide new therapeutic targets for metabolic diseases caused by obesity, which can help us better understand adipose differentiation and metabolism.

SPARCL1 is involved in the process of sheep preadipocyte differentiation and plays a role in late differentiation. In addition, interference with SPARCL1 leads to apoptosis. SPARCL1 may be a negative regulator of adipocyte differentiation and lipid droplet accumulation. Thus, SPARCL1 may be a new potential target for increasing IMF content in sheep.

Acknowledgments

We would like to thank the researchers who contributed to this work but were not included in the author list.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by basic research funding projects of Jilin Academy of Agricultural Sciences (KYJF2021ZR113), China Agriculture Research System of MOF and MARA (CARS-38).

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

Conceptualization, Y.S.Yu., and Y.Cao.; methodology, Y.Cao.; software, L.C.Zhang; validation, C.Xiao.; formal analysis, C. Xiao, and M.He.; investigation, J.Q.Liu.; resources, H.H.Ma.; data curation, C.Xiao; writing—original draft preparation, C. Xiao.; writing—review and editing, Y.Cao.; visualization, C. Xiao.; supervision, H.G.Jin.; Project administration, Y.S.Yu.; funding acquisition, H.G.Jin, and Y.Cao. All authors have read and agreed to the published version of the manuscript.
**Ethics approval and consent to participate**

All procedures involving animals such as welfare and ethical issues were approved by the Committee for the Ethics of Animal Experiments (AWEC2017A01, 9 March 2017).

**ORCID**

Yang Cao  http://orcid.org/0000-0002-8910-9576

**References**

[1] Wang J, Zhou H, Hickford J, et al. Comparison of the transcriptome of the ovine mammary gland in lactating and non-lactating small-tailed han sheep. Front Genet. 2020;11:472.

[2] Hocquette J, Gondret F, Baëza E, et al. Intramuscular fat content in meat-producing animals: development, genetic and nutritional control, and identification of putative markers. Animal: an international journal of animal bioscience. 2010;4(2):303–319.

[3] Wen Y, Liu H, Liu K, et al. Analysis of the physical meat quality in partridge (Alectoris chukar) and its relationship with intramuscular fat. Poult Sci. 2020;99(2):1225–1231.

[4] Lopez-Pajares V. Long non-coding RNA regulation of gene expression during differentiation. Pflugers Arch. 2016;468(6):971–981.

[5] Tang Q, Lane M. Adipogenesis: from stem cell to adipocyte. Annu Rev Biochem. 2012;81(1):715–736.

[6] Sá PDM, Richard AJ, Hang H, et al. Transcriptional regulation of adipogenesis. John Wiley & Sons, Inc; 2017.

[7] Yang W, Yang C, Luo J, et al. Adiponectin promotes preadipocyte differentiation via the PPARγ pathway. Mol Med Rep. 2018;17(1):428–435.

[8] Guo L, Li X, Tang Q. Transcriptional regulation of adipocyte differentiation: a central role for CCAAT/enhancer-binding protein (C/EBP) β. J Biol Chem. 2015;290(2):755–761.

[9] Rosen E, Hsu C, Wang X, et al. C/EBPα induces adipogenesis through PPARγamma: a unified pathway. Genes Dev. 2002;16(1):22–26.

[10] Mahat B, Chassé É, Mauger J, et al. Effects of acute hypoxia on human adipose tissue lipoprotein lipase activity and lipolysis. J Transl Med. 2016;14(1):212.

[11] Scavo L, Karas M, Murray M, et al. Insulin-like growth factor-I stimulates both cell growth and lipogenesis during differentiation of human mesenchymal stem cells into adipocytes. J Clin Endocrinol Metab. 2004;89(7):3543–3553.

[12] Donati G, Proserpio V, Lichtenberger B, et al. Epidermal Wnt/β-catenin signaling regulates adipocyte differentiation via secretion of adipogenic factors. Proc Natl Acad Sci U S A. 2014;111(15):E1501–1509.

[13] Benchamana A, Mori H, MacDougald O, et al. Regulation of adipocyte differentiation and metabolism by insoprazole. Life Sci. 2019;239:116897.

[14] Sullivan M, Sage E. Hevin/SC1, a matricellular glycoprotein and potential tumor-suppressor of the SPARC/ BM-40/Osteonectin family. Int J Biochem Cell Biol. 2004;36(6):991–996.

[15] Xiang Y, Qiu Q, Jiang M, et al. SPARCL1 suppresses metastasis in prostate cancer. Mol Oncol. 2013;7(6):1019–1030.

[16] Meissburger B, Perdikari A, Moest H, et al. Regulation of adipogenesis by paracrine factors from adipose stromal-vascular fraction - a link to fat depot-specific differences. Biochim Biophys Acta. 2016;1861(9):1121–1131.

[17] Xiao C, Wei T, Liu L, et al. Whole-Transcriptome analysis of preadipocyte and adipocyte and construction of regulatory networks to investigate lipid metabolism in sheep. Front Genet. 2021;12:662143.

[18] Zhao S, Jiang Y, Xu N, et al. SPARCL1 suppresses osteosarcoma metastasis and recruits macrophages by activation of canonical WNT/β-catenin signaling through stabilization of the WNT-receptor complex. Oncogene. 2018;37(8):1049–1061.

[19] Liu B, Xiang L, Ji J, et al. Sparc1 suppresses nonalcoholic steatohepatitis progression in mice through upregulation of CCL2. J Clin Invest. 2021;131(20). DOI:10.1172/jci144801

[20] Rodrigues R, Guan Y, Gao B. Targeting adipose tissue to tackle NASH: SPARCL1 as an emerging player. J Clin Invest. 2021;131(20). DOI:10.1172/jci153640

[21] de Melo K, de Oliveira F, Costa Silva R, et al. α, β-amyrin, a pentacyclic triterpenoid from Protium heptaphyllum suppresses adipocyte differentiation accompanied by down regulation of PPARγ and C/EBPα in 3T3-L1 cells. Biomed Pharmacother. 2019;109:1860–1866.

[22] Nobusue H, Kano K. Establishment and characteristics of porcine preadipocyte cell lines derived from mature adipocytes. J Cell Biochem. 2010;109(3):542–552.

[23] Ma Y, Xu Y, Li L. SPARCL1 suppresses the proliferation and migration of human ovarian cancer cells via the MEK/ERK signaling. Exp Ther Med. 2018;16(4):3195–3201.

[24] Ye H, Wang W, Cao J, et al. SPARCL1 suppresses cell migration and invasion in renal cell carcinoma. Mol Med Rep. 2017;16(5):7784–7790.

[25] Zhang S, Zhang F, Feng L. The inhibition of HeLa cells proliferation through SPARCL1 mediated by SPP1. Cytotechnology. 2021;73(1):71–78.

[26] Wang Y, Liu S, Yan Y, et al. SPARCL1 promotes C2C12 cell differentiation via BMP7-mediated BMP/TGF-β cell signaling pathway. Cell Death Dis. 2019;10(11):852.

[27] Almalki S, Agrawal D. Key transcription factors in the differentiation of mesenchymal stem cells. Differentiation. 2016;92(1–2):41–51.

[28] Yuan Z, Li Q, Luo S, et al. PPARγ and Wnt signaling in adipogenic and osteogenic differentiation of mesenchymal stem cells. Curr Stem Cell Res Ther. 2016;11(3):216–225.

[29] Fan Y, Ho B, Pang J, et al. Wnt/β-catenin-mediated signaling re-activates proliferation of matured cardiomyocytes. Stem Cell Res Ther. 2018;9(1):338.

[30] Liu D, Li S, Cui Y, et al. Podocan affects C2C12 myogenic differentiation by enhancing Wnt/β-catenin signaling. J Cell Physiol. 2019;234(7):11130–11139.
[31] Du M, Yin J, Zhu M. Cellular signaling pathways regulating the initial stage of adipogenesis and marbling of skeletal muscle. Meat Sci. 2010;86(1):103–109.

[32] Logan C, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004;20(1):781–810.

[33] Katoh M. WNT signaling in stem cell biology and regenerative medicine. Curr Drug Targets. 2008;9(7):565–570.

[34] Liu D, Pang Q, Han Q, et al. Wnt10b participates in regulating fatty acid synthesis in the muscle of Zebrafish. Cells. 2019;8(9):1011.

[35] Xie Y, Mo C, Cai Y, et al. Pygo2 regulates adiposity and glucose homeostasis via β-catenin-axin2-GSK3β signaling pathway. Diabetes. 2018;67(12):2569–2584.

[36] Prestwich T, Macdougald O. Wnt/beta-catenin signaling in adipogenesis and metabolism. Curr Opin Cell Biol. 2007;19(6):612–617.

[37] Xu C, Wang J, Zhu T, et al. Cross-talking between PPAR and WNT signaling and its regulation in mesenchymal stem cell differentiation. Curr Stem Cell Res Ther. 2016;11(3):247–254.