RESEARCH ARTICLE

J Anim Sci Technol 2021;63(4):934-953
https://doi.org/10.5187/jast.2021.e87

JAST
Journal of Animal Science and Technology
pISSN 2672-0191 eISSN 2055-0391

Effect of ciglitazone on adipogenic transdifferentiation of bovine skeletal muscle satellite cells

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Received: May 13, 2021
Revised: Jun 22, 2021
Accepted: Jun 30, 2021

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Abstract

Ciglitazone is a member of the thiazolidinedione family, and specifically binds to peroxisome proliferator-activated receptor-γ (*PPARγ*), thereby promoting adipocyte differentiation. We hypothesized that ciglitazone as a *PPARγ* ligand in the absence of an adipocyte differentiation cocktail would increase adiponectin and adipogenic gene expression in bovine satellite cells (BSC). Muscle-derived BSCs were isolated from six, 18-month-old Yanbian Yellow Cattle. The BSC were cultured for 96 h in differentiation medium containing 5 μM ciglitazone (CL), 10 μM ciglitazone (CM), or 20 μM ciglitazone (CH). Control (CON) BSC were cultured only in a differentiation medium (containing 2% horse serum). The presence of *myogenin, desmin*, and *paired box 7* (*Pax7*) proteins was confirmed in the BSC by immunofluorescence staining. The CL, CM, and CH treatments produced higher concentrations of triacylglycerol and lipid droplet accumulation in myotubes than those of the CON treatment. Ciglitazone treatments significantly increased the relative expression of *PPARγ*, *CCAAT/enhancer-binding protein alpha* (*C/EBP*α), *C/EBPβ*, *fatty acid synthase*, *stearoyl-CoA desaturase*, and *perilipin 2*. Ciglitazone treatments increased gene expression of *Pax3* and *Pax7* and decreased expression of *myogenic differentiation-1, myogenin, myogenic regulatory factor-5*, and *myogenin-4* ($p < 0.01$). Adiponectin concentration caused by ciglitazone treatments was significantly greater than CON ($p < 0.01$). RNA sequencing showed that 281 differentially expressed genes (DEGs) were found in the treatments of ciglitazone. DEGs gene ontology (GO) analysis showed that the top 10 GO enrichment significantly changed the biological processes such as protein trimerization, negative regulation of cell proliferation, adipocytes differentiation, and cellular response to external stimulus. Kyoto Encyclopedia of Genes and Genomes pathway analysis showed that DEGs were involved in the p53 signaling pathway, *PPAR* signaling pathway, biosynthesis of amino acids, tumor necrosis factor signaling pathway, non-alcoholic fatty liver disease, PI3K-Akt signaling pathway, and Wnt signaling pathway. These results indicate that ciglitazone acts as *PPARγ* agonist, effectively increases the adiponectin concentration and adipogenic gene expression, and stimulates the conversion of BSC to adipocyte-like cells in the absence of adipocyte differentiation cocktail.

Keywords: Bovine satellite cells, Ciglitazone, Adipogenesis, Myogenesis, Differentially expressed genes

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Competing interests
No potential conflict of interest relevant to this article was reported.

Funding sources
This study was funded by the National Natural Science Foundation in China (Grant number 31660667) and the Research Fund of Engineering Research Center of North-East Cold Region Beef Cattle Science & Technology Innovation, Ministry of Education and the "111" Project (D20034), China. This study also was supported by the National Research Foundation in Korea (grant number NRF-2018R1D1A3B07048219).

Acknowledgements
Not applicable.

Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Li X.
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Ethics approval and consent to participate
All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Yanbian University using the approval code YBU-20160303.

INTRODUCTION

The thiazolidinediones (TZD) family specifically bind to and activate proliferator-activated receptor-γ (*PPARγ*), increase its expression and that of the related genes (e.g., CCAAT/enhancer-binding protein alpha [*C/EBPα*]), and promote adipocyte differentiation. Yeow et al. [1] demonstrated that the TZD rosiglitazone, in the absence of supplemental fatty acids, strongly up-regulated *PPARγ* expression in C2C12 cells, and proposed that rosiglitazone inhibited myogenesis. Singh et al. [2] reported that the TZD ciglitazone up-regulated *PPARγ* and *C/EBPα* gene expression and depressed myoblast fusion in porcine satellite cells. In our previous study, it was reported that bovine muscle satellite cells (BSC) cultured in a growth medium supplemented with oleic acid (18:1n-9) increases the *PPARγ* and *C/EBPβ* gene expressions in myotubes regardless of whether ciglitazone is added or not [3]. Teboul et al. [4] first reported the almost complete conversion of C2C12 myoblasts to lipid-filled cells when incubated with linoleic acid (18:2n-6) plus the TZD pioglitazone, as indicated by a cessation of myoblast fusion, swelling of myoblasts through lipid filling, down-regulation of myogenin (*MYOG*), and up-regulation of adipocyte lipid-binding protein gene expression. In our previous study [3], we established that 10 μM ciglitizone down-regulated the expression of genes associated with myogenesis, and up-regulated genes involved in adipogenesis. However, we did not establish that 10 μM ciglitizone would elicit maximal effects on adipogenesis and/or myogenesis. Therefore, we hypothesized that a greater concentration of ciglitazone would more strongly promote the adipocyte phenotype in bovine BSC. For the current study, we selected a differentiation medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% horse serum based on the study of Singh et al. [2], which described the transdifferentiation of porcine satellite cells into adipoblasts. Here, however, we altered the adipogenic mixture of that study such as the DMEM, horse serum, penicillin/streptomycin, ascorbic acid, biotin, acetic acid, pantothenic acid, dexamethasone, isobutyl ethylxanthine, insulin, and ciglitazone to include increasing concentrations of ciglitazone (5, 10, and 20 μM). We changed the traditional cocktail of differentiation and studied the effect of ciglitazone alone on adipogenic transdifferentiation of BSC without other factors promoting lipid deposition. RNA-seq was used to further analyze and validate the changes of adipogenesis-related genes and pathways after treatment with different concentrations of ciglitazone. We added horse serum to the medium to promote BSC proliferation and myogenic gene expression to better reflect the *in situ* conditions.

MATERIAL AND METHODS

Ethics statement

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Yanbian University using the approval code YBU-20160303.

Isolation and culture of bovine satellite cells

Using sterile techniques, the semimembranosus muscle was collected immediately after the slaughter of Yanbian yellow cattle. The connective tissues and visible fats were removed, and the muscle tissues were ground using a sterile meat grinder and incubated with 1% Pronase (10165921001, Roche, Basel, Switzerland) in EBSS (Sigma-Aldrich, Louis, MO, USA) solution at 37℃ for 1h [5]. Following the enzymatic digestion was the repeated centrifugation according to the procedure previously published [3]. The obtained primary cells were suspended in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS)

(Gibco, Thermo Fisher Scientific) and 10% (vol/vol) dimethyl sulfoxide (Sigma-Aldrich), frozen, and stored in liquid nitrogen for subsequent experiments. The BSCs were cultured in DMEM containing 10% FBS, and 1× antibiotic-antimycotic (Gibco) in a humidified incubator at 37℃ with 5% CO_2. The growth medium was replaced with a differentiation medium after the cells have reached 80% confluence. The differentiation medium of Singh et al. [2] was followed omitting the insulin, pantothenic acid, and dexamethasone, and supplemented with 5 μM ciglitazone (dissolved in ethanol, Sigma-Aldrich) (CL), 10 μM ciglitazone (CM), or 20 μM ciglitazone (CH). Each experiment was replicated in three independent cultures (passage no. 2 of the BSCs were used). The average cell size and viability were determined using the Luna automatic cell counter (Luna Ⅱ, Logo Biosystems, Anyang, Korea).

Immunostaining

BSC were grown on 14 mm coverslip-bottomed dishes and fixed with 4% paraformaldehyde at ambient temperature for 20 min, washed with phosphate buffered saline (PBS), permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) at ambient temperature for 30 min, and washed with PBS again. The polyclonal anti-myogenic differentiation 1 (MYOD1) (BS-2442R, Bioss, Beijing, China), anti-paired box 7 (Pax7) (AB-528428, Abcam, Cambridge, UK), and anti-desmin Po (BS-20702R, Bioss) were diluted in PBS with 5% bovine serum albumin (BSA) at a ratio of 1:100 and incubated at ambient temperature for 2 h. The horseradish peroxidase (HRP)-labeled Goat Anti-Mouse Immunoglobulin G (IgG) (H + L) (A0216, Beyotime, Shanghai, China) or Goat Anti-Rabbit IgG-HRP antibody (BS-3550R, Bioss) was diluted in PBS with 5% BSA at a ratio of 1:200 and incubated for 2 h at ambient temperature in the dark. Finally, the BSC was incubated in the 0.1 μg/mL 4',6-diamidino-2-phenylindole, dihydrochloride solution for 30 min in the dark and observed with a fluorescence microscope (WF10X, Olympus, Tokyo, Japan).

Oil red O staining and lipid droplet area quantification

To evaluate the transdifferentiation BSCs, after 96 h of differentiation, the cells were fixed with Oil Red O (ORO) Fixative for 30 min and stained with hematoxylin staining solution for 2 min at ambient temperature. The staining protocol was performed according to the instructions provided by the ORO staining kit (G1262, Solarbio, Beijing, China). Images were collected using an inverted microscope (IX-73, Olympus) and 20 different stained areas were captured. Image analysis followed the protocol of Deutsch et al. [6] using the ImageJ software 1.43u (http://rsbweb.nih.gov/ij). The average lipid droplet size was evaluated using GraphPad Prism 6.07.

Quantitative triacylglycerol assay

The concentration of triacylglycerol (TAG) was measured using the TAG Quantitative Assay Kit (Applygen, Beijing, China) according to the manufacturer's instructions. After 96 h of differentiation, the BSC was washed with PBS to remove the medium, and the cells were lysed with Radio-ImmunoPrecipitation Assay lysis solution at ambient temperature for 10 min. Lipase is used to decompose TAG in cell lipid droplets to release glycerol, and the glycerol release amount is measured by spectrophotometric detection at 570 nm.

RNA extraction and real-time polymerase chain reaction

After BSC was induced to differentiate *in vitro* for 96 h, total RNA was extracted from cells using Trizol reagent (Thermo Fisher Scientific). The integrity (quantity and quality) of the extracted total RNA was quantified using a NanoDrop ND-100 spectrophotometer (2000C, Thermo Fisher Scientific), and the purity values (A260 / A280) of all RNA samples were greater than 1.80

https://doi.org/10.5187/jast.2021.e87

and were further verified by 1% agarose gel. The RNA samples of good quality were selected for further reverse transcription. Total RNA of 1 μg was reverse-transcribed into cDNA using the FastKing one-step kit (Tiangen Biotech, Beijing, China). Real-time polymerase chain reaction (RT-PCR) was performed using the SYBR premix SuperReal PreMix Plus Kit (SYBRGreen, Tiangen Biotech) and thermocycler Agilent Mx3000/5p system (Agilent Mx3000/5p, Agilent Technologies, Santa Clara, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase gene was used as the housekeeping gene. The genes Pax3, Pax7, MyoD1, and MyoG were selected as myogenic specific marker genes to characterize the extent of myogenesis, while the genes PPARγ, C/EBPα, C/EBPß, sterol regulatory element-binding protein (SREBP)1, stearoyl-coenzyme A desaturase (SCD), carnitine palmitoyltransferase (CPT1), adenosine 5'-monophosphate-activated protein kinase (AMPKα), lipoprotein lipase (LPL), G protein-coupled receptor-43 (GPR43), perilipin-2 (PLIN2), and fatty acid binding protein-4 (FABP4) were used to characterize the degree of adipogenic/lipogenic gene expression. The sequences of the primers are given in Table 1. The thermocycling conditions were as follows: preheating at 95℃ for 15 min, a total of 45 cycles of denaturation at 95℃ for 10 s, annealing temperature at 60℃ for 30 s and extension temperature at 72℃ for 32 s with a melting curve from 65℃ to 95℃. The quantitative PCR results were calculated using the $2^{-\Delta\Delta Ct}$ method to determine the relative mRNA expression levels.

Adiponectin in bovine satellite cell culture media

After BSC differentiation culture for 96 h, the content of adiponectin in the differentiation medium was analyzed using the bovine adiponectin ADP kit (Mlbio, Shanghai, China). The absorbance (OD value) was measured with a microplate reader (BIO-RAD, Hercules, CA, USA) at a wavelength of 450 nm, and the concentration of bovine adiponectin in the sample was calculated from the standard curve.

Construction of RNA-seq library, quality control, and sequencing

After 96 h of BSC differentiation, total RNA was extracted from each treatment using TRIzol™ Reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The isolated RNA was cleared of contaminating genomic DNA by DNase treatment (Thermo Fisher Scientific). Prior to Illumina RNA sequencing, 1% nondenaturing agarose gel electrophoresis and a Nano-Photometer® spectrophotometer (IMPLEN, Munich, Germany) were used to assess the quality and quantity of the isolated RNA, respectively. Samples satisfying the criteria of RNA integrity number > 7 were utilized for the following library preparation. RNA purification, reverse transcription, library construction, and sequencing were completed by AMOGENE. Briefly, the mRNA was enriched by oligo (dT) beads and was fragmented into short fragments through fragmentation buffer at 94℃ for 5 min. First-strand cDNA was synthesized by a random hexamer primer (Illumina) and M-MuLV Reverse Transcriptase (Invitrogen), using the short fragments as templates, at the condition of 25℃ for 10 min, followed by 42℃ for 50 min for synthesis and 70℃ for 15 min for RTase inactivation. Second strand cDNA synthesis was subsequently performed using Second Strand Master Mix (Illumina) at 16℃ for 1 h. Next, the cDNA fragments of 150–200 bp in length were treated with T4 DNA polymerase and 5′ phosphorylated, and then an adenine (A) residue was added to the 3′ ends of the DNA. Moreover, adapters were also ligated to the ends of these target template DNAs. After ligation, the cDNA library was obtained by PCR amplification (5–9 cycles). Finally, PCR products were purified, and library quality was evaluated on an Agilent. Bioanalyzer 2100 system (Agilent Technologies). The library preparations were sequenced on an Illumina Hiseq 4000 platform using paired-end technology following the manufacturer's standard guidelines. Fastp was used to filter the original sequencing data to obtain

Table 1. Forward and reverse primers for Real-time polymerase chain reaction

Genes	Accession no.	Primer Sequence (5'–3')	
Pax3	NSO_4856884_001	Forward:	GGCTGCGTCTCTAAGATCCT
		Reverse:	ATTTCCCAGCTGAACATGCC
Pax7	NSO_4856884_004	Forward:	TGCCCTCAGTGAGTTCGATT
		Reverse:	CGGGTTCTGACTCCACATCT
MYOD	8404456555	Forward:	CCGACGGCATGATGGACTA
		Reverse:	CTCGCTGTAGTAAGTGCGGT
MYOG	8404456557	Forward:	CAGTGAATGCAGCTCCCATAG
		Reverse:	GCAGATGATCCCCTGGGTTG
MYF4	8404456562	Forward:	TGGACCCCTTCAGCTACAGA
		Reverse:	ATGCTTGTCCCTCCTTCCTTG
MRF5	8404456560	Forward:	CCCACCTCAAGTTGCTCTGA
		Reverse:	CCGTGGCATATACATTTGGTACA
PPARγ	NSE_1498162_016	Forward:	ATCTGCTGCAAGCCTTGGA
		Reverse:	TGGAGCAGCTTGGCAAAGA
C/EBPß	840057464	Forward:	CCAGAAGAAGGTGGAGCAACTG
		Reverse:	TCGGGCAGCGTCTTGAAC
SREBP1	NSE_1498162_022	Forward:	CACCGAGGCCAAGTTGAATAA
		Reverse:	CCAGGTCCTTCAGCGATTTG
C/EBPα	NSO_5140066_004	Forward:	ATCGACATCAGCGCCTACAT
		Reverse:	GCCCGGGTAGTCAAAGTCG
ACC	8405780950	Forward:	CTCCAACCTCAACCACTACGG
		Reverse:	GGGGAATCACAGAAGCAGCC
FAS	8405780945	Forward:	TAAGGTTCAAATTGCTGCGT
		Reverse:	TCCAGAGCGAAGGAGAGATT
CPT1	84044456553	Forward:	ACACATCTACCTGTCCGTGATCA
		Reverse:	CCCCTGAGGATGCCATTCT
SCD	NSE_1498162_019	Forward:	TGCCCACCACAAGTTTTCAG
		Reverse:	GCCAACCCACGTGAGAGAAG
AMPKα	8404456568	Forward:	CACCAAGGTGTAAGGAAAGCA
		Reverse:	ACGGGTTTACAACCTTCCATTC
LPL	NSE_1498162_015	Forward:	ACGATTATTGCTCAGCATGG
		Reverse:	ACTTTGTACAGGCACAACCG
GPR43	NSO_5189193	Forward:	GGCTTTCCCCGTGCAGTA
		Reverse:	ATCAGAGCAGCGATCACTCCAT
FABP4	AJ_4160220	Forward:	AAACTTAGATGAAGGTGCTCTGG
		Reverse:	CATAAACTCTGGTGGCAGTGA
PLIN2	NSO_5379931	Forward:	GCGTCTGCTGGCTGATTTCT
		Reverse:	TGTAAGCCGAGGAGACCAGA
GAPDH	NSO_4761240	Forward:	ACTCTGGCAAAGTGGATGTTGTC
		Reverse:	GCATCACCCCACTTGATGTTG

Pax3, paired box 3; Pax7, paired box 7; MYOD, myogenic differentiation-1; MYOG, myogenin; MYF4, myogenin-4; MRF5, myogenic regulatory factor-5; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPß, CCAAT/enhancer-binding protein beta; SREBP1, sterol regulatory element-binding protein-1; C/EBPα, CCAAT/enhancer-binding protein alpha; ACC, acetyl-coenzyme A carboxylase; FAS, fatty acid synthetase; CPT1, carnitine palmitoyltransferase; SCD, stearoyl-coenzyme A desaturase; AMPKα, adenosine 5'-monophosphate-activated protein kinase; LPL, lipoprotein lipase; GPR43, G protein-coupled receptor-43; FABP4, fatty acid binding protein-4; PLIN2, perilipin-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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clean data, and the filtered data were evaluated with the RSeQC software package to obtain high-quality sequencing data, and then Spliced Transcripts Alignments to a Reference software was used for sequence alignment analysis with the reference genome.

Differentially expressed genes identification

Cuffdiff was used to assemble the RNA-Seq alignments and estimate gene expression and transcript. The edgeR package was used for the identification of differentially expressed genes (DEGs) within samples. Genes with statistical significance were retained first (q-value < 0.05); significant DEGs were compared with a fold change of > 1.5 than 1.5 or < 0.667 were retained [log2 (1.5) = 0.5849; log2 (0.667) = –0.5849].

Functional enrichment analysis

The DEGs were subjected to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and enrichment analysis of Gene Ontology (GO). KEGG enrichment analysis was to identify the pathways of significant enrichment of DEGs, gene function, genomic information, and functional information. The GO classification includes the following three aspects: biological process (BP), molecular function (MF), and cellular component (CC), which define and describe the functions of selected genes and corresponding proteins. KEGG was separately generated by (http://www.genome.jp/kegg).

Statistical analysis

One-way ANOVA with multiple comparisons test was used to evaluate the statistical significance of differences among treatments. All data are presented as mean ± SEM. The graphs were prepared using GraphPad Prism 6.07 (GraphPad, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

RESULTS

Identification of skeletal muscle satellite cells

MyoD1 antibodies, Desmin, and Pax7 antibodies were used to monitor myogenic differentiation of BSC, which has been characterized by immunofluorescence staining of proliferation positive BSC (Fig. 1).

Average cell size and cell viability

A total of 1,125 ± 7.82 cells were analyzed for each treatment to measure the average cell size. There was no significant difference in cell size ($p = 0.57$) and cell vitality between treatments ($p = 0.47$) (Fig. 2).

BSC differentiation and lipid accumulation

Control BSC proliferated and fuse to form myotubes. Upon incubation of BSC with ciglitazone, the cells became adipocyte-like cells, and the effect is obvious after 96 h (Fig. 3). Increasing the concentration of ciglitazone greatly accelerated the appearance of adipocyte-like cells, and strongly increased the number of BSC that differentiated into adipocytes. Exposure of post-confluent cells to the differentiation medium plus ciglitazone decreased myotube formation, with a concomitant increase in the area of cells containing lipid droplets and the formation of adipocytes.

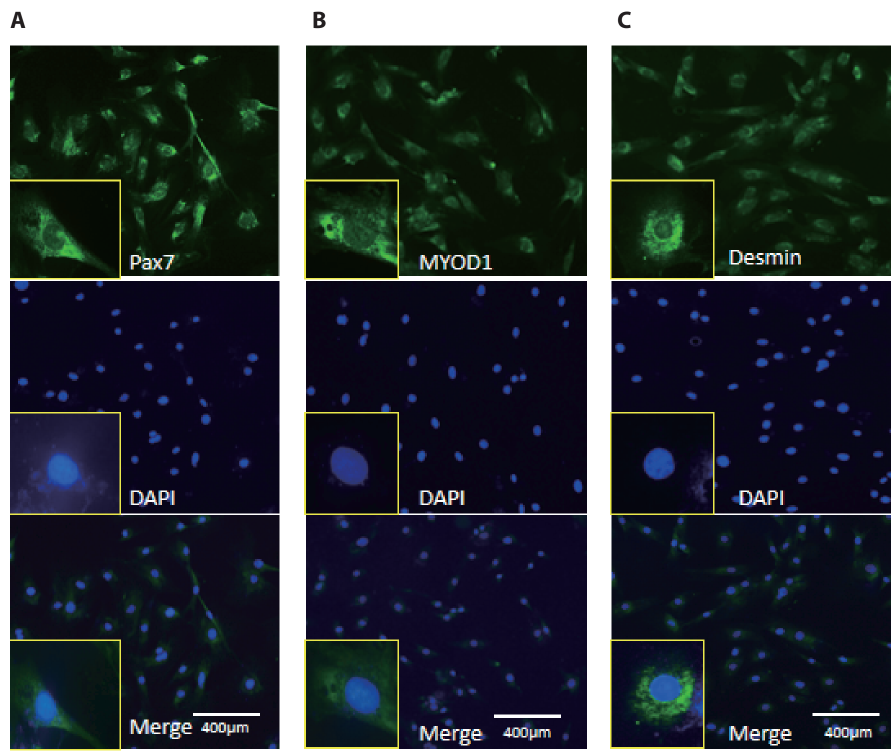

Fig. 1. Immunofluorescence. The nucleus after DAPI staining was blue, and Pax7 (A), MYOD1 (B) and Desmin (C) were green in the cytoplasm. The original magnification was 40× (scale bars = 200 μm). Results represent three independent experiments. Pax7, Paired box 7; DAPI, 4',6-Diamidino-2-Phenylindole, dihydrochloride.

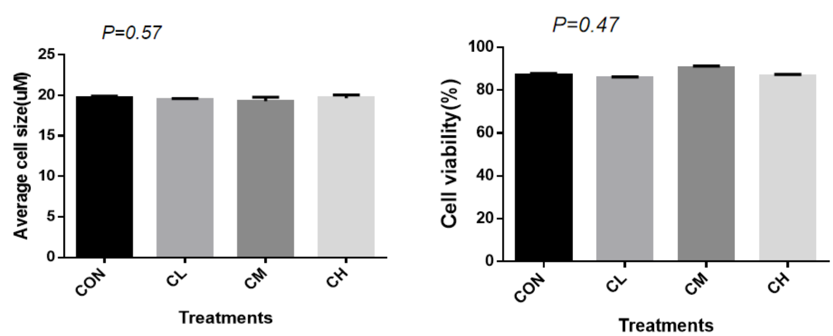

Fig. 2. Effects of different concentrations of ciglitazone on average cell size and cell viability in BSC after 96-h culture. CON, DMEM + 2% HS; CL, CON + 5 μM ciglitazone; CM, CON + 10 μM ciglitazone; CH, CON + 20 μM ciglitazone. The same superscript or no superscript on the column means that the difference is not significant ($p > 0.05$). BSC, bovine satellite cells; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum.

Triacylglycerol and lipid droplet area quantification

The concentration of cellular TAG, the main component of lipid droplets, was elevated linearly by CL, CM, and CH treatments and, correspondingly, CL, CM, and CH treatments caused a profound increase in the percent area of lipid droplets (Fig. 4) ($p < 0.01$).

Myogenic gene expression

The CL, CM, and CH treatments increased *Pax3* and *Pax7* expression and decreased *MYOD*,

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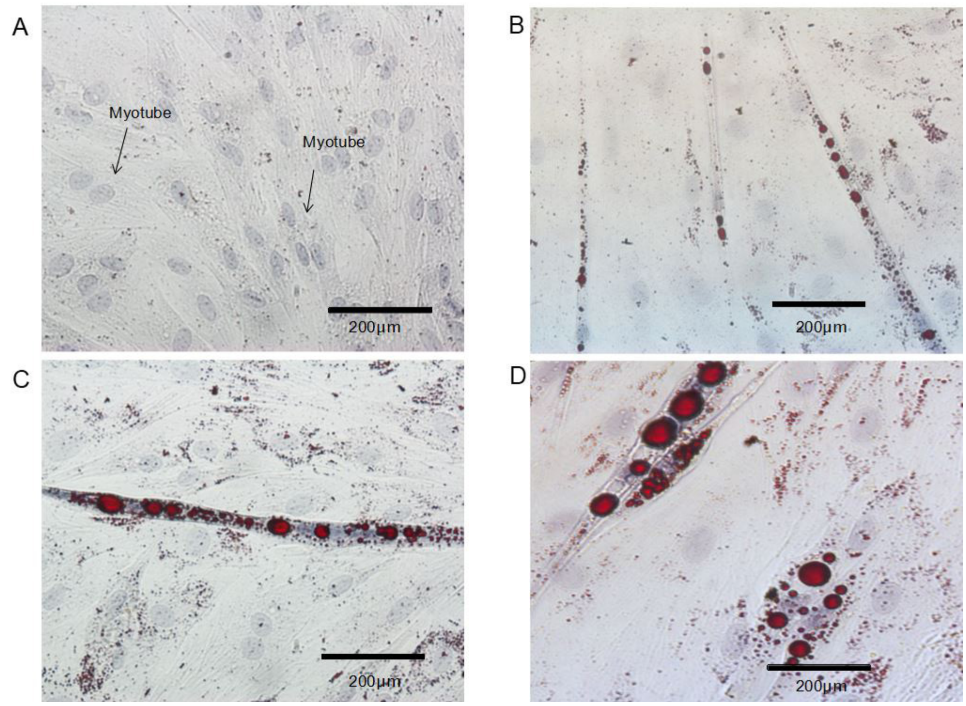

Fig. 3. Oil red O staining after 96-h incubation of BSC treated with ciglitazone. (A) CON (DMEM + 2% horse serum), (B) CL (CON + 5 μM ciglitazone), (C) CM (CON + 10 μM ciglitazone), (D) CH (CON + 20 μM ciglitazone). Scale bars = 200 μm. BSC, bovine satellite cells; DMEM, Dulbecco's modified Eagle's medium.

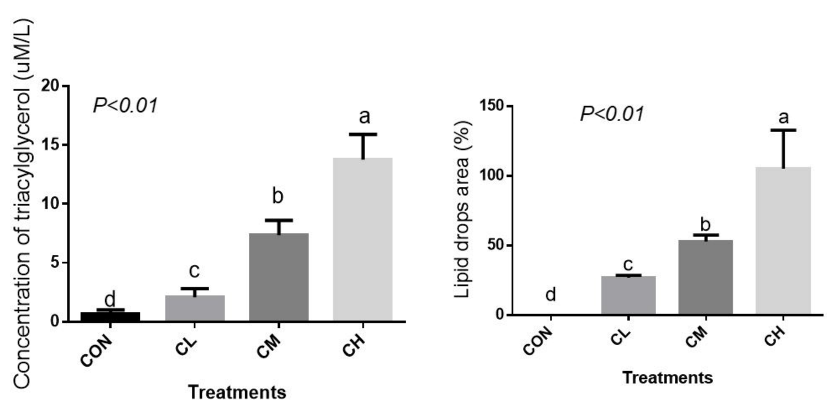

Fig. 4. Effect of ciglitazone on triacylglycerol and percent lipid droplet area in BSC after 96 h of culture. CON, DMEM + 2% HS; CL, CON + 5 μM ciglitazone; CM, CON + 10 μM ciglitazone; CH, CON + 20 μM ciglitazone. Error bars are mean ± SE. [a–d]Indicate a significant difference (p < 0.01) within an item compared with undifferentiated cells. The results are representative of six independent experiments. BSC, bovine satellite cells; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum.

MYOG, *myogenin-4* (*MYF4*), and *myogenic regulatory factor-5* (*MRF5*) expression relative to CON (p < 0.01) (Fig. 5). The expression of *MYF4* and *MRF5* were the least in the CH-treated BSC (p < 0.01).

Associated with adipogenic and lipid metabolism

There was a dose-response to ciglitazone addition for the expression of adipogenic transcription factors *PPARγ*, *SREBP1*, *C/EBPα*, *C/EBPβ*, *acetyl-coenzyme A carboxylase*, and *fatty acid*

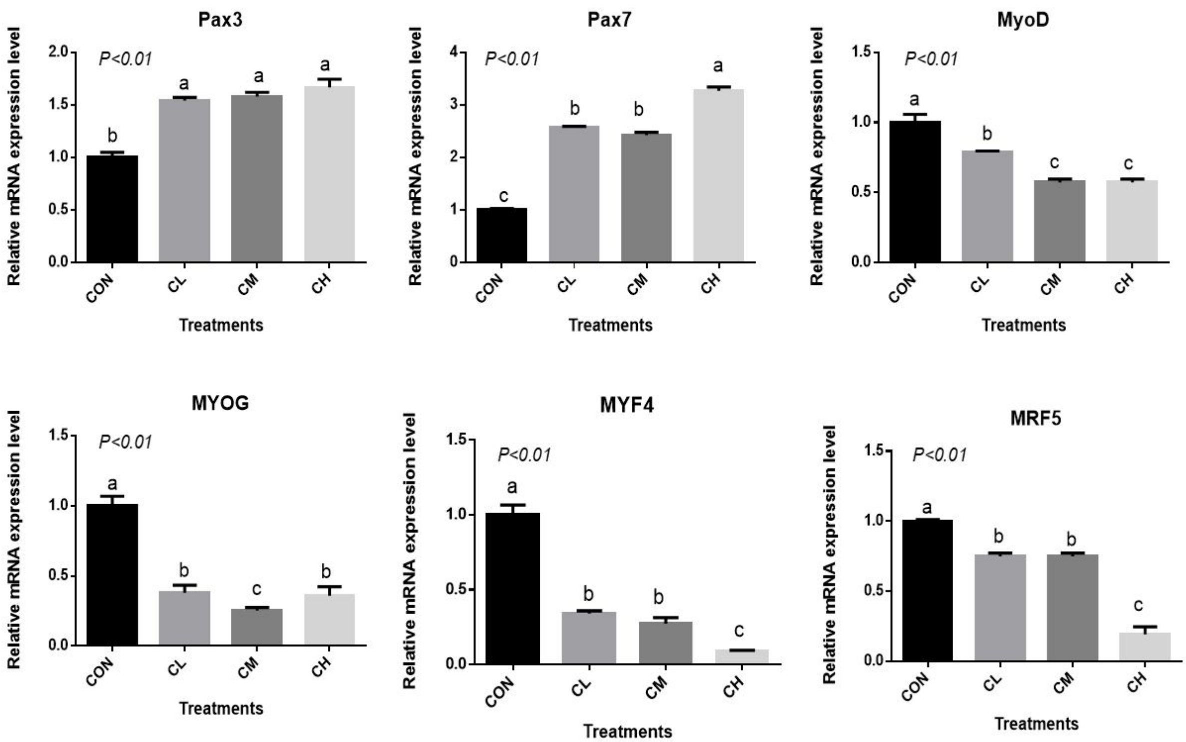

Fig. 5. Myogenic gene expression: Pax3, Pax7, MYOD, MYOG, MYF4, and MRF5 gene expression in BSC cultured with ciglitazone in DMEM containing 2% HS after 96 h of incubation. CON, DMEM + 2% HS; CL, CON + 5 μM ciglitazone; CM, CON + 10 μM ciglitazone; CH, CON + 20 μM ciglitazone. The results of gene expression were consistent with protein expression. The bars are the means ± SE. [a–d]Indicate a significant difference ($p < 0.01$) within an item compared with undifferentiated cells. The results are representative of six independent experiments. Pax3, Paired box 3; Pax7, Paired box 7; MYOD, myogenic differentiation-1; MYOG, myogenin; MYF4, myogenin-4; MRF5, myogenic regulatory factor-5; BSC, bovine satellite cells; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum.

synthetase ($p < 0.01$), and the greatest increase in the expression of these genes, were caused by CH-treated BSC (Fig. 6). Relative expression of *SCD*, *LPL*, *GPR43*, *FABP4*, and *PLIN2* was dose-dependent as the addition level of ciglitazone increased ($p < 0.01$) (Fig. 7). Expression of *CPT1* was depressed by the CL, CM, and CH-treated BSC stepwise relative to CON BSC. Expression of *AMPK α* ($p < 0.01$) was greater in CL, CM, and CH-treated BSC than in CON BSC, and the CM treatment caused the greatest increase.

Culture media adiponectin

Compared with CON BSC, the CL, CM, and CH treatments increased the amount of adiponectin (*ADP*) secretion ($p < 0.01$), and the highest concentration of *ADP* was caused by the CH treatment (Fig. 8).

Analysis of differentially expressed genes in bovine satellite cell

Whole-genome expression profiling with RNA-Sequencing analysis revealed significant *DEGs* when the BSC were treated with different levels of ciglitazone. Compared with CON, the CL treatment up-regulated 611 genes and down-regulated 510 genes; the CM treatment up-regulated 565 genes and down-regulated 527 genes; whereas the CH treatment up-regulated 501 genes and down-regulated 435 genes (Table 2). Combined with the analysis of these results of differential expressed genes, BSC treated with CL, CM, and CH had 281 *DEGs* as shown in the volcano map (Fig. 9A, B, and C).

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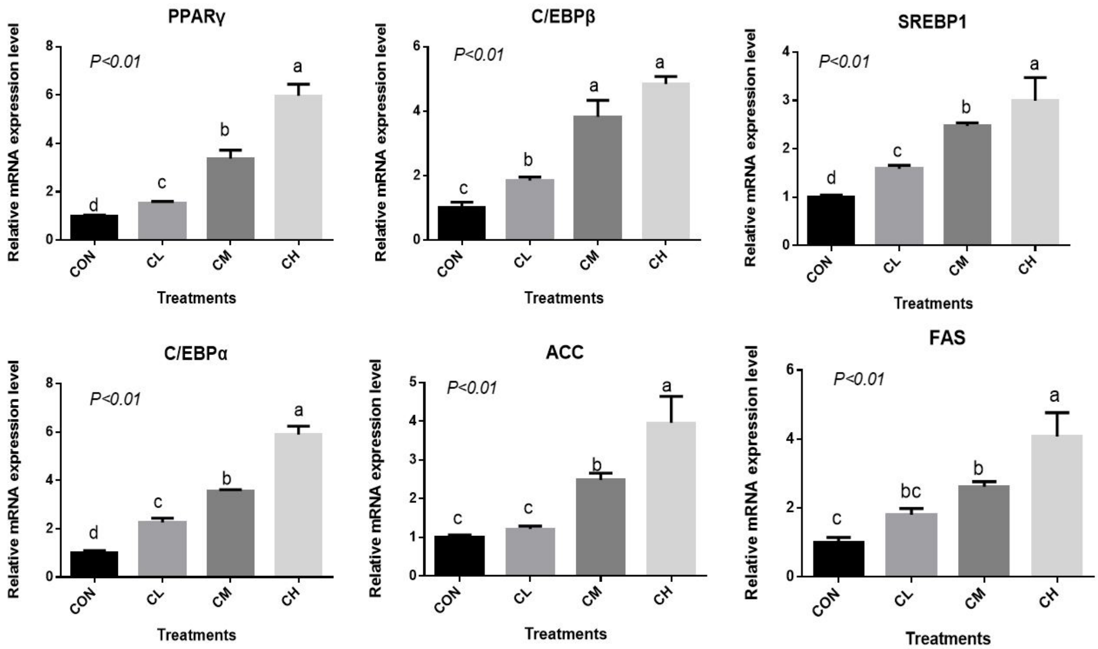

Fig. 6. Adipogenic gene expression: PPARγ, C/EBPβ, SREBP1, C/EBPα, ACC, FAS gene expression in BSC cultured with ciglitazone in DMEM containing 2% HS after 96 h of incubation. CON, DMEM + 2% HS; CL, CON + 5 μM ciglitazone; CM, CON + 10 μM ciglitazone; CH, CON + 20 μM ciglitazone. The results of gene expression were consistent with protein expression. Error bars are the means ± SE. [a–d]Indicate a significant difference ($p < 0.01$) within an item compared with undifferentiated cells. The results are representative of six separate experiments. PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPß, CCAAT/enhancer-binding protein beta; SREBP1, sterol regulatory element-binding protein-1; C/EBPα, CCAAT/enhancer-binding protein alpha; ACC, acetyl-coenzyme A carboxylase; FAS, fatty acid synthetase; BSC, bovine satellite cells; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum.

Gene ontology analysis of differentially expressed gens in bovine satellite cell

Pathway analysis of *DEGs* was performed to understand the biological relationship between pathways and molecules. The GO annotation was classified into three clusters: BP, MF, and CC. The 10 top significantly enriched GO terms in which the calculated by the hypergeometric test was Q < 0.05 were selected. As shown in Fig. 10 and (Supplementary Table S1, biological pathways were significantly changed by GO terms of ciglitazone-treated BSC. Top 10 GO enrichment analyses significantly changed cell proliferation, protein trimerization, fat cell differentiation, cellular response to external stimulus, positive regulation of release of cytochrome c from mitochondria, and type B pancreatic cell proliferation process. Molecular functions such as nuclease activity, endonuclease activity, deoxyribonuclease activity, growth factor activity, kinase inhibitor activity, biotinidase activity, and receptor-ligand activity were up-regulated by ciglitazone.

Kyoto Encyclopedia of genes and genomes enrichment of differentially expressed genes in bovine satellite cell

Pathway analysis of *DEGs* was conducted to understand the pathways and molecular interactions involved with ciglitazone treatment. The 10 top significantly enriched GO terms in which the calculated by the hypergeometric test was Q < 0.05 were selected. Enrichment degree was identified by Gene Ratio, P. adjust, and enriched genes. In the CL treated BSC, KEGG enrichment analysis indicated that a total of 465 *DGEs* were enriched in 31 pathways and mainly in glycine, serine, and threonine metabolism, tumor necrosis factor (TNF) signaling pathway, and the p53 signaling pathway. Genes with up-regulated expression were mainly enriched in mineral absorption, transcriptional misregulation in cancer, choline metabolism in cancer, glutathione metabolism.

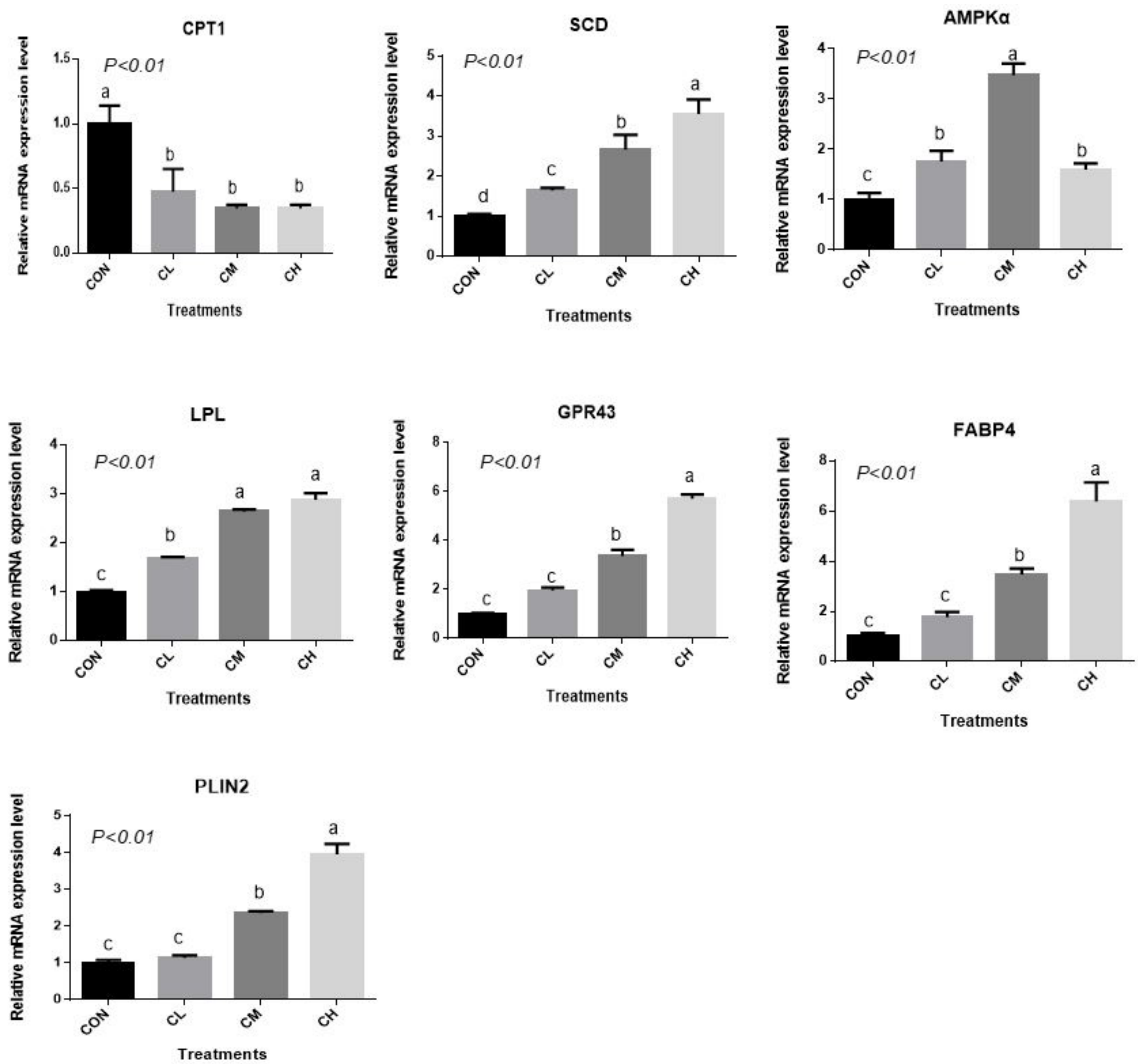

Fig. 7. Gene expression associated with lipid metabolism: CPT1, SCD, AMPKα, LPL, GPR43, FABP4, PLIN2 gene expression in BSC cultured with ciglitazone in DMEM containing 2% HS at 96 h incubation time. CON, DMEM + 2% HS; CL, CON +5 μM ciglitazone; CM, CON + 10 μM ciglitazone; CH, CON + 20 μM ciglitazone. The results of gene expression were consistent with protein expression. The bars are the means ± SE. [a–d]Indicate a significant difference ($p < 0.01$) within an item compared with undifferentiated cells. The results are representative of six independent experiments. CPT1, carnitine palmitoyltransferase; SCD, stearoyl-coenzyme A desaturase; AMPKα, adenosine 5'-monophosphate-activated protein kinase; LPL, lipoprotein lipase; GPR43, G protein-coupled receptor-43; FABP4, fatty acid binding protein-4; PLIN2, perilipin-2; BSC, bovine satellite cells; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum.

Genes with down-regulated expression were mainly enriched in the TNF signaling pathway, biosynthesis of amino acids, protein digestion, and absorption.

In the CM treated BSC, a total of 484 *DGEs* were enriched in 18 pathways and mainly in apoptosis, p53 signaling pathway, non-alcoholic fatty liver disease (NAFLD), PI3K-Akt signaling pathway, Wnt signaling pathway, and TNF signaling pathway. Genes with up-regulated expression were mainly enriched in TNF signaling pathway, the calcium signaling pathway, apoptosis, RNA degradation, and genes with down-regulated expression were mainly enriched in the biosynthesis of amino acids, p53 signaling pathway, ribosome, carbon metabolism, and the PI3K-Akt signaling pathway. In the CH treated BSC, a total of 397 *DGEs* were enriched in 30 pathways and mainly in the p53 signaling pathway, PPAR signaling pathway, biosynthesis of amino acids, pentose phosphate pathway, and TGF-beta signaling pathway. Genes with up-regulated expression were

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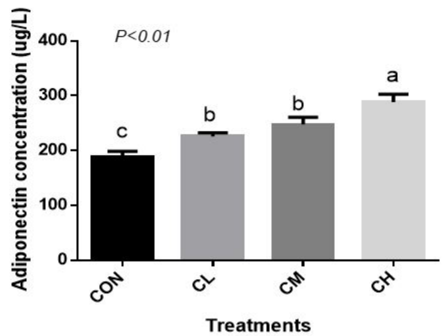

Fig. 8. Adiponectin concentration (μg/mL) in media from BSC cultured for 96 h with ciglitazone in DMEM containing 2% HS at 96 h incubation time. CON, DMEM + 2% HS; CL, CON + 5 μM ciglitazone; CM, CON + 10 μM ciglitazone; CH, CON + 20 μM ciglitazone. The bars are the means ± SE. [a–d]Indicate a significant difference (p < 0.01) within an item compared with undifferentiated cells. The results are representative of six independent experiments. BSC, bovine satellite cells; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum.

Table 2. Number of DEGs after treatment with different concentrations of ciglitazone

Type	Treatment		
	CL	CM	CH
Up	609	562	501
Down	508	526	432
Total	1,117	1,088	933

DEG, differentially expressed gene; CL, ciglitazone; CM, ciglitazone; CH, ciglitazone.

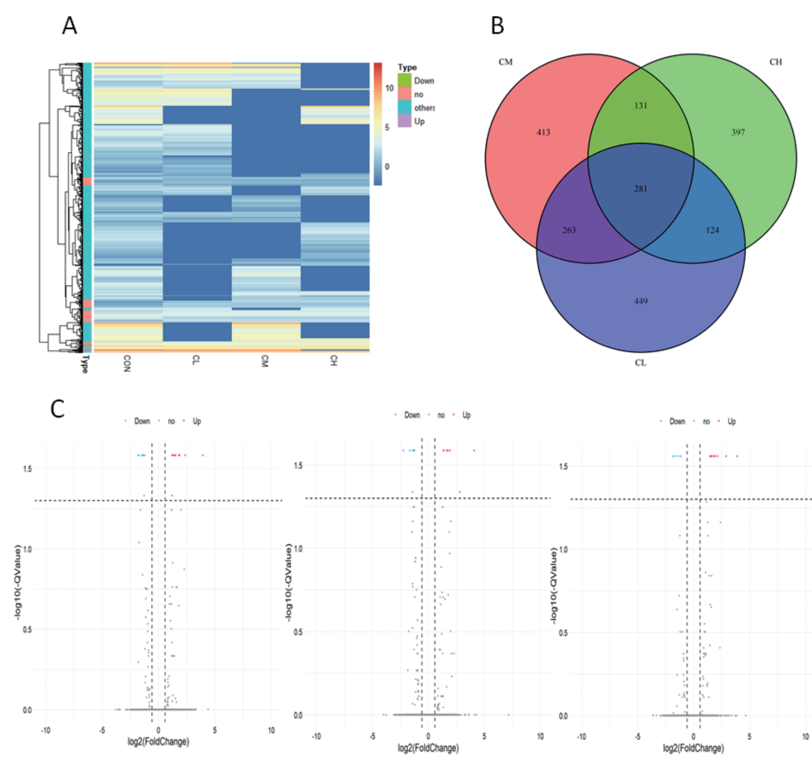

Fig. 9. Differentially Expressed Genes (DEGs) (A) Heatmap for the Figure hierarchical cluster analysis of DEGs after differentiation of BSC in Yanbian Yellow Cattle with different concentrations of ciglitazone, (B) Wayne map and (C) Volcano Plot. Red represents upregulated genes and blue represent downregulated genes. CON, DMEM + 2%HS; CL, CON + 5 μM ciglitazone; CM, CON + 10 μM ciglitazone; CH, CON + 20 μM ciglitazone. BSC, bovine satellite cells; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum.

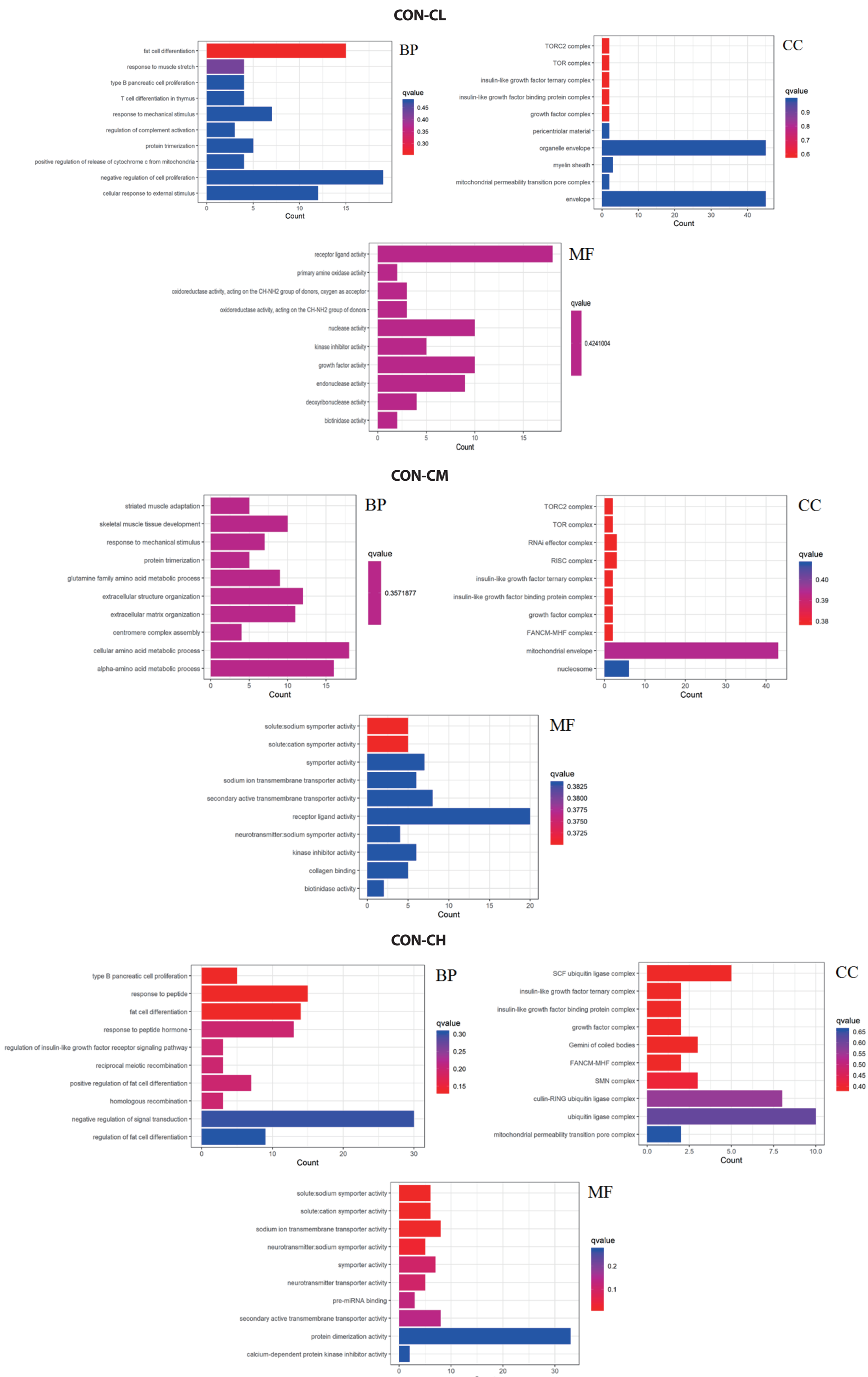

Fig. 10. Gene Ontology (GO) classification of differentially expressed genes (DEGs) after differentiation of BSC in Yanbian Yellow Cattle with different concentrations of ciglitazone. CON, DMEM + 2% HS; CL, CON + 5 μM ciglitazone; CM, CON + 10 μM ciglitazone; CH, CON + 20 μM ciglitazone. BP, biological process; CC, cellular component; MF, molecular function; BSC, bovine satellite cells; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum.

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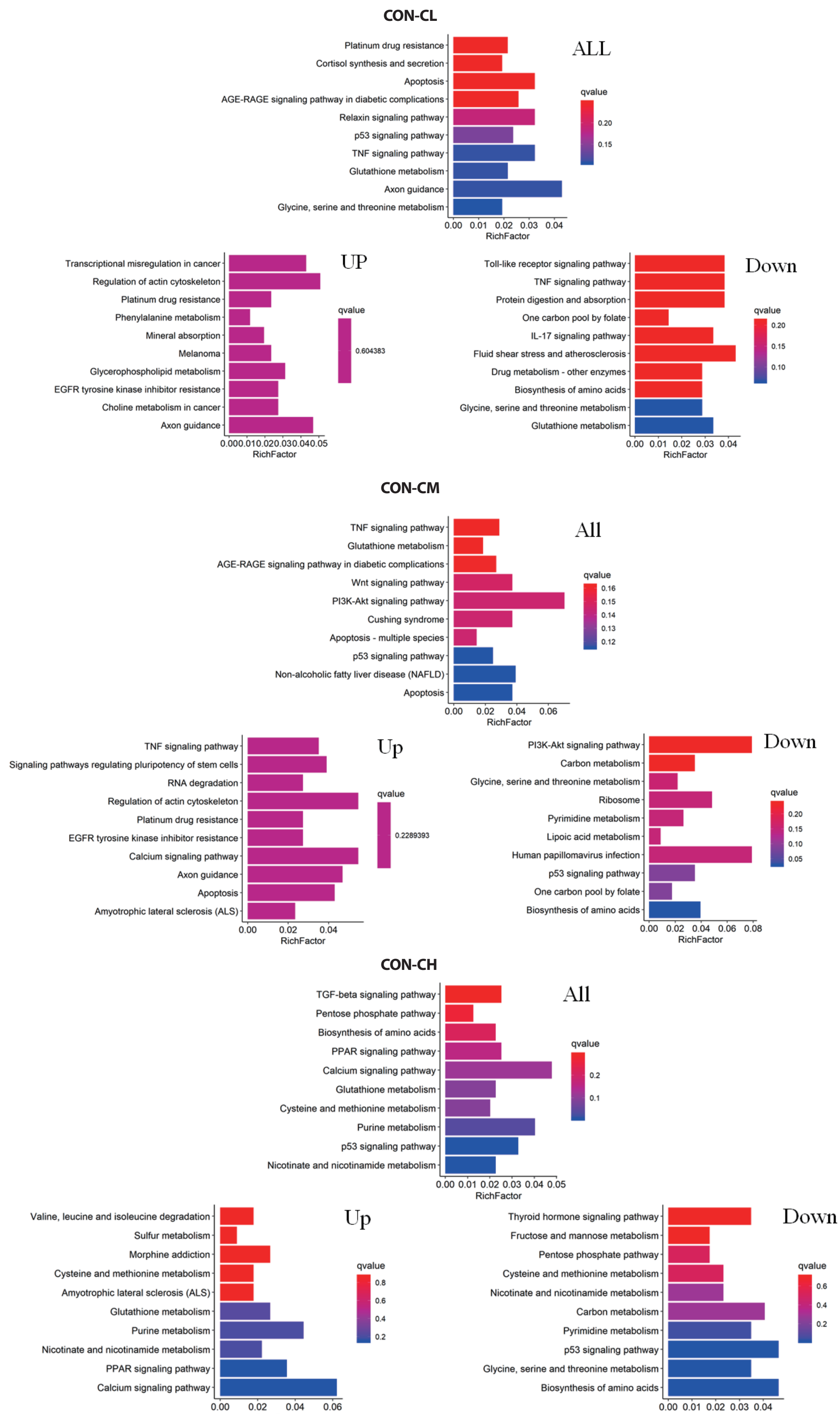

Fig. 11. KEGG functional enrichment of transcriptome from skeletal muscle satellite cells of different doses of ciglitazone. CON, DMEM + 2%HS; CON-CL, CON + 5 μM ciglitazone; CON-CM: CON + 10 μM ciglitazone; CON-CH, CON + 20 μM ciglitazone. KEGG, Kyoto Encyclopedia of Genes and Genomes; BSC, bovine satellite cells; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum.

mainly enriched in the calcium signaling pathway, PPAR signaling pathway, purine metabolism, nicotinate and nicotinamide metabolism, and genes with down-regulated expression were mainly enriched in the biosynthesis of amino acids p53 signaling pathway, glycine, serine, and threonine metabolism, pyrimidine metabolism, and carbon metabolism (Fig. 11 and Supplementary Table S2).

Kyoto Encyclopedia of genes and genomes pathway enrichment analysis of differentially expressed genes

Effects of increasing concentrations of ciglitazone on the pathway of proliferator-activated receptor-γ enrichment

As ciglitazone is a *PPARγ* agonist, we analyzed the *PPAR*-signaling pathway enriched by DEGs during adipogenic trans-differentiation of BSC induced by different concentrations of ciglitazone (Fig. 12). The high-concentration of ciglitazone significantly up-regulated the

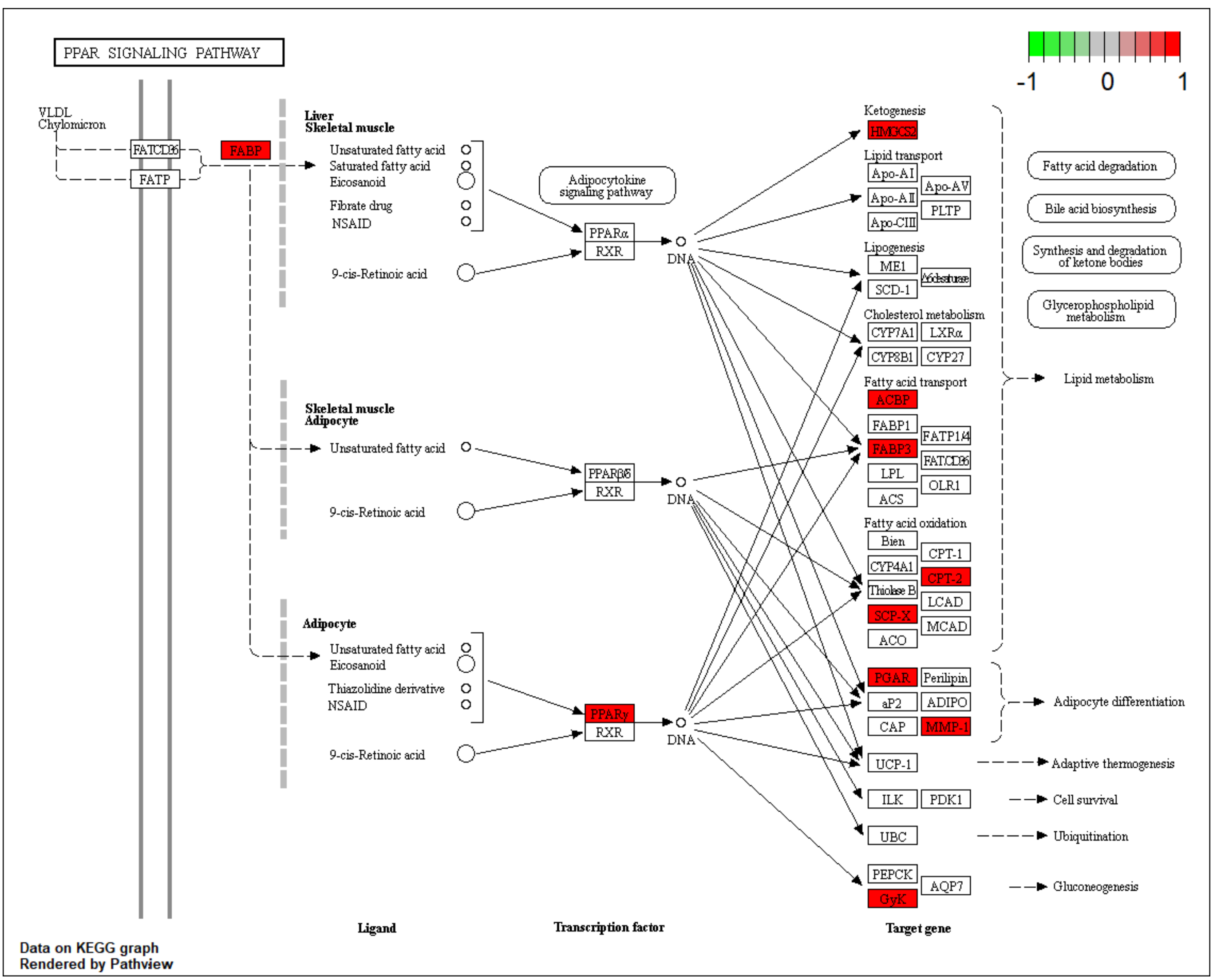

Fig. 12. PPAR signaling pathway regulated by high the concentration of ciglitazone. PPAR, proliferator-activated receptor.

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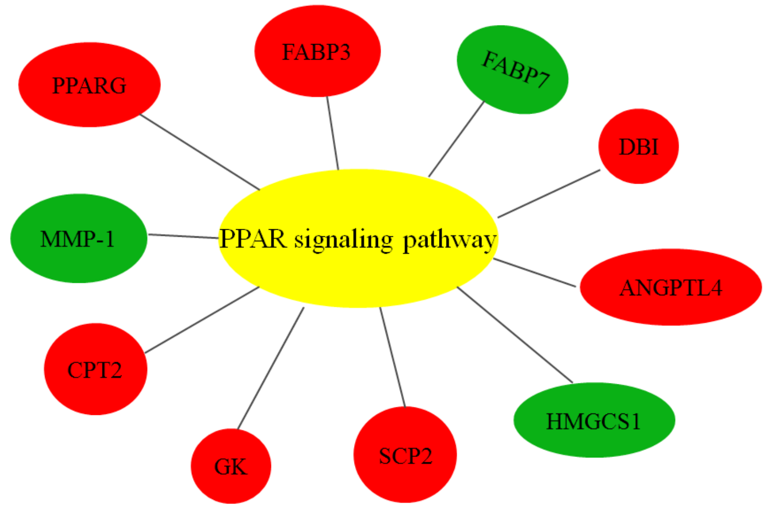

Fig. 13. Distribution of up and downregulated genes enrichment in different Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Pathway nodes (yellow), up-regulated genes (red), and down-regulated genes (green) are represented by circles. PPARG, peroxisome proliferator-activated receptor gamma; FABP4, fatty acid binding protein-4; FABP 7, fatty acid binding protein-7; DBI, diazepam binding inhibitor; ANGPTL4, angiopoietin like 4; HMGCS1, hydroxymethylglutaryl-CoA synthase; SCP-2, sterol carrier protein 2; GK; glycerolkinase; GPT2; alanine aminotransferase 2; MMP1; matrix metalloproteinase-1.

PPAR signaling pathway, there were 10 genes differentially enriched in this pathway, and the proportion of differentially enriched genes was 10/397. Pathway analysis of differentially enriched genes indicated 7 up-regulated genes and 3 down-regulated genes. Based on the up-regulated expression of *PPARγ*, the downstream fatty acid transport-related genes (diazepam binding inhibitor [*DBI*], *FABP3*), fatty acid oxidation-related genes (*CPT-2*, *sterol carrier protein 2*, *FABP3*), adipocyte differentiation-related genes (*angiopoietin like 4*, *glycogen neogenesis gene glucokinase*), and the adipocyte differentiation-related gene *FABP7* were up-regulated. The expression of *matrix metalloproteinase-1* and the ketogenic gene *hydroxymethylglutaryl-CoA synthase2* was down-regulated (Fig. 13).

DISCUSSION

This study addressed the hypothesis that ciglitazone as a *PPARγ* ligand in the absence of an adipocyte differentiation cocktail would increase adiponectin and adipogenic gene expression in BSC. Previous studies investigated TZD as an extrinsic factor that influences myogenic satellite cells by inducing the expression of several transcription factors, including *PPARγ* and *C/EBPα* [7,8]. In cases where the conditions of the adipocytes containing the *PPARγ* activator permit, these cells can differentiate into mature adipocytes [9]. Li et al. [10] assumed that myoblasts and adipocytes have similar bio-initiating sources, and the potential for trans-differentiation to produce marbling or intramuscular lipogenesis was hypothesized. Transcriptional control of adipocyte differentiation requires a series of continuous gene expression events and activation of many key signaling pathways [11]. Cell lipid accumulation and morphological changes accompanying lipogenesis are caused by the induced expression of specific genes during differentiation [12]. In the present study, the decrease in the myotube formation and the increase in the accumulation of lipid droplets caused by ciglitazone addition in BSC. This may indicate that ciglitazone could induce the BSC to differentiate into adipocytes.

Studies have reported that many transcription factors regulate adipocyte differentiation, such as *C/EBPs* and *PPARs* [9]. Muscle satellite cells can be trans-differentiated into mature adipocytes by ectopic expression of adipose trans-differentiation factors such as *PPARγ* [13]. Previous studies had documented that when the *PPARγ* gene was overexpressed in G8 myoblasts under optimal culture conditions for muscle differentiation, the expression levels of myogenic factors *MRF5*, *MYOD*, and *MYF4* were depressed, and the myoblasts could not differentiate into myotubes [14]. In mammalian cells, *PPARγ* and *C/EBPα* are major regulators of lipogenesis and be transcription targets [15]. *PPARγ* is induced during the differentiation of preadipocytes into adipocytes, which is critical for precursor cells to differentiate into mature adipocytes [16]. Adipocyte differentiation also involves the expression of several other transcription factors that interact at different the process of adipogenesis to produce mature adipocytes [17]. Teboul et al. [4] demonstrated that TZDs specifically bind to and activate *PPARγ*, increase the expression of *PPARγ* and related adipokines, and promote adipocyte differentiation. Kageyama et al. [18] demonstrated that pioglitazone increased body weight by selectively stimulating *PPARγ* and promoting lipid storage in white adipose tissue. Lizcano [19] reported that JMJD2C could inhibit *PPARγ* activity and reduce triglyceride accumulation in adipocytes. Although *PPARγ* and *C/EBPα* are highly synergistic in the adipogenic process, adipocyte genes vary in their reliance on *C/EBPα* and *PPARγ* [20].

Pax3 and *Pax7* are markers of the differentiation of mesenchymal cells toward a myogenic cell lineage. Up-regulation of the expression of *Pax3* and *Pax7* increases the expression of myogenic regulatory factors [21], which include *MYOD* and *MYOG*. Up-regulation of *MYOD* expression promotes the conversion of somite-derived cells into myoblasts; *MYOG* is involved in the fusion of myoblasts to form myotubes [22]. Cornelison et al. [23] identified *MYOD* as a key negative regulator of brown adipocyte development. Similarly, Wang et al. [24] reported that *MYOD* negatively regulated the f brown adipocyte development and concluded that loss of *MYOD* facilitates adipogenic trans-differentiation of myoblasts *in vivo*. The *PLIN2* is primarily expressed during the early stages of adipocyte differentiation, which promotes lipid droplet accumulation in muscle cells [25]. Studies with 3T3-L1 adipocytes indicated that *PLIN2* could be used as a marker gene for precursors during adipocyte differentiation [26]. In this study, ciglitazone in the absence of an adipocyte differentiation cocktail also induced an up-regulation of adipogenic gene expression accompanied by low expression of MYOD, and the higher *PLIN2* expression and adiponectin promotes the higher lipid droplets accumulation in the BSC [3].

Whole-genome RNA-Seq GO analysis and KEGG enrichment analysis o*f DEGs* showed that most *DEGs* were closely related to cell proliferation, cell differentiation, and cell metabolic processes. *PPAR* signaling pathway, P53 signaling pathway, Wnt signaling pathway, ribosome, signaling pathway regulating pluripotency of stem cells, TNF signaling pathway, and calcium signaling pathway participate in the adipogenic transdifferentiation process of BSC. The high concentration of ciglitazone significantly up-regulated the *PPAR* signaling pathway, and 7 out of 10 genes were up-regulated in this pathway. Besides, the increase in the accumulation of lipid droplets and the concentration of *TAG* and adiponectin further indicated the regulatory role of *PPARγ* in adipogenic transdifferentiation of BSC.

The PI3K-Akt signaling pathway plays an important regulatory role in adipocyte differentiation and adipogenesis. Inhibiting the activation of PI3K-Akt could depress adipogenesis and differentiation, and down-regulate the expression of adipogenic genes *PPARγ*, *C/EBPa*, *FABP4*, and *FATP1* [27,28]. Wnt signaling has been reported to inhibit adipogenesis. Adipose tissue contains adult stem cells (ADSCs) similar to mesenchymal stem cells, which inhibit adipogenic differentiation by activating the Wnt signaling pathway [29–31]. In this study, the RNA sequencing results revealed that the low concentration of ciglitazone could down-regulated the genes in the

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regulation of the Wnt signaling pathway, which revolved in adipogenesis and lipid metabolism.

TGF-β1 is a factor activated by inflammation and participates in the process of chronic fibrosis of organs. TGF-β1 transmits fibrotic signals through its downstream kinase substrate Smads protein. The TGF-β1/Smad3 signaling pathway and organ fibrosis are closely related. TGF-β1 binds to its receptor on the cell membrane causes intracellular Smad2 and Smad3 phosphorylation and Smad4 binds to form a complex that enters the nucleus and cooperates with other transcription factors to regulate the expression of genes related to cell proliferation and differentiation [32–35]. In our study, the high concentration of ciglitazone can inhibit the activation of the TGF-β1/Smad3 signaling pathway. This may suggest that ciglitazone could promote the adipocyte phenotype in BSC through these signaling pathways. These results indicate that ciglitazone as a PPARγ agonist can promote lipid droplet formation and triglyceride accumulation in BSC, and effectively increase adiponectin concentration and adipogenic gene expression. RNS-seq showed that treatment with different concentrations of ciglitazone significantly affected the p53 signaling pathway, PPAR signaling pathway, TNF signaling pathway, PI3K-Akt signaling pathway, and Wnt signaling pathway.

In conclusion, our results support at least partial conversion of BSC to adipocytes by ciglitazone in the absence of an adipocyte differentiation cocktail. Further experiments are needed to elucidate the molecular mechanisms by which *PPARγ* affects lipogenesis during the process of adipogenic differentiation of BSC and the regulatory pathways that influence other regulators of lipid production.

SUPPLEMENTARY MATERIALS

Supplementary materials are only available online from: https://doi.org/ 10.5187/jast.2021.e87.

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