Ciglitazone Increases Adiponectin and Adipogenic Gene Expression in Bovine Skeletal Muscle Satellite Cells

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Research

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**Ciglitazone increases adiponectin and adipogenic gene expression in bovine skeletal muscle
satellite cells¹**

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Running head: Ciglitazone and bovine satellite cells

Abstract

Background: Ciglitazone is a member of the thiazolidinedione (TZD) family, and specifically binds to peroxisome proliferator-activated receptor- γ (*PPAR* γ), thereby promoting adipocyte differentiation. We hypothesized that ciglitazone as a *PPAR* γ ligand in the absence of an adipocyte differentiation cocktail would increase adiponectin and adipogenic gene expression in bovine satellite cells (BSC).

Methods: Muscle-derived bovine satellite cells were isolated from six, 18-month-old Yanbian Yellow Cattle. The BSC were cultured for 96 h in differentiation medium containing 5 μ M ciglitazone (CL), 10 μ M ciglitazone (CM), or 20 μ M ciglitazone (CH). Control (CON) BSC were cultured only in differentiation medium (containing 2% horse serum).

Results: The presence of myogenin, desmin, and paired box 7 (*Pax7*) proteins were 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 *PPAR* γ , CCAAT/enhancer-binding protein alpha (*C/EBP* α), *C/EBP* β , fatty acid synthase, stearoyl-CoA desaturase (*SCD*) and perilipin 2. Ciglitazone treatments increased gene expression of paired box 3 (*Pax3*) and *Pax7* and decreased expression of myogenic differentiation-1, myogenin, myogenic regulatory factor-5 (*MRF5*), and *MYF4* ($P < 0.01$). 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. KEGG pathway analysis showed that DEGs were involved into p53 signaling pathway, *PPAR* signaling pathway, biosynthesis of amino acids, TNF signaling pathway, non-alcoholic fatty liver disease (NAFLD), PI3K-Akt signaling pathway, and Wnt signaling pathway.

Conclusion: These results indicate that ciglitazone acts as *PPAR* γ agonist, effectively increasing the adiponectin concentration and adipogenic gene expression, and stimulating 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

Background

The thiazolidinediones (TZD) family specifically bind to and activate proliferator-activated receptor- γ (*PPAR* γ), increase its expression and that of the related genes (e.g., CCAAT/enhancer-binding protein alpha (*C/EBP* α)), and promote adipocyte differentiation [1]. Yeow et al. demonstrated that the TZD rosiglitazone, in the absence of supplemental fatty acids, strongly up-regulated *PPAR* γ expression in C2C12 cells, and proposed that rosiglitazone inhibited myogenesis [2]. Singh et al. reported that the TZD ciglitazone up-regulated *PPAR* γ and *C/EBP* α gene expression and depressed myoblast fusion in porcine satellite cells [3]. Our laboratory previously reported that culture of bovine muscle satellite cells (BSC) in medium supplemented with oleic acid (18:1n-9), in the absence or presence of ciglitazone, responded with increases in *PPAR* γ and CCAAT/enhancer-binding protein beta (*C/EBP* β) gene expression in myotubes [4]. Teboul et al. 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 [5]. In our previous study [4], we established that 10 μ M ciglitazone down-regulated the expression of genes associated with myogenesis, and up-regulated genes involved in adipogenesis. However, we did not establish that 10 μ M ciglitazone would elicit maximal effects on adipogenesis and/or myogenesis. Therefore, we hypothesized that greater

concentration of ciglitazone would more strongly promote the adipocyte phenotype in bovine BSC.

For the current study, we selected a differentiation medium containing DMEM supplemented with 2% horse serum based on Singh et al. [3], who described the trans-differentiation of porcine satellite cells into adipoblasts. Here, however, we altered the adipogenic mixture of that study (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 μ M). 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 included horse serum in the media to encourage BSC proliferation and myogenic gene expression to better reflect in situ conditions.

Material and methods

Ethics statement

All experimental procedures were approved by the Yanbian University Institutional Animal Care and Use Committee.

Bovine satellite cell isolation

The isolation and culture of BSC were performed exactly as described previously [4]. Muscle-derived BSC were isolated from six 18-month-old Yanbian Yellow cattles raised at Yanbian University. Using sterile techniques, approximately 800 g of semimembranous muscle was dissected and transported to the cell culture laboratory within 10 min. Cell isolation procedures were conducted in a sterile environment in a culture hood.

After the removal of connective tissue, the muscles were grounded through a sterile meat grinder. Ground muscles were incubated with 0.1% pronase (Calbiochem, La Jolla, CA, USA) in Earl's Balanced Salt Solution (Sigma-Aldrich, Louis, MO, USA) for 1 h at 37°C with frequent mixing [6]. After incubation, the mixture was centrifuged at $1,500 \times g$ for 4 min, the pellets were suspended in phosphate-buffered saline (PBS) (140 mM NaCl, 1 mM KH_2PO_4 , 3 mM KCl, 8 mM Na_2HPO_4 ; Gibco Life Technologies, Grand Island, NY), and the suspensions were centrifuged at $500 \times g$ for 10 min. The supernates were centrifuged at $500 \times g$ for 10 min to pellet the mononucleated cells. The PBS wash and differential centrifugation steps were repeated twice. The resulting mononucleated cell preparations were suspended in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco), $1 \times$ antibiotic-antimycotic (Gibco), and 10% (vol/vol) dimethyl sulfoxide (Sigma-Aldrich) at 37°C, before being frozen and stored in liquid nitrogen.

Bovine satellite cells culture

Experiments were replicated in three independent incubations (passage no. 2 of the BSC

obtained from three Yanbian Yellow cattle for all experiments). The BSC were resuspended and maintained in the growth medium composed of DMEM, 10% FBS, and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. Upon reaching confluence, the growth medium was replaced by the differentiation medium composed of DMEM, 2% horse serum, and 1% penicillin/streptomycin.

We changed the differentiation components of Singh et al. (2007), omitting ascorbic acid, biotin, acetic acid, panthothenic acid, dexamethasone, isobutylmethylxanthine, and insulin. The BSC were cultured for 96 h in the differentiation medium containing 5 µM ciglitazone (CL), 10 µM ciglitazone (CM), or 20 µM ciglitazone (CH). Control (CON) BSC were cultured only in differentiation medium. Ciglitazone (Sigma-Aldrich) was dissolved in ethanol before addition to BSC cultures. Cell size and viability were calculated with a Luna Automated Cell Counter (Logo Biosystems, Annandale, VA) after trypan blue staining.

Immunostaining

The isolated BSC were cultured to approximately 70% confluence in DMEM with 10% FBS and 1% penicillin/streptomycin without ciglitazone. BSC were grown on coverslips for 3 d and fixed with 4% paraformaldehyde for 30 min and washed with PBS. The BSC were incubated with polyclonal anti-myogenic differentiation-1 (MYOD1; BS-2442R), anti-paired box-7 (Pax7) (AB-528428) and anti-desmin Po (BS-20702R) at 1:100 dilutions for 1 h and then exposed to (FITC)-conjugated goat anti-mouse or anti-rabbit IgG antibody (Santa Cruz Biotechnology,

USA). Finally, The BSC subsequently were incubated in 0.1 $\mu\text{g/mL}$ 4',6-diamidino-2-phenylindole (DAPI) solution for 1 min and observed with a fluorescence microscope (Olympus BX53F, Tokyo, Japan).

Morphological analysis

To assess the degree of myotube formation, the culture media was aspirated following 96 h of differentiation, and the BSC were fixed with 10% neutral buffered formalin (30 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 54.6 mM NaHPO_4 , 40% formalin) for 30 min and stained with hematoxylin solution (51275, Fluka Chemie GmbH, Switzerland) at room temperature for 5 min. After 96 h differentiation, the BSC were fixed with 10% neutral buffered formalin for 10 min and stained with an Oil Red O solution (O0625-25G, Sigma). After washing the BSC once with 60% isopropanol and twice with distilled water, the cells were photographed using a Nikon E5400 digital camera mounted on an optical microscope (Nikon TS100, Nikon Corporation, Japan).

Quantitative triacylglycerol assay

The concentration of triacylglycerol (TAG) was measured using a TAG quantification assay kit (Applygen Inc., Beijing, China) according to the manufacturer's specifications. Cultured BSC were washed with buffer to remove the residual medium and the cells lysed with a lysis buffer. Accumulated TAG was digested with lipase for 3 h to release glycerol, and glycerol was measured by spectrophotometric detection at 540 nm. The glycerol assay is linear over a

concentration range of 0 to 500 μ M.

Lipid droplet area quantification

After 96 h of differentiation, lipid droplets of BSC were stained with Oil Red O. Images of 20 different regions of the slide were captured with a Nikon E5400 digital camera, followed by computer image analysis using ImageJ software 1.43 u (<http://rsbweb.nih.gov/ij>) as describe by Deutsch et al. [7]. The analysis was performed by a threshold converting the 8-bit RGB image into a binary image, which consists only of those pixels that represented the lipid droplets. The acquired images were thresholded for the color saturation of the lipid droplet signal with the threshold command option “pass”. Following binarization, the image was subjected to watershed object separation for image processing, which is used to identify borders of adjacent lipid droplets. After separation, the binary image was manually compared with the original image for consistency and to the control for correct binary conversion. Lipid droplets that were not separated by water-shedding were corrected using the ImageJ pencil tool. After setting the scale of the image, and the number and individual size of the lipid droplets in the image displayed by ImageJ, surface area in μm^2 was calculated. Incomplete droplets located at the edge of the image were excluded.

RNA extraction and real-time PCR

Following 96 h of BSC culture, total RNA of cultured BSC was isolated (duplicate six-well culture plates for three independent experiments) with 500 μ L of TRI-Reagent (Sigma-Aldrich, St. Louis, MO, USA). Genomic DNA was removed from extracted RNA with DNase (M610A, Promega, Madison, WI) according to the manufacturer's instructions. The purity, concentration, and integrity of the total RNA from each sample were quantified using a NanoDrop spectrophotometer (Thermo Scientific, 2000C, Washington, DC) and the RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA) assessed the RNA integrity. The purity of the RNA (A260/A280) was greater than 1.85, and the A260/A230 ratio was approximately 2.0 in all samples. The concentration of RNA was determined from the absorbance at 260 nm.

Complementary DNA (cDNA) was produced from 1 μ g of RNA using Taq-Man Reverse Transcriptase Reagents (Applied Biosystems, Waltham, MA, USA) according to the protocol recommended by the manufacturer. Random hexamers were used as primers for cDNA synthesis (Table 1).

To characterize the degree of myogenesis, anti-paired box-3 (*Pax3*), *Pax7*, *MYOD1*, and *myogenin* (*MYOG*) were selected as myogenic-specific marker genes. To characterize the degree of adipogenic/lipogenic gene expression, *PPAR γ* , *C/EBP α* , *C/EBP β* , and sterol regulatory element-binding protein-1 (*SREBP1*) genes were selected. The genes stearoyl-CoA desaturase (*SCD*), carnitine palmitoyltransferase 1 (*CPT1*), AMP-activated protein kinase alpha-1 (*AMPK α*), lipoprotein lipase (*LPL*), G protein-coupled protein receptor-43 (*GPR43*), perilipin-2 (*PLIN2*),

and fatty acid-binding protein 4 (*FABP4*) were selected. Each sample of the first-strand cDNA was synthesized from 600 ng of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, cat#205313). Gene-specific primers (Table 1) were used to amplify target genes using 10 ng of the first-strand cDNA as the template in a 15 µl SYBR-green-based quantitative RT-qPCR reaction performed under the following conditions: 95 °C for 2 min, 45 cycles at 95 °C for 10 sec, 60 °C for 30 sec, 72 °C for 10 sec with a melting curve from 65 °C to 95 °C. Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene expression in each sample. Transcript data were obtained in duplicate. Relative expression was quantified by using the $2^{-\Delta\Delta C_t}$ method. The cDNA from each sample was pooled, serially diluted, quantified, and used for the creation of standard curves. Titration mRNA primers for all genes against increasing amounts of cDNA yielded linear responses with slopes between -2.8 and -3.0 . To reduce the contribution of assay-to-assay variation in the PCR assay to the final results, all values were calculated relative to a calibration standard run in every real-time PCR assay.

Adiponectin in BSC culture media

Adiponectin was measured in BSC culture media. Media samples were analyzed using a bovine adiponectin ELISA kit (CSB-E14054B, CUSABIO, Houston, TX, USA) according to the manufacturer's protocol. Adiponectin concentration was measured at 450 nm with a Biotek Epoch Microplate Reader (Winooski, VT). The amount of adiponectin in the samples was

calculated from a reference curve generated in the same assay with reference standards of known concentrations of adiponectin. Mean inter- and intra-assay precision for the adiponectin assay were < 8.4% and < 5.7%, respectively.

Construction of RNA-Seq library, quality control and sequencing

After 96 h of BSC differentiation, total RNA was extracted from each treatments using TRIzol™ Reagent (Invitrogen, USA) according to the manufacturer's instructions. The isolated RNA was cleared of contaminating genomic DNA by DNase treatment (Thermo, USA). Prior to Illumina RNA sequencing, 1% nondenaturing agarose gel electrophoresis and a Nano-Photometer® spectrophotometer (IMPLEN, USA) were used to assess the quality and quantity of the isolated RNA, respectively. Samples satisfying the criteria of RNA integrity number (RIN) > 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 °C for 5 min. First strand cDNA was synthesized by a random hexamer primer (Illumina) and M-MuLV Reverse Transcriptase (Invitrogen, USA), using the short fragments as templates, at the condition of 25 °C for 10 min, followed by 42 °C for 50 min for synthesis and 70 °C for 15 min for RTase inactivation. Second strand cDNA synthesis was subsequently performed using Second Strand Master Mix (Illumina) at 16 °C 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 polymerase chain reaction (PCR) amplification (5–9 cycles). Finally, PCR products were purified, and library quality was evaluated on an Agilent. Bioanalyzer 2100 system (Agilent Technologies, USA). 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 clean data, and the filtered data were evaluated with RSeQC software package to obtain high-quality sequencing data, and then STAR (Spliced Transcripts Alignments to a Reference, STAR) software was used for sequence alignment analysis with the reference genome.

Differentially expressed genes (DEGs) 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 DEGs within samples. Genes with statistical significance were retained first ($q\text{-value} < 0.05$); significant DEGs were compared with a fold change of >1.5 than 1.5 or < 0.667 were retained [$\log_2(1.5) = 0.5849$; $\log_2(0.667) = -0.5849$].

Functional enrichment analysis

The DEGs were subjected to 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 (www.genome.jp/kegg).

Statistical analysis

All data are presented as mean $\pm$ SEM. One-way ANOVA analysis of variance with a post hoc Turkey's multiple comparison test was used to evaluate the statistical significance of differences among the treatments. Graph Pad Prism 6.0. Statistical significance was set at $P < 0.05$.

Results

Identification of skeletal muscle satellite cells

MYOD1 antibodies were used to monitor the myogenic differentiation of BSC and desmin and Pax7 antibodies to characterize proliferation-positive BSC by immunofluorescence staining (Figure 1).

Average BSC size and cell viability

A total of $1,125 \pm 7.82$ cells for each treatment were analyzed for the measurement of average

249 cell size. There was no difference in BSC size and cell viability among treatments ($P = 0.57$)
250 (Figure 2).

251 **BSC differentiation and lipid accumulation**

252 Control BSC proliferated and subsequently fused to form myotubes. Upon incubation of BSC
253 with ciglitazone, the cells became adipocyte-like cells, an effect that was clearly evident after 96
254 h (Figure 3). Increasing the concentration of ciglitazone greatly accelerated the appearance of
255 adipocyte-like cells, and strongly increased the number of BSC that differentiated into adipocytes.
256 Exposure of post-confluent cells to the differentiation medium plus ciglitazone decreased
257 myotube formation, with a concomitant increase in the area of cells containing lipid droplets and
258 the formation of adipoblasts.

259 **Concentration of triacylglycerol and lipid droplet area**

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

263 **Myogenic gene expression**

264 The CL, CM, and CH treatments increased *Pax3* and *Pax7* expression and decreased *MYOD*,
265 *MYOG*, *MYF4*, and *MRF5* expression relative to CON ($P < 0.01$) (Figure 5). The expression of
266 *MYF4* and *MRF5* were least in the CH-treated BSC ($P < 0.01$).

Accociated with adipogenic and lipid metabolism

There was a dose-response to ciglitazone addition for the expression of adipogenic transcription factors *PPAR* γ , *C/EBP* α , *C/EBP* β , *SREBP1*, *ACC*, and *FAS* ($P < 0.01$), and the greatest increase in the expression of these genes, were caused by CH-treated BSC (Figure 6)

Relative expression of *SCD*, *LPL*, *GPR43*, *FABP4*, and *PLIN2* was dose-dependent as the addition level of ciglitazone increased ($P < 0.01$) (Figure 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 (Figure 8).

Analysis of differentially expressed genes in BSC

Whole-genome expression profiling with RNA-Sequencing analysis revealed significant differentially expressed genes (*DEGs*) when the BSC were treated with different level 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

285 CH treatment up-regulated 501 genes and down-regulated 435 genes (Table 2). Combined with
286 the analysis of these results of differential expressed genes, BSC treated with CL, CM and CH
287 had 281 *DEGs* as shown in the volcano map (Figure 9A, B, and C).

288 **Gene ontology (GO) analysis of DEGs in BSC**

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

300 **KEGG enrichment of DEGs in BSC**

301 Pathway analysis of *DEGs* was conducted to understand the pathways and molecular interactions
302 involved with ciglitazone treatment. The 10 top significantly enriched GO terms in which the

303 calculated by the hypergeometric test was $Q < 0.05$ were selected. Enrichment degree was
304 identified by Gene Ratio, P. adjust, and enriched genes. In the CL treated BSC, KEGG
305 enrichment analysis indicated that a total of 465 *DGEs* were enriched in 31 pathways and mainly
306 in glycine, serine, and threonine metabolism, TNF signaling pathway, and the p53 signaling
307 pathway. Genes with up-regulated expression were mainly enriched in mineral absorption,
308 transcriptional misregulation in cancer, choline metabolism in cancer, glutathione metabolism.
309 Genes with down-regulated expression were mainly enriched in the TNF signaling pathway,
310 biosynthesis of amino acids, protein digestion, and absorption.

311 In the CM treated BSC, a total of 484 *DGEs* were enriched in 18 pathways and mainly in
312 apoptosis, p53 signaling pathway, non-alcoholic fatty liver disease (NAFLD), PI3K-Akt
313 signaling pathway, wnt signaling pathway, and TNF signaling pathway. Genes with up-regulated
314 expression were mainly enriched in TNF signaling pathway, the calcium signaling pathway,
315 apoptosis, RNA degradation, and genes with down-regulated expression were mainly enriched in
316 the biosynthesis of amino acids, p53 signaling pathway, ribosome, carbon metabolism, and the
317 PI3K-Akt signaling pathway. In the CH treated BSC, a total of 397 *DGEs* were enriched in 30
318 pathways and mainly in the p53 signaling pathway, PPAR signaling pathway, biosynthesis of
319 amino acids, pentose phosphate pathway, and TGF-beta signaling pathway. Genes with
320 up-regulated expression were mainly enriched in the calcium signaling pathway, PPAR signaling
321 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 (Figure 11, Supplementary S2).

KEGG Pathway Enrichment Analysis of DEGs

Effects of increasing concentrations of ciglitazone on the pathway of PPAR γ enrichment

As ciglitazone is a PPAR γ agonist, we analyzed the PPAR-signaling pathway enriched by DEGs during adipogenic trans-differentiation of BSC induced by different concentrations of ciglitazone (Figure 12). The high-concentration of ciglitazone significantly up-regulated the 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 PPAR γ , the downstream fatty acid transport-related genes (*DBI*, *FABP3*), fatty acid oxidation-related genes (*CPT-2*, *SCP2*, *FABP3*), adipocyte differentiation-related genes (*ANGPTL4*, *glycogen neogenesis gene GK*), and the adipocyte differentiation-related gene *FABP7* were up-regulated. The expression of *MMP-1* and the ketogenic gene *HMGCS2* was down-regulated (Figure 13).

Discussion

This study addressed the hypothesis that ciglitazone as a PPAR γ 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 *PPAR γ* and *C/EBP α* [8, 9]. In cases where the conditions of the adipocytes containing the *PPAR γ* activator permit, these cells can differentiate into mature adipocytes [10]. Li et al. [11] 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 [12]. Cell lipid accumulation and morphological changes accompanying lipogenesis are caused by induced expression of specific genes during differentiation [13]. 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* [14]. Muscle satellite cells can be trans-differentiated into mature adipocytes by ectopic expression of adipose trans-differentiation factors such as *PPAR γ* [15]. Previous studies had documented that when the *PPAR γ* 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

359 myotubes [16]. In mammalian cells, *PPAR γ* and *C/EBP α* are major regulators of lipogenesis and
360 be transcription targets [17]. *PPAR γ* is induced during the differentiation of preadipocytes into
361 adipocytes, which is critical for precursor cells to differentiate into mature adipocytes [18].
362 Adipocyte differentiation also involves the expression of several other transcription factors that
363 interact at different the process of adipogenesis to produce mature adipocytes [19].

364 Lydia et al. demonstrated that TZDs specifically bind to and activate *PPAR γ* , increase the
365 expression of *PPAR γ* and related adipokines, and promote adipocyte differentiation [20].
366 Kageyama et al. [21] demonstrated that pioglitazone increased body weight by selectively
367 stimulating *PPAR γ* and promoting lipid storage in white adipose tissue. Lizcano et al. [22]
368 reported that JMJD2C could inhibit *PPAR γ* activity and reduce triglyceride accumulation in
369 adipocytes. Although *PPAR γ* and *C/EBP α* are highly synergistic in the adipogenic process,
370 adipocyte genes vary in their reliance on *C/EBP α* and *PPAR γ* [23].

371 *Pax3* and *Pax7* are markers of the differentiation of mesenchymal cells toward a myogenic
372 cell lineage. Up-regulation of the expression of *Pax3* and *Pax7* increases the expression of
373 myogenic regulatory factors [24], which include *MYOD* and *MYOG*. Up-regulation of *MYOD*
374 expression promotes the conversion of somite-derived cells into myoblasts; *MYOG* is involved in
375 the fusion of myoblasts to form myotubes [25]. Cornelison et al. [26] identified *MYOD* was a key
376 negative regulator of brown adipocyte development. Similarly, Wang et al. [27] reported that
377 *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 [28]. Studies with 3T3-L1 adipocytes indicated that *PLIN2* could be used as a marker gene for precursors during adipocyte differentiation [29]. 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 [4].

Whole-genome RNA-Seq GO analysis and KEGG enrichment analysis of *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 trans-differentiation 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 accumulation of lipid droplets and the concentration of *TAG* and adiponectin further indicated the regulatory role of *PPAR γ* in adipogenic trans-differentiation 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 *PPAR γ* ,

397 *C/EBP α* , *FABP4*, and *FATP1*. Wnt signaling has been reported to inhibit adipogenesis. Adipose
398 tissue contains adult stem cells (ADSCs) similar to mesenchymal stem cells, which inhibit
399 adipogenic differentiation by activating the Wnt signaling pathway [34-36]. In this study, the
400 RNA sequencing results revealed that the low-concentration of ciglitazone could down-regulated
401 the genes in the regulation of the Wnt signaling pathway, which revolved in the adipogenesis and
402 lipid metabolism.

403 TGF- β 1 is a factor activated by inflammation and participates in the process of chronic
404 fibrosis of organs. TGF- β 1 transmits fibrotic signals through its downstream kinase substrate
405 Smads protein. The TGF- β 1/Smad3 signaling pathway and organ fibrosis are closely related.
406 TGF- β 1 binds to its receptor on the cell membrane causes intracellular Smad2 and Smad3
407 phosphorylation and Smad4 binds to form a complex that enters the nucleus and cooperates with
408 other transcription factors to regulate the expression of genes related to cell proliferation and
409 differentiation [37-40]. In our study, the high concentration of ciglitazone can inhibit the
410 activation of TGF- β 1/Smad3 signaling pathway. his may suggest that ciglitazone could promote
411 the adipocyte phenotype in BSC through these signaling pathways.

412 **Conclusions**

413 These results indicate that ciglitazone as a PPAR γ agonist can promote lipid droplet
414 formation and triglyceride accumulation in BSC, and effectively increase adiponectin
415 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 presence of an adipocyte differentiation cocktail. Further experiments are needed to elucidate the molecular mechanisms by which *PPAR* γ affects lipogenesis during the process of adipogenic differentiation of BSC and the regulatory pathways that influence other regulators of lipid production.

DECLARATIONS

Abbreviations

ACC: Acetyl CoA carboxylase; **ADP:** adiponectin; **AMPK α :** Adenosine 5'-monophosphate (AMP)-activated protein kinase; **BSC:** bovine satellite cells; **C/EBP α :** CAAT/enhancer-binding protein alpha; **C/EBP β :** CCAAT/enhancer-binding protein beta; **CPT1:** carnitine palmitoyltransferase; **DMEM:** Dulbecco's modified Eagle medium; **FABP4:** fatty acid binding protein4; **FAS:** Fatty acid synthetase; **FBS:** fetal bovine serum; **GAPDH:** glyceraldehyde-3-phosphate dehydrogenase; **GPR43:** G protein-coupled protein receptor 43; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **LPL:** lipoprotein lipase; **MRF5:** Myogenic Regulatory Factor5; **MYF4:** Myogenin4; **MYOG:** myogenin; **MYOD:** myogenic differentiation; **Pax3:** Paired box 3; **Pax7:** Paired box 7; **PLIN2:** perilipin 2; **PPAR γ :** peroxisome proliferator-activated receptor gamma; **RT-qPCR:** Real-time quantitative reverse

transcription PCR; **RNA-Seq**: RNA sequencing; **SCD**: stearyl-CoA desaturase; **SREBP1**:
sterol regulatory element-binding protein 1; **TAG**: triacylglycerol; **TZD**: thiazolidinedione

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Authors' contributions

The authors contribution are as follows: X. Z. Li, S.H. Choi and S. B. Smith concieved and
designed the experiments. S. B. Smith was the principal investigator and contributed to
interpretation of the findings and editing for English usage. J. F Zhang, Y. Yan, J. F. Sun, Y.
Wang, Y. Cui and S. Naseem, performed all the laboratory works. J. F Zhang, Y. Yan and J. F. Sun
were the major contributors in writing the manuscript along with X. Z. Li. B. Sun, A. Manzoor
and J. Yu contributed to subject briefings and data collection and analyses, Critical revision was
performed by X. Z. Li, S.H. Choi and S. B. Smith. All authors read and approved the final
manuscript.

Ethics approval and consent to participate

452 All experimental procedures were approved by the Yanbian University Institutional Animal Care
453 and Use Committee.

454 **Consent for publication**

455 Not applicable.

456 **Availability of data and materials**

457 The datasets generated and/or analysed during the conduct of the study are included in this
458 published article. Upon reasonable request, the datasets of this study are available from the
459 corresponding author.

460 **Competing interests**

461 The authors declare that they have no competing interests.

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466

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Figure legends

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Figure 7. Gene Expression Associated with lipid metabolism: *CPT1*, *SCD*, *AMPK α* , *LPL*, *GPR43*, *FABP4*, *PLIN2* gene expression in BSC cultured with ciglitazone in Dulbecco's modified Eagle medium (I) containing 2% horse serum at 96 h incubation time. CON, DMEM + 2% horse serum; CL, CON + 5 μ M ciglitazone; CM, CON + 10 μ M ciglitazone; CH, CON + 20 μ M ciglitazone. The results of gene expression were consistent with protein expression. The bars are the means $\pm$ SE. The letters *a*, *b*, *c*, and *d* indicate a significant difference ($P < 0.01$) within an item

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641 circles.

Table 1. Forward and reverse primers for Real-time PCR

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
PPAR γ	NSE_1498162_016	Forward:	ATCTGCTGCAAGCCTTGGA
		Reverse:	TGGAGCAGCTTGGCAAAGA
C/EBP β	840057464	Forward:	CCAGAAGAAGGTGGAGCAACTG
		Reverse:	TCGGGCAGCGTCTTGAAC
SREBP1	NSE_1498162_022	Forward:	CACCGAGGCCAAGTTGAATAA
		Reverse:	CCAGGTCCTTCAGCGATTG
C/EBP α	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: TGCCCACCACAAGTTTTTCAG
		Reverse: GCCAACCCACGTGAGAGAAG
AMPK α	8404456568	Forward: CACCAAGGTGTAAGGAAAGCA
		Reverse: ACGGGTTTACAACCTTCCATTC
LPL	NSE_1498162_015	Forward: ACGATTATTGCTCAGCATGG
		Reverse: ACTTTGTACAGGCACAACCG
GPR43	NSO_5189193	Forward: GGCTTTCCCCGTGCAGTA
		Reverse: ATCAGAGCAGCGATCACTCCAT
FABP4	AJ_4160220	Forward: AAACCTTAGATGAAGGTGCTCTGG
		Reverse: CATAAACTCTGGTGGCAGTGA
PLIN2	NSO_5379931	Forward: GCGTCTGCTGGCTGATTCT
		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; PPAR γ , peroxisome proliferator-activated receptor gamma; C/EBP β , CCAAT/enhancer-binding protein beta; SREBP1, sterol regulatory element-binding protein-1; C/EBP α , CCAAT/enhancer-binding protein alpha; ACC, acetyl-coenzyme A carboxylase; FAS, fatty acid synthetase; CPT1, carnitine palmitoyltransferase; SCD, stearoyl-coenzyme A desaturase; AMPK α , 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.

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

Treatment Type	CL	CM	CH
Up	609	562	501
Down	508	526	432
Total	1117	1088	933

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

CON-CL

CON-CM

CON-CH

Figure 10

CON-CL

CON-CM

Figure 11

Figure 12

Figure 13

Figures

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CON-CL

CON-CM

CON-CH

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PPAR signaling pathway regulated by high the concentration of ciglitazone.

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Supplementary Files

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