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Differential gene expression of bovine intramuscular and subcutaneous preadipocytes during differentiation*

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The study suggests that Sirtuin 1 (SIRT1) may play an important role in the development of bovine adipose tissue *in vivo*. To investigate the differential mRNA expression between bovine intramuscular (IM) preadipocytes (BIPs) and subcutaneous (SC) preadipocytes (BSPs), conventional collagenase-based procedures were modified to harvest preadipocytes from the *longissimus dorsi* muscle and SC adipose tissue of Luxi cattle, respectively. The time-spatial expression of SIRT1 and associated genes, such as forkhead box O1(FoxO1), peroxisome proliferator-activated receptor-γ (PPARγ), insulin-like growth factor-1(IGF-1), glyceraldehydes-3-phosphate dehydrogenase(GAPDH), and leptin, during bovine preadipocyte differentiation were investigated using real-time quantitative PCR. The intracellular triglyceride concentration of BSPs increased more dramatically than that of BIPs during cell differentiation. The expression of SIRT1 and other genes occurred in the BSPs and BIPs during differentiation indicating that different mechanisms underlie the adipogenesis of BSPs and BIPs, and that the BSPs cultured *in vitro* show a higher differentiation capacity than BIPs.

KEY WORDS: Bovine preadipocyte differentiation / FoxO1 / GAPDH / IGF-1 / leptin / PPARγ / SIRT1

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A better understanding of the differential mechanisms that regulate the deposition and release of fat between the intramuscular (IM) and external adipose tissues, particularly the subcutaneous (SC) adipose tissues, would prove vital in developing a method to increase marbling without causing any adverse effects on beef yield grade [Bong *et al*. 2010]. Gene expression in fat depots provides further proof that the mechanisms for fat accumulation differ significantly between animal species [Hishikawa *et al*. 2005]. Some studies have reported the regional differences in the expression of individual genes among different adipose depots [Kim *et al*. 2000, Wang *et al*. 2005]. Adipocyte biology reportedly plays pivotal roles in the regulation of fat metabolism, glucose absorption, energy metabolism, and innate immune response [Taniguchi *et al*. 2008a]. However, there are no reports comparing the global gene expression pattern between bovine IM preadipocytes (BIPs) and SC preadipocytes (BSPs).

Sirtuin type 1 (SIRTl) has been hypothesized be a key candidate for reducing adiposity [Picard *et al*. 2004]. In adipose tissue, SIRT1 inhibits fat storage and increases lipolysis *via* the repression of peroxisome proliferator-activated receptor-γ (PPARγ) [Picard *et al*. 2004]. PPARγ is a key regulator in adipogenesis and fat storage, controlling the expression of many adipocyte-specific genes [Picard and Auwerx 2002]. Forkhead box O1(FoxO1) was induced in the early stages of adipocyte differentiation, but its activation was delayed until the end of the clonal expansion phase. Glyceraldehyde-3-phosphate dehydrogenase(GAPDH) was believed to be as important during the terminal phase of the differentiation as others during the mid-differentiation. Insulin-like growth factor-1 (IGF-1) is an essential factor for the differentiation of preadipocytes into adipocytes. IGF-1 produced by differentiating preadipocytes acts over their adjacent cells [Zizola *et al*. 2002]. Leptin can induce the differentiation of human [Aprath-Husmann *et al*. 2001], rat [Machinal-Quelin *et al*. 2002], and porcine preadipocytes [Ramsay 2005]. However, most current research on SIRT1 and other genes have been carried out on human rodent and little studies on other mammals have been reported yet. Finding the key factors that regulate the deposition of IM fat will contribute to the development of methods that can produce highly marbled beef.

The goal of the current study, therefore, is to investigate the molecular mechanisms of the conversion of proliferative-competent BIPs and BSPs by analysing the expression patterns and interactions among particular transcription factors of SIRTl and other genes. Understanding the genetic interaction profile (molecular markers) in these cells will provide knowledge about the biochemical pathways involved in the adipogenesis of bovine adipocytes.

Material and methods

Cell culture and induction of differentiation of preadipocytes

Cells from subcutaneous (SC) adipose tissues of Luxi cattle were isolated according to Liu *et al*. [2009]. The cells were propagated and maintained in DMEM/

F12 (Hyclone) containing 10% FBS (GIBCO) with antibiotic-antimycotic agent (containing 100 IU/mL penicillin and 100 μg/mL streptomycin, GIBCO). The cells were incubated at a density of 2×10^4 cells/cm^2 at 37°C under a humidified 5% CO_2 atmosphere. To induce the adipogenesis of bovine preadipocytes, 2-day postconfluent preadipocytes (designated Day 0) were incubated in differentiation-induction medium with DMEM/F12 containing 10 μg/mL insulin (SIGMA), 0.25 μM DEX (SIGMA), and 0.5 mM IBMX (SIGMA) for 2 days. The cells were transferred into differentiation medium with DMEM/F12 containing 1 mM octanoate (SIGMA), 10 mM acetate (SIGMA), 10 μg/mL transferrin (SIGMA), 3 μg/mL cholesterol (SIGMA), 17 mM biotin (SIGMA), 100 mM calcium pantothenate (SIGMA) and 0.5% BSA. The cells were fed with fresh medium every 2 days.

Oil red O staining

To examine lipid accumulation, the cells were seeded into 6-well culture plates at a density of 5×10^4/cm^2 [Liu *et al*. 2009]. After 8 days, the medium was removed, and the cells were washed three times with phosphate-buffered saline (PBS) and fixed with 10% formaldehyde for 30 min at room temperature. After washing three times with PBS, the cells were stained for at least 1 h with 1% filtered Oil Red O (6:4 Oil Red O stock solution-H_2O, where Oil Red O stock solution comprises 0.5% Oil Red O in isopropyl alcohol). Finally, their cell morphology was examined and photographed under a microscope.

Oil red O extraction

On Days 6 and 12, the intracytoplasmic lipid contents were determined. The steps for Oil red O extraction were similar to those for Oil red O staining. The cells were washed three times with PBS and fixed with 10% formaldehyde for 30 min at room temperature. After washing three times with PBS, the cells were stained with 1% filtered Oil red O for 40 min at room temperature. Subsequently, the Oil red O solution was removed; the cells no longer needed washing. Intracellular triglyceride levels of the cells were extracted through agitation with 100% isopropanol solution for 15 min in a shaker. Absorbance at 500 nm was measured on a Rayto RT-2100C microplate reader (Shenzhen, China) and corrected for the absorbance of blank wells containing DMSO, but without the cells.

RNA extraction and cDNA synthesis

Cells were cultured for 0, 2, 4, 6, and 8 days as described in Section Oil Red O Staining of this paper, following [Liu *et al*. 2009]. Total cellular RNA was isolated with TRIzol reagent (INVITROGEN, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using PrimeScript™ First Strand cDNA Synthesis Kit (TaKaRa, Japan) from 1 μg of RNA.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCRs were performed using the ABI Prism 7500 Sequence Detector (APPLIED BIOSYSTEMS, Foster City, CA, USA) in a final volume of 20 μL containing SYBR®Premix Ex Taq™ (Perfect Real Time) (TaKaRa). Data were analysed and the values for ΔΔCt were obtained with the ABI 7500 System SDS Software. The relative levels of gene expression were determined using the "$2^{-\Delta\Delta C(T)}$" method [Liu *al*. 2010, Livak and Schmittgen T.D., 2001] . For this experiment, *18S* was used as the housekeeping gene. The gene expression levels of the BIPs on Day 0 were set to 1, to compare the relative levels of gene expression in the experimental groups. Specific primer sequences were synthesized in BIOSUNE Biological Technology Corp (Shanghai, China). The primer sequences are shown in Table 1.

Table 1. Primer sequences of PCR

Gene	Primers	Source
SIRT1	F: CGTGCCAGAGTCCAAGTTTAG R: CTTCAAATACAGTTCCTCCAGC	Liu *et al*. [2010]
FoxO1	F: GCAACGCGTGGGGCAACCTGT R: GGGCACGCTCTTCACCATCCACTC	Liu *et al*.[2010]
PPARγ	F: ATTTACACGATGCTGGCCTC R: GAGGCCAGCATCGTGTAAAT	Liu *et al*. [2010]
Leptin	F: TCTGTCTTACGTGGAGGCTGTGC R: TCTGTTTGGAGGAGACGGACTGC	Kim *et al*. [2005]
IGF-1	F: CTGCTTCCGGAGCTGTGATCTGAG R: TCCTTCTGAGCCTTGGGCATGTC	Kim *et al*. [2005]
GAPDH	F: CACCCTCAAG ATTGTCAGCA R: GGTCATAAGTCCCTCCACGA	Komatsu *et al*. [2003]
18S	F: CGGTCGGCGTCCCCCAACTT R: GCGTGCAGCCCCGGACATCTAA	Lehnert *et al*. [2007]

Statistical

Data are expressed as least squares means (LSM) and standard errors (SEM), with at least three repeats for each experimental group. Three experiment replications were performed using BIPs and BSPs from each animal. The differences on gene expression levels for each time were analysed using a 2-way ANOVA with time and cell type as the two factors, followed by Duncan's multiple comparisons. All analyses were performed using SAS 8.0 software. Differences were considered significant at $P< 0.05$ and $P<0.01$.

Results and Discussion

The morphology and lipid accumulation of bovine preadipocytes

There is no difference between BSPs and BIPs in shape. The majority of preadipocytes presented an irregular triangular appearance in the beginning of

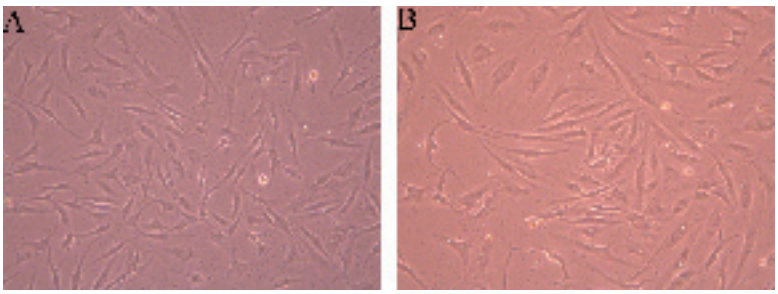

Fig. 1. Morphology of bovine preadipocytes (×100) before confluence. A – Morphology of BSPs; B – Morphology of BIPs.

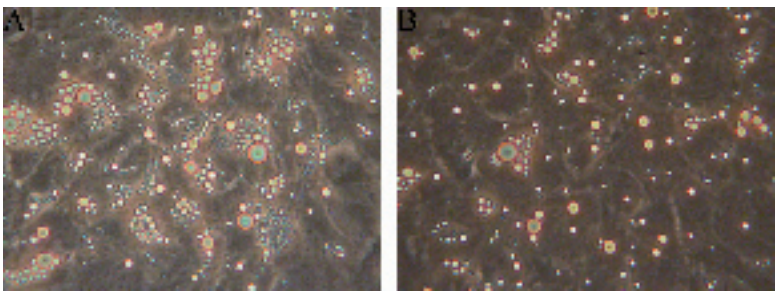

Fig. 2. Morphology of bovine preadipocytes after 12 days of differentiation (×200). A – BSPs; B – BIPs.

attachment (result not shown). When the attached cells were cultured in the growth medium, they appeared fusiform in shape (Fig. 1). The confluent cells were treated with differentiation-induction medium, and small lipid droplets began to appear on Day 2 (results not shown); the number of lipid droplets rapidly increased around the nuclei on Day 12 (Fig. 2). However, there were more intracytoplasmic lipid droplets in the BSPs than in the BIPs (Fig. 2).

Through Oil Red O staining, the BSPs were observed to differentiate into mature adipocytes, which were large, round, and filled with fat droplets. In comparison, there were significantly fewer fat droplets in the BIPs (Fig. 3). After analysing the Oil Red

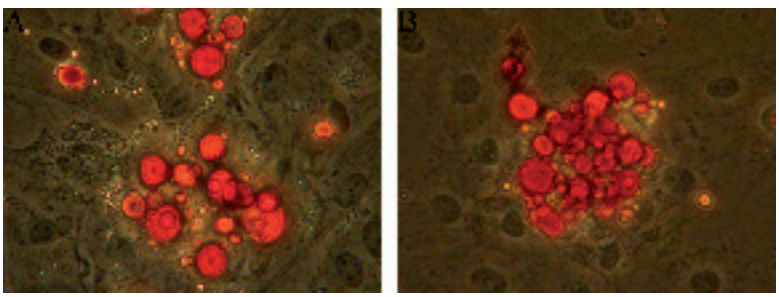

Fig. 3. Preadipocyte differentiation under Oil Red O staining (×400). Lipid droplets stained bright red. A – BSPs; B – BIPs.

Table 2. OD Value of bovine preadipocytes by Oil Red O Extraction

Cells	Day 6	Day 12
BIPs	0.376 ± 0.079^{B}	0.599 ± 0.132^{A}
BSPs	0.403 ± 0.098^{B}*	0.682 ± 0.103^{A}**

In the same row, that is in the same cell type (BIPs or BSPs), OD values of different differentiation day with different capital letter superscripts mean significant difference ($P<0.01$).
In the same column, that is in the same time (Day 6 or Day 12), OD values of different cell type with * mean significant difference ($P<0.05$), ** mean significant difference($P<0.01$).

O extraction, the OD value of the BSPs was found to be higher than that of the BIPs (Tab. 2). The results of the Oil Red O extraction also show that triglyceride (TG) content notably increased on Day 12 compared to Day 6 (Tab. 2). This shows that the intracytoplasmic lipid content of BSPs was higher than that of BIPs.

Adipose tissue plays a central role in energy balance, both as a reservoir, storing and releasing fuel, and as an endocrine organ, secreting adipokines involved in whole-body energy metabolism. The accumulation of adipose tissue occurs through both adipocyte hyperplasia (increase in number) and hypertrophy (increase in size) [Hood and Allen 1978]. Previous studies have demonstrated that regional variations in proliferation exist in rat adipocyte precursors [Djian *et al*. 1983, Wang *et al*. 1989] , and in porcine and bovine stromal-vascular cells [Aso *et al*. 1995, Zhou *et al*. 2007, Gallardo *et al*. 2008] isolated from different depots. In our experiments, the separated cells were highly homogenic and proliferative. When the confluent preadipocytes were treated with differentiation medium, small lipid droplets began to appear on Day 2, rapidly increasing around the nuclei on Day 6. Our results clearly show that bovine preadipocytes from SC have greater potential of accumulating lipid droplets than those from IM adipose tissue. This result is similar to that of an earlier study on bovine adipose tissue by Smith *et al*. [1998].

Time-spatial expression of gene mRNA during adipocyte differentiation

In the current study (Tab. 3) SIRT1 mRNA was found widely expressed throughout the entire differentiation process of bovine preadipocytes, and abundant in the middle of the term after adipocyte differentiation. During differentiation, SIRT1 mRNA levels significantly increased on Days 6 and 8 for the BIPs ($P<0.01$), and on Days 4 and 8 for the BSPs ($P<0.01$). Under normal conditions, the expression of SIRT1 mRNA gradually increases, reaching a peak on Days 2 or 4 after differentiation, and

Table 3. Sequential expression of genes during the differentiation of bovine preadipocytes

Genes	Cells	Day 0	Day 2	Day 4	Day 6	Day 8
SIRT1	BIPs	1.01 ± 0.14^{B}	2.67 ± 0.42^{B}	1.40 ± 0.18^{B}	11.10 ± 0.55^{A}	10.25 ± 2.03^{A}
	BSPs	4.48 ± 0.28^{C}**	6.70 ± 0.29^{C}**	31.92 ± 2.50^{A}**	6.31 ± 1.31^{C}**	23.31 ± 1.79^{B}**
FoxO1	BIPs	1.00 ± 0.04^{C}	8.83 ± 2.90^{B}	0.41 ± 0.10^{C}	1.25 ± 0.29^{C}	15.95 ± 1.61^{A}
	BSPs	3.72 ± 0.49^{b}**	3.95 ± 0.23^{b}**	0.52 ± 0.04^{c}	0.10 ± 0.06^{c}	16.76 ± 2.11^{a}
PPARγ	BIPs	1.02 ± 0.09^{C}	0.02 ± 0.00^{C}	1.39 ± 0.11^{C}	6.32 ± 0.15^{B}	14.56 ± 2.32^{A}
	BSPs	0.46 ± 0.04^{c}	0.03 ± 0.01^{c}	4.19 ± 0.75^{bc}**	6.37 ± 0.76^{b}	24.56 ± 3.47^{a}**
GAPDH	BIPs	1.06 ± 0.03^{DC}	0.15 ± 0.01^{D}	1.99 ± 0.31^{BC}	3.09 ± 0.47^{AB}	3.34 ± 0.41^{A}
	BSPs	0.76 ± 0.02^{d}**	0.59 ± 0.05^{d}	7.29 ± 0.96^{b}**	2.42 ± 0.63^{c}**	15.40 ± 0.41^{a}**
IGF-1	BIPs	1.01 ± 0.00^{C}	1.48 ± 0.23^{B}	0.01 ± 0.00^{D}	0.16 ± 0.02^{D}	7.38 ± 0.06^{A}
	BSPs	0.52 ± 0.07^{B}	0.01 ± 0.00^{B}	0.19 ± 0.03^{B}	0.03 ± 0.01^{B}	12.47 ± 2.48^{A}**
Leptin	BIPs	1.00 ± 0.02^{D}	0.82 ± 0.07^{D}	4.27 ± 0.16^{C}	7.04 ± 0.85^{B}	29.95 ± 0.55^{A}
	BSPs	0.55 ± 0.00^{C}	0.08 ± 0.02^{C}**	15.24 ± 3.46^{B}**	6.79 ± 1.29^{BC}	45.64 ± 4.86^{A}**

For every gene in present study, in the same row, that is in the same cell type (BIPs or BSPs), gene expression values of different differentiation day with different small letter superscripts mean significant difference ($P<0.05$), different capital letter superscripts mean significant difference ($P<0.01$), same letter or no superscript mean no significant difference ($P>0.05$).
In the same column, that is in the same time (Day0, Day 2, Day 4, Day 6 or Day 8), gene expression values of different cell type with * mean significant difference ($P<0.05$), ** mean significant difference($P<0.01$), no superscript mean no significant difference ($P>0.05$).

then declines steadily [Jing *et al*. 2007, Bai *et al*. 2008]. In this study the higher level of FoxO1 gene expression was on Day 8 and Day 6 (BIPs $P<0.01$; BSPs $P<0.05$). However, during 3T3-F442A cell differentiation, FoxO1 expression increased up to 6-fold over basal levels, peaking at Day 4 and then partially declining [Nakae *et al*. 2003]. PPARγ mRNA was found widely and increasingly expressed throughout the entire differentiation process of the bovine preadipocytes. During the first 2 days of differentiation, the PPARγ mRNA levels were low, but increased for the remainder of the differentiation period (BIPs $P < 0.01$, BSPs $P < 0.05$), while Taniguchi *et al*. [2008b] showed that bovine PPARγ was expressed only during the early stage of adipocyte cell

development. GAPDH mRNA was found to be increasingly expressed throughout the entire differentiation process of bovine preadipocytes, which is consistent with that of 3T3-F442A adipocytes [Christiaens *et al*. 2007] , and in contrast to *in vitro* cultured human adipocytes, which stably express GAPDH [Gorzelniak *et al*. 2001]. During the first 2 days of differentiation, the GAPDH mRNA levels in the BIPs were low, but increased for the remainder of the differentiation period ($P < 0.01$). On the other hand, the GAPDH mRNA levels in the BSPs increased only on Days 4 and 8 ($P<0.05$). The IGF-1 mRNA levels increased in both BIPs and BSPs on Day 8 of differentiation, but remained low for the remainder of the differentiation period ($P<0.01$).

Whether leptin is adipogenic for bovine adipose tissue-derived cells is still unknown. The present study was designed to test the hypothesis that bovine leptin inhibits the proliferation and promotes the differentiation of porcine preadipocytes. The leptin mRNA levels increased sitgnificantly ($P<0.01$) since Day 4 up to Day 8 in both BIPs and BSPs and reached a highest value on Day 8.

Difference of BSPs and BIPs

In the current study (Tab. 3), the level of expression of investigated genes was significantly higher in BSPs than in BIPs during whole period of adipocyte differentiation except Day 6 (SIRT1), on Days 4 and 8 (PPARγ, GAPDH, Leptin) and on Day 8 (IGF-1). Moreover, the FoxO1 mRNA level in BSPs was higher on Day 0 ($P<0.01$) and lower on Day 2 ($P<0.01$) than that in BIPs. On the remaining Days of investigation the differences between BIPs and BSPs occurred to be not significant**.**

The difference between the adipose tissue depots are likely manifested at the cellular level (preadipocytes and adipocytes) - [Hausman *et al*. 2009]. Bovine preadipocytes are capable of differentiation in response to combinations of insulin, serum lipids, dexamethasone (DEX), and troglitazone (TRO). Although TRO enhances the differentiation of bovine preadipocytes, it has no differential effects on the differentiation of BIPs and BSPs [Grant *et al*. 2008]. Propionate has selective adipogenic effects on BIPs and BSPs, and it contributes to the hypertrophy of adipocytes within IM adipose tissues and to the recruitment of new adipocytes within SC adipose tissues [Wan *et al*. 2009]. The differences in the results could be explained by the differences in the design of the experiments due to the age and breed of the animals, as well as the length of the differentiation period. The presented results indicate that SC preadipocytes cultured *in vitro* show a higher capacity of differentiation than do IM preadipocytes.

In summary, the presented results show that different mechanisms underlie the adipogenesis of BSPs and BIPs, and that the former when cultured *in vitro* demonstrate a higher differentiation capacity than that of BIPs. SIRT1 and remaining genes' expression profile during adipogenesis will supply a valuable information on biochemical pathways underlying bovine preadipocyte differentiation. The bovine adipocyte culture system may be a useful model in understanding the mechanisms by which the IM adipose is deposited in the tissues, and for improving the quality of local cattle breeds.

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