

Gene expression patterns of bovine perimuscular preadipocytes during adipogenesis

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Abstract

Bovine perimuscular fat (PMF) preadipocytes were induced to undergo adipogenesis *in vitro* in our recent study to define the expression patterns of genes involved in the differentiation process. Based on the understanding of the interaction among adipogenic genes, a broad overview of gene expression profile in the differentiating PMF preadipocytes was evaluated using bovine specific DNA microarray from day 2 to 8 post-differentiation induction. A total of 100 significantly differentially expressed genes were detected between differentiated and control cells including those involved in several biochemical pathways and cellular/molecular signaling. In addition, quantitative real-time PCR validated that typical adipogenic genes were up-regulated at early differentiation in the preadipocytes. These results suggest that the PMF preadipocyte system is available as a novel *in vitro* model for molecular adipogenesis studies in the bovine and that a series of genes are switched on/off during early events associated with adipogenesis.

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In beef industry, adipose tissue type and amount are directly associated with quality and value of the meat [1]. Use of molecular biological technique has provided substantial knowledge regarding molecular markers paralleling the processes of adipogenesis and lipid metabolism, both of which coordinate the overall fatness of an animal. Adipogenesis is defined as the proliferation, differentiation, and initial incorporation of lipid in cells of the adipose lineage to form lipid-assimilating adipocytes [2]. Adipogenesis within specific adipose depots in ruminants may be under somewhat different regulation than that experienced by 3T3-L1 cells [2]. In our recent findings, we confirmed the important roles of PPAR- γ and SREBP-1 in bovine perimuscular fat (PMF) preadipocyte differentiation. Moreover, we proposed that a bovine-specific gene network (different to that seen in adipogenic cell lines of the other

species) was at play in the adipogenesis process, based on comparison of the expression patterns of the key adipogenic genes [3]. However, the global gene interactions among entire molecular pathways in the adipocyte differentiation in any specific adipose depot in cattle are as yet unclear.

In this study, differentially expressed (DE) genes were profiled, during the PMF preadipocyte differentiation/conversion process to become adipocytes, by bovine specific oligo-DNA microarray analyses. Analyses on ontology and biochemical pathway of the DE genes were identified using bioinformatics tool based on obtained microarray data. Furthermore, an interaction of the DE genes involved in the key biochemical pathways was discussed with an analysis of gene clustering according to the expression patterns of the DE genes.

Materials and methods

Cell culture and differentiation. Details of tissue collection, homologous preadipocyte preparation, and the PMF cell culturing were described as

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previously outlined [3]. In brief, the PMF cell monolayer in each flask was allowed to reach confluence. Two days after confluence (day 0), the cells were separated into two groups: treated and untreated (control). The treated cells were incubated with medium including 1 µg/mL insulin, 0.25 µM dexamethasone (DEX, sigma), and 10 mM acetic acid in DMEM supplemented with 10% FBS and 1× antibiotic-antimycotic (Invitrogen). The treatment was continued using the same stimulant-supplemented medium until day 8. Control cells were cultured in DMEM supplemented with 10% FBS and 1× antibiotic-antimycotic. The cells were harvested on day 2, 4, and 8 for RNA extraction.

Total RNA extraction and complementary DNA (cDNA) synthesis. Total RNA was extracted as previously described [3]. Two micrograms of total RNA was amplified using Amino Allyl MessageAmp™ II aRNA Amplification kit (Ambion). The amplified RNA (aRNA) was coupled with Cy3 and Cy5 fluorescent dyes (GE Healthcare) according to the instruction manual. The Cy-labeled probes were hybridized onto the microarray slides customarily printed in our facility. In order for the quantitative real-time PCR (qPCR) analysis, complementary DNA (cDNA) was synthesized from 800 ng total RNA with SuperScript II reverse transcriptase and oligo (dT) priming (Invitrogen).

Microarray experimental design and data analysis. Bovine specific oligo-DNA microarray used in this study was *Bos taurus* (bovine) AROS™ VI.1 containing 70-mer oligos representing 8329 genes from *Bos taurus* genome (OPERON Biotechnologies, Inc.). Gene sequences used for probe design are obtained from TIGR Cattle Gene Index Release 11 (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=cattle) and GenBank.

In the microarray hybridization, the treated and control cells were directly compared within each of three time points including triplicates dye-swap hybridizations. Each comparison was performed with triplicate sets of hybridizations. Probes labeled with Cy3 and Cy5 fluorescent dyes were hybridized on the microarray slides at 42 °C for 18 h. Hybridized slide was washed with low stringency buffer (2× SSC and 0.5% SDS), high stringency buffer (0.5× SSC and 0.2% SDS), and 0.05× SSC. Hybridized slides were scanned with 5 micron resolution and their signal intensities were detected by Q-Scan (Genetix, UK).

Data analysis of microarray was performed using GeneSifter™ (VizX Labs). The background-corrected signal intensity for each spot was normalized by LOWESS method and base 2 log-transformed. The differences on gene expression levels in each time point were analyzed with *t*-test, followed by 2-way analysis of variance (2ANOVA) with time and treatment as the two factors. Criteria on detection of differentially expressed gene were 2-fold or greater change in expression level with $P < 0.01$ which was adjusted by the method of Bonferroni correction. Gene ontology was analyzed among the DE genes detected in major KEGG pathways such as

adipocytokine signaling, fatty acid metabolism, glycerolipid metabolism, insulin signaling, MAPK signaling, PPAR signaling, and Wnt signaling. Clustering analysis of expression pattern of DE genes in the pathways was performed using PermutMatrix software with which methods for clustering and seriation were based on average linkage criteria and multiple-fragment heuristic, respectively [4].

Quantitative real-time PCR (qPCR) and data analysis. The RNA abundance was measured by TaqMan® Universal PCR Master Mix with gene specific MGB probes labeled with FAM or VIC fluorescent dyes (Applied Biosystems). Targeted genes included PPAR-γ, adipocyte type fatty acid binding protein (FABP4), fatty acid binding protein (heart) like (FABP3), fatty acid synthase (FASN), and diacylglycerol acyltransferase 1 (DGAT1) (Table 1). As an internal control for relative gene expression analyses, 18 S ribosomal RNA (18 S rRNA) gene was the best stable reference gene in the qPCR assay detected by TaqMan assay in our facility. Each reaction was carried out with triplicates and run in ABI PRISM 7700 Sequence Detection System (SDS) with software version 1.7 (Applied Biosystems). The thermal cycling condition was as follows; 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 60 s. Relative gene expression for each gene was calculated by ratio of target gene expression to reference gene expression. Fold change of gene expression was calculated by ratio of expression levels of treated cells to the expression level of control cell. The up-regulated expression fold change was calculated by dividing the results for the treated sample by the control for each of the time point. Data on transcript quantification were simply conducted by comparisons between means of treated cells in three time points and control cells using a *t*-test.

Results and discussion

Preadipocyte differentiation is a complex process accompanied by coordinated change in cell morphology, hormone sensitivity, and gene expression. As we previously reported, the bovine PMF preadipocytes differentiated into lipid-filled adipocytes with similar cell morphology to other cell lines [5,6], even though some of the molecular markers were different to the rodent cell system [3]. However, when evaluating gene expression, bovine PMF cells were activated at relatively early stage of the conversion process, as expressions of PPAR-γ, SREBP-1, and FABP4 were up-regulated early [3]. Due to the lack of understanding

Table 1
Oligonucleotides used in qPCR

Gene	Nucleotide sequence (5'–3')	Accession No.
DGAT1	Forward: GGA ACTCCGAGTCCATCACCTA Reverse: TCTGATGCACCACTTGTGAACA Probe: TCTGGCAGAACTGGAACA	BC118146
FABP3	Forward: CACTTGTGCGGAAATGGTT Reverse: TGCA GTGCCATGGGTGAGT Probe: ACGGGAACTCATTCTG	BC102153
FABP4	Forward: GGAATGTGTCATGAATGGTGTCA Reverse: CCCTTGCTTATGCTCTCTCA Probe: TGCCACCAGAGTTT	NM_174314
FASN	Forward: GGAGGACGCTTCCGTTACA Reverse: TGCTCTTCCCTCACGTACCTGAA Probe: CCAGGGCAAACACAT	AY343889
PPAR-γ	Forward: GATGTCTCATAATGCCATCAGGTT Reverse: TCTCCGCTAACAGCTTCTCTCT Probe: CATGCCACAGGCCGA	BC116098
18S rRNA	Forward: CCGCGTTCTATTITGTGGT Reverse: CGGCCGCCCTCTTAA Probe: TTCGGA ACTGAGGCCAT	DQ222453

of overall regulation in bovine adipogenesis, the global overview of gene expression profile at early differentiation period of the bovine PMF preadipocytes was investigated by microarray analysis to study the molecular networks among factors involved in the adipogenesis.

Proportions of DE genes were shown by time points and up/down regulations in 10 categories of the molecular functions based on the gene ontology analysis (Fig. 1). Most of the DE genes were observed in binding and catalytic activity at each time point in either up- or down-regulation. The proportions of DE genes in binding activity moderately increased in up-regulated genes as the time elapses (27.3% on day 2, 28.9% on day 4, and 29.4% on day 8), whereas decreased in down-regulated genes (20.3% on day 2, 19.0% on day 4, and 18.7% on day 8). The same tendency in the proportion of DE genes was observed in catalytic activity genes (Fig. 1). The similar characteristics of gene expression profiles were outlined in a study of bovine bone marrow derived preadipocyte development [7], suggesting the result reflected genetic aspect of differentiating bovine preadipocytes.

A total of 100 DE genes were identified by 2ANOVA analysis upon the microarray result (Fig. 2). Fifteen DE genes were detected in the multiple biochemical pathways defined by Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/>) (Table 2). The transcription factor PPAR-γ was involved in the moderately up-regulated gene cluster as well as the other adipogenic factors such as FASN and FABPs and DGAT1 showed similar up-regulation expression patterns (Fig. 2). Most of the genes involved in multiple pathways, shown with asterisk, were observed in down-regulated gene cluster (Table 2, Fig. 2). The DE genes in the pathways associated with adipogenesis, lipogenesis, and fat metabolism were included in PPAR signaling, adipocytokine signaling, insu-

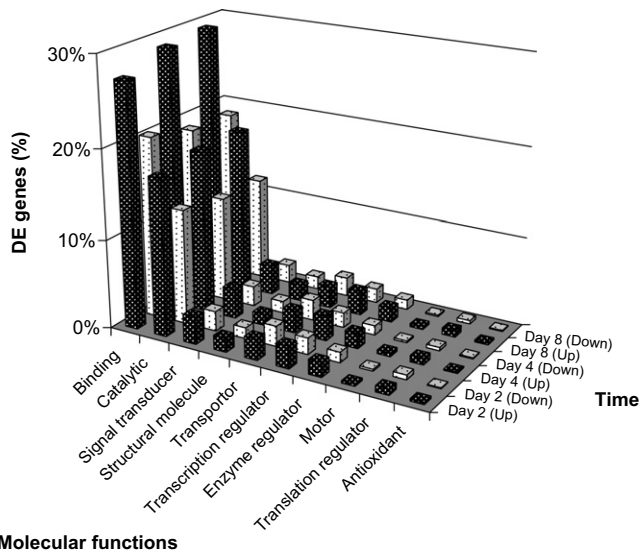


Fig. 1. DE genes ontology analysis during the PMF preadipocytes differentiation. The x-, y-, and z-axes indicate molecular functions, time, and percentages of the DE genes, respectively.

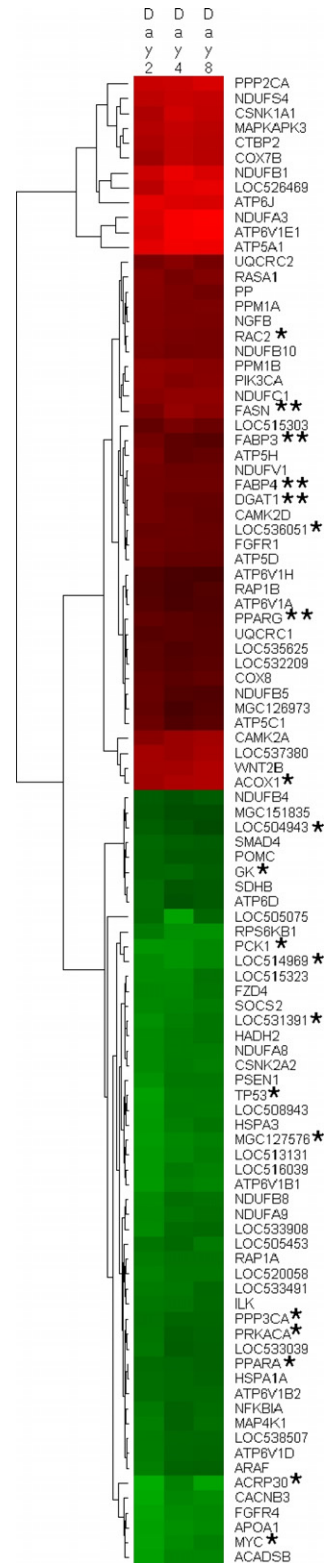


Fig. 2. Clustering analysis of the DE genes during the PMF preadipocytes differentiation. Data measurements were based on fold change of each gene's expression. As shown by the color scale bar, increasing green and red signal intensities indicate genes that decrease and increase in expression of treated PMF adipocytes during the rime course. *Detected in multiple KEGG pathways. **Validated by qPCR.

Table 2
Differentially expressed (DE) genes detected in differentiating of PMF preadipocyte

Differentially expressed genes (symbol)	KEGG ^a	Accession No.	Day2	Day4	Day8
Adipocyte complement related protein of 30 kDa (ACRP30)	A, P	AF269230	−9.01	−5.00	−7.88
Peroxisome proliferator-activated receptor alpha (PPARA)	A, P	AF229356	−3.52	−3.29	−2.93
Phosphoenolpyruvate carboxykinase 1 (PCK1)	A, I, P	BC112664	−6.70	−6.51	−5.57
Acyl-Coenzyme A oxidase 1, palmitoyl (ACOX1)	F, P	BC102761	7.57	8.94	9.45
Glycerol kinase (GK)	G, P	BC122692	−3.10	−3.23	−2.69
Protein kinase, cAMP-dependent, catalytic, alpha (PRKACA)	I, M, W	NM_174584	−4.07	−2.83	−3.04
Mitogen-activated protein kinase 3 (LOC531391)	I, M	XM_609884	−6.00	−4.71	−4.13
v-raf murine sarcoma viral oncogene homolog B1 (LOC536051)	I, M	XM_864068	3.26	3.42	3.18
Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (PPP3CA)	M, W	NM_174787	−3.97	−3.14	−3.14
Ras-related C3 botulinum toxin substrate 2 (RAC2)	M, W	NM_001099179	4.87	4.34	4.12
Growth factor receptor-bound protein 2 (MGC127576)	I, M	BC102411	−7.14	−5.37	−4.82
Tumor protein p53 (Li-Fraumeni syndrome) (TP53)	M, W	NM_001034350	−7.45	−4.58	−4.45
V-myc myelocytomatosis viral oncogene homolog (MYC)	M, W	NM_001046074	−7.74	−5.72	−4.87
branched chain acyl-CoA oxidase (LOC514969)	F, P	XM_874903	−5.71	−6.48	−5.52
retinoid X receptor beta (LOC504943)	A, P	XM_877602	−2.79	−2.20	−1.77

^a Abbreviations indicate adipocytokine signaling (A), fatty acid metabolism (F), glycerolipid metabolism (G), insulin signaling (I), MAPK signaling (M), PPAR signaling (P), and Wnt signaling (W).

lin signaling, fatty acid metabolism, and glycerolipid metabolism. The other DE genes were categorized in MAPK signaling and Wnt signaling pathways (Table 2). As a key biochemical pathway relevant to the fat metabolism and development, the PPAR signaling pathway in *bos taurus* from KEGG (http://www.genome.jp/dbget-bin/get_pathway?org_name=bta&mapno=03320) indicates that PPARs trigger inductions of various down-stream biological pathways such as lipid metabolism, adipocyte differentiation, and gluconeogenesis. Six DE genes (ACOX1, ACRP30, GK, PPAR- α , PCK1, and LOC504943) in the PPAR pathway are known to be involved in the other pathways including adipocytokine signaling, insulin signaling, fatty acid metabolism, and glycerolipid metabolisms. This suggests that the PPAR signaling may be an essential pathway in cooperation with the other pathways for the differentiation of the preadipocytes. This also agrees with that PPAR- γ , the primary transcription regulator in the PPAR signaling, can be a key factor to regulate transcription of many genes which are involved in a system of biochemical pathways underlying the adipogenesis as suggested by previous studies [2,8].

Interestingly, the DE genes involved in multiple biochemical pathways were mostly detected to be down-regulated (Fig. 2). Remarkably, six genes are detected in MAPK signaling with Wnt or insulin signaling genes (Table 2). It is reported that MAPK pathway possibly blocks or promotes adipogenesis according to a timing of MAPK activation [9], suggesting that the observed inactive MAPK genes may result in adipogenesis in the bovine preadipocytes. The Wnt signaling is considered to play inhibitory role in differentiation of 3T3-L1 preadipocytes through mediations of receptors including frizzled proteins 1, 2, and 5, and then inhibits PPAR- γ induction [10]. Observation of down-regulation of similar to FZD2 protein (LOC538507) gene may partly reflect the Wnt-inhibition did not repress the differentiation of the bovine

adipocytes (Fig. 2). In the down-regulated DE genes, adiponectin (ACRP30) was detected in adipocytokine and PPAR signaling pathways (Table 2). It has been known that ACRP30, one of the biomarkers of metabolic syndromes (hypo adiponectinaemia), is associated with increased fat mass [11,12]. This suggests that down-regulation of ACRP30 may be associated in part with the induction of adipogenesis and/or differentiation of the bovine PMF preadipocytes.

On the other hand, in the up-regulated DE genes, ATP synthase alpha subunit (ATP5A1) and cytochrome c (COX7B and COX8) were reported to be up-regulated together with PPAR- γ expression in 3T3-L1 preadipocyte [13] and were included in highly up-regulated gene cluster (Fig. 2). In addition, up-regulation of DGAT1 gene was validated to be co-expressed with PPAR- γ and FABPs (Fig. 3). Previous studies revealed that DGAT1 gene was significantly associated with fat related traits such as back-fat thickness and marbling in beef and fat content and composition in dairy cattle [14–16]. These indicate that up-regulation of DGAT1 gene may have certain functional effect upon the adipocyte development in beef cattle. Furthermore, PIK3CA (phosphoinositide-3-kinase catalytic, alpha polypeptide) in insulin signaling is detected as one of the up-regulated genes as well as known to be a down-stream effect of insulin stimulated adipogenesis through the activation of insulin receptor [17]. PPAR- γ has been considered to regulate adipogenesis by imparting insulin sensitivity to fat cells [18]. The up-regulation gene expression patterns of PIK3CA as well as PPAR- γ in the PMF preadipocytes suggest that insulin signaling was appropriately stimulated for the differentiation during the process of the cell treatment.

Five genes including DGAT1, FABP3, FABP4, FASN, and PPAR- γ were selected for further validation of qPCR analysis, since the function of these genes were considered to be associated with preadipocytes differentiation and fat

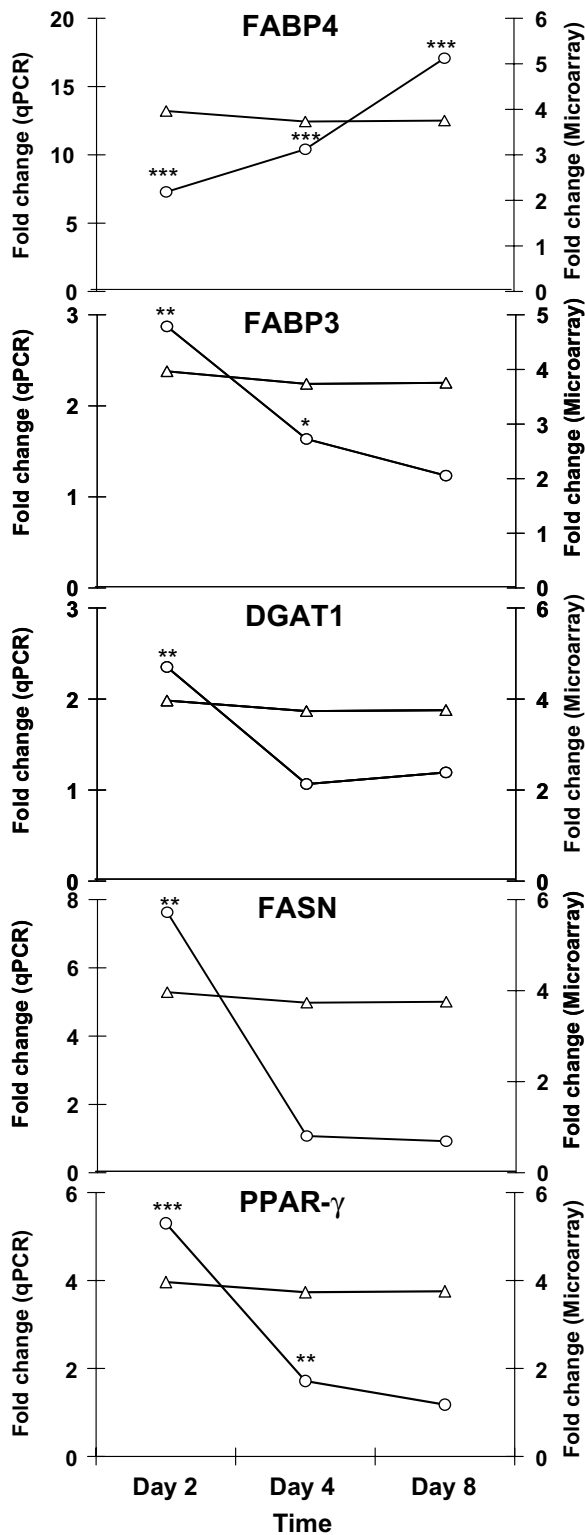


Fig. 3. Comparison of gene expression patterns between qPCR and DNA microarray. The x-axis shows the 3 time points of differentiation of preadipocytes. Circles and triangles indicate qPCR and microarray, respectively. Gene expression fold changes of qPCR and microarray were indicated on the left y-axis and the right y-axis, respectively. Asterisks show the level of significant differences between control and the treated cells in qPCR analysis (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

metabolism. The qPCR results revealed that the early up-regulation of adipogenic genes confirmed the importance of early development of the PMF preadipocytes (Fig. 3). Up-regulated expression pattern of those genes reconfirmed that the key adipogenic factors were co-activated at early stage of the culture (Figs. 2 and 3).

Our results suggest that, during the times evaluated, the bovine PMF cells were still transiting the adipogenesis barrier. Genes involved in intracellular energy flux, calcium mobilization, and PPAR signaling were up-regulated, while genes associated only with terminally-differentiated and metabolically-active adipocytes (adipocyte signaling via ACRP30-type protein) were not being expressed. Also of note was the rather inactive set of insulin-related genes, which would be active (even in the bovine) following PPAR- γ signaling [2]. However, as part of the insulin signaling system involves C/EBP- α [2] and this product was not expressed in the bovine PMF adipocytes [3] may help explain the absence of strong gene expression of the insulin system. Other gene products were altered during the adipogenesis in PMF adipocytes *in vitro*.

In summary, the gene expression profile shown in this study revealed that the DE genes identified in the multiple pathways such as PPAR, insulin, and MAPK signaling were proven to play the pivotal role in the re-differentiating bovine preadipocytes into mature adipocytes.

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