Global gene expression patterns spanning 3T3-L1 preadipocyte differentiation

C. Hansen[1], A. Fu, C. Li, W. T. Dixon, R. Christopherson, and S. S. Moore

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5 (e-mail: christiane.hansen@ualberta.ca). *Received 8 December 2003, accepted 4 March 2004.*

Hansen, C., Fu, A., Li, C., Dixon, W. T., Christopherson, R. and Moore, S. S. 2004. **Global gene expression patterns spanning 3T3-L1 preadipocyte differentiation**. Can. J. Anim. Sci. **84**: 367–376. Adipogenesis is of significant relevance from an agricultural perspective. Traits such as subcutaneous fat thickness, marbling and waste fat are of substantial economic importance in animal production. In order to discover more about the genetic basis of this process, a study was undertaken to examine the changes that occur daily in global gene expression as 3T3-L1 cells differentiate from preadipocyte to adipocyte. Duplicate RNA samples were collected daily during the differentiation process and probed with the Affymetrix U74Av2 GeneChip® microarray to allow the time-course analysis of the gene expression profile in these differentiating cells. Self-organizing maps (SOM) clustering was performed to extract patterns of expression over the course of the experiment (day 0 to day 6). The clustering generated nine distinct expression patterns containing between 74 and 420 genes/ESTs. Functional clusters and important chronological changes in the expression of key genes and gene groups were identified. The pattern of expression observed for many genes not only confirmed what has been shown previously for the early stages of differentiation, but also expanded this pattern to cover the whole differentiation process thus giving a very comprehensive overview of patterns and changes in gene expression over the time course of adipocyte differentiation.

Key words: Adipocyte differentiation, gene expression, SOM clustering

Hansen, C., Fu, A., Li, C., Dixon, W. T., Christopherson, R. et Moore, S. S. 2004. **Modes généraux d'expression des gènes codant la différenciation des pré-adipocytes 3T3-L1.** Can. J. Anim. Sci. **84**: 367–376. L'adipogénèse revêt une importance particulière en agriculture. En effet l'épaisseur du gras sous-cutané, le persillé et la graisse superflue sont des caractères d'une importance économique appréciable pour les éleveurs. Pour en savoir plus sur les fondements génétiques de ces caractères, les auteurs ont entrepris d'examiner les changements quotidiens dans l'expression de gènes quand les cellules 3T3-L1 se transforment de préadipocytes en adipocytes. Pour cela, ils ont recueilli des énchantillons doubles d'ARN chaque jour durant la différenciation et les ont examinés avec le système de microéchantillonnage U74Av2 GeneChip® d'Affymetrix, ce qui a permis une analyse chronologique de l'expression de gènes pendant la différenciation. Les chercheurs ont préparé des cartes topologiques auto-adaptatives des groupes génétiques afin des déceler leurs modes d'expression durant l'experience (du jour 0 au jour 6). Le regroupement a révélé neuf modes d'expression de 74 à 420 gènes par séquence génomique exprimée. Les auteurs on identifié le groupes fonctionnels et les changements chronologiques importants qui affectent l'expressiont des principaux gènes et groupes de gènes. Le mode d'expression de nombreux gènes non seulement confirme ce qu'on savait déjà pour les primiers stades de la différenciation, mais a aussi permis d'entendre ces modes à la totalité du processus le différenciation, ce qui nous procure une vue d'ensemble fort complète des modes d'expression génétique et des changements qui surviennent pendant la différenciation des adipocytes.

Mots cles: Différenciation de adipocytes, expression de gènes, regroupement par cartes topologiques auto-adaptives

Adipose tissue is one of the major types of connective tissue in mammalian species. Adipose tissue and its constituent adipocytes play a central role in energy metabolism and homeostasis through endocrine, paracrine and autocrine mechanisms (Mohamed-Ali et al. 1998) and can influence energy metabolism in numerous other tissues including the brain, liver and muscle (Kim and Moustaid-Moussa 2000). It has been suggested that adipocyte functions can be grouped into three categories: lipid metabolism, glucose metabolism and endocrine functions (Morrison and Farmer 2000). The many processes making up these three large functional groupings are of considerable interest due not only to their physiological effects, but also to their potential effects on such economically important traits in cattle as fat depth and fat distribution, including marbling, as well as the overall efficiency of nutrient use.

The ultimate aim of beef producers in Canada, and worldwide, is to deliver a product that is desired by consumers in as cost effective a manner as possible. A reduction in waste fat would be of tremendous economic savings to producers since the total cost of excess fat to the US beef industry has been shown to be US$ 4.4 billion; US$ 2 billion to produce it and US$2.4 to remove and ship it (Ritchie et al. 1993). In addition, since marbling has been shown to influence con-

Abbreviations: **DMEM**, Dulbecco's modified Eagle's medium; **cDNA**, DNA complementary to RNA; **CBP**, cAMP-reponse element binding protein-binding protein; **C/EBP**, CCAAT/enhancer binding protein; **cRNA**, RNA complementary to cDNA; **EST(s)**, expressed sequence tag(s); **FBS**, foetal bovine serum; **LPL**, lipoprotein lipase; **PPARγ**, peroxisome proliferator activated receptor γ; **SOM**, self-organizing map(s)

sumer preferences for a beef product, understanding the mechanisms underlying adipocyte differentiation and adipogenesis could eventually help producers to increase the amount of beef the consumer purchases. To achieve these economic advantages for the beef producer it is therefore imperative that we understand the genes involved in adipocyte differentiation more completely.

Adipocyte development/differentiation has been intensively studied over the last two and a half decades (Cowherd et al. 1999). Traditional methods of examining differential gene expression have allowed the study of only a few genes at a time. Recent advances in microarray technology (Sendera et al. 2002) have opened the door for the study of global gene expression changes during the differentiation of cells from preadipocytes to adipocytes. At the same time, preadipocyte cell lines, such as the murine 3T3-L1 cell line, present a unique opportunity to study preadipocyte differentiation in cell culture. This cell line has been extensively studied and characterized both functionally and histologically (Plaas et al. 1981; Fan et al. 1983; MacDougald and Lane 1995; Tontonoz et al. 1994; Smas and Sul 1995; Gregoire et al. 1998). It has been shown (Reed and Lane 1980; Student et al. 1980) that 3T3-L1 preadipocytes can be induced to differentiate into adipocytes by treatment with insulin, dexamethasone and 3-isobutyl-1-methylxanthine and that this differentiation involves changes in the expression of a variety of different genes (Green and Kehinde 1975; MacDougald and Lane 1995; Gregoire et al. 1998; Cowherd et al. 1999; Guo and Liao 2000; Burton et al. 2002).

To date the studies of preadipocyte differentiation that have examined differential and/or global gene expression have focussed on individual or small groups of genes (e.g. Torrejon-Escribano et al. 2002; Nishizuka et al. 2002; Okuno et al. 2002; Takahashi et al. 2002; Villalonga et al. 2002) or on the beginning and end of the differentiation process (Guo and Liao 2000; Gerhold et al. 2002). Recently, Burton et al. (2002) examined the first 24 h of 3T3-L1 cell differentiation in detail using microarray analysis. In the present study we further expand the expression data available for this model cell line by examining global gene expression in 3T3-L1 cells on each day (day 0 to day 6) of the differentiation process using the Affymetrix U74Av2 GeneChip® microarray and characterizing the expression patterns and gene expression changes that are observed.

MATERIALS AND METHODS

Cell Culture and Differentiation

Cell culture and differentiation were carried out as previously described by Guo and Liao (2000). Briefly, frozen 3T3-L1 cells were thawed and cultured for 1 d in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (vol/vol) foetal bovine serum (FBS) at 37°C / 5% (vol/vol) CO2. The medium was then replaced with DMEM/10% (vol/vol) FBS and cells were split and cultured until enough flasks had been produced to carry out the study. The cell monolayer in each flask was allowed to reach confluence. Two days post confluence (day 0), differentiation was initiated in all treatment flasks with 1 μg mL^{-1} insulin, 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine in DMEM supplemented with 10% (vol/vol) FBS. On day 2, the culture medium was replaced with DMEM/10% (vol/vol) FBS and supplemented with 1 μg mL^{-1} insulin. On day 4, the culture medium was replaced with DMEM/10% (vol/vol) FBS. As was shown by Guo and Liao (2000), cells were fully differentiated by day 6. Untreated control cells were collected on day 0, and treated, differentiating cells were harvested daily, in duplicate, from day 1 until day 6.

Isolation of Total RNA and Hybridization with the Murine U74Av2 Microarray

Total RNA was isolated from approximately 5×10^7 cells collected from two flasks per day, in duplicate, using the RNeasy mini kit (Qiagen Inc., Mississauga, ON). Isolated total RNA was sent to the Ottawa Health Research Institute's Gene Expression Facility where the subsequent processing, hybridizations and analysis were performed using the Affymetrix GeneChip® system and the murine U74Av2 GeneChip® microarray following the recommendations of the Affymetrix GeneChip® Expression Analysis Manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Briefly, cDNA was prepared from the total RNA that had been extracted from the cells. An in vitro transcription reaction was then carried out to produce biotin-labelled cRNA from cDNA. The cRNA was fragmented by incubation at 94ºC for 35 min. in 5× Fragmentation Buffer (Affymetrix Inc., Santa Clara, CA) and a hybridization cocktail (see Affymetrix GeneChip® Expression Analysis Manual for exact composition) containing the fragmented target, probe array controls, BSA and herring sperm DNA was prepared. The hybridization was then carried out using the standard hybridization process recommended for the U74Av2 chip (Affymetrix GeneChip® Expression Analysis Manual). Subsequently the probe array was subjected to an automated washing and streptavidin phycoerythrin staining protocol. Finally, the probe array was scanned twice to improve assay sensitivity and reduce background noise. Using Affymetrix Microarray Suite 5.0 software, an average of the two scans was calculated, the probe cells (specific areas on the probe array that contain oligonucleotides of a specific sequence) were defined and the intensity of each cell was determined. The data were then analyzed for probe intensities using the same software. Duplicate arrays were run for each time point in the differentiation process, resulting in 14 individual arrays for analysis (i.e., two each for day 0 to day 6). Single array analyses were carried out for the various chips. Finally, self-organizing maps (SOM) clustering was performed (Tamayo et al. 1999). This clustering process has been validated previously for use with Affymetrix GeneChip® microarrays and differentiating 3T3-L1 cells (Burton et al. 2002).

Statistical Algorithms and GeneChip® Expression Analysis

A detailed description of the algorithms and methods used by the Affymetrix system to analyze GeneChips® can once

again be found in the Affymetrix GeneChip® Expression Analysis Manual (see above). The approach is summarized below.

Single array analysis

This analysis allows one to build data bases of gene expression profiles, facilitates sample classification and transcript clustering and allows one to monitor gross expression characteristics. The detection algorithm used in this analysis generates a Detection *P* value using probe pair intensities and then assigns a "present", "marginal" or "absent" designation. The Detection *P* value is determined by calculating the Discrimination score (*R*) for each probe pair and then testing *R* against the threshold Tau (0.015). The discrimination score measures the target-specific intensity difference of the probe pair relative to its overall hybridization intensity. Detection *P* value cut-offs were set at 0.04 and 0.06 for this experiment and defined the boundaries for the present, marginal and absent designations. The Signal algorithm, on the other hand, calculates the Signal value, which represents the relative level of expression of a transcript. The Signal value is calculated using the One-Step Tukey's Biweight Estimate and is an estimate of the real signal due to hybridization of the target.

Comparison analysis

In a comparison analysis two GeneChips® are compared with one another in order to detect and quantify changes in expression. Arrays are first scaled or normalized to account for variations between the two arrays that are not treatment related. A change algorithm generates a Change *P* value and an associated Change. In other words, each probe set on the experimental array is compared to its counterpart on the baseline array and a Change *P* value is calculated indicating an increase, decrease or no change in gene expression. Cut-offs defining the boundaries of the change calls were 0.0025 and 0.003. A second algorithm quantifies the estimate of the change in gene expression in the form of the Signal Log Ratio. The magnitude and direction of change of a transcript between the two arrays is therefore determined.

SOM Clustering Analysis

SOM clustering was performed using the Affymetrix Data Mining Tool software in order to extract patterns of expression over the course of the experiment. Each day of differentiation was considered a time point. The software filter eliminated genes whose expression did not vary significantly. The following parameters: Filters: Threshold: (min) 20.00, (max) 20 000.00; Row variation: Fold = 3.00, Difference =100.00; Row normalization: Mean = 0.00, Variance = 1.00; Settings: Parameters: Rows = 3, Columns = 3, Epochs = 50 and Seeds = 1; Initialization: Random vectors; Neighbour Function: Gaussian; Learning Rate: Initial = 0.1, Final = 0.005; Neighbourhood Size: Initial = 5.000, Final = 0.200. Further information about all of these parameters can be found in the Affymetrix Data Mining Tool Users Guide, version 3 (http://www.affymetrix.com/Download/manuals/dmt_manual.pdf).

FatiGO Data Mining

Individual genes found within each of the clusters generated through SOM clustering were analyzed using the web-based FatiGO data mining program (http://fatigo.bioinfo.cnio.es/). This program not only allows the user to extract Gene Ontology (GO) terms for a set of genes, but also allows two sets of genes to be compared in order to analyze the differential distribution of GO terms between sets. Using Fisher's exact test for 2×2 contingency tables, the program compares the two sets of genes and extracts a list of GO terms that are significantly different between the two. FatiGO uses the step-down minP method of Westfall and Young (1993) to adjust p-values for multiple testing.

RESULTS AND DISCUSSION

The murine U74Av2 array contains all functionally characterized sequences (~6000) in the mouse UniGene database (Build 74) and an additional ~6000 EST clusters. It therefore is a valuable tool for the identification of gene associations in cell types or tissues and in defining signalling pathway targets of key importance. As such, it was the ideal choice for the present study.

Figure 1 illustrates the changes that occurred in the morphology of the 3T3-L1 cells between day 0 and day 6 of culture. As expected, cells transformed from elongated fibroblast-like entities at the beginning of the experiment to lipid-filled adipocytes by the end, confirming the change in phenotype anticipated (Ntambi and Kim 2000). Untreated control cultures were also monitored for possible morphological changes on each day of the differentiation process. None were detected however.

A SOM approach was used to analyze the data in order to get a better picture of the patterns of gene expression exhibited by genes that showed appreciable changes in expression over the course of the differentiation process. All analysis was based on the average expression on a particular day. The analysis software discarded from the analysis genes and ESTs that were absent as well as those that did not show changes in expression or whose changes in expression were inconclusive or did not fit a common pattern. The clusters that resulted from the SOM analysis of the data as well as the number of genes/ESTs in each cluster are shown in Fig. 2. Nine different clusters were generated, each showing a varying pattern of gene expression between day 0 and day 6 with the expression patterns for the time period between day 1 and day 6 having never been documented before. Due to space constraints, Table 1 contains a small sample of the hundreds of genes that were found in the clusters shown in Fig. 2. A full list can be found at http://www.afns.ualberta.ca/Hosted/Bovine%20Genomics.

Cluster 1 contains the largest number of genes of any of the clusters (420). As can be seen, expression of the genes contained therein generally increases after day 1. Initially however there is a slight decrease in expression between day 0, the day that differentiation was induced, and day 1. This cluster contains many of the genes known to be associated with adipocytes and adipogenesis. These are genes such as those involved in lipid catabolism, fatty acid metabolism and catabolism as well as fatty acid and steroid biosynthe-

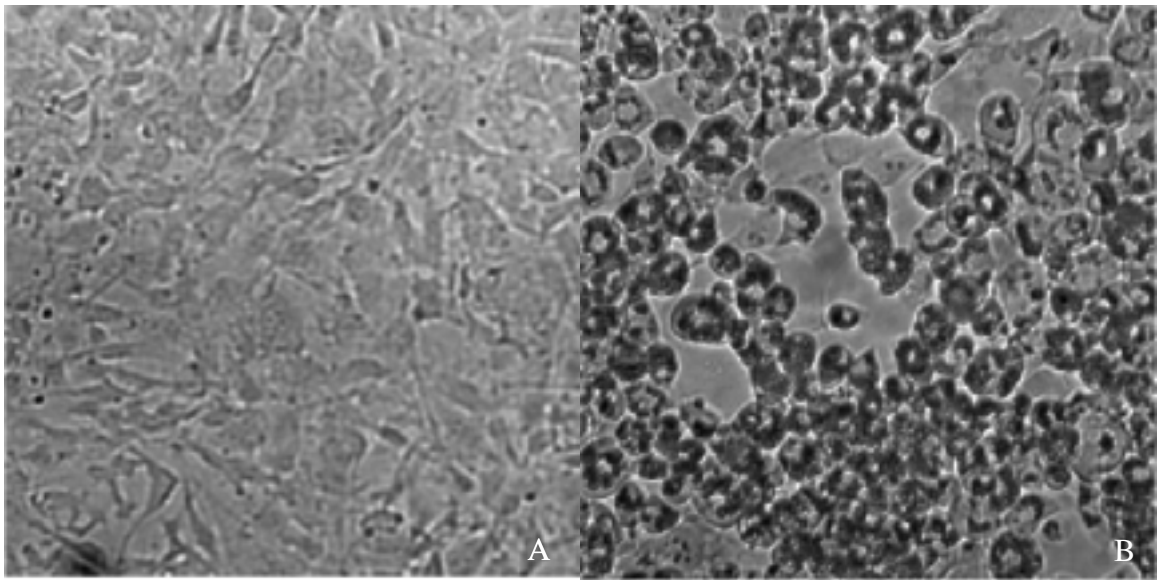

Fig. 1. Differentiation of 3T3-L1 cells. Panel A shows the preadipocyte cells on day 0 of culture. Panel B shows the differentiated adipocytes on day 6 of culture.

sis. In addition, this cluster contains many genes that are classified as being generally involved in central metabolism, genes such as those that are involved in transport, regulation of transcription, a variety of signalling pathways, the cell cycle and electron transport. Peroxisome proliferator activated receptor γ (PPARγ), a transcription factor that has been shown to be important in regulating adipogenesis (Tontonoz et al. 1994; Brun et al. 1996), is one such gene found in this cluster. Comparison analysis showed that expression of PPARγ increased eightfold between day 0 and day 6. This is similar to the 11-fold increase reported by Guo and Liao (2000) for this gene over the same time period. In addition, Takahashi et al. (2002) showed that expression of lipoprotein lipase (LPL) found in this cluster was suppressed in 3T3-L1 adipocytes expressing cAMP-response element binding protein-binding protein (CBP) and p300. Interestingly, the expression of these genes did not change during the present experiment (data not shown). LPL expression was thus not affected by these genes, increasing steadily, and thus triacylglyceride accumulation in the cells occurred (Fig. 1).

The patterns of expression exhibited by the remaining eight clusters are also illustrated in Fig. 2. Clusters 3 and 6 contain genes that have their highest level of relative expression in the preadipocyte. Cluster 7, on the other hand, contains all those genes whose expression peaked on day 2 of the differentiation process. The functional category, assigned by the analysis software, with the largest number of members in this latter cluster was regulation of transcription. Other functional categories of importance were DNA replication, ribosome biogenesis, protein folding, and development. This cluster therefore seems to contain many genes that were related to protein production or protein modification. Cluster 8 was found to contain many of the genes that can be associated with DNA and its replication and transcription. Functional categories of importance in this cluster included cell cycle related genes (the largest category by far), DNA replication, DNA repair, DNA methylation, nucleosome assembly, regulation of transcription and protein amino acid phosphorylation. It is interesting to note that relative peak gene expression for genes in this cluster was on day 1 of differentiation. This precedes the peak in relative gene expression for genes in Cluster 7 by 1 d. As was mentioned earlier, many genes in Cluster 7 had functions related to transcription and protein modification. The patterns of peak expression seen therefore make functional sense with expression of many DNA-related genes peaking prior to protein-related genes.

As mentioned above, the largest functional category in Cluster 8 was the cell cycle-related genes. For example, many of the cyclins (B1, B2, E2, F and A2) were found in this cluster. This pattern confirms what has been postulated about the process of 3T3-L1 cell differentiation. As outlined by Morrison and Farmer (1999), after 3T3-L1 cells reach confluence and thus a state of growth arrest, they can be induced to differentiate as was done in the current experiment. Immediately after exposure to the differentiation-inducing agents, the cells will re-enter the cell cycle for a limited period of cell proliferation (clonal expansion) and then go into a state of post-mitotic growth arrest during which subsequent differentiation is possible. Having such a preponderance of cell cycle-related genes in Cluster 8 with its peak in relative gene expression on day 1 and the subsequent decrease is consistent with the known biological process.

Table 2 summarizes the results obtained when the genes found in each of the individual clusters were subjected to data mining with FatiGO. It was hoped that an examination of the individual clusters in this way might further help to highlight functional differences between genes associated

Table 1. GenBank accession numbers for a subset of genes found in the clusters resulting from the SOM analysis

Cluster 1			
U10374	PPARγ	X13135	Fatty acid synthase
AF078752	DGAT1	M21285	Stearoyl-Coenzyme A desaturase 1[z]
M20497	Fatty acid binding protein 4, adipocyte	M83749	Cyclin D2
U69543	Hormone sensitive lipase	U95826	Cyclin G2[z]
M63335	Lipoprotein lipase	AI846600	Monoglyceride lipase
U07159	Acetyl CoA dehydrogenase, medium chain	U88624	Phospholipase A2, group VI
U15977	Fatty acid CoA ligase, long chain 2	D17571	P450 (cytochrome) oxidoreductase[z]
M93275	Adipose differentiation related protein[z]	M64292	B-cell translocation gene 2, anti-proliferative
M95200	Vascular endothelial growth factor A[z]	U67187	Regulator of G-protein signaling 2[z]
Y11666	Hexokinase 2	AJ002390	Annexin A8[z]
Cluster 2			
AF109906	Neuraminidase 1	AW060401	Ras-like protein Tc10
L34111	Iduronidase, alpha-L-	AW047343	D site albumin promoter binding protein
AW123396	Glucosamine-6-phospate deaminase	U00937	Growth arrest and DNA damage inducible 45 alpha[z]
AI462105	Vinculin	U90435	Flotillin 1[z]
AA717826	Dermatopontin	U77630	Adrenomedullin[z]
X79199	Tetranectin		
Cluster 3			
AI849928	Cyclin D1	M21828	Growth arrest specific 2[z]
AF026124	Phospholipase D3	M21952	Colony stimulating factor 1 (macrophage)[z]
U34277	Phospholipase A2, group VII	M32745	Transforming growth factor, beta 3[z]
M84487	Vascular cell adhesion molecule 1	X04367	Platelet derived growth factor receptor, beta polypeptide[z]
AF053943	AE binding protein 1	X13297	Actin, alpha 2, smooth muscle, aorta[z]
M34476	Retinoic acid receptor gamma	X56602	Interferon-stimulated protein (15 kDa)[z]
X06086	Cathepsin L preproprotein	U26437	Tissue inhibitor of metalloproteinase 3
J03520	Plasminogen activator, tissue	AF000236	Chemokine orphan receptor 1[z]
D31951	Osteoglycin[z]	M62470	Thrombospondin 1[z]
D38162	Procollagen, type XI, alpha 1[z]	U20344	Kruppel-like factor 4 (gut)[z]
L07803	Thrombospondin 2[z]		
Cluster 4			
D16503	Lymphoid enhancer binding factor 1	AB017189	Solute carrier family 7, member 5
Z11886	Notch gene homolog 1	M62362	CCAAT/enhancer binding protein (C/EBP) alpha
AV368209	Pyruvate kinase 3	AF017128	Fos-like antigen 1[z]
J02935	Protein kinase, cAMP dependent regulatory, type II	M33960	Serine (or cysteine) proteinase inhibitor, clade E, member 1[z]
AV338260	Retinoblastoma 1	X99963	Ras homolog gene family, member AB[z]
Cluster 5			
D11091	Protein kinase C, theta	AW121438	Regulator of G protein signalling 19
U19118	Activating transcription factor 3	M22832	Keratin complex 1, acidic, gene 18
U20282	Transcription factor 20	M15501	Actin, alpha, cardiac
AF020737	Fibroblast growth factor 13	AI153959	Sialyltransferase 10 (alpha-2,3-sialyltransferase VI)
Cluster 6			
M57966	Transcription factor 1	M12481	Actin, beta, cytoplasmic
X75330	H6 homeobox 3	AV225659	Calmodulin 2
AI853714	Cathepsin B preproprotein	X5317	Integrin alpha 4
U66079	Coagulation factor VIII	M32490	Cysteine rich protein 61[z]
D13695	Interleukin 1 receptor-like 1	U73620	Vascular endothelial growth factor C[z]
X70058	Chemokine (C-C motif) ligand 7	U86783	Steroid/thyroid hormone nuclear receptor[z]
AA672499	Chemokine (C-C motif) ligand 27		
Cluster 7			
X61800	CCAAT/enhancer binding protein (C/EBP), delta[z]	X85994	Sema domain, immunoglobulin domain (Ig)[z] short basic domain, secreted, (semaphorin) 3C
M31419	Interferon activated gene 204		
D00812	Replication protein A2	X79003	Integrin alpha 5 (fibronectin receptor alpha)[z]
D13545	DNA primase, p58 subunit	M31419	Interferon activated gene 204[z]
AV170770	Chaperonin subunit 5 (epsilon)	D45889	Chondroitin sulfate proteoglycan 2[z]
AI845934	EBNA1 binding protein 2	M14223	Ribonucleotide reductase M2[z]
U20257	Alcohol dehydrogenase 7	X66091	M.musculus ASF mRNA[z]
L00039	Myelocytomatosis oncogene[z]	X81627	Lipocalin 2[z]
M31885	Inhibitor of DNA binding 1[z]		

Table 1. Continued

Cluster 8			
X75483	Cyclin A2	U83903	Tumor necrosis factor induced protein 6[z]
X64713	Cyclin B1, related sequence 1[z]	X15591	Cytotoxic T lymphocyte-associated protein 2 alpha
X66032	Cyclin B2	D26089	Mini chromosome maintenance deficient 4 homolog (S. cerevisiae)[z]
AF091432	Cyclin E2	D26090	Mini chromosome maintenance deficient 5 (S. cerevisiae)[z]
Z47766	Cyclin F	D26091	Mini chromosome maintenance deficient 7 (S. cerevisiae)[z]
AF028009	Protein kinase C, epsilon	L26320	Flap structure specific endonuclease 1[z]
K02927	Ribonucleotide reductase M1	M64086	Serine (or cysteine) proteinase inhibitor, clade A, member 3N[z]
D13543	Polymerase (DNA directed) alpha 1	U25691	Helicase, lymphoid specific[z]
AF024570	Polymerase (DNA directed) delta 1, catalytic subunit	U42385	Fibroblast growth factor inducible 16[z]
Z72486	Polymerase (DNA directed) delta 2, regulatory subunit	AF002823	Budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)[z]
AF036898	Polymerase (DNA directed) epsilon 2	D55720	Karyopherin (importin) alpha 2[z]
AF036008	DNA methyltransferase (cytosine 5) 1	K02245	Proliferin[z]
L32836	S-adenosylhomocysteine hydrolase	K03235	Proliferin 2[z]
AW107230	Nucleosome assembly protein 1-like	K02927	Ribonucleotide reductase M1[z]
AA611766	Krupple-like factor 5	L02914	Aquaporin 1[z]
U20735	Jun B oncogene[z]	U08110	RAN GTPase activating protein 1[z]
AV236858	Tripeptidyl peptidase II	Y09632	Rab6, kinesin-like[z]
AW050256	Tubulin, beta 3		
AW061016	Vitamin D receptor		
AF077861	Inhibitor of DNA binding 2		
M60523	Inhibitor of DNA binding 3[z]		
D30743	Wee 1 homolog (S. pombe)		
Cluster 9			
X76653	Nuclear receptor subfamily 2, group F, member 2	AB010742	Uncoupling protein 3, mitochondrial
L35236	Mitogen activated protein kinase 10	J04596	Chemokine (C-X-C motif) ligand 1[z]
AV028184	Colony stimulating factor 1 receptor	Y07519	Interleukin 1 receptor-like 1
L25890	Eph receptor B2	X99915	High mobility group AT-hook 2[z]
AV294354	Uncoupling protein 1, mitochondrial		

[z]Indicates genes that were also found in clusters produced by Burton et al. (2002).

with each cluster and thus with each pattern of gene expression. As can be seen, the subheadings associated with the majority of genes in each cluster were generally the same, although the proportion of genes associated with a given subheading or GO term changed. Several significant differences between clusters can be identified from the table. For example, a comparison of Cluster 1, containing genes having their highest level of expression in mature adipocytes, and Cluster 3, containing genes whose relative level of expression was highest in preadipocytes, revealed that Cluster 1 had a significantly smaller proportion of genes associated with the GO term "extracellular space" ($P = 0.001$) and a significantly higher proportion associated with the term "intracellular" ($P = 0.0125$). Likewise, the proportions of genes associated with the GO terms "cell communication" and "metabolism" were significantly lower ($P = 0.0003$) and higher ($P = 0.0337$), respectively, for Cluster 1 than Cluster 3. This may relate to the fact that genes peaking in relative expression in the mature adipocyte are more active in processes leading to the manufacture of lipids than those that peak in the preadipocyte, a cell essentially proliferating and awaiting the stimulus to differentiate. FatiGO analysis furthermore showed that the proportion of genes associated with the GO term "oxidoreductase activity" was significantly higher in Cluster 1 than in Cluster 3 ($P = 0.0001$; not shown in Table 2). Oxidoreductase is the systematic name for a class of enzymes which comprises dehydrogenases, hydroxylases, oxidases, and reductases. This class of enzymes is important in many of the processes making up adipocyte metabolism and a higher proportion of oxidoreductase activity-associated genes in Cluster 1 is therefore not surprising.

When Cluster 1 was compared to Cluster 6, again a cluster containing genes whose peak in relative expression is in the preadipocyte, the only differences found were with respect to genes associated with the GO terms "morphogenesis" and "nucleic acid binding". In both cases Cluster 6 contained significantly more genes associated with these terms ($P = 0.0009$ and $P = 0.0206$, respectively). Morphogenesis, which pertains to the creation of form, is something that, purely from a definition point of view, could be associated with proliferating cells or cells very early in the differentiation process and therefore a cluster, such as Cluster 6, that contains genes that peak in expression in the proliferating preadipocyte would likely contain more genes associated with this GO term than a cluster containing genes that peak in the mature, fully differentiated adipocyte. Interestingly, no significant differences were found between Clusters 3 and 6 for any of the GO terms ($P > 0.05$). Obviously the differences between them therefore stem from the specific genes making up each of these clusters rather than the generalizations represented by the GO terms. These are presented, in part, in Table 1.

The current model for adipogenesis describes a cascade of expression of C/EBPβ and C/EBPδ followed by C/EBPα

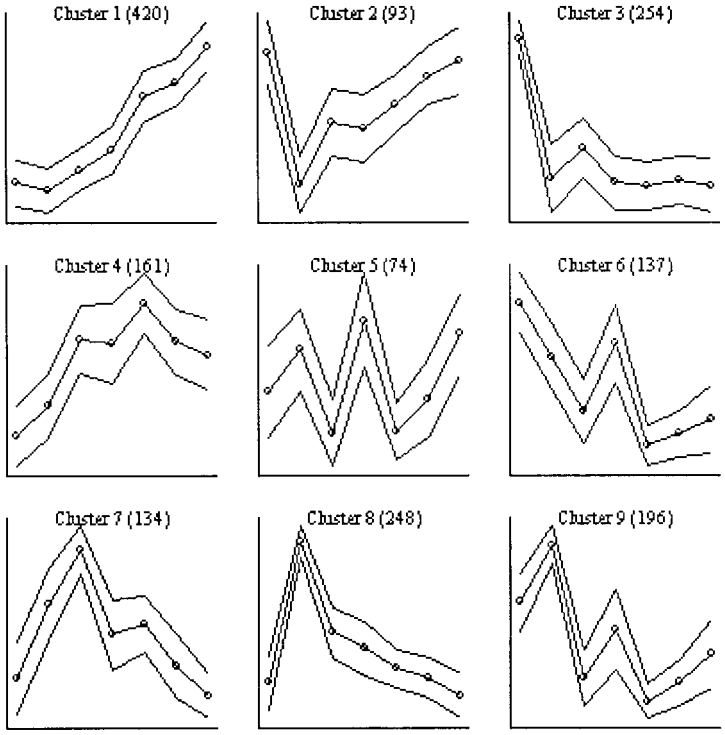

Fig. 2. SOM clusters representing average gene expression patterns of the genes/ESTs exhibiting the greatest change between day 0 and day 6. Numbers in parentheses indicate the number of genes/ESTs in each cluster.

and PPARγ. This cascade is thought to precede the expression of many of the genes that are characteristic of the mature adipocyte (Morrison and Farmer 1999). Clusters formed in the present study place C/EBPδ in Cluster 7 with a peak in relative expression on day 2, C/EBPα in Cluster 4 with a peak of relative expression on day 4 and PPARγ in Cluster 1 with a peak of relative expression on day 6, thus supporting what is postulated in the model. Furthermore, as was mentioned previously, many of the genes that we associate with adipocytes and adipogenesis are found in Cluster 1 along with PPARγ. Since levels of expression were not measured after day 6 nor at short time intervals, it may well be that the levels of many of these genes actually peak after PPARγ. Present results are therefore not inconsistent with the model.

A comparison of the results of this experiment with what was documented by Burton and colleagues (2002) for the first 24 h of differentiation shows that, for the most part, similar expression patterns were obtained for this period of experimental overlap. The only genes whose expression patterns were not consistent between the two studies were adrenomedullin, found in Cluster 2 of our study, and two of the genes in Cluster 6, cysteine rich protein 61 and steroid/thyroid hormone nuclear receptor, both belonging to the same cluster as adrenomedullin in the Burton study. Genes in this group appeared to have an initial sharp increase in relative expression 2 h after induction with levels then declining to levels slightly higher than at 0 h by 24 h post-induction. The sharp relative decrease in expression of these genes seen at day 1 and days 1 and 2, respectively, of the current study was therefore contrary to that of the previous study. The reasons for these apparent incongruities are unclear. Since the Burton paper was not yet available when these experiments were carried out, it is possible that small, unintentional differences in induction procedures or the make-up of the culture medium between experiments are reflected here. It is important to note that other laboratories (unpublished data) have shown that adrenomedullin expression, for example, decreases during induction of preadipocyte differentiation, which agrees with the findings of the present study. All genes found in the various clusters generated in this study that were previously documented by Burton and colleagues (2002) are indicated with an asterisk in Table 1.

While the microarrays used in the study by Guo and Liao (2000) and the present study contained many of the same genes, only a few of the results obtained in that study were confirmed by the present study. The expression of PPARγ has already been mentioned. In addition, the present study showed a decrease in the expression of cadherin 11, cathepsin L, Slug and beta actin on day 6 relative to day 0 as was reported by Guo and Liao (2000). The changes in expression demonstrated by the latter group for such genes as Janus kinase 2, cadherin 1, angiopoeitin 2, yolk sac gene 2, capping protein beta 2, munc-18c and glutathione S transferase theta 2, for example, could not, however, be confirmed. All of these genes showed no change in expression on day 6 relative to day 0. While different microarrays (cDNA versus

Table 2. The four principle ontology subheadings in each GO ontology category that were generated through FatiGO data mining of the individual clusters generated by the SOM clustering analysis

	SOM cluster								
GO ontology	1	2	3	4	5	6	7	8	9
Molecular function	Transferase activity (22.22)	Transferase activity (18.18)	Hydrolase activity (24.00)	Nucleic acid binding (21.88)	Nucleotide binding (19.44)	Nucleic acid binding (26.58)	Nucleic acid binding (33.33)	Nucleic acid binding (36.67)	Transferase activity (22.22)
	Oxidoreductase activity (21.79)	Hydrolase activity (16.36)	Metal ion binding (18.67)	Nucleotide binding (20.83)	Receptor binding (16.67)	Hydrolase activity (20.25)	Nucleotide binding (22.22)	Nucleotide binding (35.83)	Oxidoreductase activity (21.79)
	Nucleotide binding (15.38)	Receptor activity (16.36)	Protein binding (17.33)	Hydrolase activity (18.75)	Hydrolase activity (13.89)	Nucleotide binding (18.99)	Transferase activity (20.83)	Transferase activity (25.83)	Nucleotide binding (15.38)
	Hydrolase activity (14.96)	Protein binding (14.55)	Nucleic acid binding (14.67)	Transferase activity (16.67)	Nucleic acid binding (13.89)	Protein binding (16.46)	Protein binding (12.50)	Hydrolase activity (20.83)	Hydrolase activity (14.96)
Biological process	Metabolism (68.02)	Metabolism (57.14)	Metabolism (52.99)	Metabolism (68.60)	Metabolism (51.52)	Metabolism (53.03)	Metabolism (81.16)	Metabolism (64.10)	Metabolism (46.32)
	Cell growth/ maintenance (24.32)	Cell growth/ maintenance (32.65)	Cell communication (29.85)	Cell growth/ maintenance (34.88)	Cell communication (33.33)	Cell growth/ maintenance (33.33)	Cell growth/ maintenance (30.43)	Cell growth/ maintenance (54.70)	Cell growth/ maintenance (35.79)
	Cell communication (11.71)	Response to external stimulus (18.37)	Cell growth/ maintenance (23.88)	Cell communication (19.77)	Cell growth/ maintenance (27.27)	Morphogenesis (19.70)	Cell communication (11.59)	Response to stress (11.11)	Cell communication (29.47)
	Response to external stimulus (7.21)	Cell communication (12.24)	Response to external stimulus (12.69)	Morphogenesis (11.63)	Morphogenesis (18.18)	Cell communication (19.70)	Morphogenesis (7.25)	Cell communication (8.55)	Morphogenesis (13.68)
Cellular component	Intracellular (63.33)	Intracellular (51.02)	Extracellular space (47.30)	Intracellular (64.86)	Intracellular (50.00)	Intracellular (60.32)	Intracellular (78.69)	Intracellular (83.49)	Intracellular (55.56)
	Membrane (43.81)	Extracellular space (40.82)	Intracellular (47.30)	Extracellular space (35.14)	Membrane (36.67)	Membrane (36.51)	Membrane (16.39)	Membrane (21.10)	Membrane (40.40)
	Extracellular space (28.10)	Membrane (38.78)	Membrane (37.16)	Membrane (32.43)	Extracellular space (36.67)	Extracellular space (36.51)	Extracellular space (13.11)	Extracellular space (9.17)	Extracellular space (33.33)
	Cell fraction (5.24)	Cell fraction (6.12)	Extacellular matrix (7.43)	Cell fraction (5.41)	Extacellular matrix (6.67)	Extacellular matrix (4.76)	Viral capsid (3.28)	Viral capsid (0.92)	Extacellular matrix (7.07)

oligonucleotide) and different microarray technology (Genome Systems Inc. versus Affymetrix) were used in the two studies, the exact reasons for this lack of agreement remain unclear. However, Li et al. (2002) have shown that oligonucleotide-based microarrays are more reliable for global gene expression screening compared to long cDNA arrays primarily due to differences in the amount of cross-hybridization occurring.

CONCLUSIONS

The data in this study present a very broad, yet comprehensive, overview of gene expression patterns over the course of adipocyte differentiation. More work can naturally be done to define further the gene expression patterns during the course of each day. While this has been carried out for the first 24 h (Burton et al. 2002) with excellent results, the present experiment reveals that an in-depth study of time periods during the entirety of the differentiation process is equally vital. Work is also underway to establish bovine cell lines in the laboratory so that these results can be confirmed and expanded in the bovine. Finally, it should also be noted that because of the nature of the experiment performed, it is impossible to separate the effects of the differentiation treatment entirely from the actual downstream differentiation process. Because of the necessity for treatment at the various stages of the differentiation process, it is impossible to deduce with absolute certainty whether a change in gene expression is due to a change in treatment (i.e., a medium change) or due to the differentiation process itself. This must naturally be kept in mind when we evaluate individual gene expression changes in an in vivo context.

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