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Temporal gene expression changes during adipogenesis in human mesenchymal stem cells

Takanori Nakamura,[a] Satoshi Shiojima,[b] Yuichi Hirai,[a] Takehisa Iwama,[a] Nobutomo Tsuruzoe,[a] Akira Hirasawa,[b] Susumu Katsuma,[b] and Gozoh Tsujimoto[b,*]

[a] *Phamaceutical Research Department, Biological Research Laboratories, Nissan Chemical Industries Ltd., 1470, Shiraoka, Minamisaitama, Saitama 349-0294, Japan*
[b] *Department of Molecular, Cell Pharmacology, National Center for Child Health and Development Research Institute, 3-35-31, Taishido, Setagaya-Ku, Tokyo 154-8567, Japan*

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Abstract

Human bone marrow mesenchymal stem cells (hMSCs) give rise to adipocytes in response to adipogenic hormones. An in-house cDNA microarray representing 3400 genes was employed to characterize the modulation of genes involved in this process. A total of 197 genes showed temporal gene expression changes during adipogenesis, including genes encoding transcriptional regulators and signaling molecules. Semi-quantitative RT-PCR analyses confirmed differential expression at the transcriptional level of several genes identified by cDNA microarray screening. Cluster analysis of the genes regulated during the late phase (from day 7 to day 14) of hMSC adipogenesis indicated that these changes are well correlated with data previously reported for murine preadipocytes. However, during the early phase (day 1–day 5), the modulations of genes differed from those reported for the preadipocytes. These data provide novel information on the molecular mechanisms required for lineage commitment and maturation accompanying adipogenesis of hMSC.

Keywords: Mesenchymal stem cells; Adipogenesis; cDNA microarray; Gene expression; Cluster analysis

Adipogenesis has been studied extensively in vitro using a number of preadipocyte cell lines including 3T3-L1 and 3T3-F442A cells [1–4]. When cultured in defined media, these preadipocyte cells differentiate to form adipocytes, with accumulation of lipid vesicles and expression of genes that are also expressed in adipocytes in vivo [1,5]. These phenotypes have been recorded from 3 to 7 days after stimulation in murine preadipocyte cell lines [1–4]. Several key regulatory genes that are necessary and/or sufficient for the transition from preadipocytes to adipocytes in vitro, including the CCAAT/enhancer binding proteins (C/EBPs) α, β, and δ, as well as the peroxisome proliferator-activated receptor (PPAR) γ, were identified [5,6]. Studies of these transcription factors have suggested that adipogenesis is the result of a temporally ordered pattern characterizing distinct phases of gene expression [1–4].

Human bone marrow mesenchymal stem cells (hMSCs) are known as multipotent stem cells, and they give rise to adipocytes, osteoblasts, chondrocytes, and myoblasts when cultured under defined in vitro conditions [7,8]. Suitable culturing conditions drive the lineage commitment and maturation systems required for differentiation to each mature cell type [7–9]. When cultured with dexamethasone, indomethacin, 3-isobutyl-1-methyl-xanthine, and insulin (adipogenic hormones), most of the hMSCs differentiate to the adipocyte lineage [7]. Hence, hMSCs can be differentiated into adipocytes in a similar fashion as 3T3-L1 and 3T3-F442A cells when stimulated by adipogenic hormones; however, phenotypic changes, such as lipid vesicles, can be observed at the later stage (generally after culturing for 7 days) [7]. These observations indicate that 3T3-L1 and 3T3-F442A cells are committed to adipogenesis, while

* Corresponding author. Fax: +81-3-3419-1252.
E-mail address: gtsujimoto@nch.go.jp (G. Tsujimoto).

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hMSCs may be in a more undifferentiated state. As the same treatment with adipogenic hormones leads these cells to adipogenic differentiation, hMSCs may be a better model to explore the earlier molecular events triggering this transformation.

To obtain more detailed insight into human adipogenesis, we examined gene expression profiles of hMSCs that were in the process of adipogenic differentiation using cDNA microarrays. The temporal gene expression patterns indicated genes that were differentially expressed during the late phase of hMSC adipogenesis are similar to those previously identified as "adipose-specific genes" in the differentiation processes of preadipocytes other than hMSCs. By using hMSCs, the present study for the first time identifies genes involved in the early phase of adipogenesis. The genes encode transcriptional regulators and signaling molecules, many of which have not been previously identified as factors relating to adipogenesis.

Materials and methods

Cells. Human bone marrow mesenchymal stem cells (hMSCs) were purchased from BioWhittaker (Walkersville, MD). The cells were seeded at 5000 cells per cm^2 of surface area in MSCGM medium (BioWhittaker) containing 10% fetal bovine serum (FBS: BioWhittaker) and were cultured at 37 °C, 5% CO_2 for 7 days. During the adipocyte differentiation process, hMSCs were used for passages six times. After they reached confluence, hMSCs were stimulated in the induction medium [Dulbecco's modified Eagle's medium (DMEM)-high glucose (4.5 g glucose/L: Asahi Techno Glass, Chiba, Japan) containing 10% FBS, 1 μM dexamethasone (Wako, Osaka, Japan), 0.2 mM indomethacin (Sigma, St. Louis, MO), 0.5 mM of 3-isobutyl-1-methyl-xanthine (Sigma), and 0.01 mg/ml of insulin (Sigma)] for 3 days, followed by one day of culture in maintenance medium (DMEM-high glucose containing 10% FBS and 0.01 mg/ml of insulin). After repeating this cycle of induction/maintenance three times, the cells were cultured for a further 3 days in the maintenance medium.

The adipocyte differentiation of hMSCs was confirmed by phase-constructed microscopic analysis and flow-cytometer analysis after staining the lipid vesicles with Nile red fluorescent dye (Molecular Probes, Eugene, OR) [10]. Cells grown in 6-well plates were fixed with 0.5% paraformaldehyde (Wako) and stained with Nile red at a final concentration of 1 μg/ml. Cells were analyzed on an EPICS-XL multiparameter flow cytometer (Beckman-Coulter, Fullerton, CA).

Construction of hMSC cDNA microarrays. Two cDNA libraries were purchased from Incyte Genomics (St. Louis, MO) and from Invitrogen (Carlsbad, CA). cDNA clones were selected according to the following strategy. First, the clones that were expressed in the early phase of hMSC adipogenesis were selected by filter array hybridization using the GeneFilter kit (GF200-GF205: Invitrogen), which contains approximately 30,000 (30 K) clones. Hybridization probes for the filter were prepared using poly(A)+ RNA isolated from hMSCs that were cultured under three conditions of adipocyte induction for 0, 24, and 96 h. The poly(A)+ RNA was labeled with [^{33}P]dCTP (Amersham Biosciences, Piscataway, NJ) during reverse transcription and hybridized against the filter as per the manufacturer's protocol. Approximately 2400 clones were selected by this approach. Second, approximately 1000 clones were selected by employing an information-based approach. A public domain gene database was searched using the keywords 'adipocyte' and 'adipogenesis,' and the resulting clones were selected from 30 K clones. In total, approximately 3400 cDNA clones were selected, amplified by PCR, and spotted onto glass slides as described before [11,12].

Fluorescent probe labeling and hybridization. Poly(A)+ RNA was isolated from hMSCs cultured under pretreatment conditions. Adipocyte induction was carried out for 1, 3, 5, 7, 9, and 14 days, using ISOGEN (Nippon Gene, Toyama, Japan) and Oligotex-dT30 (Takara, Kyoto, Japan), according to the manufacturer's protocol. Two micrograms of extracted poly(A)+ RNA was reverse transcribed with Cy3- or Cy5-conjugated dUTP (Amersham Biosciences, Piscataway, NJ) using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers (Takara, Kyoto, Japan). After 2 h of incubation at 42 °C, the labeled probes were concentrated in a Microcon filter device (Millipore, Bedford, MA) and diluted in 15 μl hybridization solution (3.4× SSC containing 0.3% SDS, 20 μg poly(A) DNA, and 20 μg yeast tRNA). Using Cy5-labeled samples taken at each time point, and a Cy3-labeled pretreatment control, two probes were mixed and hybridized against cDNA microarrays with overnight incubation at 65 °C. After hybridization, the glass slides were washed twice with 2× SSC containing 0.1% SDS for 5 min at room temperature, twice with 0.2× SSC containing 0.1% SDS for 5 min at 40 °C, and finally with 0.2× SSC for 3 min at room temperature. Hybridized images were scanned by a fluorescence laser scanning device (GenePix 4000A, AXON, Foster City, CA) at 532 nm for Cy3 and at 635 nm for Cy5. For duplication, the procedure of RNA extraction, fluorescent dye labeling, and array hybridization and scanning were independently executed.

Semi-quantitative RT-PCR. The cDNA was synthesized from total RNA using Superscript II reverse transcriptase. The cDNA fragments were amplified for 25–35 cycles and the exponential phase was determined to allow a semi-quantitative analysis of each reaction. The amplification cycles consisted of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The primer sets [Forward (F) and Reverse (R)] used were: 5′-CCT CTT CTC CGA CGA CTA CG-3′ (C/EBP β, F), 5′-TAC TCG TCG CTG TGC TTG TC-3′ (R); 5′-TGT CTG TGA GGG AAG CAG TG-3′ (LIFR, F), 5′-TTC TGG TTT TGC TTG AGG CT-3′ (R); 5′-TGC ATT TTC TGA CAC GCT TC-3′ (SREBP-1c, F), 5′-CTG TCC TGC AGG TAC CCA CT-3′ (R); 5′-TTC CAT GCT GTT ATG GGT GA-3′ (PPAR γ2, F), 5′-ACC CTT GCA TCC TTC ACA AG-3′ (R); 5′-TGC AGC TTC CTT CTC ACC TT-3′ (FABP-a, F), 5′-TGG TTG ATT TTC CAT CCC AT-3′ (R); 5′-GTC CGT GGC TAC CTG TCA TT-3′ (LPL, F), 5′-AGC CCT TTC TCA AAG GCT TC-3′ (R); 5′-GTT TAT GGC ACT CTG GGC AT-3′ (OSF-2os, F), 5′-AGA TCC GTG AAG GTG GTT TG-3′ (R); and 5′-CAG AGC AAG AGA GGC ATC C-3′ (β-actin, F), 5′-CTC CTT AAT GTC ACG CAC GAT-3′ (R).

Data processing. Raw scanned images were processed using GenePix Pro 3.0 microarray image analysis software (AXON, Foster City, CA). The data accumulation and statistical analyses were executed by Microsoft Excel and Pirouette version 2.6 software (Infometrix, Woodinville, WA). The analyzed data consist of seven time points (Pre = 0, 1, 3, 5, 7, 9, and 14 days) and RNA derived from two independent cell cultures of each time point for duplication. The fold-change seen at the time points was calculated as follows: The ratio of signal intensities at each point (Cy5 intensity/Cy3 intensity) was calculated and defined as Ratios—Pre, -1, -3, -5, -7, -9, and -14, indicating the time points described above. The fold-change of each time point was defined as the average of the duplication of relative ratio to Ratio-Pre (i.e., $Fold_1 = Ratio\text{-}1/Ratio\text{-}Pre$). Clones that did not show differential expression levels either more than 2-fold or less than 0.5-fold from at least one time point were excluded from further analysis. For the purpose of monitoring differential expression, the expression changes were displayed as percentages of the maximum change seen in Fig. 2. The profile of differentially expressed genes was analyzed by an agglomerate hierarchical clustering method with Euclidean distance matrices. The cluster nodes indicating correlations greater than 0.4 are displayed in Fig. 2.

Results and discussion

The hMSCs differentiate to adipocytes in response to the administration of dexamethasone, indomethacin, 3-isobutyl-1-methyl-xanthine, and insulin [7]. As shown in Figs. 1A–E, few cells accumulating lipid vesicles were observed until the fifth day following stimulation (Figs. 1A–C), and then the lipid droplet-containing cell population increased in a time-dependent manner up to day 14. At the fourteenth day following stimulation (Fig. 1E), flow-cytometer analysis with Nile red dye showed that approximately 40% of the hMSCs had differentiated into adipocytes (Fig. 1F).

To investigate patterns of gene expression during the process of hMSC adipogenesis, cDNA microarray analysis was performed. Labeled cDNA targets were prepared from hMSC poly(A)+ RNA of control and adipogenic hormone-treated cells. These were hybridized with the microarray, and 207 clones (corresponding to 197 non-redundant genes) that differed by more than 2-fold intensity in at least one pairwise comparison were selected. The number of differentially regulated clones found at each time point is shown in Fig. 1G. This result shows that the number of up-regulated genes peaked at day 3 in the early stage (0–6 days) and at days 7 and 9 in the late stage (7–14 days), respectively.

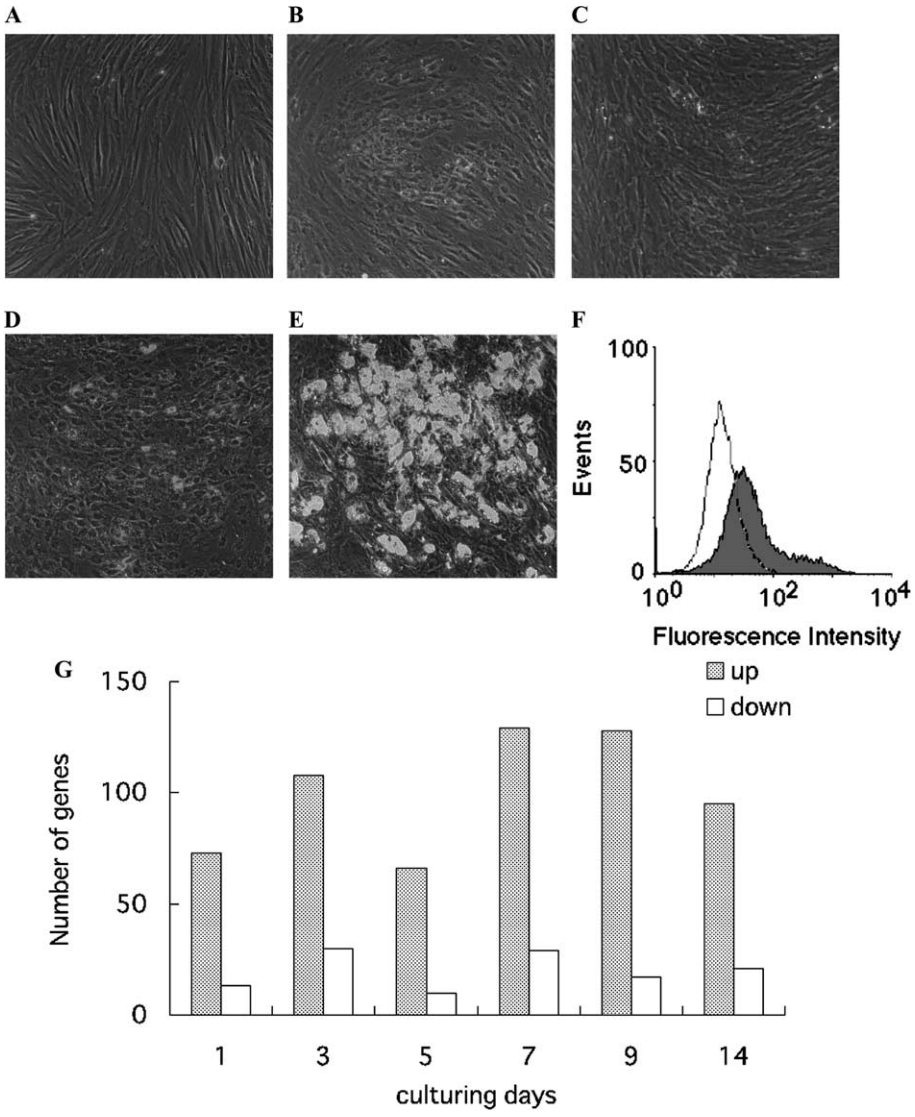

Fig. 1. Histological analysis and number of modulated genes revealed by cDNA microarray analysis of hMSC differentiation. (A–E) The hMSCs were cultured in the presence of adipogenic hormones. Histological observation by phase-constructed analysis indicated that the cells began to accumulate lipid vesicles by 7–14 days. A, control; B, day 3; C, day 5; D, day 7; and E, day 14 are indicated. (F) The accumulation of lipid vesicles was assayed by staining with Nile red and carrying out flow cytometry experiments. The open histogram indicates the control and the shaded histogram indicates results after 14 days of culturing. (G) The number of differentially regulated genes revealed by cDNA microarray analysis. The number of genes at each time point that were up- or down-regulated at least 2-fold is shown.

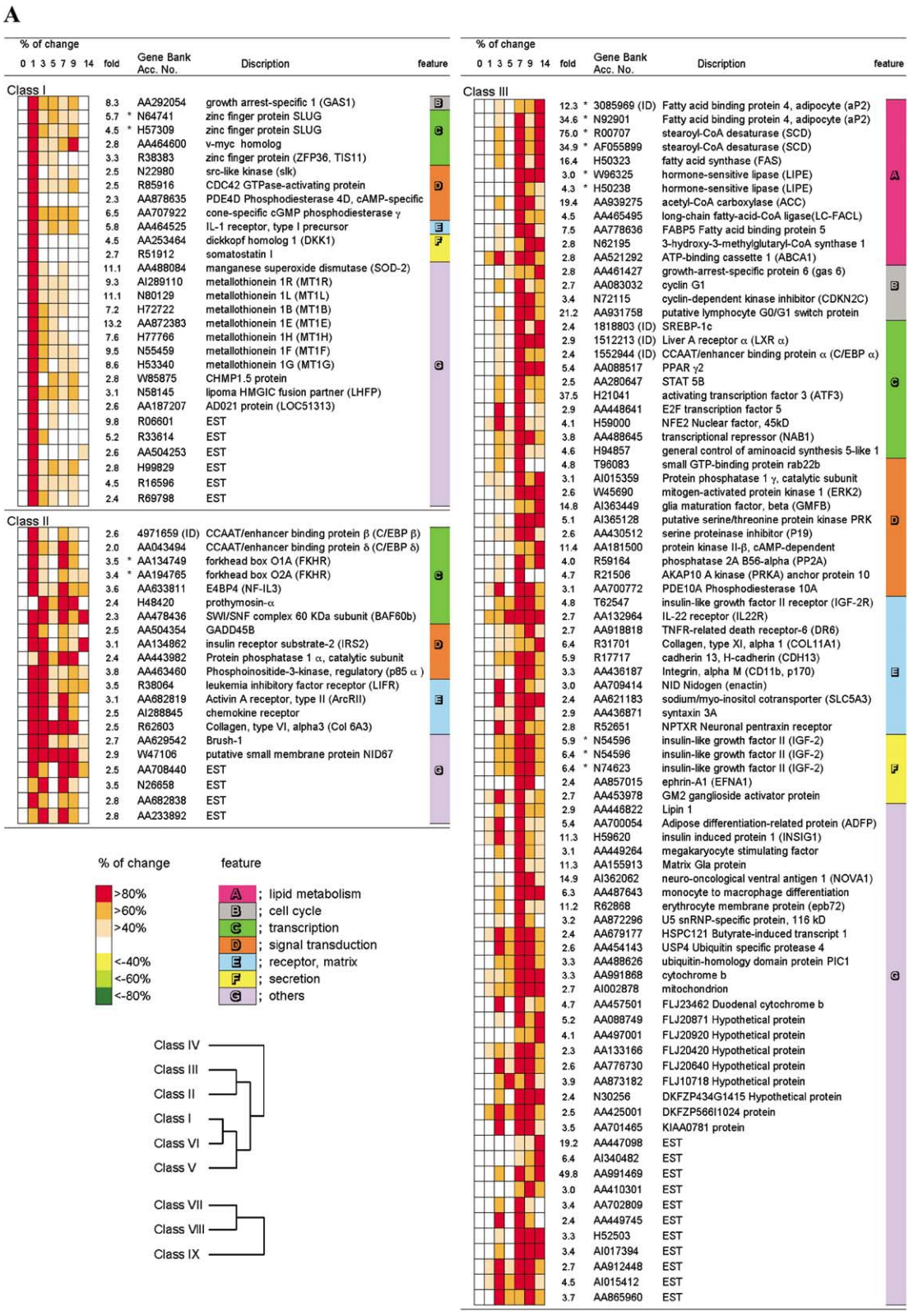

Fig. 2. Cluster analysis of genes that are differentially regulated during the differentiation of hMSCs into adipocytes. The 197 genes were grouped into nine clusters according to their expression profiles across seven time points, using Euclidean distance measurement. Experiments are ordered along the x axis, which shows the % change, and genes are ordered along the y axis. The % change relative to the pretreatment control is shown colorimetrically, as indicated at the bottom left. Each value represents the mean of two independent experiments. A representative feature of each description is labeled A–G with a color bar as indicated. The ID number in the "GenBank Accession No." column indicates the clone identification number, as given by Incyte Genomics. The redundant gene is labeled with a *.

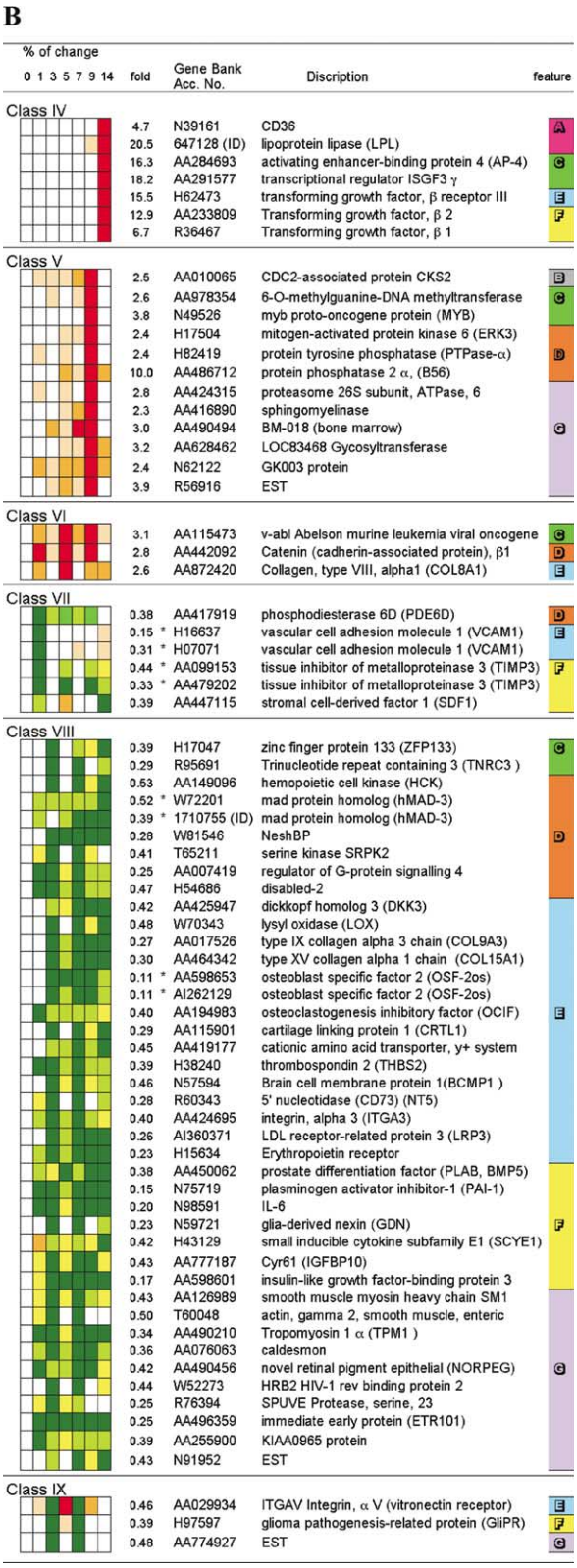

Fig. 2. (*continued*)

The differentially expressed genes were next classified into nine clusters (Class I–IX) using a hierarchical clustering method (Fig. 2). Class I included genes for which expression was up-regulated one day after treatment with adipogenic hormones, and expression then returned to the basal level (Fig. 2I). Class II contained genes that were up-regulated from day 1 to day 3, and at day 7 (Fig. 2II). Class III genes were up-regulated at day 3, and from day 7 to day 14 (Fig. 2III). The genes in Class IV were induced throughout the 14-day period examined and Class V displayed a peak in up-regulation at day 9 (Figs. 2IV and V). Class VI contained genes that peaked at days 3, 5, and 9 (Fig. 2VI). Class VII included genes for which expression was down-regulated at days 1, 3, and 9 (Fig. 2VII). Class VIII included genes that displayed a decreased expression level 1 or 3 days after adipogenic hormone treatment (Fig. 2VIII). Class IX contained genes for which down-regulation reached its lowest point at days 3 and 7 (Fig. 2IX).

Two of the clusters (Classes III and IV) included 44% (87) of the 197 differentially regulated genes that are known to have specific functions in adipocytes, such as lipid metabolism and gene transcription (Figs. 2A and C). Several key genes in the metabolism of fatty acid and triglycerides, such as lipoprotein lipase (LPL), fatty acid binding protein-adipocyte (FABP-a), stearyl CoA desaturase, fatty acid synthase, hormone-sensitive lipase, acetyl-CoA carboxylase, and fatty acid translocase, were included in these clusters [4,13–16]. Also, several genes that have been reported to be expressed in the differentiated adipocyte were assigned to these clusters. These included insulin-like growth factor 2, Lipin 1, adipose differentiation-related protein, and transforming growth factor β [4,17–20]. Furthermore, the transcription factors PPAR γ, C/EBP α, sterol regulatory element binding protein (SREBP-1c), signal transducer, and activator of transcription 5, and liver A receptor α were included in these clusters. These transcription factors are known to be intimately involved in the regulation of the genes in the above clusters [4–6,21–23].

Both Class I and Class II clusters included genes that were up-regulated at the early stage of adipogenesis. Among these genes, C/EBP β and δ have been reported to serve as transcriptional activators of many adipocyte genes whose expression produces the adipocyte phenotype [5,6]. Also, a SWI/SNF complex (BAF60b) gene, which is known to cooperate with C/EBP β [24], was included in Class II. Class V contained genes related to cell cycle progression such as CDC2-associated protein and signal transduction such as mitogen-activated protein kinases, which were previously reported to be differentially regulated in adipogenesis [1–3]. Class VI included a cytoplasmic component, β-catenin. Three of the clusters (Classes VII–IX) were composed primarily of genes comprising the cytoskeleton and the extracellular matrix. Markers of cell lineages other than the adipocyte were also included in these clusters. For example, a putative bone adhesion protein (osteoblast-specific factor-2os; OSF-2os), which is expressed in

osteoblasts [23], and an osteoclastogenesis inhibitory factor that is a marker for mallow stroma cells [25] were included, as were Type IX collagen and a cartilage linking protein that is a marker for chondrocytes [26,27]. The genes in these clusters were down-regulated during progression of hMSC adipogenesis, probably due to a loss of their ability to differentiate into other cell lineages.

To validate the differential gene expression revealed by cDNA microarray-based profiling of hMSC adipogenesis, semi-quantitative RT-PCR was carried out for C/EBP β and leukemia inhibitory factor receptor (LIFR) genes in Class II, SREBP-1c, PPAR γ 2, and FABP-a genes in Class III, the LPL gene in Class IV, and the OSF-2os gene in Class VII. As shown in Fig. 3, this analysis confirmed the accuracy of the results regarding transcriptional regulation that were obtained from the cDNA microarray experiments.

Cluster analysis identified two distinct major groups of genes involved in hMSC adipogenesis; thus, the genes identified could be classified into two groups, those participating in the early stage (days 0–6) and in the late stage (days 7–14) of adipogenesis. Genes participating in the late stage of adipogenesis (such as C/EBP β and δ, mitogen-activated protein kinases, CDC2-associated protein, Myb, cycline G1, PPAR γ, C/EBP α, and FABP-a) were previously reported to be differentially regulated in the differentiation processes of murine preadipocytes 3T3-L1 [1–3]. In contrast to the late stage of adipogenesis, several genes identified here as being regulated differentially in the early stage were not included among those previously associated with the differentiation process of the murine preadipocyte cells 3T3-L1 [1–3]. Interestingly, genes that were transiently up-regulated in the early stage of hMSC adipogenesis, such as the transcription factors SLUG, FKHR, and a cytoplasmic component, β-catenin, were reported to be genes showing only down-regulation in 3T3-L1 adipogenesis [1–3].

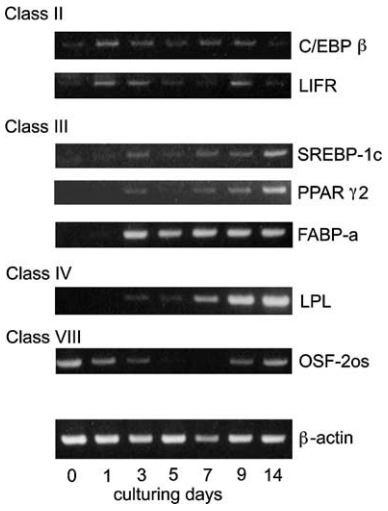

Fig. 3. Semi-quantitative RT-PCR analysis of the differentially expressed genes identified by cDNA microarray-based gene expression profiling. Using varied cycle numbers, the exponential phase for each primer pair was determined and used to allow semi-quantitative analysis of the respective reactions. β-Actin was used as an internal control.

Furthermore, the temporal change in C/EBP β and δ expression highlighted a unique feature of hMSC adipogenesis. The vital roles played by C/EBP β and δ in adipogenesis are well defined; thus, C/EBP β and δ initially activate transcription of the C/EBP α gene, after which C/EBP α coordinately activates the expression of adipocyte genes, producing the terminal differentiation state [1–3,5,6]. Interestingly, the present study shows that this temporal change in C/EBP β and δ can be observed not only in the late stage, but also in the early stage of hMSC adipogenesis. As described above, the expression profile obtained in the late stage of hMSC adipogenesis appears to be similar to that reported in the differentiation process of murine preadipocyte 3T3-L1 cells. In both cases, C/EBP β and δ are expressed early, while C/EBP α is expressed later. A similar wave of C/EBP β and δ and C/EBP α expression during hMSC adipogenesis was observed even in its early stage. Hence, our results show that the hMSC does not proceed to the terminal differentiation stage of adipogenesis, even though C/EBP β and δ expression is activated. Intrigued by this observation, we further examined the effect of the enhanced expression of C/EBP β and δ on hMSC adipogenesis. hMSCs were stimulated by 100 ng/ml of LIF at day 1 and day 3 after treatment with adipogenic hormones. Although an enhanced expression of C/EBP β by LIF was confirmed, up-regulation of PPAR γ 2 and FABP-a gene expression was not detected, nor was the accumulation of lipid vesicles, until the cells had been cultured for 5 days with LIF treatment (data not shown). Taken together, the results described above may indicate that hMSC adipogenesis consists of two distinct stages in the cell differentiation program. It is our view that the similarity in expression profiles for the late stage hMSC adipogenesis and for the 3T3-L1 cells may indicate that both cell types are in a postcommitment state and are proceeding to the terminal differentiation stage. On the other hand, the early stage of hMSC adipogenesis may reflect its precommitment state.

In conclusion, genome-wide expression analysis of adipogenesis was performed using hMSC as a model, and a unique gene expression signature was observed in the early stage of hMSC adipogenesis. Further studies are clearly required to examine the functional significance of the differentially regulated genes that were identified in the early stage of hMSC adipogenesis, in particular. hMSCs would also be of value in studying novel molecular cascades that accompany adipogenesis.

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