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Functional Classification of Gene Expression Profiles During Differentiation of Mouse Embryonic Cells on Monolayer Culture

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Abstract: Embryonic stem (ES) cells have a capability to generate all types of cells. However, the mechanism by which ES cells differentiate into specific cell is still unclear. Using microarray technology, the differentiation process in mouse embryonic stem cells was characterized by temporal gene expression changes of mouse ES cells during differentiation in a monolayer culture. A large number of genes were differentially regulated from 1 day to 14 days, and less number of genes were differentially expressed from 14 days to 28 days. The number of up-regulated genes was linearly increased throughout the 28 days of in vitro differentiation, while the number of down-regulated genes reached the plateau from 14 days to 28 days. Most differentially expressed genes were functionally classified into transcriptional regulation, development, extra cellular matrix (ECM), cytoskeleton organization, cytokines, receptors, RNA processing, DNA replication, chromatin assembly, proliferation and apoptosis related genes. While genes encoding ECM proteins were up-regulated, most of the genes related to proliferation, chromatin assembly, DNA replication, RNA processing, and cytoskeleton organization were down-regulated at 14 days. Genes known to be associated with embryo development or transcriptional regulation were differentially expressed mostly after 14 days of differentiation. These results indicate that the altered expression of ECM genes constitute an early event during the spontaneous differentiation, followed by the inhibition of proliferation and lineage specification. Our study might identify useful time-points for applying selective treatments for directed differentiation of mouse ES cells.

Key words: embryonic stem cells, differentiation, cDNA microarray

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INTRODUCTION

Embryonic stem (ES) cells have the ability to generate all cell types derived from the three germ layers of the embryo and has drawn attention to ES cells as a novel cell source for the therapeutic cell transplantation. Mouse ES cells derived from inner cell mass of blastocysts (Martin, 1981; Evans and Kaufman, 1981) are maintained in an undifferentiated state by the presence of leukemia inhibitory factor (LIF) in culture medium (Smith et al., 1988; Williams et al., 1988). The removal of LIF from the medium can induce the spontaneous differentiation of ES cells (Mummery et al., 1990) and the formation of embryoid bodies (EBs) by the aggregation of ES cells can generate heterogeneous population of cells lineages derived from all three germ layers (Itskovitz-Eldor et al., 2000). In addition, the culture of ES cells under the appropriate conditions can direct the differentiation of ES cells into specialized cells such as hematopoietic (Keller et al., 1993), myogenic (Rohwedel et al., 1994), neuronal (Bain et al., 1996), and hepatic cells (Hamazaki et al., 2001), which suggest that in vitro differentiation process of ES cells may reflect the differentiation progress during mouse embryo development in vivo. Therefore, ES cells may provide a systemic experimental model to study the process driving tissue differentiation in developing embryo *in vivo*.

Much effort have been applied to understand the mechanisms determining lineage commitments, which makes it possible to manipulate the differentiation of ES cells into specific cell types. Differentiation of ES cells is very complicated process involving multifactorial interactions. Although the differential expression levels of several specific genes have been used to characterize in vitro differentiation of ES cells into certain types of cells (Bain et al., 1996; Fairchild et al., 2000; Itskovitz-Eldor et al., 2000;

Keller et al., 1993), it has a limit to study systemically in vitro differentiation of ES cells because the differentiation of ES cells in culture is controlled by dynamic and accurate regulation in the expression of numerous genes.

cDNA microarray makes it possible to define gene expression profile of a large number of a genes in a sensitive, quantitative and efficient manner, which provides powerful potential for characterizing gene expression patterns during biological processes. This technology has been used to compare the undifferentiated ES cells and differentiated cells (Kelly et al., 2000), to find genes associated with pluripotency and lineage specificity (Tanaka et al., 2002), to uncover self-renewal related signaling pathway (Zhu et al., 2007), and to dissect the events of early differentiation during EB formation (Mansergh et al.,2009). However, the time sequential events during differentiation of ES cells can also be characterized by global gene expression profiles, which may provide better understanding the mechanisms involved in the differentiation of ES cells.

Therefore, the purpose of this study was to characterize the sequential events during differentiation of mouse ES cells using temporal gene expression profiles in monolayer culture system. Moreover the differential expressed genes during differentiation were classified into biological functions, which may provides better understanding the differentiation process in mouse ES cells. We found that the number of up-regulated genes linearly increased throughout the 28 days, but the number of down-regulated genes reached the plateau from 14 days to 28 days. Functional classification of most differentially expressed genes revealed that the altered expression of ECM genes constitute an early event during the spontaneous differentiation, followed by the inhibition of proliferation and lineage specification.

MATERIALS AND METHODS

ES cell culture and induction

The hypoxanthine phosphoribosyltransferase-deficient ES cell line, HM-1, were maintained in undifferentiation state on 0.1% gelatin-coated plate in Glasgow Minimum Essential Medium (Invitrogen, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 5% heat-inactivated newborn calf serum (Gemini Bioproduct), 1% MEM nonessential amino acid (Invitrogen), 1% MEM sodium pyruvate solution (Invitrogen), 100 U/mL of penicillin/streptomycin (Biofluids), 1% β-mercaptoethnol (Sigma, St. Louis, MO) and 1000 U/mL of leukemia inhibitory factor (LIF; CHEMICON International, Inc., Temecula, CA)]. The differentiation of ES cells was induced by changing the culture media with Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (HyClone), 1% glutamine (Biofluids), 100 U/mL of penicillin/streptomycin (Biofluids) and 300 μM monothioglycerol (Sigma). Media were changed every two days.

Isolation of RNA and cDNA microarray

Total RNA was prepared from the cells harvested at 1, 7, 14, 21, or 28 days after induction of differentiation using Trizol (Invitrogen) as described in the supplier's instruction. Total RNA for reference RNA was also extracted from undifferentiated ES cells on 2 days after the culture. The mouse 36 K cDNA microarrays used in this study were produced using 15,000 cDNAs obtained from National Institute of Aging and 21,000 cDNAs from Brain Molecular Anatomy Project. These represented sequences from 9836 named genes and 26,487 ESTs.

Microarray experiments

Labeling of total RNA for microarray analysis was performed using indirect methods as described below. Twenty micrograms of total RNA was mixed with 4 μ of oligo dT 20-mer (Qiagen, Valencia, CA) in a 25-μL reaction, denatured at 70°C for 10 min, and then primed while cooling to room temperature. The primed total RNA was reverse transcribed for 1 hr at 42°C in a 50-μL reaction containing 2.5 μL of 20X dNTP mix [10 mM dATP, 10 mM dGTP, 10 mM dCTP, 4 mM dTTP, and 6 mM aminoallyl-dUTP (Sigma)], 10 μL of 5X first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 5 μL of 0.1 M DDT, 2μL of Superscript II reverse transcriptase (200 μg/μL, Invitrogen), and 1μL of SUPERase. In^{TM} (10,000 Unit, Ambion Austin, TX). The reaction was stopped by heating at 75°C for 5 min and the RNA was degraded with 2 μL of RNase H (2 μg/μL; Invitrogen) at 37°C for 20 min. cDNAs were cleaned up QuickCleanTM Enzyme Removal Resin (Clontech, Palo Alto, CA), precipitated with ethanol, and resuspended in 10 μL of 0.1 M $NaHCO_3$. Chemical coupling of aminoally groups in cDNAs were performed by incubating cDNAs with monoreactive Cy3 or Cy5 dye (Amersham Pharmacia Biotech, Piscataway, NJ) that has been resuspended in 10 μL of 0.1 M $NaHCO_3$ overnight at room temperature in dark place. The labeled cDNA probes were precipitated with ethanol and dissolved in 50 μL of TE buffer. Both probe solutions were combined, cleaned up with Nucleospin Extraction kit (Clontech), and then concentrated to a volume of 17 μL using a Centricon-30 micro-concentrator (Millipore, Bedford, MA). The hybridization mixture consisted of 17 μL of the combined probe, 1 μL of mouse COT-1 DNA (1 μg/μL, Invitrogen), 1 μL of yeast tRNA (4 μg/μl, Sigma), and 1 μL of poly A (10 μg/ μL, Amersham Pharmacia Biotech.) in a final volume of 40 μL containing 5X SSC, 0.1% SDS, and 25% formamide. The cDNA microarrays were prehybridized with the buffer (5X SSC, 0.1% SDS, and 1% BSA) at 42°C for 1 hr in water bath and

hybridized with the hybridization mixture at 42°C for 48 hrs in water bath. The hybridized microarrys were washed in 2x SSC with 0.1% SDS, 1x SSC, 0.2x SSC, and then 0.05x SSC each for 1 min, sequentially. The washed hybridized microarrays were scanned at 10 mm resolution on a GenePix 4000A scanner (Axon Instrument, Union City, CA) at variable PMT voltage to obtain maximal signal intensities with less than 1% probe saturation. Resulting images were analyzed via GenePix Pro v3.0 (Axon Instrument) as described in the manufacturer's manual. Throughout the experiments, any bad spots in terms of excessive noise, printing artifacts, or a net total intensity less than 200 in either channel were eliminated from further calculations. Each channel (Cy3 and Cy5) was normalized using the average spot signal intensity across the whole intensity. To minimize the random and systematic variations, each sample was examined twice at least by switching dye Cy5 and Cy3.

Quantitative real time RT-PCR

For the confirmation of microarray data, quantitative real-time PCR analysis was conducted to quantify the expression levels of *Afp*, *Mest,* and *Sox2* in undifferentiated ES cells and differentiated cells at 1, 7, 14, 21, and 28 days using the ABI Prizm 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) in a two-step RT-PCR. First-strand of cDNA was synthesized from 1 μg of total RNA with Superscript II reverse transcriptase (Invitrogen) as described in manufacturer's manual. PCR primers for Afp (forward: AACTCTGGCGATGGGTGTTTA, reverse: AC ACTGATGTCTTTCCACTCCA), Mest (forward; GGCTG ACCCTGAGGTTCCAT, reverse; TGATGTGGTCTCGGC TTGTC) and Sox2 (forward; GCGGAGTGGAAACTTTT GTCC, reverse; CGGGA AGCGTGTACTTATCCTT) were designed with Primer Express software version 2.0. PCR amplification was performed with SYBR Green PCR Core Reagents kit (Applied Biosystems) as described in manufacturer's manual. The mRNA abundances were determined by normalization of the data to the expression levels of glyceraldehydes-3-phosphate dehydrogenase mRNA, and represented by $\log_2$ ratio of the normalized values in samples to those in undifferentiated ES cells. Pearson correlation coefficient between PCR data and microarray data was calculated to validate microarray experiments.

Microarray data analysis

Raw data set that had been preprocessed via GenePix Pro v3.0 was imported into BRB Array Tools version 3.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) and then filtered to exclude spots based on channel intensity, flag values, spot size and/or missing values. The expression level of each gene was represented by a relative ratio of its intensity in the differentiated samples to that in the undifferentiated reference. The intensity ratio values were transformed into $\log_2$ for further analysis. To determine the distribution pattern of gene expression levels between two adjacent time points, scatter plot analysis has been conducted using all genes that have been filtered in. Only genes whose expression levels were different more than 2-fold from those of undifferentiated ES cells at least at one time point has been considered as differentially regulated genes. The temporal expression patterns of the differentially regulated genes were analyzed using the combination of K-mean clustering methods. The known genes in the differentially regulated genes were classified into functional categories based on Gene Ontology system and published reports.

RESULTS

Overview of characteristics of transcriptional change during differentiation of ES cells on monolayer culture

To characterize the differentiation events occurring in ES cells on monolayer culture, overall gene expression changes were measured at 1 day, 7, 14, 21, and 28 days after induction of differentiation by the withdrawal of LIF from culture media. For analysis of differentially regulated genes, the genes whose expression levels were different more than 2-fold from those of undifferentiated ES cells at least on one time point has been considered as the differentially regulated genes. Five thousands seven hundreds two genes of 33,075 cDNAs on the microarray were differentially regulated during 28 days differentiation periods. The percentage of differentially regulated genes increased from 0.09% on 1 day to 17.17% on 28 days (Fig. 1). While the percentage of up-regulated genes increased linearly from 1 day (0.06%) to 28 days (13.09%), the percentage of down-regulated genes increased from 1 day (0.03%) to 14 days (4.37%) and then did not increase any more on 21 and 28 days.

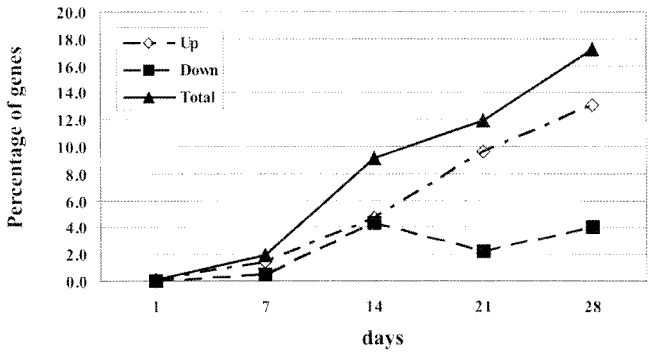

Fig. 1. Temporal occurrence of the differentially expressed genes during spontaneous differentiation of HM1 ES cells in the monolayer culture system. Up; the genes expressed 2 fold or greater than in undifferentiated HM1 ES cells on each time points, Down; the genes expressed 2 fold or less than in undifferentiatied HM1 ES cells, Total; 2 fold or greater up- or down-regulated genes.

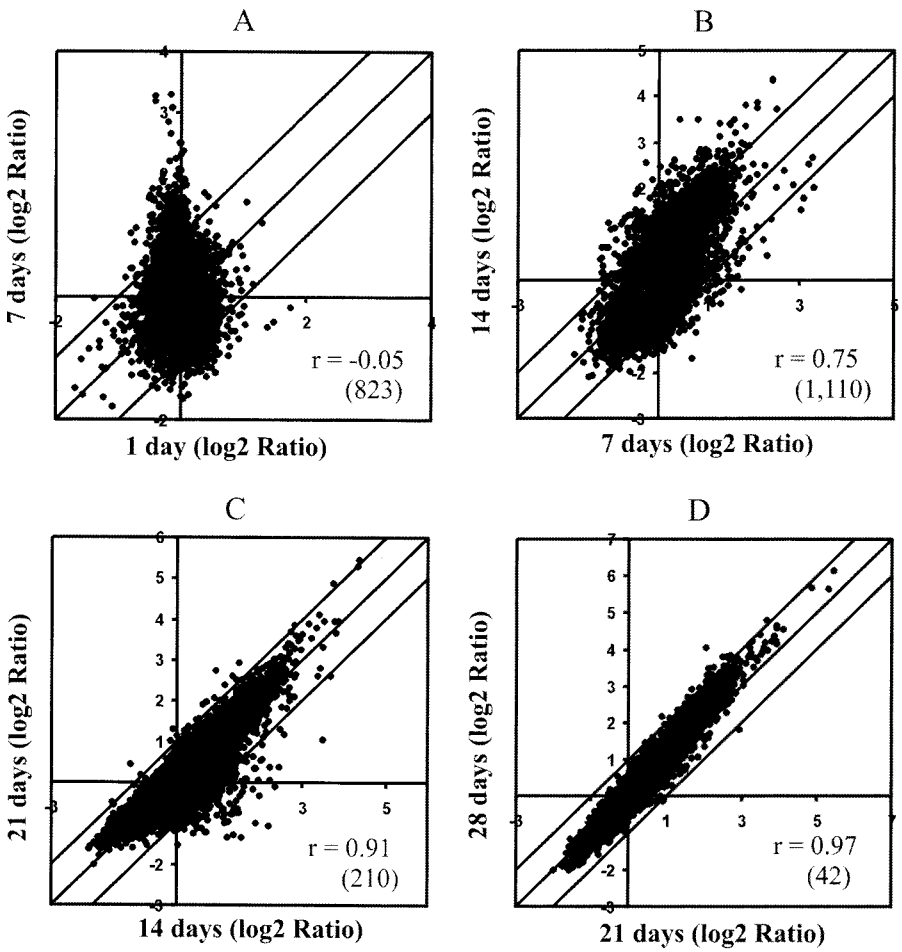

Fig. 2. Scatter plot analysis of log_2-transformed gene expression data between two adjacent time points (A; 1 day vs. 7 days, B; 7 days vs14 days, C; 14 days vs. 21 days, D; 21 days vs. 28 days). Each point represents the normalized expression level of an individual gene within mRNA populations of two adjacent time points. The upper or bottom line represents the range of a 2-fold difference between two adjacent time points. Numbers in parenthesis indicates the number of genes that were differentially expressed 2 fold or greater between two adjacent time points. r is correlation coefficient.

Scatter plotting analysis of the gene expression levels between adjacent time points revealed the gradual changes of gene expression patterns during spontaneous differentiation (Fig. 2). The scatter plat of 1 day vs 7 days displayed almost oval shape (Fig. 2A), indicating that the distribution pattern of gene expression levels on 1 day was not related (r=−0.05) to that on 7 days. 823 genes were differentially expressed more than 2 fold between 1 day and 7 days. The scatter plot of 7 days and 14 days displayed elliptic shape (Fig. 2B), indicating that gene expression pattern on 7 days was related (r=0.75) that on 14 days. 1,110 genes were more than 2 fold differentially expressed between 7 days and 14 days. In the scatter plot of 14 days and 21 days, however, many spots placed close to the diagonal line (Fig. 2C), indicating that the gene expression pattern on 14 days was tightly related (r=0.91) to that on 21 days. Two hundred ten genes were expressed differentially between 14 days and 21 days. The distribution pattern of gene expression levels on 21 days were almost identical (r=0.97) with that on 28 days except 40 genes that were expressed differentially more than 2 fold between 21 days and 28 days (Fig. 2D).

Distinct kinetic patterns in genes differentially regulated during differentiation

The 5,702 genes differentially regulated during spontaneous differentiation were classified into 7 clusters with distinct temporal expression patterns by K-mean clustering methods (Fig. 3). Of 7 clusters, six clusters displayed the up-regulated patterns with distinct kinetics and one cluster displayed the down-regulated pattern. Three clusters (Fig. 3A, 3B, 3C) consist of genes whose expression was up-regulated at 14 days. After up-regulation at 14 days, the expression of 3,175 genes in cluster A was continued to be up-regulated throughout differentiation, the expression of 122 genes in cluster B was returned to initial levels at 21 days, and the expression of 173 genes in cluster C was reached the plateau after second week of differentiation. The other three clusters (Fig. 3D, 3E, 3F) displayed the

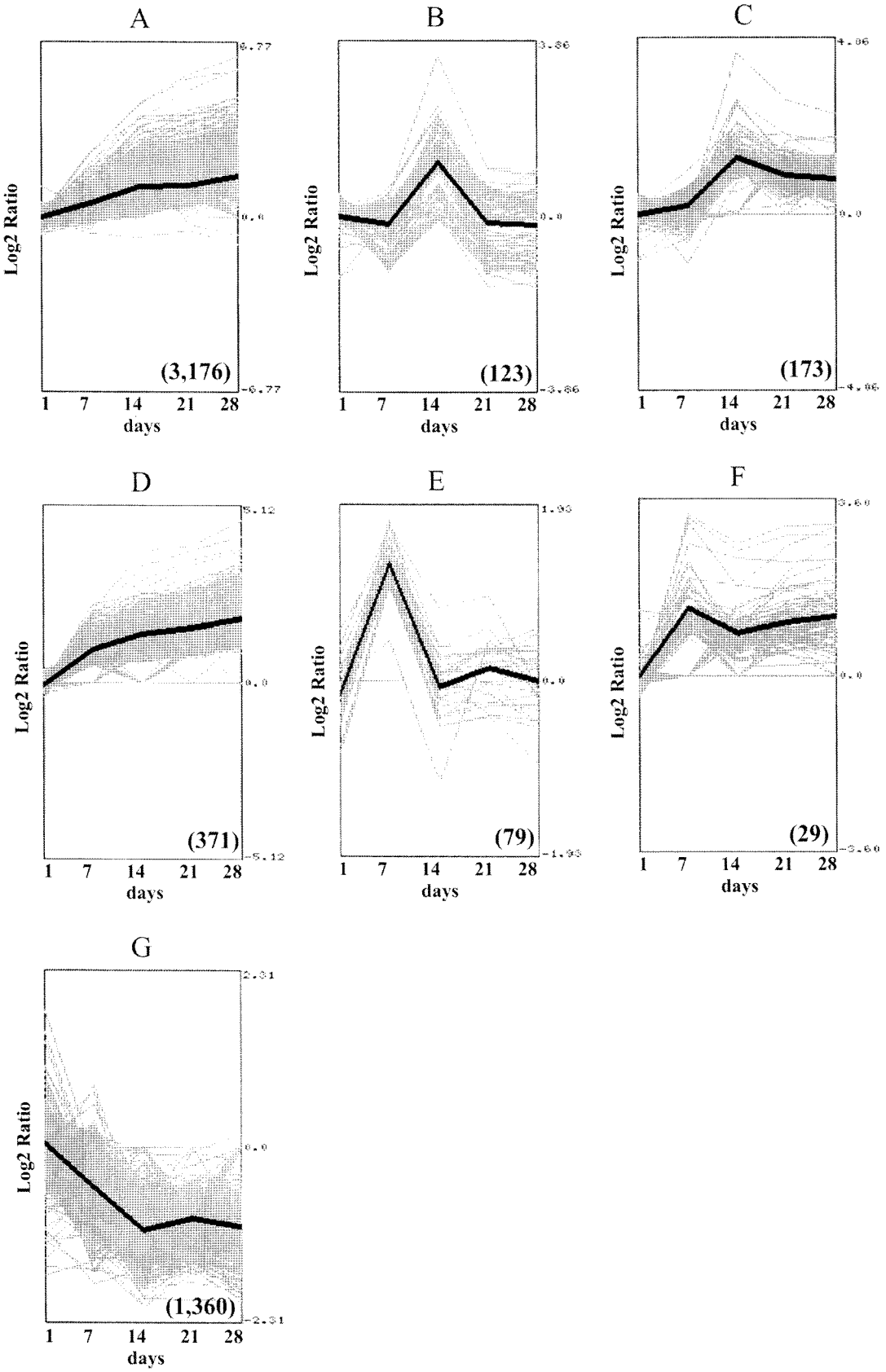

Fig. 3. Temporal cluster analysis of genes differentially expressed during spontaneous differentiation using K-means algorithms applied to the log2 values for the ratio of each gene's expression. Each cluster represents the average pattern for genes within the cluster. Numbers in parenthesis indicates the number of genes that were differentially expressed 2 fold or greater at each time point.

Fig. 4. Functional classification of the genes differentially regulated genes during spontaneous differentiation. A; apoptosis related genes, B; proliferation related genes, C; chromatin assembly or binding related genes, D; DNA replication, repair, binding or methylation related genes, E; RNA processing or binding related genes, F; cytoskeleton structure or organization related genes, G; cytokines or receptors related genes, H; ECM related genes, I; transcriptional regulation related genes, J; development related genes. The intensity of color red or green corresponds to the degree of up- or down-regulation, respectively. The first, second, third column represent gene symbol, gene bank accession number, and cluster, respectively.

expression pattern that was up-regulated at 7 days. After up-regulation at 7 days, the expression of 372 genes in cluster D was continued to be up-regulated throughout the differentiation, the expression of 29 genes in cluster E was returned to the initial level at 14 days, and the expressions of 79 genes in cluster F was remained constantly with a slight decrease at 14 days. In cluster G which displayed the down-regulated pattern, the expression levels of 1,360 genes decreased linearly by 14 days and then remained constant.

Functional classification of genes differentially regulated during differentiation

Among the 5,702 genes regulated differentially during spontaneous differentiation of ES cells in monolayer culture system, 939 genes are known genes and 4,374 cDNA clones are ESTs. To understand the process of the spontaneous differentiation, the known 939 genes were classified into functional groups, based on Gene Ontology system and published reports. The major functional groups identified in this study include apoptosis, proliferation, chromatin, DNA, RNA, cytoskeleton, ECM, cytokine, receptor, development, and transcription related genes (Fig. 4). Apoptosis related genes displayed both up- and down-regulated expression patterns (Fig. 4A). *Bnip3* and *Peg3* were up-regulated at 7 days. The expression of *Birc6*, *Pdcd4*, and *Bcl2* was up-regulated at 14 days. The expression of *Sgk*, *Perp*, *Mrps30*, *Birc5*, *Pdcd8,* and *Api5* were down-

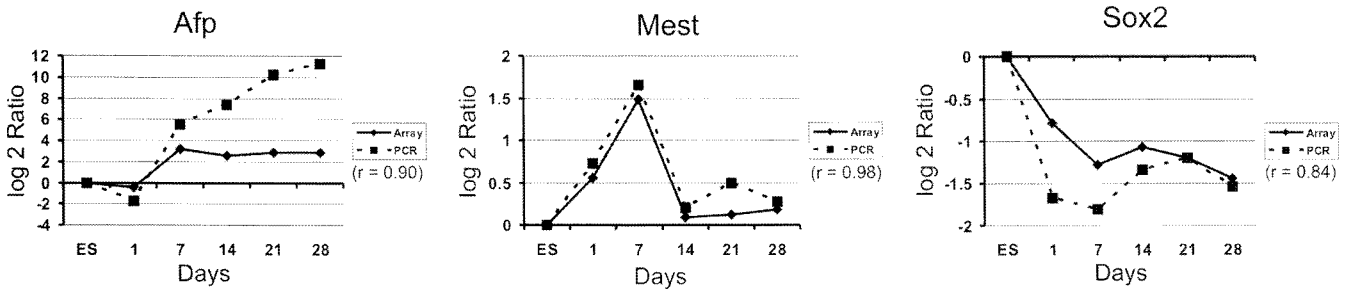

Fig. 5. Comparison of *Afp*, *Mest*, and *Sox2* expression data from real-time PCR and microarray experiments. Gene expression level in PCR was normalized to the expression level of *Gapdh* and represented as log2 ratio of those in undifferentiated ES cells (ES). r is Pearson correlation coefficient between PCR data and microarray data.

regulated at 7 days or 14 days. The genes that are related to proliferation such as *Ccnd2*, *Cdkn1b*, *Cdc25b*, *Cdc25c*, and *Mdm2* were up-regulated at 14 days (Fig. 4B). The expression of *Cul1*, *Cdkap1*, *Hdac1*, *Ccne1*, *Ccnb1*, *Pa2g4*, *Gspt1*, *Phb*, *Brd4*, and *Cdk4* were down-regulated at 7 or 14 days. The chromosome assembly or binding related genes such as nucleosome assembly proteins (*Napl1l*, *Napl1l2*), chromobox homolog (*Cbx1*, *Cbx2*, *Cbx5*), and histone family (*H3f3a*, *H2afz*) were mostly down-regulated at 14 days (Fig. 4C). Also, most of DNA replication, repair or binding related genes was down-regulated during spontaneous differentiation (Fig. 4D). Most of RNA processing, binding, or splicing related genes were down-regulated at 14 days (Fig. 4E). Most cytoskeleton related genes such as actins (*Acta1*, *Acta2*, *Actb*, *Actg*, *Actn4*), capping proteins (*Capza2*, *Capzb*), chaperonin subunits (*Cct3*, *Cct6a*, *Cct8*), *Dnclc1*, myosins (*Myln*, *Mylpf*), *Sdc1*, tubulin (*Tuba2*, *Tuba6*, *Tuba8*, *Tubb5*), and utrophin (*Utrn*) were down-regulated at 14 days (Fig. 4F). However, the expression of kerain complexes (*Krt1-18*, *Krt2-7*, *Krt2-8*) increased 2 fold or greater at 7 days and then returned to the undifferentiated expression levels at 14 days. In cytokine and receptor related genes, the expression levels of *Igf2*, *Afp*, *Adrb2*, and *Tgfbr1* were up-regulated at 7 days (Fig. 4G). While the expression levels of *Tgfa*, *Vegfa*, *Fzd2*, *Fzd4*, *Ifngr2*, *Igfbp4*, *Il2ra*, *Tnfrsf 19*, and *Tnfrsf 22* were up-regulated at 14 days, the expression levels of *Bmp1*, *Fin14*, *Hdgf*, *Mif*, *Fgfr2*, *Lamr1*, *Pdgfc*, and *Lrp2* were down-regulated during spontaneous differentiation. The expression of *Ctgf*, *Epha4*, *Csf3r*, and *Tccr* were induced 2 fold or greater at 21 days. The expression of *Tgfbr3* was induced at 14 days and reached to more than 2 fold increase at 28 days. Most ECM related genes displayed the up-regulated pattern during spontaneous differentiation (Fig. 4H). The expression of *Col1a1*, *Cola2*, *Col2a1*, *Col3a1*, *Col4a1*, *Col4a2*, *Col5a2*, *Osf2-pending*, *Sgce*, and *Sparc* were up-regulated at 7 days. The expression of *Lama1*, *lamb1-1*, *Lamc1*, *Nid1*, *Prg*, *Psap*, *Spt1*, *Dyt1*, *Fga*, *Bgn*, and *Cdh5* were up-regulated at 14 days or 21 days. In the transcription regulation related genes, the expression of *Klf4*, *Zfp42*, *Sox2*, *Klf5*, *Crtr1-pending*, and *Nanog* were down-regulated at 1 day (Fig. 4I). Transcription related genes that were down-regulated at 7 days were *Myc*, *Mybl2*, *Tfdp1*, *Trp53*, *Klf9*, *Pou5f1*, *Eed*, *Sirt1*, *Rest*, *Sall1*, *Ifrd1*, *Trim28*, and *Gtf2h1*. The expression of *Nrbf2*, *Zfp216*, *Lmo4*, *Mybbp1a*, *Edf1*, *Klf13*, *Nsep1*, *Sox1*, and *Zfp162* were down-regulated at 14 days. Among the activated transcription related genes, *Fos*, *Meis1*, *Mrg1*, *Zfp110*, and *Psx1*, *Nr2f2* and *Nrip1* were up-regulated at 7 days, and *E2f1*, *Rb1*, *Hoxc4*, *Hoxa5*, *Zfp46*, *Foxc1*, *Arx*, *Nkx6-1*, *Pou3f4*, *Mesp2*, *Gata6*, *Tcfe3*, and *Msc* were up-regulated in expression at 14 days and retained to be up-regulated throughout the remained time course. During the spontaneous differentiation in the monolayer culture system, the development-related genes that were up-regulated at 7 days were *Tagln*, *Mest*, *Psg19*, and *Cryab* (Fig. 4J) The expression of *Gna13*, *Qk*, *Sept3*, *Tesc*-pending, *Madcam1*, *Tm7sf1*, *Dyrk1a*, *Fmn2*, *Hpca*, *Ndr2*, *Gdap1*, *Cdh11*, *Tnni2*, *Ptn*, *Ttr*, *Gata6*, and *Smo* were up-regulated at 14 days or later. In the down-regulated development-related genes, *Edr1*, *Prei3*, *Rab23*, *Nrg3*, *Stmn2* and *Efnb2* were down-regulated at 7 days, and the other development-related genes were down-regulated at 14 days, which include *Narg1*, *Naca*, *Rex3*, *Itga7 Itgb1*, *Nedd4*, *Socs2*, *Slit2*, *Nif3l1*, *Ntn3*, *Nte*, *Nudc*, *Neurl*, and *Odz3*.

To verify the microarray data, we conducted real-time PCR for *Afp*, *Mest*, and *Sox2* at five different time points (Fig. 5). There was high correlation (r=0.84~0.98) between PCR and microarray data, which indicates that gene expression data from microarray were an accurate reflection of the transcription levels.

DISCUSSION

Unlike the previous studies in which microarray has been used to compare or find specific genes related to differentiation of ES (Kelly et al., 2000; Tanaka et al., 2002; Zhu et al., 2007; Mansergh et al.,2009, we characterized time-sequential *in vitro* differentiation by the functional classification of overall genes differentially expressed

during spontaneous differentiation of ES cells in monolayer culture. Our study revealed that the 5,702 genes were differentially regulated during 28 days differentiation of mouse ES cells in monolayer culture, and most genes were differentially regulated at 7 and 14 days, which reflect that most dramatic changes in the ES cells differentiation occurs during this period.

Several distinct kinetic patterns in the gene expression have been observed in the temporal cluster analyses of changes in global gene expression during hepatic differentiation (Plescia et al., 2001), trophoblast differentiation (Aronow et al., 2001), and neural differentiation (Wei et al., 2002), which may reflect the existence of the distinct phases in differentiation of different cell lineages. Our study also revealed that the 5,702 differentially regulated genes during the spontaneous differentiation were grouped into 7 distinct temporal kinetic patterns. Most interestingly, the down-regulated genes showed single kinetic pattern in which most dramatic changes of expression occurred at 7 days and 14 days of differentiation, while the up-regulated genes were subdivided into complicated six different kinetic patterns determined by the expression patterns after the 14 days of differentiation. It might indicate that the loss of stemness of ES cells is largely associated with the repression of gene expression that maintained pluripotent stemness at the initial stage of differentiation, while commitment of differentiation into various lineages is largely associated with activation of genes regulated with different kinetic patterns at later stage of differentiation.

When knowledge-based annotation of genes were applied, we noticed that certain functional groups displayed synchronized unilateral (either up- or down-regulation) expression pattern, while other functional groups showed mixed (both up- and down-regulation) gene expression patterns. These data suggest that unilateral expression pattern in certain biological functions may play an important role in the progress of differentiation. While apoptosis related genes displayed mixed expression pattern, most of proliferation, cell cycle, chromatin assembly and binding, DNA replication and repair, RNA processing and splicing, and cytoskeleton organization related genes were down-regulated, which might result in the overall inhibition of cell growth during the spontaneous differentiation of ES cells. Likewise, the down-regulation of cell cycle, DNA damage and cytoskeleton organization related genes has been demonstrated in microarray analysis of trophoblast differentiation (Aronow et al., 2001), and the retinoic acid-induced differentiation of ES cells also resulted in the down-regulation of cell cycle regulator such as cyclin E1 and prothymosin alpha (Kelly et al., 2000). Therefore, the inhibition of cell growth during the spontaneous differentiation of ES cells might be determined by the inhibition of proliferation rather than the activation of apoptosis. Furthermore, the repression of cytoskeleton organization related genes might be associated with the inhibition of cell growth during the spontaneous differentiation of ES cells.

It has been known that ECM plays an important role in regulating differentiation and development in collaboration with growth factors (Kelly et al., 2000). Furthermore, the ECM constituents can modulate the shape of cells in culture (Watt, 1986) and determine the developmental fate of differentiating pluripotent of stem cells (Kelly et al., 2000). Taken together these data suggest that changes in ECM composition and organization have profound effects on cell differentiation. In the present study, most of ECM related genes were up-regulated with distinct kinetic expression patterns during the spontaneous differentiation. Interestingly, while a family of procollagens (*Col1a1, Col1a2, Col2a1, Col4a1, Col4a2,Col5a2, Col3a1*) was up-regulated at 7 days, most of ECM components expressed during development in a variety of tissues such as *Bgn* (Scholzen et al., 1994), *Cdh5* (Breier et al., 1996), *Lamc1* and *Nid1* (Magnaldo et al., 1995) were up-regulated mainly at 14 or 21 days, which suggests that the up-regulation of a variety of ECM molecules might be associated with the appearance of different morphological patterns during the spontaneous differentiation.

The present study revealed that among several cytokines and receptors that were differentially regulated during spontaneous differentiation, most of the up-regulated genes were receptors rather than cytokines during the spontaneous differentiation, which suggests that the major signaling pathways are induced by the up-regulation of receptors rather than cytokines. The up-regulation of *Tgfbr1* and *Tgfbr3* at 7 days and of *Fzd2* and *Fzd4* at 14 days might result in the activation of Smad and Wnt pathway, respectively, which have profound effects on the process of differentiation. *Vegf* has a direct role in chondrocyte maturation and the osteoblastic activity regulation (Zelzer et al., 2002), and *Ctgf* is a crucial regulator of cartilage extracellular matrix remodeling during chondrogenesis (Ivkovic et al., 2003). *Tgfb*, a multifunctional cytokine, plays an important role in stimulating the synthesis of individual matrix components including proteoglycans, collagens and glycoproteins (Noble et al., 1992). Therefore, the up-regulation of these growth factors may influence on the development of ECM during the spontaneous differentiation.

Interestingly, the earlier repression of transcription regulation-related genes suggests that the initial events of the spontaneous differentiation are induced by the repression rather than activation of transcription regulation-related genes. It has been demonstrated that the expressions of Klf4 (Kelly et al., 2000), *Zfp42* (Hosler., 1989), *Sox2* (Avilion et al., 2003), and *Nanog* (Chambers et al., 2003) are high in

the undifferentiated pluripotent cells and low during the differentiation, which is concomitant with the present results showing the early down-regulation in the expression of *Klf4, Zfp42, Sox2,* and *Nanog* during the spontaneous differentiation. Therefore, the early down-regulation of transcription regulation-related genes such as *Klf4, Zfp42, Sox2* and *Nanog* might be responsible for the initiation of ES cell differentiation into multiple lineages. Interestingly, while the transcription regulator related to differentiation or development including *Klf9* (Imhof et al., 1999), *Pou5f1* (Shimozaki et al.,2003), *Rarg* (Kastner et al., 1990), *Sall1* (Ott et al., 2001), *Sirt1* (McBurney et al., 2003), *Trim28* (Cammas et al., 2002), *Etv1* (Arber et al., 2000), and *Eed* (Morin-Kensicki et al., 2001) were down-regulated mostly at 7 days, the transcription regulator related to the tissue specific development including *Mesp2* (Normura-Kitabayashi et al., 2002), *Meis1* and *Mrg1* (Zhang et al., 2002), *Psx1* (Han et al., 1998), *Arx* (Kitamura et al., 2002), Msc (Lu et al., 2002), *Nkx6-1* (Watada et al., 2000), and Gata6 (Morrisey et al., 1998) were up-regulated mainly at 7, 14 or 21 days, which might be responsible for the determination of cell fate into tissue specific lineages.

We also observed that the group of the development related genes displayed both of up-and down-regulated expression patterns. The activation or repression in the expression of the development related genes was mainly induced at 14 days, which reflect that most developmental decision might occur in this period. Most of them were the genes that are involved in the development of different tissues, which indicate that the complex and heterogeneous cell populations are developed during the spontaneous differentiation in the monolayer culture system. It is notable that among development related genes up-regulated at 7 or 14 days, *Mest* (King et al., 2002), *Tagln* (Li et al., 1996), *Cdh11* (Kimura et al., 1995), *Tnni2* (Koppe et al., 1989), might be involved in the mesoderm lineage development. *Ttr* (Makover et al., 1989) and *Gata6* (Morrisey et al., 1998) that are related to endoderm development were significantly up-regulated at 21 days. This result might indicate earlier development of mesoderm lineage than of endoderm lineage during spontaneous differentiation. Most of genes that were down-regulated at 14 days in the present study were the genes that are involved in the neural differentiation or neurogenesis, including *Nrg3* (Zhang et al., 1997), *Stmn2* (Okazaki et al., 1993), *Socs2* (Turnley et al., 2002), *Slit2* (Piper et al., 2002), which indicate that most neuro-ectoderm development might be repressed during the spontaneous differentiation in the monolayer culture system. Although ES cell has pluripotency to give rise to many cell types, the lineage-specific differentiation of ES cells might be influenced by a variety of culture conditions (Nishikawa et al., 1998; Keller et al., 1995; Rathjen and Rathjen, 2001). Therefore, the monolayer culture system used in the present study might lead to predominant mesoderm lineage and late endoderm lineage developments during the spontaneous differentiation.

This study characterized the differentiation of mouse ES cells in the monolayer culture system by functional classification of global gene expression profiles. Our results indicate that the activation of genes related to ECM was an early event, followed by the inhibition of proliferation and lineage specification during in vitro differentiation of ES cells in monolayer culture. Furthermore, a predominant mesoderm development, the late appearance of endoderm lineages, and the repression of neuro-ectoderm lineage development might occur during ES cell differentiation in monolayer culture. These results might provide a useful reference to identify time-points for applying selective treatments for manipulation of ES cells differentiation.

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