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Temporal Expression Changes During Differentiation of Neural Stem Cells Derived From Mouse Embryonic Stem Cell

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Abstract Temporal analysis in gene expression during differentiation of neural stem cells (NSCs) was performed by using in-house microarrays composed of 10,368 genes. The changes in mRNA level were measured during differentiation day 1, 2, 3, 6, 12, and 15. Out of 10,368 genes analyzed, 259 genes were up-regulated or down-regulated by 2-fold or more at least at one time-point during differentiation, and were classified into six clusters based on their expression patterns by K-means clustering. Clusters characterized by gradual increase have large numbers of genes involved in transport and cell adhesion; those which showed gradual decrease have much of genes in nucleic acid metabolism, cell cycle, transcription factor, and RNA processing. In situ hybridization (ISH) validated microarray data and it also showed that Fox M1, cyclin D2, and CDK4 were highly expressed in CNS germinal zones and ectonucleotide pyrophosphatase/phosphodiesterase 2 (Enpp2) was highly expressed in choroid plexus where stem/progenitor cells are possibly located. Together, this clustering analysis of expression patterns of functionally classified genes may give insight into understanding of CNS development and mechanisms of NSCs proliferation and differentiation. J. Cell. Biochem. 93: 563–578, 2004.
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Key words: microarray; proliferation; in situ hybridization; germinal zone; choroid plexus

Neural stem cells (NSCs) are multipotent and self-renewing cell population able to generate the three major central nervous system (CNS) lineages neurons, astrocytes, and oligodendrocytes. Therefore, NSCs is thought of as a reservoir with the potential to become any one of the thousands of cell types within the CNS [McKay, 1997; Ross et al., 2003].

Analyses of a variety of mammalian model systems have identified helix-loop-helix (HLH) transcription factors as a regulator of early differentiation of NSCs. The different classes of HLH factors cross-regulate one another to mediate the effect of several signaling systems on a large number of target genes. HLH transcription factors participate at different stages of NSC differentiation, such as formation of progenitor cells, initiation of differentiation, cell fate determination, neurite outgrowth, and synaptogenesis, and the timing of differentiation is regulated by the balance of these factors [Morrison, 2001; Ross et al., 2003].

The signaling molecules that regulate the transition of NSCs from proliferation to differentiation are beginning to be identified, with several growth factors, including basic fibroblast growth factor, epidermal growth factor, brain-derived neurotrophic factor, and notch ligands [Vescovi et al., 1993; Zigova et al., 1998; Benraiss et al., 2001; Caldwell et al., 2001]. In addition to these factors on the stem cell proliferation and differentiation, identification of other factors will advance the understanding of CNS development and the potential for the use of stem cells as therapeutic agents [Karsten et al., 2003].

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To identify regulatory genes that are involved in NSCs proliferation and differentiation, we have performed in-house-made cDNA microarray, which allows simultaneous assessment of the expression of potentially thousands of genes. Recently, some microarray analyses were performed to investigate the molecular mechanism underlying NSC proliferation and pluripotency [Geschwind et al., 2001; Luo et al., 2002]. However, most genes identified in these microarrays do not overlap with each other. Elucidation of common genetic factor in various stem cell populations is hampered by different experimental design and analysis. To highlight conserved genes expressed during differentiation in NSCs, we compared list of genes identified in this embryonic stem cell (ES)-derived NSCs population with genes identified in other stem cell populations, such as primary neural progenitor cells [Karsten et al., 2003].

MATERIALS AND METHODS

ES Cell-Derived Neural Stem Cells and Differentiation

The NSCs were generated through five-stage method as described [Lee et al., 2000]. Nestin-positive cells (NSCs, stage 4) were expanded for 4 days. The medium was changed every 2 days. Differentiation (stage 5) was induced by removal of bFGF. The cells were incubated under differentiation conditions for 1, 2, 3, 6, 12, and 15 days. The total RNA of each time-point was extracted using Tri-Reagent (MRC, Inc., Cincinnati, OH) according to the manufacturer's instructions.

Microarray Fabrication and Hybridization

A total of 10,368 cDNA clones were spotted onto SuperAldehyde slides (Telechem, Sunnyvale, CA) using a MicroGrid TAS arrayer (Genomic solutions, Huntingdon, United Kingdom) as previously described [Lee et al., 2002]. The microarray consisted of 6,531 clones from the National Institute of Aging (NIA), 1,243 clones from the Brain Molecular Anatomy Project (BMAP), 2,060 clones from Incyte, and houskeeping genes and yeast DNA as negative controls.

Probes were made from 2 μg of total RNA from NSCs (stage 4) for control and 2 μg of RNA from each differentiated cells RNA (stages 5), using a dendrimer labeling kit (3DNA 350RP; Genisphere, Hatfield, PA). For each experiment, at least four replicates were performed, and two of these were repeated with the fluorophores reversed to eliminate false-positive results.

Scanning and Data Analysis

The two fluorescent images from each slide were scanned separately using a GMS 418 scanner (Affymetrix, Santa Clara, CA). The images were analyzed using ImaGene 4.2 (Biodiscovery, El Segundo, CA) and MAAS (Gaiagene, Seoul, Korea) software. Cy3:Cy5 intensity ratios from each gene were calculated and subsequently normalized with nonlinear lowess normalization. To filter out unreliable data, spots with signal-to-noise ratios below 2 were flagged. The non-flagged genes in at least three out of four experiments were used for further analysis. We took the median value from the gene expression ratio of each of four independent experiments and selected genes whose expression level differed from their mean expression level by at least 2-fold at one or more differentiation time. K-means clustering was applied to genes using the Euclidean distance as a similarity measurement, as implemented in the software program Genesis (http://genome.tugraz.at, Sturn et al. [2002]). Genes showing more than a 2-fold difference in expression level were divided into categories according to their molecular function using the classification scheme in Gene Ontology™. To examine statistical significance for frequencies of genes of each functional group in each cluster, two-sided Fisher's exact test was performed.

Immunocytochemistry

The cells were grown on polyornithine (15 μg/ml, Sigma, St. Louis, MO)/fibronectin (1 μg/ml, Sigma) coated coverslips and fixed with 4% paraformaldehyde/0.15% picric acid in 0.1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS). Subsequently, the cells were washed three-times with BSA/PBS and permeabilized with Triton X-100/PBS. The cells were incubated for 12 h at 4°C with polyclonal nestin antibodies (1:50) (Matha Marvin and Ron McKay, National Institute of Health, Bethesda, MD), polyclonal neuron-specific class III β-tubulin (Tuj1) antibodies (1:2,000) (Babco, Richmond, CA) and polyclonal glial fibrillary acidic protein (GFAP) antibodies (1:400) (DAKO, Glostrup, Denmark). For detection of primary antibodies, fluorescence labeled (FITC or Cy3) secondary antibodies (Jackson Immunore-

search Laboratories, West Grove, PA) were used according to the specifications of the manufacturer. Cells were mounted in Vectashield containing 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and analyzed under an epifluorescence microscope (Nikon, Tokyo, Japan).

Semi-Quantitative and Real-Time Quantitative RT-PCR

For semi-quantitative and real-time quantitative RT-PCR, beta-actin was used as an internal control with the following primer sets; 5′-ctt tat ggt gtg gtc gca ga-3′ and 5′-tca ggg tag tca gcc atg tg-3′. For each sample from each time-point, the same total RNA used for cDNA microarray hybridization was reverse-transcribed using 1 μM oligo (dT) primer with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The thermal profiles consisted of 95°C for 5 min for initial denaturing, followed by 25–30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. All RT-PCR reactions were repeated at least three-times. Triplicate real-time RT-PCR experiments were performed using iCycler iQ system (Bio-Rad, Hercules, CA) and the SYBR Green I dye (Molecular probes, Eugene, OR). A melting curve was obtained for each PCR product after each run to confirm that the signal corresponded to a unique amplicon of the product size. The following primers were used: 5′-ggagtgtcgcttagaggtgc-3′ and 5′-tccagaaagccaagagaagc-3′ for nestin; 5′-tgtcagaggagcccgaggtc-3′ and 5′-ccaagagcagcccatcaaag-3′ for tyrosine hydroxylase (TH); 5′-aaggtagccgtgtgtgacatc-3′ and 5′-accaggtcattcatgttgctc-3′ for Tuj1; 5′-ccaaactggctgatgtctacc-3′ and 5′-gcttcatgtgcctcctgtcta-3′ for GFAP; 5′-tccctacataacgggagcag-3′ and 5′-aactcaggccaagcgataga-3′ for insulin-like growth factor 2 (Igf2); 5′-ttttcatcttggcagctgtg-3′ and 5′-acactccactgccattctcc-3′ for pleiotrophin; 5′-ttccccagaagcgaaatatg-3′ and 5′-tgaccccattcctttctgac-3′ for ectonucleotide pyrophosphatase/phosphodiesterase 2 (Enpp2); 5′-gatgatttccgagggagaca-3′ and 5′-catgaggaatgtcagccaga-3′ for *N*-myc downstream regulated 2 (Ndr2); 5′-gaggaaacagcaccttcagc-3′and 5′-aggcaatgtctccttgatgg-3′ for forkhead box M1 (FoxM1); 5′-ggaagagactgttggaagagga-3′and 5′-ctgataagcccaggctagaaga-3′ for thymopoietin.

In Situ Hybridization

ISH were performed on E13.5, E18.5, P0, P7, and P14 BL6 mouse brains and embryos sectioned at 12 μm. Sections were thaw-mounted onto gelatin-coated slides, fixed in 4% paraformaldehyde, treated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8.0), dehydrated and defatted in ethanol and chloroform, and finally air-dried. Transcription of antisense probes was carried out using the Riboprobe (Promega, Madison, WI) in the presence of α-^{35}S-UTP (Amersham, Buckinghamshire, England). Sections were hybridized overnight at 53°C with 5×10^5 cpm of labeled probe per slide, treated with RNase A (20 mg/ml, Boehringer-Mannheim, Mannheim, Germany) for 30 min at 25°C, washed sequentially for 60 min in 2× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.2) at 50°C, 60 min in 0.2× SSC at 55°C, and 60 min in 0.2× SSC at 60 × °C, briefly rinsed in a graded series of ethanol containing 0.3 M ammonium acetate and dried. Hybridized radioactivity was visualized after 6 days' exposure using-max film (Amersham, Arlington Heights, IL).

RESULTS

Generation and Differentiation of Neural Stem Cells

To generate NSCs, we adopted lineage selection (five-stage) method that leads to the efficient selection and proliferation of NSCs from ES cell. We performed immunocytochemistry to evaluate generation and differentiation of NSCs. As shown in Figure 1A, a majority of cells in the stage 4 were labeled with nestin, which is commonly used marker for NSCs. After 6 day of bFGF removal, differentiated cells were immunostained with Tuj1 and GFAP which is specific marker for neurons and astrocytes, respectively. Furthermore, morphological changes were observed. The NSCs began extension of neurite-like structures at day 2 after removal bFGF. The number and length of neurite-like structures were increased as the differentiation time of cultures evolves (Fig. 1B). We also performed semiquantitative RT-PCR analysis for neuronal and glial phenotypic genes (Fig. 1C). Nestin showed little expression since differentiation day 12. Tuj1 and GFAP were gradually increased during differentiation. Additionally, TH was also gradually expressed, because our culture method generates dopaminergic neurons in high yield [Lee et al., 2000].

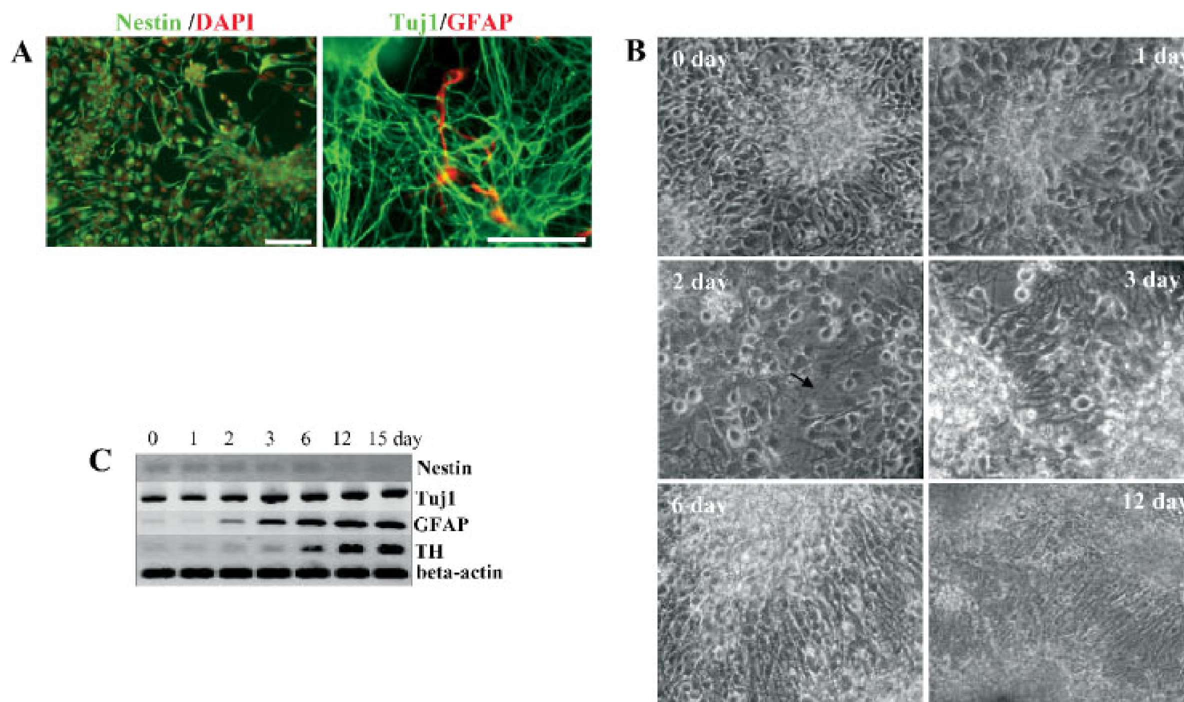

Fig. 1. Differentiation of neural stem cells (NSCs). **A**: Immunocytochemistry for NSCs, neurons, and astrocytes. Most cells (DAPI nuclear staining, red) at stage 4 were immunostained with the intermediate filament nestin (green) which is marker of NSCs. Differentiation of NSCs was induced by withdrawal of bFGF. After 6 day of bFGF removal, differentiated cells were immunostained with Tuj1 (green) and GFAP (red). Scale bar, 20 μm. **B**: Phase-contrast images illustrate the morphological changes during differentiation of NSCs. The neurite-like structures were first detected at differentiation day 2. Arrow indicates the neurite-like structures at day 2. **C**: The generation and differentiation of NSCs were further validated by RT-PCR analysis for phenotypic genes (Nestin, Tuj1, GFAP, and TH). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Identification of Significantly Expressed Genes

To examine the temporal changes in gene expression during differentiation comprehensively, we performed gene expression profiling. Total RNA derived from differentiated cell at 1, 2, 3, 6, 12, or 15 days and the control (NSCs) were subjected to cDNA synthesis. Both cDNAs were subsequently mixed and hybridized with a microarray. After hybridization, specific Cy3 and Cy5 dye labeling for each cDNA (NSCs vs. differentiating cells) were carried out in chips. The flagged genes at more than one time-point were not subjected to further analysis (K-means clustering). The data set of differentially expressed genes including missing value is available at; http://neurogenomics.hanyang.ac.kr. Out of the 10,368 genes represented on the chip, 259 genes were up- or down-regulated 2-fold or more at least at one time-point during the differentiation (Table I).

To determine the validity of results obtained by the microarray analysis, six randomly selected genes (*Igf2*, *pleiotrophin*, *thymopoietin*, *Enpp2*, *FoxM1*, *Ndr2*) were subjected to real-time quantitative PCR analysis. These expression pattern observed by PCR showed a good concordance with the pattern assayed by microarray (Fig. 2).

Temporal Cluster Analysis

To characterize patterns of transcripts expression during the time course of differentiation, K-means clustering was performed on the 259 genes, and these genes were classified into six clusters (Fig. 3). The temporal cluster analysis revealed that four clusters of transcripts whose the expression was either upregulated (cluster A, 26 genes and B, 75 genes) or down-regulated (cluster D, 71 genes and E, 39 genes). Cluster F (45 genes) showed gradual increase by day 6 and slight decrease on day 15. Cluster C represented only three genes (*RIKEN cDNA 1700011I03* gene, *exportin 4*, *BRF2*) that were markedly decreased by day 3, but returned to undifferentiated level on day 12 and increased at day 15.

TABLE I. Significantly Expressed Genes During Differentiation of NSCs

No.	Cluster	Fold change (logarithm, base 2)						GenBank	Title	Result of Karsten et al. [2003]
		1 day	2 day	3 day	6 day	12 day	15 day			
1	A	−0.029	0.040	−0.089	0.461	1.691	1.325	BG073613	Insulin-like growth factor 2	
2	A	−0.031	0.047	0.521	0.875	2.129	2.065	AA444490	Tissue inhibitor of metalloproteinase 2	Up-regulated in DC
3	A	−0.146	0.105	0.148	0.331	1.877	1.874	BG069465	Cd63 antigen	
4	A	−0.196	0.008	0.199	0.494	1.767	1.603	BG086970	Glutathione *S*-transferase, mu 1	
5	A	0.070	0.117	0.207	0.804	2.186	2.005	BG074103	H19 fetal liver mRNA	
6	A	0.295	0.070	0.285	0.973	1.365	1.376	BG079167	Zinc finger protein 42	
7	A	0.354	0.604	0.853	0.604	1.190	1.461	BG080229	Aldolase 3, C isoform	Up-regulated in DC
8	A	0.101	0.299	0.640	0.821	2.478	2.179	AA063753	ATP-binding cassette, sub-family A (ABC1), member 1	
9	A	−0.228	0.410	0.851	1.013	2.459	2.203	BG070737	Solute carrier family 1, member 3	
10	A	−0.158	−0.135	−0.116	0.125	1.600	1.664	BG087410	CD9 antigen	
11	A	−0.059	0.238	0.190	0.595	1.789	1.610	BG072683	Prion protein	Up-regulated in DC
12	A	−0.130	0.041	0.359	0.591	1.782	1.786	W83974	Carboxypeptidase E	Up-regulated in DC
13	A	0.038	0.330	0.569	1.044	1.397	1.182	AW060338	Gamma-aminobutyric acid receptor, subunit beta 3	
14	A	0.040	0.228	0.433	1.087	1.839	1.587	BG077733	ATPase, Na+/K+ transporting, beta 1 polypeptide	
15	A	0.063	−0.108	0.431	0.494	1.410	1.416	AA518639	Aldolase 1, A isoform	
16	A	−0.132	−0.035	0.107	0.662	1.624	1.401	BG085991	Integral membrane protein 2B	
17	A	0.242	0.045	0.274	0.546	2.186	1.956	AI854310	Cocaine and amphetamine regulated transcript	
18	A	0.499	0.279	0.140	1.021	1.549	1.609	BG076160	Membrane-spanning 4-domains, subfamily A, member 6D	
19	A	−0.018	0.052	−0.153	0.264	1.637	1.776	W83447	Serine (or cysteine) proteinase inhibitor, clade A, member 3G	
20	A	0.138	−0.275	0.042	0.262	2.151	2.305	W78651	Cystatin C	Up-regulated in DC
21	A	−0.007	0.626	0.109	0.864	1.670	1.591	AI853802	Phosphofructokinase, platelet	
22	A	−0.066	0.508	0.917	1.295	1.788	1.838	AI844824	Endothelin receptor type B	
23	A	−0.037	−0.040	0.088	0.809	1.565	1.535	BG070501	Glutathione *S*-transferase, mu 2	
24	A	−0.451	0.145	0.272	0.996	2.550	2.420	BG081834	ATPase, Na+/K+ transporting, beta 2 polypeptide	
25	A	−0.257	−0.057	−0.133	0.622	1.486	1.786	BG075757	CD 81 antigen	
26	A	−0.005	−0.477	−0.091	1.475	1.102	1.513	BG077818	Metallothionein 1	
27	B	−0.256	−0.143	−0.029	0.109	0.767	1.156	AA002439	Annexin A5	
28	B	−0.134	0.014	0.075	−0.388	1.678	1.591	BG064768	*RIKEN cDNA 1810020C02* gene	
29	B	0.334	0.568	−0.140	0.611	0.967	1.004	AU022809	Deleted in polyposis 1	
30	B	−0.367	−0.215	−0.175	0.024	0.464	1.137	W89518	Annexin A2	
31	B	−0.268	0.204	0.601	0.536	0.983	1.242	W75931	Secretogranin III	Up-regulated in DC
32	B	−0.079	−0.162	0.127	−0.041	0.489	1.106	AA038826	Keratin complex 2, basic, gene 4	
33	B	−0.071	0.140	−0.002	0.280	0.765	1.082	BG084347	Developmental pluripotency associated 5	
34	B	0.016	0.212	−0.003	0.350	1.247	1.628	BG074398	SPARC-like 1 (mast9, hevin)	
35	B	0.105	0.057	0.227	0.371	1.093	1.041	AI854816	Bone morphogenetic protein receptor, type 1B	
36	B	−0.235	0.162	0.350	0.331	1.153	1.010	BG083549	Protein tyrosine phosphatase 4a3	
37	B	−0.139	−0.095	0.106	0.679	1.342	1.161	BG086357	Procollagen, type III, alpha 1	
38	B	−0.246	0.136	0.119	0.551	0.776	1.191	BG088450	Low density lipoprotein receptor-related protein associated protein 1	
39	B	−0.054	−0.070	0.095	0.123	1.058	1.648	AA108928	*RIKEN cDNA 9530006B08* gene	
40	B	−0.043	0.009	0.349	0.171	0.871	1.730	BG069468	F-box only protein 15	
41	B	−0.068	0.256	0.497	0.077	1.085	0.740	BG083306	Interleukin 6 signal transducer	
42	B	−0.449	−0.255	−0.025	−0.041	0.776	1.078	BG086434	Phospholipase A2, group VII	Up-regulated in DC
43	B	0.164	0.117	0.334	0.562	0.929	1.152	AA198668	Acid beta glucosidase	
44	B	0.270	−0.066	0.054	0.373	0.291	1.101	AU017674	Mus musculus transcribed sequence	
45	B	−0.247	−0.154	−0.097	0.017	0.624	1.082	BG072874	Secreted acidic cysteine rich glycoprotein	
46	B	−0.017	0.002	0.202	0.678	1.027	0.948	BG087506	Glutamate dehydrogenase	
47	B	−0.060	−0.106	0.186	0.347	1.520	1.328	W16221	Procollagen, type VI, alpha 1	

(*Continued*)

TABLE I. (*Continued*)

No.	Cluster	Fold change (logarithm, base 2) 1 day	2 day	3 day	6 day	12 day	15 day	GenBank	Title	Result of Karsten et al. [2003]
48	B	−0.382	−0.309	0.132	−0.330	0.818	1.269	AA030447	Peripherin 1	
49	B	0.099	0.041	0.157	0.346	1.009	1.098	BG085219	*RIKEN cDNA 1810009M01* gene	
50	B	−0.341	−0.151	−0.154	−0.143	1.374	1.945	W29607	Enoyl coenzyme A hydratase 1, peroxisomal	
51	B	−0.319	−0.800	−0.186	−1.116	0.316	0.892	AA122925	Carbonic anhydrase 2	Up-regulated in NS
52	B	−0.240	0.136	0.015	−0.341	1.420	1.518	AA105295	BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3[a]	Up-regulated in NS
53	B	0.218	0.141	0.136	0.675	1.011	1.376	BG079209	*RIKEN cDNA 1700029I01* gene	
54	B	0.021	0.030	0.306	0.341	1.302	1.253	BG065432	ATP-binding cassette, sub-family F (GCN20), member 3	
55	B	−0.009	0.139	0.162	0.616	1.218	0.876	BG065450	Unknown EST	
56	B	0.049	0.146	0.219	0.686	0.717	1.152	BG071897	Fatty acid binding protein 3, muscle and heart	
57	B	−0.170	0.043	0.239	0.219	0.789	1.110	AI836517	Pleiotrophin[a]	Up-regulated in NS
58	B	0.007	0.092	0.071	0.083	1.159	1.274	BG064176	Lysosomal-associated protein transmembrane 5	
59	B	−0.162	−0.204	−0.197	0.260	1.046	1.135	BG064802	Secreted acidic cysteine rich glycoprotein	
60	B	0.188	0.273	0.281	0.489	0.719	1.003	BG079424	Ornithine aminotransferase	
61	B	0.050	−0.196	−0.102	0.305	1.328	1.816	BG073601	Diazepam binding inhibitor	
62	B	−0.224	−0.611	−0.236	0.211	0.780	1.317	AA276440	Selenoprotein P, plasma, 1	Up-regulated in DC
63	B	−0.039	−0.147	−0.134	0.442	1.050	1.112	BG070071	Heat shock protein 1	
64	B	−0.045	−0.022	−0.027	0.024	0.973	1.306	AI835702	GM2 ganglioside activator protein	
65	B	0.150	0.308	0.265	0.752	1.118	1.164	BG084031	Similar to hypothetical protein FLJ90036	
66	B	0.087	−0.001	−0.379	0.206	0.775	1.093	BG087985	Laminin B1 subunit 1	
67	B	−0.065	0.354	−0.137	0.562	1.187	0.954	AA023786	Polycystic kidney disease 2	
68	B	0.122	−0.059	−0.111	0.620	1.086	1.577	BG085352	Procollagen, type IV, alpha 1	
69	B	0.038	−0.142	−0.149	0.242	0.942	1.205	BG084610	Microrchidia	
70	B	0.029	−0.155	−0.244	−0.017	0.898	1.315	BG066605	Laminin, gamma 1	
71	B	0.084	0.096	−0.424	−0.239	1.214	1.023	BG073394	Adducin 3 (gamma)	Up-regulated in DC
72	B	−0.012	−0.203	−0.044	0.721	0.999	1.344	BG087341	Procollagen, type IV, alpha 2	
73	B	−0.125	−0.779	−0.827	−0.350	1.282	1.289	AW557873	Inhibitor of DNA binding 3	
74	B	0.026	−0.204	−0.191	0.366	1.516	1.231	AA034564	Procollagen, type V, alpha 2	
75	B	0.131	−0.018	−0.389	0.203	0.424	1.085	BG067246	PCTAIRE-motif protein kinase 3	
76	B	0.125	0.207	0.158	0.320	1.325	1.466	BG070449	*RIKEN cDNA 4933436C10* gene	
77	B	0.060	0.082	0.018	0.555	0.814	1.071	AA230924	Myosin light chain, alkali, nonmuscle	
78	B	0.114	0.270	0.328	0.623	0.632	1.199	BG067269	Adenylate kinase 2	
79	B	0.166	0.213	0.090	0.518	1.055	0.963	BG087365	Stromal cell derived factor receptor 1	
80	B	0.027	−0.031	−0.130	0.326	1.125	1.800	BG082965	Cyclin-dependent kinase 8	
81	B	−0.019	0.077	−0.052	0.060	0.805	1.038	BG069516	Solute carrier family 6, member 8	
82	B	−0.012	−0.010	0.153	0.182	0.976	1.046	BG071424	Integral membrane protein 2C	
83	B	0.055	0.148	0.176	0.856	0.882	1.201	BG083801	*RIKEN cDNA 2610036A22* gene	
84	B	−0.131	−0.297	−0.573	−0.134	0.985	1.115	BG085415	Gap junction membrane channel protein alpha 1	
85	B	0.229	−0.133	−0.029	0.221	1.267	0.901	AA030540	Adenine phosphoribosyl transferase	
86	B	0.049	−0.516	−1.004	−0.146	1.213	1.598	AA109951	Beta-2 microglobulin	
87	B	0.327	−0.225	−0.216	−0.020	1.170	0.917	BG084290	Inhibitor of DNA binding 2	
88	B	0.198	0.058	0.056	0.449	0.762	1.028	BG086136	Zinc finger protein 51	
89	B	−0.158	0.250	−0.119	−0.561	1.312	1.199	BG086605	Thioredoxin interacting protein	Up-regulated in DC
90	B	−0.391	−0.714	−0.780	−0.182	0.586	1.467	BG078028	Lectin, galactose binding, soluble 1	
91	B	0.081	−0.041	0.014	0.493	1.046	1.307	BG079624	Serine (or cysteine) proteinase inhibitor, clade E, member 2	
92	B	−0.009	−0.023	−0.118	0.365	1.235	1.137	AW491453	Calpain 6	
93	B	−0.211	−0.023	0.095	0.397	0.787	1.051	BG084582	Lectin, galactose binding, soluble 8	
94	B	−0.072	0.193	−0.043	0.663	1.308	0.974	AI849826	Catenin delta 2	
95	B	−0.230	−0.490	0.178	−0.523	0.975	1.451	AI835385	Lactate dehydrogenase 1, A chain	

96	B	0.285	0.147	−0.350	−0.269	1.052	0.837	BG070902	Nuclear protein 1	
97	B	−0.069	0.048	0.053	1.008	1.033	1.045	AI385510	Cadherin 13[a]	Up-regulated in NS
98	B	−0.006	−0.025	−0.309	0.199	0.372	1.108	BG067443	Mus musculus transcribed sequences	
99	B	0.249	0.146	−0.083	0.178	0.837	1.188	BG085167	Lysosomal membrane glycoprotein 2	
100	B	0.130	−0.103	−0.207	0.222	0.755	1.048	BG080373	Expressed sequence AW743433	
101	B	−0.199	0.108	0.252	0.452	1.024	0.728	BG088310	Prosaposin	
102	C	0.986	0.843	−1.324	0.272	−0.373	1.051	AU022767	Exportin 4	
103	C	1.308	0.777	−0.947	0.436	0.132	1.006	BG068317	*RIKEN cDNA 1700011I03* gene	
104	C	1.220	1.120	−1.131	0.431	0.061	0.862	BG068328	BRF2, subunit of RNA polymerase III transcription initiation factor	
105	D	−0.143	−0.264	−0.492	−1.193	−1.136	−0.817	AA466087	H2A histone family, member Z	Up-regulated in NS
106	D	−0.011	−0.267	−0.509	−1.489	−1.217	−0.661	AW490674	*ect2* oncogene	Up-regulated in NS
107	D	−0.135	−0.082	−0.352	−1.026	−1.364	−0.517	BG066442	Karyopherin (importin) alpha 2	
108	D	−0.243	−0.074	−0.454	−1.107	−0.991	−0.770	BG068146	Calmodulin binding protein 1	
109	D	−0.104	−0.085	−0.705	−1.486	−1.542	−0.603	BG079172	Topoisomerase (DNA) II alpha	
110	D	−0.061	−0.364	−0.437	−0.677	−1.351	−1.155	BG081202	Spermine synthase	
111	D	−0.546	−0.431	−0.280	−1.048	−0.397	−0.286	BG065190	Adenosine kinase	
112	D	−0.224	0.229	−0.295	−1.108	−1.153	−1.078	BG080962	cDNA sequence BC034753	
113	D	−0.072	0.045	−0.416	−0.832	−1.054	−0.571	BG082035	Activator of S phase kinase	
114	D	−0.121	−0.095	−0.493	−0.844	−1.366	−1.286	BG075630	*RIKEN cDNA 2310035M22* gene	
115	D	−0.072	−0.286	−0.425	−1.268	−0.885	−0.698	BG068666	Kinesin family member 23	
116	D	−0.110	−0.345	−0.390	−0.679	−1.223	−0.735	AI836129	High mobility group nucleosomal binding domain 2	
117	D	0.005	−0.308	−0.638	−1.614	−1.650	−0.744	BG082403	Histone 1, H4m	
118	D	−0.110	−0.266	−0.579	−1.116	−0.594	−0.432	AA060205	Zinc finger protein 36, C3H type-like 1	
119	D	−0.085	−0.280	−0.363	−0.925	−1.088	−0.607	BG074248	Kinesin family member 22, pseudogene	
120	D	0.089	−0.277	−0.530	−1.057	−1.324	−0.933	BG087310	*DEK* oncogene (DNA binding)	
121	D	0.011	−0.254	−0.649	−1.327	−1.055	−0.868	BG072056	Centromere autoantigen A	
122	D	−0.035	−0.259	−0.729	−1.376	−1.192	−0.568	AA396324	Cyclin B1	Up-regulated in NS
123	D	0.124	−0.263	−0.507	−0.517	−1.131	−0.695	BG066359	Ubiquitin specific protease 1	
124	D	0.045	−0.416	−0.819	−1.311	−1.594	−1.525	BG066232	High mobility group AT-hook 2	
125	D	0.073	−0.375	−0.657	−0.791	−1.198	−0.753	BG072545	CDC28 protein kinase 1	Up-regulated in NS
126	D	−0.085	−0.199	−0.356	−0.566	−1.096	−1.082	BG063923	H3 histone, family 3B	
127	D	−0.081	−0.157	−0.549	−0.708	−0.780	−1.071	BG085335	*RIKEN cDNA 2600016F06* gene	
128	D	−0.018	−0.317	−0.613	−0.996	−0.803	−1.020	W81792	Cytochrome P450, family 2, subfamily d, polypeptide 10	
129	D	−0.201	−0.278	−0.862	−2.048	−1.827	−1.504	AI836520	Histone 3, H2bb	
130	D	0.032	−0.205	−0.357	−1.512	−0.889	−0.841	BG066499	Gene rich cluster, *C8* gene	
131	D	0.131	−0.293	−0.562	−0.809	−1.286	−1.028	BG076805	Nuclear autoantigenic sperm protein (histone-binding)	
132	D	−0.174	−0.154	−0.779	−1.380	−1.249	−0.897	BG082508	DNA segment, Chr 2, ERATO Doi 750, expressed	
133	D	0.061	−0.152	−0.500	−1.600	−1.410	−1.026	BG085939	*RIKEN cDNA A730011O11* gene	
134	D	−0.151	−0.148	−0.512	−0.509	−1.042	−1.074	AW547625	Cyclin D2	Up-regulated in NS
135	D	0.122	−0.354	−0.177	−0.838	−1.342	−0.860	AA030433	Bloom syndrome homolog (human)	
136	D	0.194	−0.193	−0.506	−0.941	−1.430	−1.274	AI835559	Thymopoietin	
137	D	0.010	−0.382	−0.667	−1.081	−1.213	−0.908	BG078138	Ribonucleotide reductase M2	
138	D	0.006	−0.185	−0.700	−1.199	−1.125	−0.338	BG072979	*RIKEN cDNA 2610201A12* gene	
139	D	0.227	−0.308	−0.373	−0.819	−1.110	−0.804	AW553739	Ttk protein kinase	
140	D	0.066	−0.534	−0.336	−0.376	−1.822	−1.176	AA034857	RNA binding motif protein 3	
141	D	0.011	0.521	−0.420	−1.184	−0.666	−0.957	AI844331	Pyrroline-5-carboxylate synthetase	Up-regulated in NS
142	D	0.144	−0.112	−0.650	−1.201	−1.606	−1.029	BG069688	Cyclin A2	
143	D	−0.158	−0.327	−0.686	−1.241	−1.209	−0.476	BG069168	Pituitary tumor-transforming 1	Up-regulated in NS
144	D	0.151	−0.197	−0.531	−0.953	−0.839	−1.212	BG087468	Forkhead box M1	Up-regulated in NS
145	D	0.221	−0.352	−0.417	−0.770	−1.041	−0.904	AA544948	Minichromosome maintenance deficient 7 (*S. cerevisiae*)	Up-regulated in NS
146	D	0.131	−0.350	−0.745	−1.335	−1.026	−1.123	BG068759	Transforming, acidic coiled-coil containing protein 3	Up-regulated in NS
147	D	0.263	−0.425	−0.669	−0.755	−1.024	−0.945	AA153012	Chromatin assembly factor 1, subunit A (p150)	Up-regulated in NS
148	D	−0.062	−0.205	−0.618	−1.865	−1.370	−1.157	AA049416	Histone 1, H1c	
149	D	0.077	−0.152	−0.860	−1.304	−1.240	−0.596	BG079289	*RIKEN cDNA 2700099C18* gene	
150	D	−0.204	−0.404	−0.778	−0.454	−1.014	−0.097	AA120637	*RIKEN cDNA 5430410E06* gene	

(*Continued*)

TABLE I. (*Continued*)

No.	Cluster	Fold change (logarithm, base 2) 1 day	2 day	3 day	6 day	12 day	15 day	GenBank	Title	Result of Karsten et al. [2003]
151	D	0.251	−0.407	−0.596	−1.133	−1.395	−1.128	AA276998	Serine/threonine kinase 18	
152	D	−0.123	−0.307	−1.091	−1.907	−2.380	−1.825	AI847223	Sema domain, transmembrane domain and cytoplasmic domain,6C	
153	D	−0.046	−0.412	−0.951	−1.524	−1.290	−0.442	BG071965	Cyclin B1, related sequence 1	
154	D	0.257	−0.418	−0.526	−0.958	−1.103	−0.905	BG071804	Budding uninhibited by benzimidazoles 1 homolog, beta (*S. cerevisiae*)	
155	D	0.175	−0.264	−0.675	−0.751	−1.060	−0.942	BG088398	Glucocorticoid induced transcript 1	
156	D	−0.332	0.146	−0.361	−1.075	−0.272	−0.418	BG076333	Methylenetetrahydrofolate dehydrogenase (NAD+ dependent)	Up-regulated in NS
157	D	−0.015	−0.247	−0.558	−0.920	−1.013	−0.877	AI853169	Hypothetical protein C530022J18	
158	D	−0.024	−0.492	−0.617	−1.103	−0.967	−0.868	BG064686	Chorionic somatomammotropin hormone 1	
159	D	−0.062	−0.546	−0.510	−0.396	−1.054	−0.799	BG069969	Tcra enhancer-binding factor interacting protein 1	
160	D	−0.029	−0.395	−0.715	−0.753	−1.204	−0.528	BG085427	High mobility group box 2	Up-regulated in NS
161	D	−0.041	−0.225	−0.114	−0.597	−1.301	−0.890	BG086151	CDK2 (cyclin-dependent kinase 2)-associated protein 1	
162	D	0.072	−0.538	−0.407	−0.575	−1.536	−1.068	BG077092	*RIKEN cDNA 2600016C11* gene	
163	D	0.330	−0.033	−0.860	−1.061	−1.044	−0.345	BG081629	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	
164	D	0.252	−0.360	−0.909	−1.108	−1.386	−0.901	BG067860	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)	Up-regulated in NS
165	D	0.229	−0.130	−0.606	−1.106	−1.167	−1.118	BG083621	Poliovirus receptor-related 3	
166	D	−0.156	−0.417	−0.606	−0.529	−1.111	−1.137	AA498281	Lamin B1	
167	D	0.010	−0.619	−0.818	−1.223	−1.052	−0.478	BG064846	Cell division cycle 2 homolog A (*S. pombe*)	Up-regulated in NS
168	D	−0.182	−0.376	−0.321	−0.529	−1.024	−0.380	BG085860	Small nuclear ribonucleoprotein polypeptide G	
169	D	0.267	−0.271	−0.807	−1.600	−0.993	−0.546	BG086805	Budding uninhibited by benzimidazoles 1 homolog (*S. cerevisiae*)	
170	D	−0.042	−0.479	−0.462	−0.647	−1.732	−0.994	BG064598	Proliferating cell nuclear antigen	Up-regulated in NS
171	D	−0.208	−0.420	−0.605	−1.280	−1.106	−0.636	BG080690	Shc SH2-domain binding protein 1	Up-regulated in NS
172	D	−0.182	−0.480	−0.654	−1.732	−1.498	−1.048	BG069986	Polymyositis/scleroderma autoantigen 1	
173	D	−0.009	−0.215	−0.286	−0.671	−1.014	−0.011	BG083522	CDC28 protein kinase regulatory subunit 2	
174	D	0.140	−0.240	−0.547	−1.140	−0.559	−0.620	AI893902	Sperm associated antigen 5	Up-regulated in NS
175	D	0.040	−0.362	−0.723	−1.134	−0.867	−0.313	BG077844	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	
176	E	−0.436	0.290	0.372	0.005	−0.940	−1.309	BG071846	Transducin-like enhancer of split 4, E(spl) homolog (*Drosophila*)	
177	E	−0.031	0.170	0.209	0.090	−0.809	−1.303	BG085705	*RIKEN cDNA 4933432H23* gene	
178	E	−0.020	0.207	0.378	0.188	−1.180	−0.786	AW554258	Midnolin	
179	E	−0.012	0.106	0.036	−0.307	−1.151	−1.029	BG074931	Enhancer of zeste homolog 2 (*Drosophila*)	
180	E	−0.049	0.083	0.016	−0.656	−1.280	−1.464	AA498495	Erythrocyte protein band 4.1	
181	E	0.280	−0.065	0.290	−0.096	−0.963	−1.018	BG079889	Isopentenyl-diphosphate delta isomerase	Up-regulated in NS
182	E	−0.053	−0.151	−0.165	−0.521	−1.303	−1.740	AA242226	Cadherin 2	
183	E	0.045	0.370	0.729	0.558	−0.777	−1.451	AI846983	Microtubule-associated protein 1 B	
184	E	0.027	0.594	−0.009	0.061	−1.021	−0.683	BG081111	Topoisomerase (DNA) I	
185	E	−0.032	0.295	0.323	−0.302	−1.005	−1.080	BG086306	Polyhomeotic-like 2 (*Drosophila*)	
186	E	0.091	−0.298	−0.104	0.285	−1.271	−1.421	AI836508	Splicing factor, arginine/serine-rich 2 (SC-35)	
187	E	0.225	0.069	0.066	0.053	−1.166	−1.550	BG086100	*RIKEN cDNA 1110055B05* gene	
188	E	0.062	−0.016	−0.070	−0.463	−1.083	−0.643	BG072534	Heterogeneous nuclear ribonucleoprotein A1	
189	E	0.074	−0.207	−0.444	−0.388	−1.182	−0.971	BG064278	Nucleosome assembly protein 1-like 1	
190	E	0.014	0.427	0.101	−0.368	−0.708	−1.304	BG071313	Cyclin-dependent kinase inhibitor 1C (P57)	
191	E	0.097	−0.029	−0.268	−0.035	−1.433	−1.308	BG074524	Heterogeneous nuclear ribonucleoprotein A/B	
192	E	0.054	−0.088	−0.235	−0.516	−1.289	−1.285	BG085009	Cyclin-dependent kinase 4	
193	E	0.016	−0.058	−0.085	−0.192	−1.071	−1.247	BG087161	Latrophilin 2	
194	E	0.109	−0.027	−0.013	−0.315	−0.715	−1.011	AA048831	High mobility group box 3	
195	E	0.051	−0.011	−0.248	−0.374	−1.054	−0.845	AI848377	cDNA sequence BC010348	
196	E	0.272	−0.116	0.182	−0.525	−0.929	−1.070	AI323209	Makorin, ring finger protein, 3	
197	E	0.435	−0.230	−0.405	−0.298	−0.870	−1.021	BG078653	Heterogeneous nuclear ribonucleoprotein D	

198	E	0.058	−0.030	−0.182	−0.025	−0.677	−1.093	AA212717	*C*-terminal binding protein 1	
199	E	0.111	0.438	0.447	0.243	−0.618	−1.013	AU045483	Ubiquilin 2	
200	E	0.120	−0.138	−0.123	−0.568	−0.729	−1.009	AA059891	Upstream regulatory element binding protein 1	
201	E	−0.079	0.136	0.273	0.133	−0.308	−1.263	BG070656	SRY-box containing gene 4	
202	E	0.052	−0.288	−0.369	−0.166	−1.011	−1.410	BG087241	Mesoderm specific transcript	
203	E	0.080	0.155	−0.173	−0.272	−1.006	−0.790	BG085781	Chromobox homolog 1 (*Drosophila* HP1 beta)	
204	E	−0.001	−0.219	−0.155	0.273	−1.171	−0.744	BG078157	Splicing factor, arginine/serine-rich 3 (SRp20)	Up-regulated in NS
205	E	0.250	0.155	0.216	0.425	−1.351	−1.165	AA125197	Actin dependent regulator of chromatin, subfamily e, member 1	
206	E	0.076	−0.036	−0.057	−0.508	−1.051	−0.706	BG079001	G elongation factor	
207	E	−0.027	−0.174	0.242	0.296	−0.447	−1.084	AA120351	Ubiquitin-conjugating enzyme E2I	
208	E	−0.197	−0.304	−0.420	−0.365	−1.211	−1.397	AA032448	Zinc finger protein 162	
209	E	0.010	0.328	0.546	0.491	−0.688	−1.425	AI840073	Neuronatin	
210	E	0.112	0.273	0.091	0.364	−1.320	−1.393	BG072739	SRY-box containing gene 11	
211	E	−0.070	0.163	0.565	0.400	−0.645	−1.102	AI838805	Kruppel-like factor 15	
212	E	−0.026	0.201	0.139	−0.293	−1.071	−1.026	BG087208	H1 histone family, member 0	
213	E	0.047	0.062	−0.227	−0.111	−0.918	−1.161	BG087103	KH domain containing, RNA binding, signal transduction associated 1	
214	E	0.024	−0.232	−0.084	−0.276	−0.904	−1.002	AA209061	Thioredoxin reductase 1	
215	F	−0.058	0.668	1.329	1.175	0.847	0.281	W18828	Dihydropyrimidinase-like 3	
216	F	−0.098	0.607	1.024	0.657	1.248	0.638	AI842333	Double cortin and calcium/calmodulin-dependent protein kinase-like 1	
217	F	0.145	0.474	0.150	1.121	0.298	0.223	BG064500	Heat shock protein	
218	F	0.007	0.567	0.788	1.177	1.157	0.868	BG086292	Amyloid beta (A4) precursor protein	
219	F	−0.048	0.386	1.080	1.200	0.371	0.145	AA275865	ALL1-fused gene from chromosome 1q	
220	F	−0.014	0.612	1.017	0.803	0.712	0.402	BG083182	Guanine deaminase	
221	F	−0.077	0.070	0.345	0.697	1.200	0.821	AA547428	Protein kinase, cAMP dependent, catalytic, beta	
222	F	0.142	0.081	0.788	0.989	1.057	0.799	AI854566	Synaptotagmin 7	
223	F	0.169	0.552	1.067	1.295	0.280	0.169	BG087115	Myeloid ecotropic viral integration site-related gene 1	
224	F	−0.071	0.479	0.757	0.768	1.112	0.578	AI323062	Guanine nucleotide binding protein, alpha o	
225	F	0.108	0.488	0.820	1.165	0.244	−0.349	AA231471	Sialyltransferase 8 (alpha-2, 8-sialyltransferase) B	
226	F	−0.122	0.310	0.754	0.714	1.011	0.556	AA269845	Glycoprotein m6a	
227	F	−0.100	0.719	0.791	0.834	1.032	0.597	AI835125	Pleckstrin homology, Sec7 and coiled-coil domains 2	
228	F	0.123	0.591	1.023	0.636	0.439	0.423	BG087300	p21 (CDKN1A)-activated kinase 4	
229	F	0.138	0.381	0.648	1.167	1.327	1.008	BG086955	Brain protein 44-like	
230	F	0.085	0.474	0.837	0.983	1.133	0.836	BG082280	*RIKEN cDNA 9430072K23* gene	
231	F	0.063	0.302	0.557	0.725	0.595	1.049	BG070401	Cyclin M2	
232	F	−0.050	0.514	1.065	1.061	1.121	0.806	W42241	Stathmin-like 4	
233	F	−0.235	0.192	0.867	0.480	1.556	0.745	AI840345	Gamma-aminobutyric acid (GABA-A) transporter 1	
234	F	0.078	0.695	1.327	2.075	2.036	1.150	AA028410	Microtubule-associated protein tau	Up-uregulated in DC
235	F	0.134	−0.129	−0.065	1.758	0.592	0.927	BG063925	Metallothionein 2	
236	F	0.077	0.557	0.863	1.160	1.252	0.838	AI853686	Glutamate receptor, ionotropic, AMPA3 (alpha 3)	
237	F	0.203	0.366	0.635	1.303	1.353	0.884	AA521764	Receptor (calcitonin) activity modifying protein 2	Up-regulated in DC
238	F	0.040	0.679	0.725	1.311	1.026	0.242	BG075834	*RIKEN cDNA 3110039L19* gene	
239	F	−0.190	0.429	0.338	1.144	0.838	0.685	BG076172	Calmodulin 1	
240	F	0.362	0.540	0.697	1.015	0.488	0.314	AI844249	*RIKEN cDNA 1110003P13* gene	
241	F	0.073	0.321	0.736	1.082	0.310	−0.015	W66622	TEA domain family member 2	
242	F	−0.274	−0.166	0.059	0.751	1.094	0.395	AI837858	Protein kinase C, beta	
243	F	0.234	0.237	0.709	1.273	1.139	0.729	AI835523	Protein kinase inhibitor, alpha	
244	F	0.113	0.320	0.456	1.306	0.551	0.275	BG081562	Regulator of G-protein signaling 2	
245	F	0.009	0.227	0.611	1.039	1.068	0.972	BG067399	Tropomodulin 2	
246	F	0.239	0.578	0.620	0.829	1.190	0.887	BG084093	*N*-myc downstream regulated 2	
247	F	0.023	0.505	0.660	1.141	0.685	0.380	BG086006	Ephrin B3	
248	F	0.400	0.573	0.490	1.058	0.822	0.624	BG080627	Nuclear respiratory factor 1	
249	F	0.206	0.266	1.176	1.538	0.905	0.215	AI849758	Microtubule-associated protein 2	
250	F	0.287	0.355	0.323	0.797	0.876	1.012	BG069134	Mus musculus transcribed sequences	
251	F	0.435	0.548	0.575	1.193	0.685	0.733	BG066404	Mus musculus transcribed sequences	

(*Continued*)

TABLE I. (*Continued*)

No.	Cluster	Fold change (logarithm, base 2)						GenBank	Title	Result of Karsten et al. [2003]
		1 day	2 day	3 day	6 day	12 day	15 day			
252	F	0.249	0.465	0.723	1.001	0.505	0.534	BG069707	CD1d1 antigen	
253	F	−0.026	0.091	0.134	0.961	1.272	0.836	BG086024	Activated leukocyte cell adhesion molecule	
254	F	−0.174	0.396	0.376	0.607	1.004	0.383	BG073000	Protocadherin gamma subfamily B, 4	
255	F	−0.254	0.022	0.328	1.259	0.915	0.726	AI852463	Protein tyrosine phosphatase, receptor type, T	
256	F	0.015	0.063	0.088	1.094	0.721	0.924	AI323177	Neurofibromatosis 2	
257	F	0.165	0.388	0.738	1.039	0.531	0.250	AA463173	Solute carrier family 11, member 2	
258	F	0.134	0.162	0.531	1.005	−0.094	−0.262	BG077853	Coatomer protein complex, subunit gamma 2	
259	F	0.173	0.067	0.056	1.048	−0.156	−0.358	BG067712	*RIKEN cDNA 6720463E02* gene	

[a]Genes with opposite expression pattern in overlapped genes of both systems.

We also categorized the 259 genes based on their functions, referring to Gene Ontology^{TM} and classified them into 14 groups (Fig. 4). Frequencies of these functionally classified genes in each cluster are shown in Figure 4, with statistical significance evaluated by Fisher's exact test for high and low frequencies. High frequencies were observed as follows: "Transport"-related genes in the cluster A; "cell adhesion"-related genes in the cluster B; both "nucleic acid metabolism"- and "cell cycle"-related genes in the cluster D; both "transcription"- and "RNA processing"-related genes in the cluster E.

Transport-related genes. Among genes in the cluster A with properties of gradual increase during differentiation, significantly frequent were members of the category "transport"-related genes. These genes are a ATP-binding cassette, sub-family A, member1(ABCA1); solute carrier family 1, member 3; gamma-aminobutyric acid receptor, subunit beta 3; ATPase, Na+/K+ transporting, beta 1 and cocaine and amphetamine regulated transcript (CART). Of them, *ABCA1* gene was reported to be highly expressed in CA1 and CA3 pyramidal neurons [Koldamova et al., 2003] and *CART* gene, expressed in hypothalamus neuron [Larsen et al., 2003] and may selectively regulate certain central dopamine neuronal activities [Shieh, 2003].

Cell adhesion-related genes. Genes of "cell adhesion" were significantly enriched in cluster B. These included procollagen, type VI, alpha 1; procollagen, type IV, alpha 1; procollagen, type IV, alpha 2; laminin B1 subunit 1; laminin, gamma 1; catenin delta 2; cadherin 13. Specially, mRNA level of procollagen, type IV, alpha 1 was up-regulated after differentiation day 12 (2.8-fold) and this gene previously reported as a marker of neural differentiation [Clifford et al., 1996] and a promoting factor for the differentiation of neuronal progenitors [Ali et al., 1998].

Cell cycle-related genes. The majority of "cell cycle" related genes was included in cluster D and was down-regulated during differentiation. With regard to cell cycle checkpoint transition, the expression of cyclin A2, B1, D2, and cell division cycle 2 homolog A (cdc2A) were repressed. In addition, this cluster included activator of S phase kinase; CDC28 protein kinase 1; transforming, acidic coiled-coil containing protein 3; budding uninhibited by benzimidazoles 1 homolog beta (Mm.29133); budding uninhibited by benzimidazoles 1 homo-

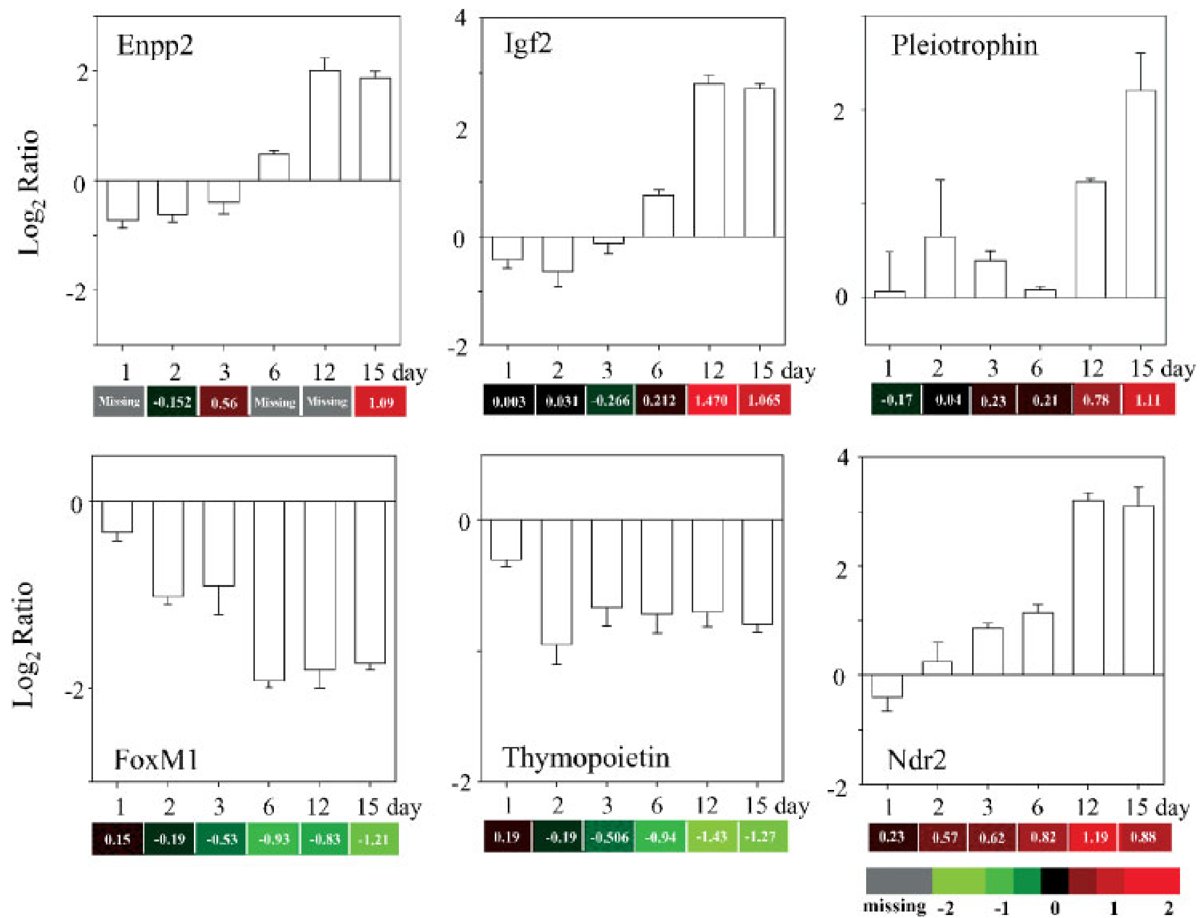

Fig. 2. Verification of microarray data. Real-time RT-PCR was used to verify the data from the microarray. Each value is expressed as fold change (logarithm, base 2) in mRNA levels at each differentiation time-point compared to the control NSCs, and represents the mean ± SEM of three independent experiments. Microarray data of each gene was displayed as a horizontal strip. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

log (Mm.2185); MAD2 (mitotic arrest deficient, homolog)-like 1 and CDC28 protein kinase regulatory subunit 2.

Nucleic acid metabolism-related genes. Among genes in the cluster D with properties of decrease during differentiation, significantly frequent were also members of the category "nucleic acid metabolism"-related genes. There was a decrease in the expression of genes involved in DNA packaging, replication, repair, nucleosome assembly, and chromosome segregation, such as H2A histone family, member Z; topoisomerase II alpha; high mobility group nucleosomal binding domain 2; centromere autoantigen A; Bloom syndrome homolog; ribonucleotide reductase M2; minichromosome maintenance deficient 7; chromatin assembly factor 1; proliferating cell nuclear antigen (PCNA); high mobility group box 2 and SMC2 structural maintenance of chromosomes 2-like 1. Altered expressions of these functional genes were concomitant with the decrease in expression of G1/S cell cycle regulatory genes.

Transcription regulators. Transcription regulators which may be involved in NSCs proliferation and maintenance showed high frequency in cluster E and included transducin-like enhancer of split 4 (TLE 4); enhancer of zeste homolog 2 (EZL2); ring finger protein 3; SRY-box containing gene 4, 11 (Sox 4, 11); actin dependent regulator of chromatin, subfamily e, member 1 and Kruppel-like factor 15. Both Sox4 and 11 were previously reported to be highly expressed in ventricular zones and down-regulated in neurons as they become mature [Cheung et al., 2000].

In Vivo Expression Patterns

We performed ISH for four genes. Of them, three genes (*Fox M1*, *cyclin D2*, and *CDK4*) were

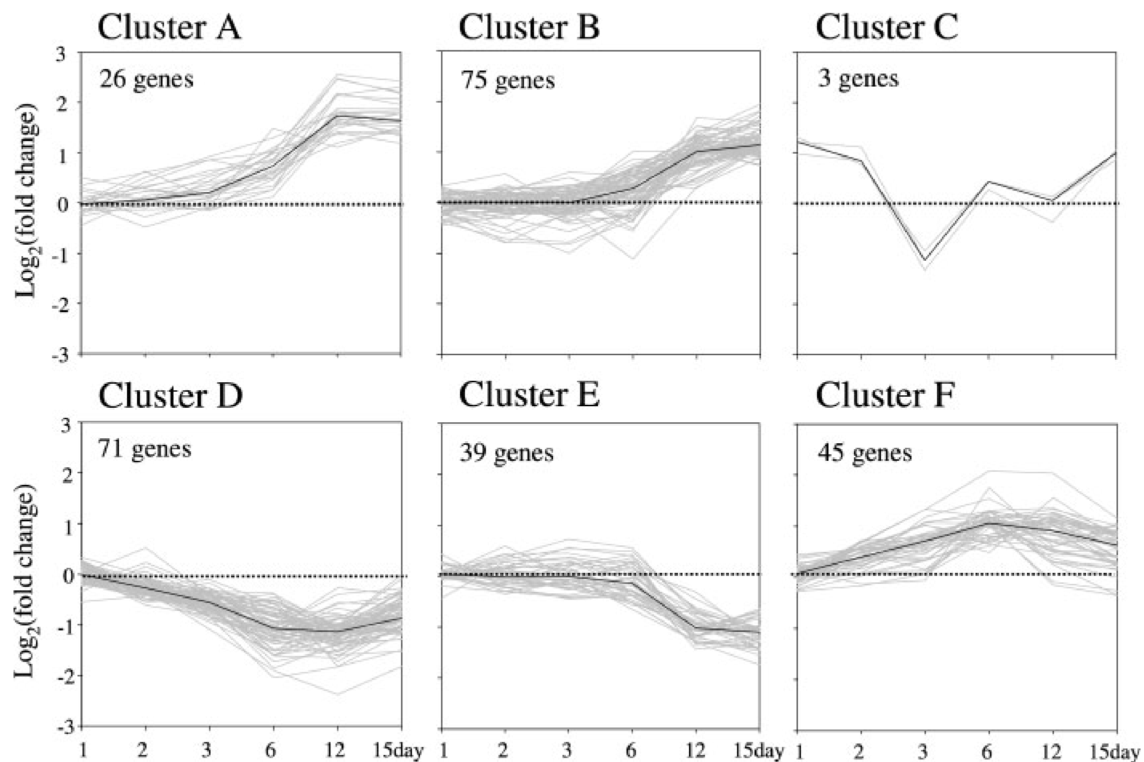

Fig. 3. K-means clustering of genes with expression levels that changed during NSCs differentiation. K-means clustering was used to group the 259 identified genes into clusters based on similar expression over the six time-point. These 259 genes were classified into 6 clusters (**A–F**).

highly expressed in the NSCs, while Enpp2 showed high expression level in differentiated cells as shown by RT-PCR. In ISH, Fox M1, cyclin D2, and CDK4 were highly expressed in the germinal zone during embryonic development. Enpp2 showed high and specific expression in the choroid plexus of brain from E13.5 to P14 (Fig. 5A,B). The choroid plexus has recently been shown to be essential organizing centers for inducing dorsal neuron fates and sustaining neuron function [Awatramani et al., 2003] and was identified as a possible location of stem/progenitor cells [Li et al., 2002]. Moreover, lysophosphatidic acid (LPA), catalyzed by Enpp2, was shown to increase terminal mitosis of neural precursor cells and promote their cell cycle exit [Kingsbury et al., 2003].

DISCUSSION

Comparision of Two Expression Profiles

Our experiment used a comprehensive molecular approach to identify temporal changes in gene expression that occur during differentiation of NSCs. A total of 259 genes were regulated during differentiation. The 259 gene expression profiles provided us with an opportunity to compare or contrast our analysis with those of other investigators who have used similar methodologies. Karsten et al. [2003] reported a data set consisting of 318 significantly changed expression profiles in proliferating NSCs (NS) and its 24 h differentiated cells (DC) in vitro. There were 36 overlapped genes (defined as Unigene IDs) represented in both data sets (Table I). In addition, 33 of 36 overlapped genes were found to have very similar expression patterns in both systems, which suggest a good overall agreement (91.6%) between the results of these two groups. However, these genes accounted for very small proportion in total overlapped genes of our 10K chip and UCLA 9K array which had been used in Karsten et al. experiment (36/2,312 genes). There could be several reasons to explain these consequences. One major possibility is difference in the determination of significantly expressed genes in microarray data set (stringent 2-fold change cutoff method vs. false discovery rate method). Other reasons are differences in NSC population,

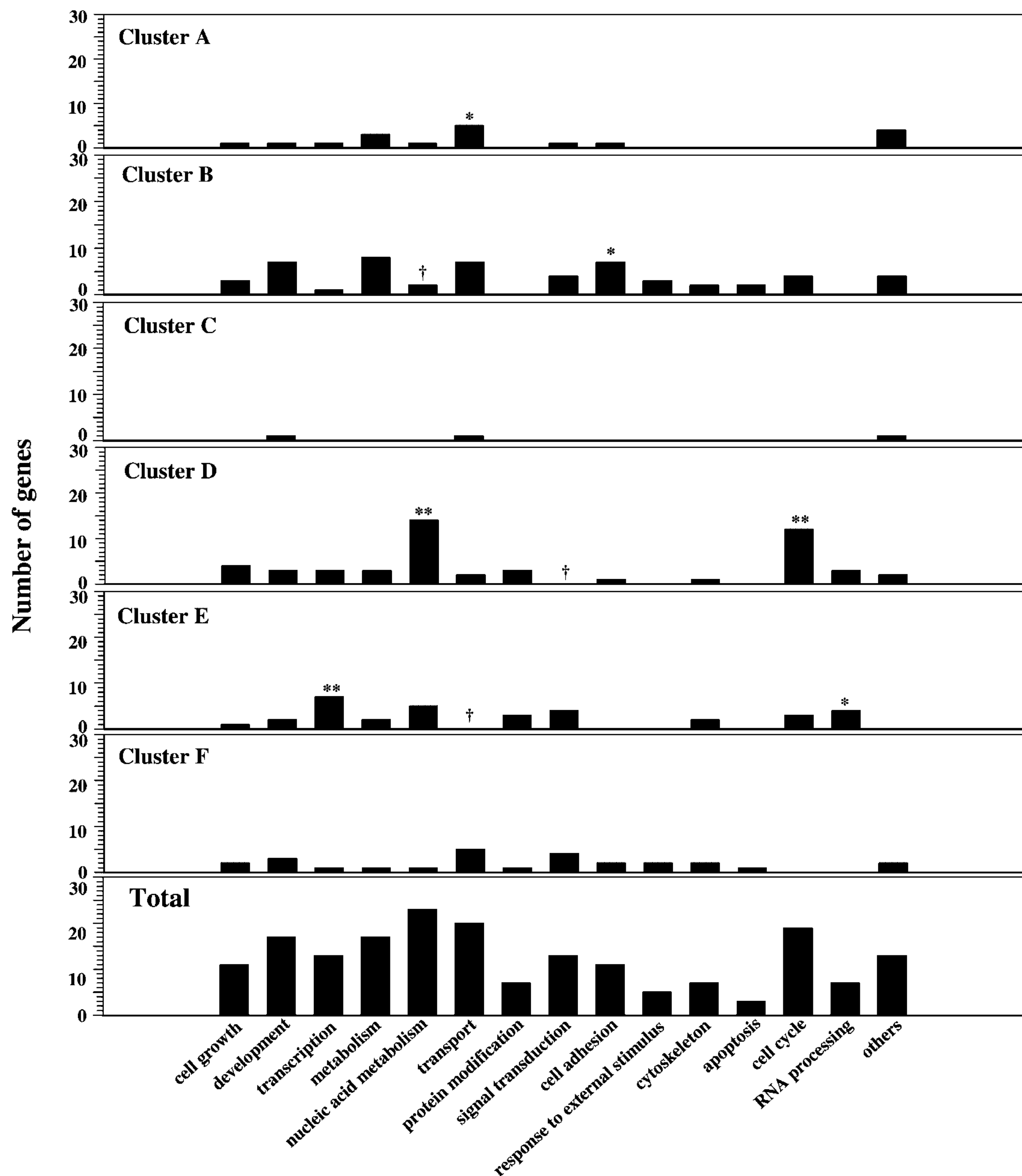

Fig. 4. Distribution of functionally categorized genes in each cluster. Frequencies of genes of each functional category are shown. Significantly high (**, $P < 0.01$; *, $P < 0.05$) and low (†, $P < 0.05$) frequencies were evaluated by Fisher's exact test.

differentiation protocol, and microarray labeling method. Although we used NSCs derived from ES cells, Karsten et al. used neural progenitor cells which were obtained from P0 mouse.

Cell Cycle Regulation and Inhibition of Proneuronal Genes in NSC Proliferation

The clustering analysis of expression patterns of 259 genes and following statistical analysis based on functional classification revealed remarkable features of changes in gene expression during NSC differentiation. The majority of cell cycle, nucleic acid metabolism, transcription, and RNA processing related genes were overexpressed in NSCs. Notably, the up-regulation of G1-S cell cycle regulation, nucleic acid synthesis, DNA replication, packaging, and repair genes in NSCs were consistent with their self-renewal properties. These changes also indicate a progressive switch from biosynthetic

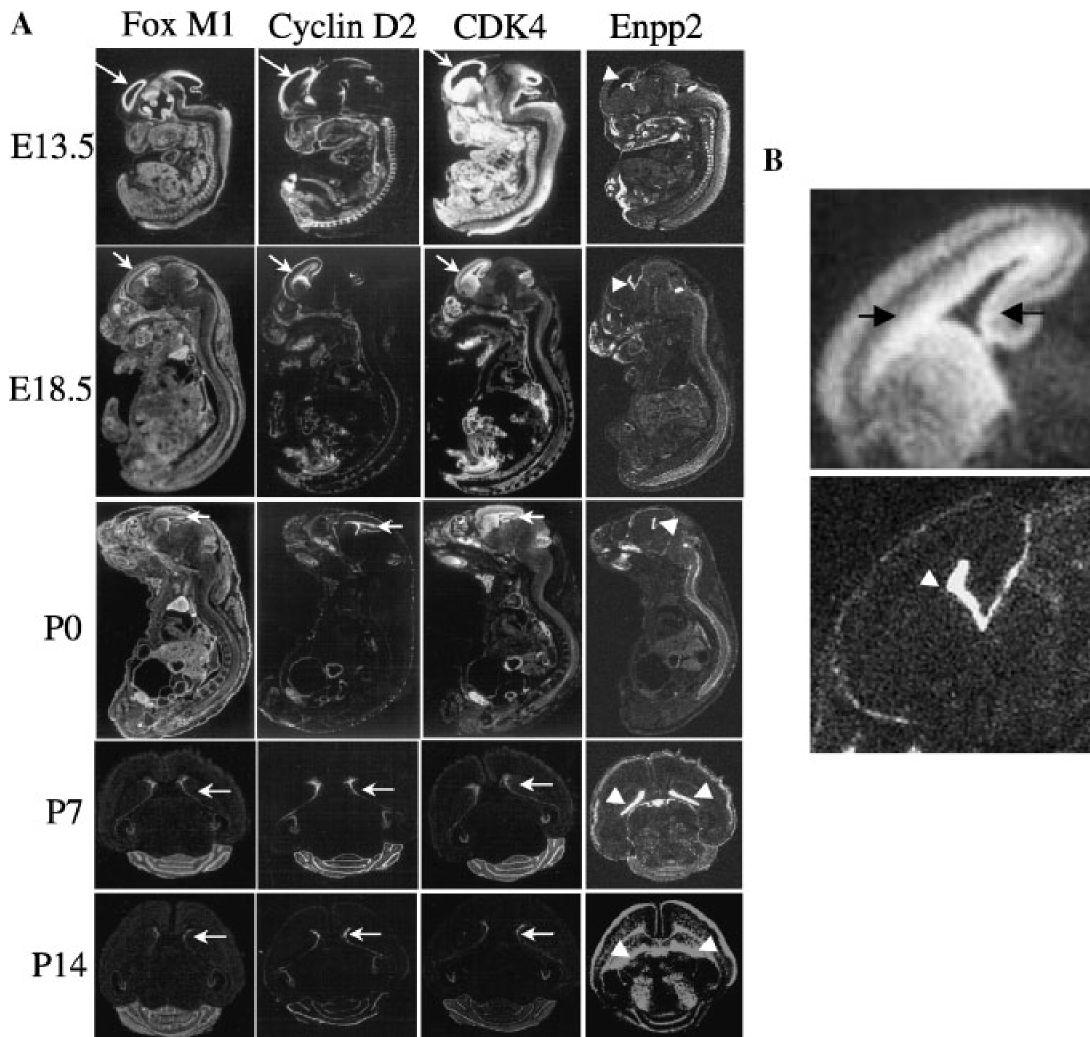

Fig. 5. In situ hybridization of selected transcripts that showed altered expression level during NSCs differentiation. **A**: Sagittal and coronal sections of each age were hybridized with ^{35}S labeled antisense cRNAs. The arrows indicate germinal zone and arrowheads indicates choroid plexus at each age. **B**: Higher magnification of germinal zone (cdk4) and choroid plexus expression (Enpp2).

activity towards more functional activity in the differentiating NSCs.

A number of transcription factors required for NSCs proliferation through cell cycle regulation were suppressed as cells differentiated. A good example is Fox M1 mRNA, which activates cyclin B1 [Leung et al., 2001]. Cyclin B1 is a key regulatory component of the G2/M phase transition in the cell cycle. Germinal zone expression and cell cycle regulator activation of Fox M1 support its important role in NSCs proliferation. Another transcription factor is EZL2. Concordance with the hypothesis that retinoblastoma protein complex (pRb) may play a central role in NSC proliferation [Karsten et al., 2003], we observed a down-regulation of EZL2 at 12 and 15 day. Previous study reported that EZL2 is a downstream target of pRb-E2F pathway and essential for proliferating cells [Bracken et al., 2003]. Additionally, there were increases in mRNA of inhibitor of DNA binding (Id) 3 at differentiation day 12 and 15. Ids promote cell cycle progression by interacting with components of the cell cycle machinery and inhibit the precocious differentiation of neural progenitors into neurons and oligodendrocytes. Though Id3 is necessary for proliferating NSCs, previous study showed that Id3 may play an important role in the regulation of astrocyte proliferation [Tzeng and de Vellis, 1997]. Interestingly, only one member of cyclin family, cyclin M2, exhibited an alteration in differen-

tiated cells and an opposite expression pattern against other cyclins. The precise role of this gene during differentiation remains to be confirmed through function-based assays.

In addition to cell cycle regulation, our analysis showed that inhibition of proneuronal genes is continued during proliferation (before terminal differentiation). TLE 4, a mammalian homologue of *Drosophila groucho*, was significantly down-regulated at differentiation day 15. Notch stimulation leads to activation of the expression of Enhancer of split complex [E (spl)-C] ranscripts. Their protein products then mediate the negative regulation of the expression of proneural genes. The transcriptional repressor function of E (spl)-C proteins requires the activity of the product of groucho [Grbavec et al., 1998]. Taken together, these altered expression levels strongly suggest that cell cycle regulation and negative regulation of proneuronal genes have high influences on NSC proliferation.

Up-Regulation of Neurite Outgrowth and Axon Guidance Related Genes in Differentiated Cells

A variety of extracellular cues, consisting of secreted molecules, cell–cell contact and cell–extracellular matrix (ECM) interaction, are responsible for neurite outgrowth and axon guidance [Wei et al., 2002]. Genes encoding members of a number of these signaling pathways are up-regulated. Pleiotrophin, a cytokine that induce neurite outgrowth [Deuel et al., 2002], is overexpressed during differentiation and also reported to exhibit a trophic effect on survival of dopaminergic neurons [Hida et al., 2003]. Another secreted molecule, annexin A2, induce neuritogenesis and differentiation of the cell line PC12, suggesting involvement in NSC differentiation [Jacovina et al., 2001]. Ephrin B3, guidance cue molecule, was also expressed. Ephrin-Eph receptor system has been shown to mediate contact-dependent repulsion involved in axon guidance [Cook et al., 1998].

Many molecules have been isolated from the ECM and found to be very good at supporting neurite out growth. ECM proteins, such as laminins and various forms of collagens in our chip, are also up-regulated. Most growth cones, especially those in the CNS, grow along the surfaces of other cells and axons. In this function, they are supported largely by class of growth-promoting molecules that are expressed on the cell surfaces and known as cell adhesion molecules (CAM) [Walsh and Doherty, 1997]. mRNA for activated leukocyte CAM (Alcam) in the cluster F was also up-regulated. Neurite formation requires extensive cytoskeleton remodeling. Microtubules provide structural support and act as substrate for the fast axonal transport of vesicles [Signor and Scholey, 2000]. Microtubule proteins including microtubule associated protein (MAP) 2 and MAP tau, were overexpressed.

Cellular signaling pathways that make use of CAMs, repulsive factors, attractive factors, and growth factors are received by receptors on the surface of the growth cone. Receptors that have tyrosine phosphatase activity have been found in abundance on growth cones [Holland et al., 1998]. Tyrosine phosphatase 4a3 in the cluster B was up-regulated at differentiation day 12 and 15 and tyrosine phosphatase, receptor type T in the cluster F was up-regulated at day 6.

In conclusion, we used microarray analysis during differentiation of NSCs to identify regulatory molecules. We identified 259 modulated genes, which could be divided into 14 functional groups. The further statistical analysis showed that cell cycle regulation, nucleic acid metabolism, and negative regulation of proneuronal genes play crucial roles in NSCs proliferation and its self-renewal. Also, in differentiated cells, a variety of neurite outgrowth and axon guidance related genes were detected as significantly expressed genes. Genes that we identified in this study will advance the understanding of mechanisms underlying proliferation/differentiation of NSCs and the potential for the use of stem cells as therapeutic agents.

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