Analysis of Gene Expression in Mouse Spinal Cord-derived Neural Precursor Cells During Neuronal Differentiation

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Abstract

The differentiation of neural precursor cells (NPCs) into neurons and astrocytes is a process that is tightly controlled by complicated and ill-defined gene networks. To extend our knowledge to gene networks, we performed a temporal analysis of gene expression during the differentiation (2, 4, and 8 days) of spinal cord-derived NPCs using oligonucleotide microarray technology. Out of 32,996 genes analyzed, 1878 exhibited significant changes in expression level (fold change > 2, $p < 0.05$) at least once throughout the differentiation process. These 1878 genes were classified into 12 groups by k-means clustering, based on their expression patterns. K-means clustering analysis revealed that the genes involved in astrogenesis were categorized into the clusters containing constantly upregulated genes, whereas the genes involved in neurogenesis were grouped to the cluster showing a sudden decrease in gene expression on Day 8. Functional analysis of the differentially expressed genes indicated the enrichment of genes for Pax6-NeuroD signaling—TGFB-SMAD and BMP-SMAD—which suggest the implication of these genes in the differentiation of NPCs and, in particular, key roles for Nova1 and TGFBR1 in the neurogenesis/astrogenesis of mouse spinal cord.

Keywords: astrogenesis, microarray, neural precursor cells, neurogenesis, spinal cord

Introduction

Genetic programming of multipotent neural precursor cells (NPCs) of the embryonic spinal cord is a critical prerequisite to the generation of neurons, oligoden-

drocytes, and astrocytes *in vivo*. The differentiation of NPCs occurs in highly specific manners both in temporal and spatial aspects (Lee *et al.*, 2003) and results in production of neuronal-restricted precursors (NRPs) and glial-restricted precursors (GRPs), which subsequently develop into mature neurons and glial cells, respectively (Kalyani *et al.*, 1998). *in vivo*, NPCs generate neurons first and then produce mainly astrocytes (Sun *et al.*, 2003; Cheng *et al.*, 2006). This unvarying sequential generation, the so-called gliogenic switch, is reported to be conserved across vertebrates having a central nervous system (CNS), suggesting its fundamental role in neuronal differentiation mechanisms (Deneen *et al.*, 2006). This sequential generation of neurons and astrocytes can be repeated *in vitro*. It has been reported that when incubated in growth factor-free culture medium, NPCs from early developmental stages differentiate into neurons, whereas those from late embryonic and early postnatal stages differentiate into astrocytes (Qian *et al.*, 2000).

Proliferation of NPCs occurs and stays in the ventricular zone of the neural tube at an early developmental stage. In the presence of intrinsic and environmental signals, some of these precursor cells exit the proliferation cycle and express young neuronal and glial characteristics. The postmitotic cell bodies and differentiating cells are confined within the mantle layer and give rise to a distinct grey appearance. Within this layer, some neurons develop local interconnections, while others elaborate axons to distal targets and form the marginal layer during the process. Glial cells gradually encapsulate the axons producing a marginal layer, which demonstrates a distinctive white appearance in the adult spinal cord. In this way, neuronal and glial cells follow a developmental program that depends on temporal and regional patterning (Poh *et al.*, 2002).

Recent progress in the field has improved our understanding of the genetic control involved in neurogenesis and gliogenesis. For example, the identification of the basic helix-loop-helix (bHLH) transcription factor has allowed us to further understand the molecular regulation of vertebrate neurogenesis and gliogenesis (Bertrand *et al.*, 2002; Kageyama *et al.*, 2005; Lu *et al.*, 2000; Zhou and Anderson, 2002). Despite the identification of intrinsic cellular factors and extracellular cues, the molecular bases of general programs, including sequential generation, are not yet fully understood. In this study, we employed microarray analysis to monitor the tempo-

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Accepted 29 April 2009

ral changes of gene expression during NPC differentiation and to identify the genes playing pivotal roles in the process.

Methods

Cell culture

Spinal cords of C57BL/6 mouse embryos at embryonic day 11.5 (E11.5) were dissected in Hank's buffered saline solution (Invitrogen, Carlsbad, CA, USA). Cells from spinal cords were mechanically dissociated and plated on T-25 or 6-well culture dishes. For neurosphere formation, cells were grown in DMEM/F12 (1:1, Invitrogen) media with N2 supplement (Invitrogen) at 37°C in a 5% CO₂ humidified atmosphere in the presence of 20 ng/ml epidermal growth factor (EGF; Invitrogen) and basic fibroblast growth factor (bFGF, Invitrogen) for 4 days. EGF and bFGF were added to the culture medium every 2 days. Subsequently, the culture medium containing floating neurospheres was collected in sterile 15 ml tubes and centrifuged at 110 relative centrifugal forces for 5 min. The neurosphere pellets were dissociated with trypsin, and single cells were seeded at a density of approximately 4×10^4 cells/cm² in 6-well culture dishes or 12-mm glass cover slips coated with poly-L-ornithine (0.1 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) and fibronectin (1 µg/ml, Sigma-Aldrich). Cells in the culture dishes were allowed to attach during growth in the same medium for 2 days. Differentiation was induced by withdrawal of bFGF and EGF. These spinal cord precursor cells were incubated under the differentiation conditions for 8 days.

Immunocytochemistry

Spinal cord precursor and differentiated cells were fixed in 4% paraformaldehyde-containing phosphate-buffered saline (PBS) at room temperature for 10 min. After rinsing several times with PBS, the cells were incubated for 10 min in PBS containing 0.4% Triton X-100 and re-rinsed with PBS containing 0.1% Tween-20 (TPBS). Then, the cells were incubated for 2 h with 10% horse serum or 10% goat serum in TPBS. To reduce non-specific antibody binding, the cells were incubated overnight at 4°C with primary antibodies depending on conditions; mouse monoclonal anti-nestin (1:100, Chemicon, Temecula, CA, USA), mouse monoclonal Tuj1 (1:100, Covance, East Lansing, MI, USA), low-affinity neurotrophin receptor (p75, 1:100, Chemicon), and GFAP (1:100, DAKO, Glostrup, Denmark). After rinsing with TPBS 3 times, the cells were incubated with fluorescein isothiocyanate- or Texas red-conjugated secondary anti-

body (1:100, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. Cell nuclei were counter-stained with 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories) for 10 min at room temperature. Fluorescent images were captured with a Leica DM IRB microscope (Leica Microsystems, Wetzlar, Germany)

RNA isolation and DNA microarray

Total RNA was extracted using Trizol reagent (Invitrogen) in accordance with the manufacturer's manual. Quantification and determination of purity (260/280 ratio) of RNA were performed using an ND-1000 UV/VIS spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). A commercial mouse genome survey array was used for differential gene expression profiling (Applied Biosystems, Foster City, CA, USA). The array consists of 60-mer oligonucleotide probes, representing a set of 32,996 individual mouse genes and more than 1000 control probes. Microarray experiments were performed following the manufacturer's instructions. Briefly, digoxigenin (DIG)-UTP-labeled cRNA was generated by amplification of 5 µg of total RNA using a chemiluminescent reverse transcription *in vitro* transcription labeling kit (Applied Biosystems). Each microarray was prehybridized in hybridization buffer with blocking reagent at 55°C for 1 h. Hybridization of arrays was then carried out using DIG-labeled cRNA (10 µg), fragmented into sizes of 100-400 bp, at 55°C for 16 h. The arrays were washed with hybridization wash buffer, followed by a subsequent wash with chemiluminescence rinse buffer. Chemiluminescent signals were generated by incubating the arrays with anti-DIG alkaline phosphatase and chemiluminescence substrate. Images were collected for each microarray using the Model 1700 Chemiluminescent Microarray Analyzer (Applied Biosystems). Microarray images were autogridded, and the chemiluminescent signals were quantified, corrected for background, and spatially normalized.

Analysis of microarray expression data

Signal intensities were imported into GenPlex software (Istech, Korea) and normalized to reduce interarray variation in hybridization intensity after filtering out probe sets with unreliable intensity values (flag value < 100 and S/N < 2). Expression changes were presented as fold-changes in comparison with intensities from the control group. The expression intensities were then log₂-transformed. We took the average value from the gene expression ratio obtained in 3 biological replicates. DEGs were selected on the basis of ratios (fold change > 2) and Welch's t-test (p < 0.05). For further analysis,

Table 1. RT-PCR primer sequences

Gene name	Forward	Reverse
Nkx2_2	GGAGGACTCGATCCTTACCAC	AGTCAATGGAATCTGCCA
Pax6	GGACCACTTCAACAGGACTCA	TTTCTCTCTCGATCACATGC
Neurod6	GAAAGTACCTCCCCTGAGTGTG	CTGCGCAGATGTAAGTCGTAAG
Ntrk2	TTCCGCTAGGATTGGTGATC	GAGCAGCCAGACGTGCAGATG
BMP4	TGTGAGGAGTTTCCATCACG	TTATTCTTCTTCTGGACCG
Ncam2	GGCATCAGAAACCTGGAAAA	CCTCCATCATCTTGCTTGGT
Nova1	CTAGCAGCCAGTGCAGTTTCTA	GGGCAAACCTCAGAGGTTAACAG
TGFBR1	TGAGTCACTGGGTGTTATGAGG	ACTTAAGTCTGCAGCGAGAACC
β -actin	CCATCATGAAGTGTGACGTTG	GTGCTAGGAGCCAGAGCAGTA

Fig. 1. Differentiation of spinal cord-derived NPCs. (A) Immunocytochemistry of NPCs, neurons, astrocytes, and motoneurons. (B) DAPI nuclear staining results. Most cells at Day 0 were immunostained in green with the intermediate filament nestin, an NPC marker. Differentiation of NPCs was induced by withdrawal of bFGF and EGF. After 2, 4, and 8 days of bFGF and EGF removal, differentiated cells were immunostained with Tuj1 (green), the neuron marker GFAP (red), and the astrocyte and motoneuron marker p75 (green). The results shown in the graph are percentages (mean $\pm$ SEM; $n=3$) of nestin-, Tuj1-, GFAP-, and p75-immunoreactive cells relative to total cell numbers. Scale bar, 50 μ m. * $p<0.05$ and $^{\dagger}p<0.05$, significantly different from differentiation at Day 0. One-way analysis of variance (ANOVA) was applied.

DEGs were divided into gene clusters according to expression levels at each time point (Chung *et al.*, 2007; Ko *et al.*, 2008). K-means clustering was applied to genes using the Euclidean distance as a similarity measurement, as implemented in the Genesis software

program (<http://genome.tugraz.at>). Additionally, DEGs were categorized according to their biological processes using the PANTHER (Protein ANALYSIS THrough Evolutionary Relationships) protein classification system (Applied Biosystems, <https://panther.appliedbiosystems.com>).

Protein interaction network analysis

To further define the molecular mechanism involved in the differentiation of spinal cord precursor cells, Cytoscape ver. 2.5 software was used for protein interaction analysis to identify potential regulatory genes and pathways. The molecular interaction data were downloaded from the BIND and BIOGRID websites. Each dataset was loaded separately to the software, and 2 distinct protein-protein interaction networks were generated. For each network, the DEGs were mapped, and the proteins that interacted directly with the DEGs were selected to generate groups of subnetworks. The subnetworks were merged into a single subnetwork using the "union" function in Cytoscape, and the nodes interacting with more than 9 proteins were selected.

Verification of microarray

For semiquantitative RT-PCR, beta-actin was used as an internal control. For each sample, the same total RNA that was used for microarray hybridization was reverse-transcribed using 1 μ M oligo (dT) primer with

Superscript II reverse transcriptase (Invitrogen). The thermal profiles consisted of 95°C for 5 min for initial denaturing, followed by 25~30 cycles of 95°C for 30 s, 55~60°C for 30 s, and 72°C for 30 s to 1 min. All RT-PCR reactions were repeated at least 3 times. The primers used in this study are listed in Table 1.

Results and Discussion

Verification of spinal cord precursor cells into neuronal cells by immunocytochemistry

Prior to gene expression study of spinal cord precursor cells, their differentiation process was monitored by immunocytochemical detection using the most representative neural markers, such as nestin, Tuj1, and GFAP. The differentiation was initiated by removing basic fibroblast growth factor (bFGF) from the medium on Day 0, and the process was monitored on Days 2, 4, and 8 after the initiation (Fig. 1).

On Day 0, most cells in the culture ($92.6 \pm 1.1\%$) were nestin-positive, a common characteristic for NPCs, whereas only a small portion (less than 2.5% and 3.5%)

Fig. 2. (A) Hierarchical clustering of 1878 genes. These genes were clustered hierarchically on the basis of the similarity of their expression profiles. The intensity of red and green is proportional to the relative gene induction (red) or repression (green). (B) Number of differentially expressed genes on each differentiation day. Black bar represents downregulation and grey bar represents upregulation.

of the total cells were found to be tubulin β III (Tuj1)-positive and glial fibrillary acidic protein (GFAP)-positive, specific characteristics for neurons and astrocytes, respectively. As the differentiation progressed, the proportion of nestin-positive cells in the culture decreased subsequently to $71.0 \pm 9.8\%$, $6.3 \pm 2.5\%$, and $3.7 \pm 1.5\%$ on Days 2, 4, and 8, respectively. In the meantime, the proportion of Tuj1-positive cells reached $5.7 \pm 1.7\%$ of total cells on Day 2 and decreased gradually thereafter ($3.9 \pm 1.4\%$ on Day 4 and $3.3 \pm 0.4\%$ on Day 8). It was noticed that a small portion of the Tuj1-positive cells were also immunoreactive to anti-p75 antibody on Days 2, 4, and 8, which is a characteristic of motoneurons in the developing neural tube (Camu and Henderson, 1992). The number of GFAP-positive cells increased along the differentiation process, representing $8.9 \pm 0.3\%$ on Day 2, $75.9 \pm 6.0\%$ on Day 4, and $86.5 \pm 0.8\%$ of total cells on Day 8. This observation coincides well with a previous report (Kalyani *et al.*, 1997), in which GFAP-positive cells constituted the largest portion ($73 \pm 6.0\%$) of the total population of differentiated neuroepithelial cells after 5 days of differentiation.

Based on the immunocytochemical observation pre-

sented in this study, it was confirmed that spinal cord precursor cells sequentially generate neurons and astrocytes along the process, the so-called gliogenic switch, as described in a previous report (Qian *et al.*, 2000).

Identification of differentially expressed genes

To examine the temporal changes in gene expression along the differentiation, the expression level of the whole genome was profiled using a microarray. Total RNA from cells on Day 0, Day 2, Day 4, and Day 8 of differentiation was subjected to microarray analysis. Gene expression levels in NPCs (Day 0) were compared with those in differentiated cells (Days 2, 4, and 8). Out of the 32,996 genes represented on the chip, 1878 were differentially expressed more than 2-fold at least at one point during the experiment (Fig. 2A and Supplemental data). On Day 2, 517 genes were upregulated and 379 genes were downregulated. On Day 4, 658 genes were upregulated and 403 genes were downregulated. On Day 8, when most spinal cord precursor cells were differentiated into astrocytes, 1554 genes showed modu-

Fig. 3. K-means clustering of genes exhibiting altered expression levels during NPC differentiation. The 1878 genes were classified into 12 clusters based on similar expression over the 3 time points. The number at the center of each cluster inset represents the number of genes in that particular cluster. Lines indicate the mean expression value.

lated expression levels (Fig. 2B).

K-means clustering analysis of differentially expressed genes

To characterize the patterns of gene expression along the time course of differentiation, k-means clustering was performed on the 1878 differentially expressed genes. The genes were classified into 12 clusters (Fig. 3). Six clusters showed a gradual increase by Day 8.

The expression of genes in clusters A and B was upregulated more than 2-fold at all time points. In particular, Cluster B comprised highly upregulated genes (>eight folds) and included the astrocyte-specific marker GFAP. Clusters C and D consisted of genes that were constantly upregulated more than 2-fold at all time points. Four clusters contained genes with decreasing expression as the differentiation progressed. Clusters G and K comprised genes whose expression decreased more than 2-fold at all time points.

Table 2. Abundance of differentially expressed genes categorized by their biological function in each cluster

PANTHER classification category	Cluster	Number of DEGs in array	Number of changed genes in each cluster	Expected value	p value
Neuromuscular synaptic transmission	A	4	2	0,17	0,012
Antioxidation	A	5	2	0,21	0,018
Transport	B	91	3	0,74	0,034
Apoptosis	B	39	2	0,32	0,039
Neuronal activities	C	61	6	2,13	0,019
Homeostasis	C	18	3	0,63	0,025
Intracellular signaling	C	21	3	0,73	0,037
Cell structure	C	54	5	1,88	0,039
Neuronal activities	D	61	12	4,99	4,5e-3
Cell adhesion-mediated signaling	D	34	7	2,78	0,022
Cell adhesion	D	65	10	5,32	0,041
Synaptic transmission	D	31	6	2,54	0,043
Developmental processes	D	207	24	16,95	0,043
Coenzyme metabolism	E	12	8	2,07	1,2e-3
Lipid metabolism	E	75	25	12,94	1,4e-3
Lipid and fatty acid transport	E	16	9	2,76	2,0e-3
Immunity and defense	F	97	22	9,46	2,1e-4
Carbohydrate metabolism	F	53	15	5,17	2,4e-4
Detoxification	F	7	5	0,68	6,7e-4
Sulfur metabolism	F	7	4	0,68	5,1e-3
Phospholipid metabolism	F	18	5	1,76	0,032
Cell cycle	G	110	36	8,69	1,7e-13
DNA metabolism	G	47	12	3,71	3,7e-4
Extracellular matrix-mediated signaling	G	9	4	0,71	5,9e-3
Protein phosphorylation	G	63	10	4,98	0,028
Nucleic acid metabolism	H	266	75	41,55	1,4e-7
Oncogene	I	9	3	0,5	0,014
Immunity and defense	I	97	11	5,41	0,019
Immunity and defense	J	97	16	7,6	4,0e-3
Proteolysis	J	64	11	5,02	0,012
Nucleic acid metabolism	K	266	65	35,99	9,2e-7
DNA metabolism	K	47	20	6,36	7,9e-6
Cell cycle	K	110	32	14,88	4,0e-5
Nuclear transport	K	13	6	1,76	9,0e-3
Cell structure and motility	L	98	19	5,75	3,9e-6
Cell adhesion	L	65	13	3,81	1,1e-4
Neurogenesis	L	77	12	4,52	1,8e-3
Signal transduction	L	270	24	15,84	0,022

Representative groups with p value lower than 0,05 in each cluster are listed. Expected value is the number of genes that would be expected for a particular PANTHER category, based on 1878 differentially expressed genes (reference list). p value was determined by the binomial statistic.

Functional enrichment analysis of K-means clustered genes

To identify genes involved in the differentiation of spinal cord precursor cells, it was of interest to apply an ontological study to the clustered genes. For this purpose, functional enrichment analyses were performed on the differentially expressed genes grouped in each cluster. Table 2 summarizes the list of biological functions that were overrepresented among the differentially expressed genes in each cluster. Based on the enrichment analysis, only clusters A, C, D, and L showed significant enrichment ($p < 0.05$) of biological functions related to neuronal development, such as development/developmental processes, neuronal activities, neurogenesis, and synaptic transmission. The list of genes that contributed to enrichment of biological function in clusters A, C, D, and L is summarized in Table 3.

In cluster C, in which most genes were constantly up-regulated more than 2-fold at all time points, we noticed the presence of basic helix-loop-helix domain-containing class B5 (Bhlhb5). In a previous report, the Bhlhb5 gene was identified as a member of a new subclass of the bHLH family (Bramblett *et al.*, 2002; McLellan *et al.*, 2002). The proteins in this class, as well as members of

the HES and ID families, are considered negative regulators of the function and/or transcription of other bHLH proteins (e.g., Mash1, Math, and Neurogenin2) that are necessary and sufficient to promote neuronal differentiation (Kageyama *et al.*, 2005). It has been also reported that the inhibitory HLH factors Id1 and Hes1 stimulate astrogenesis *in vitro* (Sugimori *et al.*, 2007) and that mouse Bhlhb5 represses a human Pax6 promoter (Xu *et al.*, 2002). Bhlhb5, constantly upregulated along the differentiation, suggests a possible implication of this protein during gliogenic differentiation as a transcriptional regulator.

Cluster D, which consisted of 168 constantly upregulated genes, showed significant enrichment of genes related to biological functions, such as neuronal activities, synaptic transmission, and developmental processes (Table 2). Among the genes that belong to enriched biological functions in cluster D, Nkx2.2 and BMP/SMAD have been shown to promote gliogenesis (Lee *et al.*, 2003; Nakashima *et al.*, 2001; Fukuda *et al.*, 2007). In addition, the expression of truncated tyrosine kinase B (trkB-t) has been reported to play a pivotal role in the developmental transition from neurogenesis to gliogenesis (Cheng *et al.*, 2006; Climent *et al.*, 2000). These reports strongly suggest a possible association of the

Table 3. List of neuromuscular synaptic transmission-, neuronal activity-, synaptic transmission-, developmental process-, and neurogenesis-related genes in each cluster

PANTHER classification category	Cluster	Probe id	Gene name	Symbol
Neuromuscular synaptic transmission	A	377521	Syntrophin, acidic 1	Snta1
	A	629403	Dystrobrevin alpha	Dtna
Neuronal activities	C	338995	Solute carrier family 1, member 1	Slc1a1
	C	749790	Syntrophin, gamma 2	Sntg2
	C	494196	Doublecortin and calcium/calmodulin-dependent protein kinase-like 1	Dcamk1
	C	336928	Basic helix-loop-helix domain containing, class B5	Bhlhb5
	C	718185	Cerebellin 2 precursor protein	Cbln2
	C	533127	SH3-domain GRB2-like 3	Sh3gl3
Neuronal activities	D	776947	Phosphatase and actin regulator 1	Phactr1
	D	452059	Gamma-aminobutyric acid (GABA-B) receptor, 1	Gabbr1
	D	925263	Synaptic vesicle glycoprotein 2 a	Sv2a
	D	854132	Synaptoporin	Synpr
	D	889212	RAB3A interacting protein (rabin3)-like 1	Rab3il1
	D	833456	Neural cell adhesion molecule 2	Ncam2
	D	786106	Sortilin-related VPS10 domain containing receptor 3	Sorcs3
	D	377670	Fasciculation and elongation protein zeta 1	Fez1
	D	803105	Neuroigin 1	Nlgn1
	D	608088	Syntrophin, gamma 1	Sntg1
	D	931645	Plasma membrane proteolipid	Plip
	D	852817	Neurexin III	Nrxn3
	D	452059	Gamma-aminobutyric acid (GABA-B) receptor, 1	Gabbr1
Synaptic transmission	D	925263	Synaptic vesicle glycoprotein 2 a	Sv2a
	D	854132	Synaptoporin	Synpr
	D	608088	Syntrophin, gamma 1	Sntg1
	D	889212	RAB3A interacting protein (rabin3)-like 1	Rab3il1

Table 3. Continued

PANTHER classification category	Cluster	Probe id	Gene name	Symbol
Developmental processes	D	852817	Neurexin III	Nrxn3
	D	930146	Four and a half LIM domains 1	Fhl1
	D	370481	Down syndrome cell adhesion molecule-like 1	Dscaml1
	D	530589	Bone morphogenetic protein 4	Bmp4
	D	785886	Cytoplasmic polyadenylation element binding protein 2	Cpeb2
	D	803105	Neuroigin 1	Nlgn1
	D	827822	Very low density lipoprotein receptor	Vldlr
	D	744253	Tensin 3	Tns3
	D	506452	Prickle like 1 (Drosophila)	Prickle1
	D	350633	Amyloid beta (A4) precursor-like protein 1	Aplp1
	D	753186	Tripartite motif protein 2	Trim2
	D	827245	Thrombospondin, type I, domain containing 7A	Thsd7a
	D	550580	Cytoplasmic FMR1 interacting protein 2	Cyfp2
	D	870866	Leucine rich repeat neuronal 6C	Lrrn6c
	D	352965	t-complex 11 (mouse) like 2	Tcp11l2
	D	437292	MAD homolog 7 (Drosophila)	Smad7
	D	930146	Four and a half LIM domains 1	Fhl1
	D	641040	Protocadherin beta 14	Pcdhb14
	D	729136	Protocadherin beta 15	Pcdhb15
	D	651940	Protocadherin beta 20	Pcdhb20
	D	833456	Neural cell adhesion molecule 2	Ncam2
	D	626399	Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	Sema6a
	D	342710	Neurotrophic tyrosine kinase, receptor, type 2	Ntrk2
	D	371284	NK2 transcription factor related, locus 2 (Drosophila)	Nkx2-2
	D	586957	DIX domain containing 1	Dixdc1
Neurogenesis	L	586936	Reticulon 4	Rtn4
	L	612748	Neurogenic differentiation 6	Neurod6
	L	456676	Sema domain (semaphorin) 5A	Sema5a
	L	681156	Roundabout homolog 2 (Drosophila)	Robo2
	L	621590	Eph receptor B1	Ephb1
	L	760825	Cadherin EGF LAG seven-pass G-type receptor 1	Celsr1
	L	508826	Contactin 5	Cntn5
	L	596198	Sidekick homolog 2 (chicken)	Sdk2
	L	331509	Kelch-like 25 (Drosophila)	Klhl25
	L	923718	Cadherin 11	Cdh11
	L	646184	Brevican	Bcan
	L	377059	Sema domain (semaphorin) 5A	Sema5a

upregulation of these genes (Nkx2.2, BMP4, Smad7, Ntrk-2) with gliogenic differentiation of spinal cord precursor cells.

Neurogenesis-related genes were found to be highly enriched in cluster L, which showed declining upregulation patterns along the differentiation time. In particular, the Neurod6 gene was upregulated on Days 2 and 4 but decreased on Day 8 in our experiments. Neurod6, also called Nex, Atoh2, and Math2, is a member of the NeuroD family of bHLH transcription factors (Guo *et al.*, 2002) and is a critical effector of the nerve growth factor (NGF) pathway (Schwab *et al.*, 2000). It is also involved in neuronal differentiation in PC12 cells and may be one of the executors of neurogenic function of Pax6 (Holm *et al.*, 2007). It has been reported that Pax6 may be in-

volved in the neuronal commitment of the spinal cord and induces the expression of Ngn2, a proneuronal gene, in the mouse spinal cord (Scardigli *et al.*, 2001). Robo2 and Sema5a, known as axon guidance molecules (Yue *et al.*, 2006; Kantor *et al.*, 2004), are reported to be regulated by Pax6 function (Jones *et al.*, 2002; Jiménez *et al.*, 2002). Based on these early reports, Pax6-NeuroD6 and its downstream signaling seem to have important roles in neurogenic differentiation of the spinal cord.

The immunocytochemistry study in the earlier section revealed that there was significant differentiation of the astrocyte on Day 4. But the expression analysis in cluster D showed genes involved in astrocyte differentiation, such as Nkx2.2, BMP, Smad7, and NtrK-2, on Day 2 as

well as on Day 4. This observation implies the existence of a repression mechanism of astrocyte differentiation in the early stage between Day 2 and Day 4. It was also in agreement with a previous report in which Ngn1 was suggested to inhibit astrocyte differentiation and promote neurogenesis in midgestational NPCs (Sun *et al.*, 2001). Any significant change in expression of Ngn1 was not observed in the experiment, but Neurod6, one of the bHLH factors, showed significantly increased expression in the early stage of differentiation. To confirm whether neurod6 is able to repress astrocyte differentiation, further investigation might be necessary.

To verify the expression of genes, including those mentioned in this section, 5 selected genes (Lmo2, Nkx2.2, BMP4, Ncam2, NeuroD6) were subjected to semiquantitative RT-PCR analysis. The expression pattern of each gene was consistent with the results of the microarray (Fig. 4).

Computational biomolecule interaction network analysis

For a better understanding of the underlying mechanism of spinal cord precursor cell differentiation, a biological interaction network was constructed using the Biomolecular Interaction Network Database (BIND; <http://bond.unleashedinformatics.com/>) and the Biological General Repository for Interaction Datasets (BIOGRID; <http://www.thebiogrid.org/>) and visualized with Cytoscape v2.5 open source software (<http://www.cytoscape.org>). The network obtained from BIND was composed of 2584 edges (interactions) and 2054 nodes (genes), and the network from BIOGRID was composed of 658 interactions and 424 genes. A single subnetwork was constructed initially by mapping both differentially ex-

pressed genes (DEGs) and their first neighbors for each time point. Up- and downregulated genes were colored

Fig. 4. Verification of microarray data by RT-PCR. Semiquantitative RT-PCR was performed to verify the relative mRNA levels of genes measured by microarray hybridization. Five genes analyzed by microarray and 3 other genes of interest (*) were assayed by RT-PCR. Lmo2, LIM domain only 2; Nkx2.2, NK2 transcription factor related, locus 2; BMP4, bone morphogenetic protein 4; Ncam2, neural cell adhesion molecule 2; NeuroD6, neurogenic differentiation 6; Pax6, paired box gene 6; Nova1, neuro-oncological ventral antigen 1; TGFBR1, transforming growth factor beta receptor I.

Fig. 5. Cytoscape-mediated protein interaction analysis and mapping of subnetwork involved in NPC differentiation at each time point. The network was filtered by showing nodes that were significantly upregulated (red) or downregulated (green) following 2, 4, and 8 differentiation days (>2 fold, $p < 0.05$) and their first neighbors (white).

Fig. 6. Identification of nodes interacting with more than 9 genes in the subnetworks. (A) Nova1 interaction network, (B) TGFBR1 interaction network. Up- and down-regulated genes are colored in red and green, respectively.

in red and green, respectively. Finally, the subnetworks containing 370, 435, and 508 genes were identified for differentiation Day 2, 4, and 8, respectively (Fig. 5). As the key node (gene) among these genes, we identified 2 genes, neuro-oncological ventral antigen 1 (Nova1) and transforming growth factor beta receptor 1 (TGFBR1), which interact with more than 9 proteins in the networks.

Nova1 is reported to be necessary for the development of the motoneuron and for control of the alternative splicing of pre-mRNA involved in synapse formation and activity (Ratti *et al.*, 2008). Because Nova1 is a neuron-specific splicing factor, the genes in the first neighbors of Nova1 were expected to be downregulated on Day 8, the late stage of neuronal differentiation. However, most of the genes in the first neighbor of Nova1 were upregulated in a time-dependent manner along the differentiation (Fig. 6A) according to the expression analysis. Meanwhile, the expression of 6 genes (Sorcs3, Purg, Phactr1, Fgd4, Nrnx3, and Rap1gds1) in clusters 10 and 12 were found to be slightly decreased on Day 8. The network analysis showed that Nova1 has direct interactions with Pax6, BTB, and CNC homology 2 (Bach2). But Pax6 and Nova1 were filtered out in the process of DEG-finding procedures, when we performed semiquantitative reverse transcription polymerase chain reaction (RT-PCR) for each gene to check the expression level of the genes. As shown in Fig. 4, the expression of Pax6 significantly decreased on Days 4 and 8, and the expression of Nova1 was slightly down-regulated on Day 8. The network analysis indicated that Nova1 may play a pivotal role in neuronal development in the spinal cord.

The number of upregulated genes that connected with another node (TGFBR1) also increased in a time-dependent manner, through the differentiation period (Fig. 6B). This result was correlated with gene expression levels monitored by RT-PCR (Fig. 4). Recent studies have shown that TGF-beta1/SMAD signaling induces astrocyte fate commitment of neuronal precursor cells *in vitro* (Stipursky and Gomes, 2007; de Sampaio e Spohr *et al.*, 2002). These observations imply that TGFBR1 has important roles in the astrogenesis of spinal cord precursor cells.

In summary, in order to identify genes involved in regulatory functions of the differentiation process, we analyzed the levels of whole gene expression during the differentiation of spinal cord NPCs. We identified 1878 genes whose expression levels were significantly changed during differentiation and categorized these genes into 12 clusters based on their patterns of expression. Computational analyses on biological function suggested that the Pax6-NeuroD6 pathways may be important in the generation of neurons and astrocytes. The interaction network analysis also sheds light on the possible pivotal roles of Nova1 and TGFbeta in neurogenesis and astrogenesis in the mouse spinal cord, respectively. In particular, further molecular studies on Nova1 will be of great help for elucidating the underlying regulation mechanism of neurogenesis/astrogenesis of NPCs.

Acknowledgments

This work was supported by a grant (06121KFDA424) from the Korea Food & Drug Administration.

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