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Expression profile identifies novel genes involved in neuronal differentiation

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In the presence of NGF, PC12 cells extend neuronal processes, cease cell division, become electrically excitable, and undergo several biochemical changes that are detectable in developing sympathetic neurons. We investigated the expression pattern of the apoptosis-related genes at each stage of neuronal differentiation using a cDNA microarray containing 320 apoptosis-related rat genes. By comparing the expression patterns through time-series analysis, we identified candidate genes that appear to regulate neuronal differentiation. Among the candidate genes, HO2 was selected by real-time PCR and Western blot analysis. To identify the roles of selected genes in the stages of neuronal differentiation, transfection of HO2 siRNA in PC12 cells was performed. Down-regulation of HO2 expression causes a reduction in neuronal differentiation in PC12 cells. Our results suggest that the HO2 gene could be related to the regulation of neuronal differentiation levels. [BMB reports 2008; 41(2): 146-152]

INTRODUCTION

Rat pheochromocytoma-derived PC12 cells have been utilized in the characterization of neurotrophic factors such as nerve growth factor (NGF). NGF treatment induces differentiation of PC12 cells into sympathetic-like neurons, and this process is characterized by neurite outgrowth, electrical excitability, and the presence of synaptic vesicles (1). In addition, PC12 cells have been used widely as a cellular model system for studying the intracellular signaling mechanisms associated with neuronal differentiation and proliferation or apoptosis, and to show neuronal differentiation upon treatment with nerve growth factor (NGF) (2, 3).

Nerve growth factor protein (NGF) is known to have a number of effects on responsive sympathetic and sensory ganglionic neurons. These effects include stimulation of neurite outgrowth, increases in cell size, and induction of certain enzymes involved in neurotransmitter synthesis. In addition, NGF also appears to play a role in supporting the survival of such neurons. For example, sympathetic and responsive sensory neurons fail to survive and extend neurites *in vitro* unless exogenous NGF is added to the culture medium. A clonal cell line known as PC12 has been established from transplantable rat pheochromocytoma, and this line is particularly promising as a model with which to study the mechanism(s) of action of NGF (4).

Stimulation with differentiating factors, including nerve growth factor (NGF), was demonstrated to initiate a variety of signaling events that led to the cessation of growth, the acquirement of electrical excitability, the expression of neuron-specific marker proteins, and the extension of long and branching neurites (5-7). It is well accepted that the differentiation of neurons and neurite outgrowth involve alterations in gene expression (8).

The objective of the present study was to investigate candidate genes related to the neuronal differentiation expression level in PC12 cells by microarray technology, RT-PCR, and real-time PCR. In addition, we selected the HO2 gene among a number of candidate genes, and observed the effect of HO2 siRNA treatment on neuronal differentiation in PC12 cells.

RESULTS

Morphological characteristics of PC12 cells

To study gene expression during neuronal differentiation, we performed differential screening of the arrays of 332 cDNA clones related to rat brain development. Complex cDNA targets of PC12 cells treated with NGF were compared with untreated control PC12 cells. PC12 cells were treated with NGF, and were found to respond rapidly to NGF treatment. One day after NGF treatment, cells began to extend neurites. On the second day of NGF treatment, neurites reached the diameter of the cell body. The morphological characteristics of differentiated PC12 cells treated with 100 ng/ml NGF for 7 days were compared with those of control cells, and the neurites of NGF-treated cells were found to be elongated (data not shown). We compared the pattern of gene expression between differentiated PC12 cells and undifferentiated PC12 cells using rat cDNA microarrays. The expression microarray consisted of

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duplicated cDNAs for 332 genes.

Cluster graph analysis of the gene expression of NGF-treated PC12 cells by the gene shaving method

To comprehensively analyze the change of gene expression, we applied the clustering format, and the gene expression data derived from cDNA microarray analysis were then explored. Fig. 1(a), (b), (c) showed the cluster images of different gene expression profiles. From the data, we selected differentially expressed genes at various time points. The normalized log ratios that corresponded to each time point were exported to the software for analysis by clustering algorithm and gene shaving. To estimate the number of clusters in a dataset, we also used gap statistics in Acuity 3.1. The cluster number that had been estimated by gap statistics was used as the input parameter in gene shaving. Genes involved in cluster 1 were down-regulated 3 days after treatment with NGF. The patterns of clusters 2 and 3 were similar.

To characterize the gene expression patterns, we used another clustering method that is used to determine correlation as a measure of similarity. Fig. 1(d) shows that the hierarchical clustering method identifies the pair of genes that are most similar, joins them together, and identifies the next-most similar pair of genes. For this purpose, we applied average-linkage hierarchical clustering analysis to a new data matrix that was joined by 'gene shaving' clusters. Our data showed that genes were clustered into two groups on a large scale. We have observed that the genes in 'gene shaving' clusters 2 and 3 are involved in the same cluster, as shown in Fig. 1(a), (b), (c), which indicates that clusters 2 and 3 are very similar. Most of the 'gene shaving' cluster 1 genes were originally clustered in one cluster.

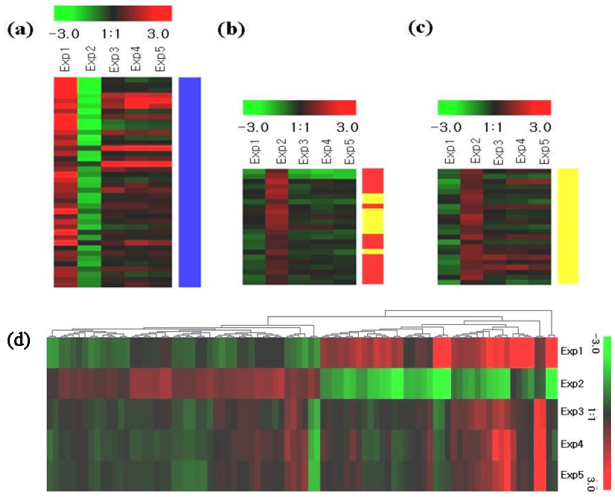

Fig. 1. Cluster graph analysis of the gene expression of NGF-treated PC12 cells by the gene shaving method and hierarchical clustering. The changes in the expression level from time 0 to a later time point (1, 3, 5, 7, and 9 days) after NGF treatment. Genes were clustered into three groups on a large scale using the gene shaving method: (a) Genes in cluster 1; (b) Genes in cluster 2; (c) Genes in cluster 3. Lane 1 is the expression level from time 0 to 1 day; lane 2 is the expression level from time 0 to 3 days; lane 3 is the expression level from time 0 to 5 days; lane 4 is the expression level from time 0 to 7 days, and lane 5 is the expression level from time 0 to 9 days. (d) Gene shaving clusters are joined into a new data matrix. The average-linkage hierarchical clustering method divides three gene shaving clusters into two clusters on a large scale. Lane 1 is the expression level from time 0 to 1 day; lane 2 is the expression level from time 0 to 3 days; lane 3 is the expression level from time 0 to 5 days; lane 4 is the expression level from time 0 to 7 days, and lane 5 is the expression level from time 0 to 9 days.

Here, we confirm that 'gene shaving' clustering is reliable. This finding is supported by hierarchical clustering. To verify the results obtained by microarray analysis, reverse transcription PCR (RT-PCR) analyzes differentially expressed transcripts that represent up-regulated or down-regulated genes for differential expression patterns. In addition, we conducted another gene analysis and searched about 180 genes that were associated with up- and down-regulation in PC12 cells for 1-9 days during NGF treatment. Table 1 shows changes in the expression of genes related to neuronal differentiation in PC12 cells.

Gene selection in up-regulated genes related to neuronal differentiation in PC12 cells

We chose five genes among 180 genes identified by the cDNA microarray, and performed RT-PCR analysis using RNA isolated from NGF-treated PC12 cells. To add to the certainty of microarray data, we conducted real-time quantitative RT-PCR, and varied the time courses of the expression patterns. Time points were 1 day, 3 days, 5 days, 7 days, and 9 days. Changes in mRNA levels of genes were determined by real-time quantitative RT-PCR for 9 days with NGF in PC12 cells. Most genes were investigated in duplicate. The genes shown included the (a) Insulin-like growth factor II (IGFII), (b) Metallothionein-2 and metallothionein-1 genes, (c) Casein kinase alpha subunit II (CKII), (d) Heme oxygenase gene (HO), and (e) Metallothionein-1 (mt-1) (Fig. 2).

Gene selection and down-regulation of HO2 expression

We selected one candidate gene that corresponded well to the results of real-time PCR in terms of the expression of neuronal differentiation. The HO2 (Heme oxygenase 2; BC062061) gene was determined to be up-regulated according to microarray data. In order to induce down-regulation of the HO2 gene, HO2 siRNA treatment of PC12 cells was performed.

In the results, we found that the down-regulation of HO2 resulted in a reduction of mRNA expression levels (Fig. 3A) and protein expression levels (Fig. 3B) in PC12 cells. It turned out that, under the conditions of the experiments, the RNA and protein expression levels were reduced when HO2 siRNA was used to treat PC12 cells.

Moreover, to investigate changes in the expression of neuro-

Table 1. Changes in the expression of genes related to neuronal differentiation in PC12 cells

Gene ID	Up- and down-regulated genes	1 day	3 day	5 day	7 day	9 day
L15618	Rat casein kinase II alpha subunit (CK2) mRNA, complete cds	1.2731	−0.2513	−0.4767	−0.1396	0.2213
M69055	Rat insulin-like growth factor binding protein (rIGFBP-6) mRNA, complete cds	0.7525	0.57	0.5316	0.3377	−0.1044
X75207	R.norvegicus CCND1 mRNA for cyclin D1	0.0213	0.5663	0.8908	1.3249	1.1377
M62781	Rat insulin-like growth factor binding protein 5 (IGFBP-5) mRNA, complete cds	0.4866	0.658	0.4395	0.5808	0.6717
M16459	Rat cellular retinol binding protein (CRBP) mRNA, complete cds	0.4516	−2.1549	−1.3121	−1.0927	0.195
M15480	Rat insulin-like growth factor I (IGF-I) mRNA, complete cds	0.3236	0.2779	−0.1529	0.165	0.1619
D30035	Rat mRNA for HBP23 (heme-binding protein 23 kDa), complete cds	0.2844	−0.4251	−0.2764	−0.2452	−0.2789
J02722	Rat heme oxygenase gene, complete cds	0.2173	1.1265	−1.0549	−1.3999	−1.4624
M31837	Rat insulin-like growth factor-binding protein (IGF-BP3) mRNA, complete cds	0.1081	0.2766	0.1048	−0.0391	0.1258
J04112	Rattus norvegicu liver fructose-1,6-bisphosphatase (Fru-1,6-P2-ase) mRNA. complete cds	0.4334	−0.62	0.3555	0.559	0.4433
D26180	Rat mRNA for novel protein kinase PKN, complete cds	0.2898	−0.5216	−0.1193	−0.0031	0.2295
U28938	Rattus norvegicus protein tyrosine phosphatase D30 mRNA, complete cds	0.5828	−0.7087	−0.0007	0.0817	0.2157
L26288	Rattus norvegicus CaM-like protein kinase mRNA, complete cds	0.1653	−0.0234	0.2804	0.3376	0.2778
M57664	Rat cretine kinase-B (CKB) mRNA, 3' end	0.2735	−0.0519	−0.2826	−0.2605	−0.2586
J05470	Rat mitochondrial carnitine palmitoyltransferase II (CPT II) mRNA, complete cds	0.681	0.4919	0.3903	0.4155	−0.1163
J00750	Rat metallothionein-i (mt-1) mrna	−1.3851	−1.6862	−1.1029	−1.9159	−2.2779
U77582	Rattus norvegicus casein kinase 1 alpha (CKIa) mRNA, complete cds	−1.1177	0.1988	−0.284	−0.1409	−0.3001
U59809	Rattus norvegicus mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2r) mRNA, complete cds	−0.6195	0.1559	−0.4029	−0.2937	−0.3884
AH002243	Rat retinol-binding protein (RBP) gene	−0.5014	1.7535	−1.3797	−1.5803	−1.0474
J05405	Rat heme oxygenase-2 (HO2) mRNA, complete cds	−0.4746	0.2471	0.2067	0.168	0.0943
LOC691570	GAPDH (marker)	−0.4628	−0.5335	0.5606	0.5872	0.146
L07578	Rat casein kinase I delta mRNA, complete cds	−0.4414	−0.1511	0.1501	0.1017	0.0605
U22321	Rattus norvegicus casein kinase 1 gamma 3 isoform mRNA, complete cds	−0.3835	0.234	0.2082	0.1653	−0.071
81822	Actin (marker)	−0.3227	−0.4149	0.2723	0.3821	−0.1223
L15619	Rat casein kinase II beta subunit (CK2) mRNA, complete cds	−0.1414	−0.0037	0.1123	−0.2007	−0.4411
M11794	Rat metallothionein-2 and metallothionein-1 genes, complete cds	−0.9941	−0.9115	−1.8478	−2.3514	−2.6057
NM_031511.1	R.norvegicus insulin-like growth factor II mRNA	−1.2081	−3.0357	−0.8729	0.054	0.4782
M13979	Rat brain glucose-transporter protein mRNA, complete cds	−1.6112	−1.389	−1.0002	−1.1953	−0.8958
J03179	Rat D-binding protein mRNA, complete cds	−0.4991	−0.5089	−0.2926	−0.2972	−0.1568
U62316	Rattus norvegicus monocarboxylate transporter 2 (MCT2) mRNA, complete cds	−0.2437	0.4287	0.172	0.227	0.0818

nal differentiation in PC12 cells, we took a photograph to assess the morphology of PC12 cells. Visual inspection of the images shown in Fig. 3C was applied to three cases: (a) before transfection in PC12 cells, (b) in a control state, and (c) after HO2 siRNA transfection. The figures were photographed in a transfection state after 48 hr.

Therefore, our results indicated that HO2 significantly influenced the neuronal differentiation expression level, which suggested that the HO2 protein is related to the neuronal differentiation expression level and confirmed that the down-regulation of HO2 in the treated PC12 cells represented the differentiation of neurites in PC12 cells.

DISCUSSION

PC12 cells have frequently been used to understand the different aspects of neuronal differentiation. Undifferentiated PC12 cells proliferate in the presence of serum. The addition of NGF results in sympathetic-like neuronal differentiation such as complete mitotic arrest (9, 10).

To monitor candidate genes in neuronal differentiation, we used a rat cDNA microarray containing 320 genes related to the brain.

We selected some differentially expressed genes such as insulin-like growth factor II (IGFII), metallothionein-2 and metallothionein-1 genes, casein kinase alpha subunit II (CKII), heme oxygenase (HO), and metallothionein-1 (MT-1) through time courses.

Insulin-like growth factor II (IGF II) in the brain has been reported to stimulate the proliferation of neuronal and glial precursors, as well as their phenotypic differentiation. IGF II exerts potent neurotrophic effects on cultured chicken ciliary ganglionic neurons (11), and enhanced neurite formation and survival in primary cultured sensory and sympathetic neurons

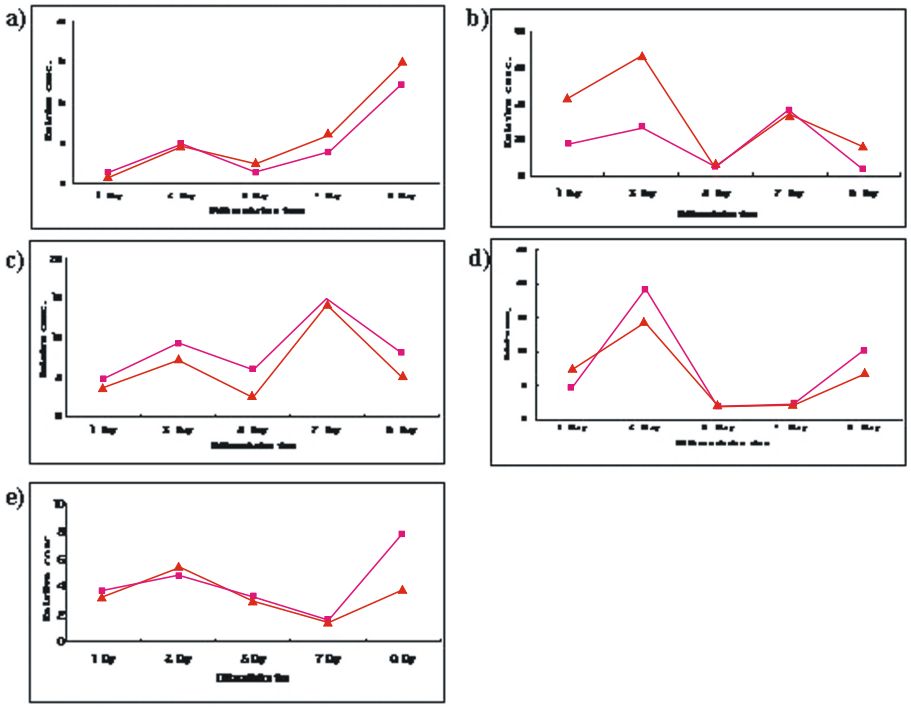

Fig. 2. Real-time PCR analysis. (a) Insulin-like growth factor II (IGFII), (b) metallothionein-2 and metallothionein-1 genes, (c) casein kinase alpha subunit II (CKII), (d) heme oxygenase gene (HO) and (e) metallothionein-1 (mt-1). The time course of the expression pattern varied at time points of 1, 3, 5, 7, and 9 days. The graph represents changes in mRNA levels of respective genes by real-time PCR analysis. The experiments were conducted in genes in duplicate.

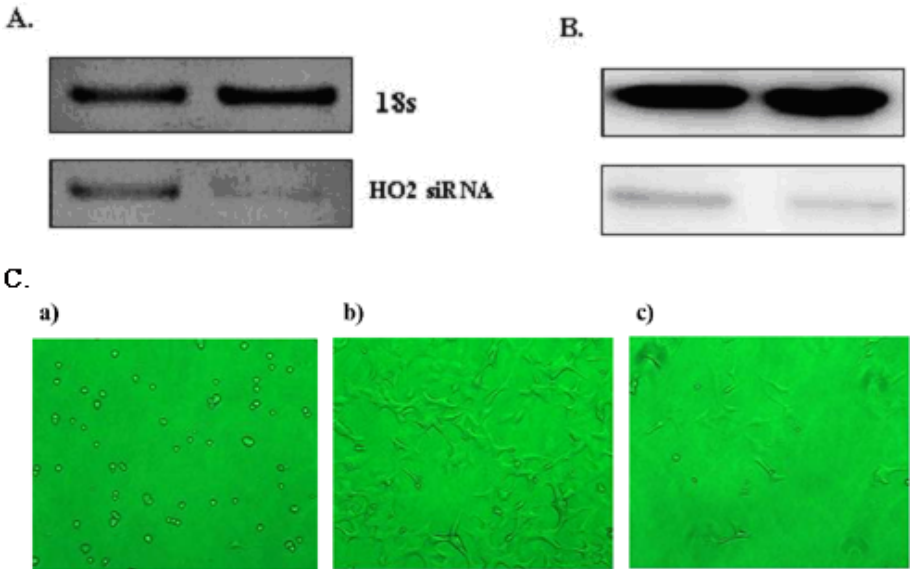

Fig. 3. RT-PCR, Western blot analysis, and morphological characteristics after HO2 siRNA treatment in PC12 cells. Panel A represents RT-PCR analysis after HO2 siRNA treatment. Left column, control that was transfected with only transfection reagent in PC12 cells; right column, HO2 50 nM siRNA transfected in PC12 cells. The bars represent the percentage of the mRNA level in PC12 cells. A clear difference was shown by the data following control and HO2 siRNA treatment in PC12 cells. Panel B represents Western blot analysis after HO2 siRNA treatment. Left column, control that was transfected with only transfection reagent in PC12 cells; right column, 50nM HO2 siRNA transfected in PC12 cells. The bars represent the percentage of the protein level in PC12 cells. A difference was shown by the data following control and HO2 siRNA treatment in PC12 cells. Furthermore, to investigate the morphological characteristics of HO2 siRNA treatment in PC12 cells, we took a photograph of PC12 cells. The figures were applied in three cases, respectively: (a) before transfection in PC12 cells, (b) in a control state, and (c) after HO2 siRNA transfection.

from chicken embryonic ganglia (12).

The metallothionein-2 and metallothionein-1 genes act as antioxidants and tissue protective factors. They are important inhibitors of apoptotic cell death in the central nervous system (CNS), and are known to be expressed in glial cells. Mice with metallothionein-2 and metallothionein-1 gene deficiencies showed increased oxidative stress and neuronal apoptosis during epileptic seizures and experimental autoimmune encephalomyelitis, and following traumatic brain injury. Similarly, transgenic mice overexpressing MT-1 showed significantly reduced oxidative tissue damage and cell death during traumatic brain injury. Hence, MT-1 appears to regulate the survival and neurite extension of dopaminergic and hippocampal neurons (13).

Casein kinase alpha subunit II (CKII) is very abundant in the rat brain compared with other rat tissues. Phosphorylation of the MAP1B substrate by CKII may be required for the assembly of microtubules within neurites. The other substrates of CKII are neuronal cytoskeletal proteins, including MAP1A and tau. This indicates a role of the enzyme in the regulation of cytoskeletal function and of mature neurons.

The heme oxygenase 2 (HO2) gene catalyzes the conversion of heme to biliverdin, and is involved in the cellular response to oxidative stress. The HO2 protein plays an important role in the regulation of muscle contractility and in the defense against sepsis-induced oxidative stress (14). HO2, the main enzyme to degrade the pro-oxidant heme, was tested for its neuroprotective ability in postnatal neuronal cell cultures and in a model of collagenase-induced intracerebral hemorrhage. In this report, HO2 is a crucial neuroprotective enzyme in the detoxification of high levels of heme from the brain (15).

Although many of the genes identified by cDNA array as highly and consistently elevated were confirmed by semi-quantitative RT-PCR, many more genes remain unconfirmed, and their roles in neuronal differentiation have not yet been characterized.

Initially, in order to analyze the genome-wide molecular events that occur in neuronal differentiation, we used a cDNA microarray that contained the probes of 322 brain-related genes and expressed sequence tags (ESTs) to profile comprehensive molecular and genetic programs underlying mammalian neuronal differentiation. This model is particularly suitable for establishing the gene expression profile during neuronal differentiation because the neuronal cell population is synchronized and homogeneous, unlike brain tissues or primary neuronal cultures.

We also investigated the degree of changes in neuronal differentiation with NGF in the PC12 cell line. In a previous report, HO2 was identified as an oxygen sensor; down-regulation of HO2 causes certain physiological changes that are important in the maintenance of cellular heme homeostasis (16).

In summary, NGF was related to neurite outgrowth, and HO2 genes were found to be candidate genes that may be related to neuronal differentiation expression levels in PC12 cells. Therefore, we assume that the HO2 gene may have affected neuronal differentiation in PC12 cells, since the neuronal differentiation levels in PC12 cells were altered by treatment with HO2 siRNA.

MATERIALS AND METHODS

Cell culture and differentiation

Undifferentiated PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (WelGENE Inc.) containing 10% horse serum (HS) (Invitrogen), 5% fetal bovine serum (FBS) (WelGENE Inc.), 100 units/mL penicillin, and 100 ug/mL streptomycin (GIBCO BRL) at 37°C with 95% air and 5% CO2. Culture medium was replaced every 1-2 days, and cells were subcultured. PC12 cells were differentiated by culturing cells in DMEM supplemented with 1% FBS, penicillin/streptomycin, and L-glutamine for 24 hours, and subsequent exposure of the cells to 100 ng/mL 2.5S nerve growth factor (grade II, Promega) for 1, 3, 5, 7, or 9 days. NGF-differentiated PC12 cells were re-plated at 5×10^6 cells in T75 flasks. We then replaced media with RPMI 1640 (WelGENE Inc.) containing 2% horse serum and 1% fetal bovine serum upon the expressions of full-length genes and siRNA in PC12 cells.

RNA preparation and semi-quantitative RT-PCR

Total RNA was isolated from PC12 cells. Confluent cells were trypsinized with trypsin, washed with 10 ml PBS, and centrifuged at 1,500 rpm for 5 minutes. Total RNA was prepared by using TRIZOL as directed by the manufacturer (Invitrogen). The purity of isolated RNA was evaluated spectrophotometrically at an absorbance ratio of *A*260 and 280. Five ug of total RNA was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen). For reverse transcription, RNA was mixed with a mixture of 0.5 ug oligo(dt) 12-18 and 10 mM dNTP, incubated at 65°C for 5 minutes, and placed on ice for 1 minute. Two hundred units of SuperScript II reverse transcriptase were then added to the reaction solution to reach a final volume of 20 ul (Invitrogen); the solution also contained 4 units RNase inhibitor, 5 × reaction buffer, and 0.1 M DTT. The mixture was incubated at 42°C for 50 minutes, and was inactivated by heating to 70°C for 15 min for inactivation of the SuperScript II reverse transcriptase. The microarray expression profile identified novel genes involved in PC12 differentiation. Primers used for PCR amplification included: for HO2 (accession number NM_024387), 5'-GGCCACCACTGCACTTTACTTCA-3' as the forward primer and 5'-CCTCCAGGGTTTCTTTTGTTAGCA-3' as the reverse primer; Forward and reverse primers were designed using the DNAStar program.

Array hybridization and data analysis

After washing normal or differentiated PC12 cells with PBS, total RNA was prepared by using TRIZOL reagent according to the instructions of the manufacturer (Invitrogen). Using a

3DNA 50 Expression array detection kit (Geni-sphere), 10-20 g of total RNA was reverse-transcribed, labeled with Cy3 and/or Cy5, and hybridized to the microarray according to the instructions of the manufacturer. Slides were covered with a coverslip and hybridized at 65°C for 16 hours in a sealed chamber (Telechem), washed with 1 × SSC and 0.1% SDS solution at 65°C for 10 minutes, and washed twice with 0.2 × SSC at room temperature for 10 minutes. Slides were dried by centrifugation at a low speed prior to scanning. Images were obtained by scanning the arrays in an ArrayWorx scanner (ArrayWorx). The signal intensity of the Cy3- and Cy5-labeled probe was extracted by using default settings and auto-image segmentation using the ImaGene software package, version 5.0 (BioDiscovery, Inc.). The mean and median intensities of the signal and the background, as well the quality characteristics of the spots ("empty" or "poor"), were obtained. The threshold for "empty" spots was achieved by raising the threshold to the point where all blank spots were flagged (17) Data were analyzed using the GeneSight software package, version 3.5 (BioDiscovery). Data preparation consisted of the following steps: using mean signal and background intensities, background correction was performed by subtracting the local background value from each spot; flagged spots, considered to be empty and poor, were omitted from the analysis; the intensity ratio of two channels was calculated; log2 was calculated; and the two channels were normalized by subtracting the log2 intensity mean of all the exons from each individual exon intensity value. Finally, the replicate experiments were combined and the mean between the intensity ratios of the series of experiments was calculated (18).

Clustering algorithm

The normalized log ratio corresponding to each time point was exported to the software to create a clustering algorithm. We used Axon's Acuity 3.1 for the 'gene shaving' algorithm. To estimate the number of clusters in a dataset, we used the 'gap statistics' function in Acuity 3.1. The cluster number that had been estimated by gap statistics was used as an input parameter in gene shaving. After gene shaving, we used single linkage hierarchical clustering.

Real-time PCR

Real-time PCR was performed on obtained cDNA in the presence of 1 · SYBR Green Mastermix (Applied Biosystems, Courtaboeuf, France) containing preset concentrations of dNTPs, $MgCl_2$, and buffers, along with adequate concentrations of forward and reverse primers that were designed by the DNAStar program.

Cell transfection

The PC12 cells were placed in 100 pie plates (2.0×10^6 cell per plate) and cultured for 24 hr. The medium was RPMI1640 (JBI) supplemented with 10% horse serum (HS; Invitrogen) and 5% fetal bovine serum (FBS; JBI). For transfection, the medium was changed to RPMI1640 (JBI) supplemented with 2% horse serum (HS; Invitrogen) and 1% fetal bovine serum (FBS; JBI) prior to transfection for 2 hr. HO2 siRNA (#SCRO-061127-021, #SCRO-061127-022; order for Samchully Pharm.) was used to treat PC12 cells (19). Thus, we treated the PC12 cells with 50 nM HO2 siRNA.

Protein extraction and Western blot analysis

Total protein was isolated from transfection with HO2 siRNA and MT-1 in PC12 cells. Each PC12 cell sample was homogenized on ice for 40 minutes with pro-prep protein extraction solution (Intron, Cat. No. 17081). Western blot analysis was performed on 30 μg of protein from each cell lysate. Total cell extracts were prepared and separated on a 12% gel. The gel was then electrophoretically transferred to a nitrocellulose membrane (Amersham). The membranes were blocked with 5% skim milk in PBST (1 × PBS by Welgene, 0.1% Tween 20) and incubated with a 1 : 1,000 dilution of polyclonal antibody against HO2 (purchased from Stressgene Bioreagents) and/or a 1 : 4,000 dilution of monoclonal antibody against anti-actin (purchased from Santa Cruz) for 1 hr at room temperature, with constant shaking. The membranes were then washed with 1 × PBST and probed with anti-rabbit IgG (purchased from Upstate) at a dilution of 1 : 5,000. Chemiluminescence detection was performed by using the Amersham ECL detection kit according to the instructions of the manufacturer.

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