Directed neuronal differentiation of mouse embryonic and induced pluripotent stem cells and their gene expression profiles

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Abstract. Embryonic stem cells (ESCs) may be useful as a therapeutic source of cells for the production of healthy tissue; however, they are associated with certain challenges including immunorejection as well as ethical issues. Induced pluripotent stem cells (iPSCs) are a promising substitute since a patient's own adult cells would serve as tissue precursors. Ethical concerns prevent a full evaluation of the developmental potency of human ESCs and iPSCs, therefore, mouse iPSC models are required for protocol development and safety assessments. We used a modified culturing protocol to differentiate pluripotent cells from a mouse iPS cell line and two mouse ES cell lines into neurons. Our results indicated that all three pluripotent stem cell lines underwent nearly the same differentiation process when induced to form neurons *in vitro*. Genomic expression microarray profiling and single-cell RT-qPCR were used to analyze the neural lineage differentiation process, and more than one thousand differentially expressed genes involved in multiple molecular processes relevant to neural development were identified.

Introduction

Neurons and glial cells constitute most of the central and peripheral nervous systems in the vertebrate that regulate and control a wide range of thinking and behaviors; their malfunction can cause serious nervous system diseases. Embryonic stem cells (ESCs) provide significant therapeutic promise as they can be induced to differentiate *in vitro* into various tissues and organs originated from all three germ layers, including neural cell lineages. Significant issues such as immunorejection, ethical concerns and safety have inhibited the advancement of ESCs toward clinical treatments, while induced pluripotent stem (iPS) cells have become an attractive option for regenerative medicine (1) and personalized medicine. Therapeutic use of iPS cells will require greater understanding and control of the reprogramming process, and demonstrations of safety such as lack of tumorigenesis, through studies that cannot be risked in humans. Mouse iPS cells are therefore a suitable model for studying basic mechanisms of development and differentiation and for evaluating the similarities between ES and iPS cells.

Several directed neuronal differentiation methods have been developed, including via embryoid bodies (EBs) (2,3), monolayer cultures (4), and stromal cell-derived inducing activity (SDIA) (1,5). In our study, we utilized a modified neuronal differentiation method (2) to induce a tetraploid complementation competent iPS cell line and two different ES cell lines to differentiate into neurons. Neurons derived from all three sources exhibited nearly the same differentiation patterns during approximately 20 days of *in vitro* culture. Derived cell populations are mixtures, and most cells are positive for the neuron-specific marker protein MAP2. Several neural differentiation stage marker genes were expressed by these cell mixtures as well, including *Blbp* (*Fabp7*), *Nestin* and *Tuj1*. Microarray profiling and single-cell PCR were employed to further analyze the neural lineage differentiation process. Following statistical comparisons and gene ontology analysis, 1,324 differentially expressed genes were identified, some of which are involved in cell morphology, synaptic transmission, neurogenesis and neuron recognition. The genes identified may be useful for investigating important signaling factors and pathways regulating neuronal differentiation.

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Materials and methods

Cell culture. The mouse ES cell line CGR8.8 was a gift from Dr Yanru Chen (Stanford University), and the mouse iPSC line IP14D-1 was derived from B6/DBA2F1 fetal fibroblasts and was confirmed to be capable of developing into a complete embryo using the tetraploid complementation assay (6,7). Both R1 and IP14D-1 were cultured on mouse embryonic fibroblasts (MEFs) with mitotic inactivation, while CGR8.8 was cultured under feeder-free conditions using only 0.1% gelatin-coated culture dishes. The complete culture medium utilized for mouse pluripotent stem cells contained high glucose Dulbecco's modified Eagle's medium with 15% fetal bovine serum tested for ES, 2 mM L-glutamine, 1 mM MEM sodium pyruvate, 1 mM MEM non-essential amino acids, 0.1 mM β-mercaptoethanol and 10^3 U/ml leukemia inhibitory factor (LIF) (ESG1107; Millipore). Culture medium was changed daily and cells were split every 2-3 days. E13.5 ICR mice were sterilely dissected and harvested cortices were made into cell suspension. They were cultured using Neurobasal culture medium containing 1% B27 supplement in a 37˚C, 5% CO_2 incubator ~7-10 days for further usage.

Induction of neural-specific embryoid body formation in conditioned culture medium. R1 and IP14D-1 were passaged on 0.1% gelatin-coated culture dishes prior to EB formation to avoid any effect of MEF cells. EBs of all three pluripotent stem cell clones were digested into single cells and cultured in suspension for the first four days in pluripotent stem cell culture medium without LIF. For further neural-specific induction, EBs were then transferred into neural induction culture medium (NIM) for the following 3 or 4 days. EBs were then transferred into culture plates coated with poly-D-lysine (PDL) and laminin for further adhesion culture with EB culture medium using the following method: coating with a 100 μg/ml PDL solution on clean coverslips in 6-well plates overnight at 37˚C, then washing twice with water to remove PDL and adding laminin solution (5 μg/ml in Hank's media) for a few hours to overnight, and rinsing once with Hank's media prior to use.

Twelve hours later, EB culture medium was replaced with NIM and cultured for one week. NIM was a condition culture medium which included Neurobasal Medium (21103-049) with L-glutamine, NEAA, N2 (17502-048), B27 (17504-044) (Gibco), bFGF, EGF and all-trans retinoic acid (R2625; Sigma) for another week. bFGF and EGF were removed from NIM for subsequent culturing.

Samples of the original R1, CGR8.8, IP14D-1 lines, derived neuronal cells (R1_Ne, CGR8.8_Ne and IP14D-1_Ne), and primary cultured neurons as positive control were harvested in TRIzol for RNA isolation. Reverse transcription of 0.5 μg total RNA produced cDNA for PCR of *Pax6*, *Sox1*, *Blbp*, *Nestin*, *Tuj1*, *Ncam*, *Map2*, which test whether the pluripotent stem cells differentiate into neurons. All PCR primers used are listed in Table I.

Immunocytochemistry confirmation. Mouse pluripotent stem cells grown on coverslips were washed with PBS twice and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were washed again with PBS and permeabilized with 0.5% Triton X-100 in PBS for 15 min. The cells were then

Table I. Primers for pluripotent stem cell directed neuronal differentiation.

	Forward primer	Reverse primer	Size (bp)	Cycles	Parameters
Pax6	5'-GAAATCCGAGACAGATTATTATCCGAG-3'	5'-CCATTTGGCCCTTCGATTAGA-3'	495	35	94˚C-40 sec; 58˚C-30 sec; 72˚C-40 sec
Sox1	5'-CCAAGAGACTGCGCGCGCTG-3'	5'-GGGTGCGCCGGGTGTGCGTG-3'	381	35	94˚C-40 sec; 62˚C-30 sec; 72˚C-40 sec
Blbp	5'-TGAGTACATGAAAGCTCTGGGCGT-3'	5'-TGAGCTTGTCTCCATCCAACCGAA-3'	224	35	94˚C-40 sec; 58˚C-30 sec; 72˚C-30 sec
Nestin	5'-CTGGAACAGAGATTGGAAGGCCGCT-3'	5'-GGATCCTGTGTCTTCAGAAAGGCTGTCAC-3'	403	35	94˚C-40 sec; 59˚C-30 sec; 72˚C-40 sec
Tuj1	5'-ATCCACCTTCATTGGCAACAGCAC-3'	5'-ACTCGGACACCAGGTCATTCATGT-3'	173	35	94˚C-40 sec; 58˚C-30 sec; 72˚C-30 sec
Ncam	5'-TTCCTGTGTCAAGTGGCAGGAGAT-3'	5'-AGATCTTCACGTTGACAGTGGCCT-3'	229	35	94˚C-40 sec; 60˚C-30 sec; 72˚C-30 sec
Map2	5'-AGCCGCAACGCCAATGGATT-3'	5'-TTTGTTCCGAGGCTGGCGAT-3'	313	35	94˚C-40 sec; 58˚C-30 sec; 72˚C-40 sec
Gapdh	5'-GCAAATTCAACGGCACAGTC-3'	5'-TCTTCTGGGTGGCAGTGATG-3'	399	35	94˚C-40 sec; 59˚C-30 sec; 72˚C-40 sec

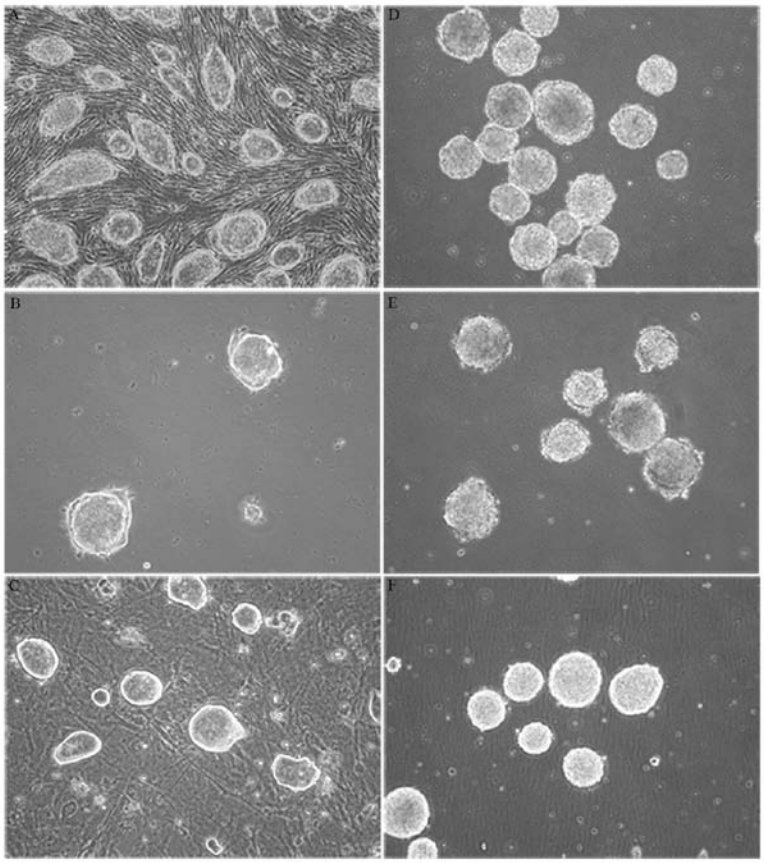

Figure 1. Morphology of the three pluripotent stem cell types and embryoid body formation. Representative cells are shown at 20x magnification from cultures of R1 (A), CGR8.8 (B), and IP14D-1 (C), and for embryoid bodies derived from these cultures (D, E and F), respectively.

blocked with 1% BSA, 0.5% Triton X-100 in PBS for 1 h at room temperature. Mouse anti-mouse MAP2 primary antibodies were diluted 1:200 in 1% BSA, 0.5% Triton X-100 in PBS and incubated with fixed cells at 4˚C overnight. After triple washing with PBS, cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (1:1,000) secondary antibody for 1 h at room temperature. DAPI counter-staining was used for cell nuclei.

Microarray assays of gene expression. Total RNA was extracted from three replicates of each cell population, including primary cultured neurons, three pluripotent stem cells and corresponding neuron populations. cDNA was hybridized to Affymetrix Mouse Gene 1.0 ST Arrays following reverse transcription, *in vitro* transcription amplification and quality control using the manufacturer's standard protocols.

Single cell PCR

Single cell isolation and cDNA synthesis. A micromanipulator was employed to transfer single cells into individual RNase-free EP tubes with 12 μl reverse transcriptase mixture on ice. Reverse transcription reactions were immediately performed to synthesize cDNA. Glycogen, ammonium acetate and cold ethanol were added and stored at -80˚C overnight for precipitation.

In vitro transcription and cDNA synthesis. Single-cell cDNA was amplified by *in vitro* transcription (8,9), and the resulting aRNA was isolated using high-speed low-temperature centrifugal sedimentation. The amplified RNA was converted to single-stranded cDNA for PCR assays of gene expression. PCR conditions were as described above.

Microarray data analysis. We applied the RMA algorithm in Affymetrix Expression Console with default parameters to normalize and summarize probe signals. We utilized the online NIA Array Analysis Tool (http://lgsun.grc.nia.nih.gov/ANOVA/index.html) for hierarchical clustering and statistical testing (6,10,11). For identification of significantly different gene expression levels between pluripotent cell populations and differentiated cells, we set cutoff thresholds at 5% false discovery rate (FDR) and 2-fold magnitude of difference and conducted pairwise comparisons using ANOVA with multiple testing correction (6,10,11). Finally, the DAVID online database and tools (http://david.abcc.ncifcrf.gov/) were employed to annotate the differentially expressed genes by Gene Ontology categories, and affected pathways were examined using Ingenuity Pathway Analysis (http://www.ingenuity.com/products/pathways_analysis.html).

Results

Pluripotent stem cell lines remain viable with shared culture conditions. The ES cell line R1 was established in 1991 from a blastocyst produced by crossing two 129 substrains (129S1/SvImJ and 129X1/SvJ) (12). R1 cells were cultured on inactivated MEF cells as recommended by the ATCC. The CGR8.8 mouse ES cell line was derived from the 129/Ola mouse

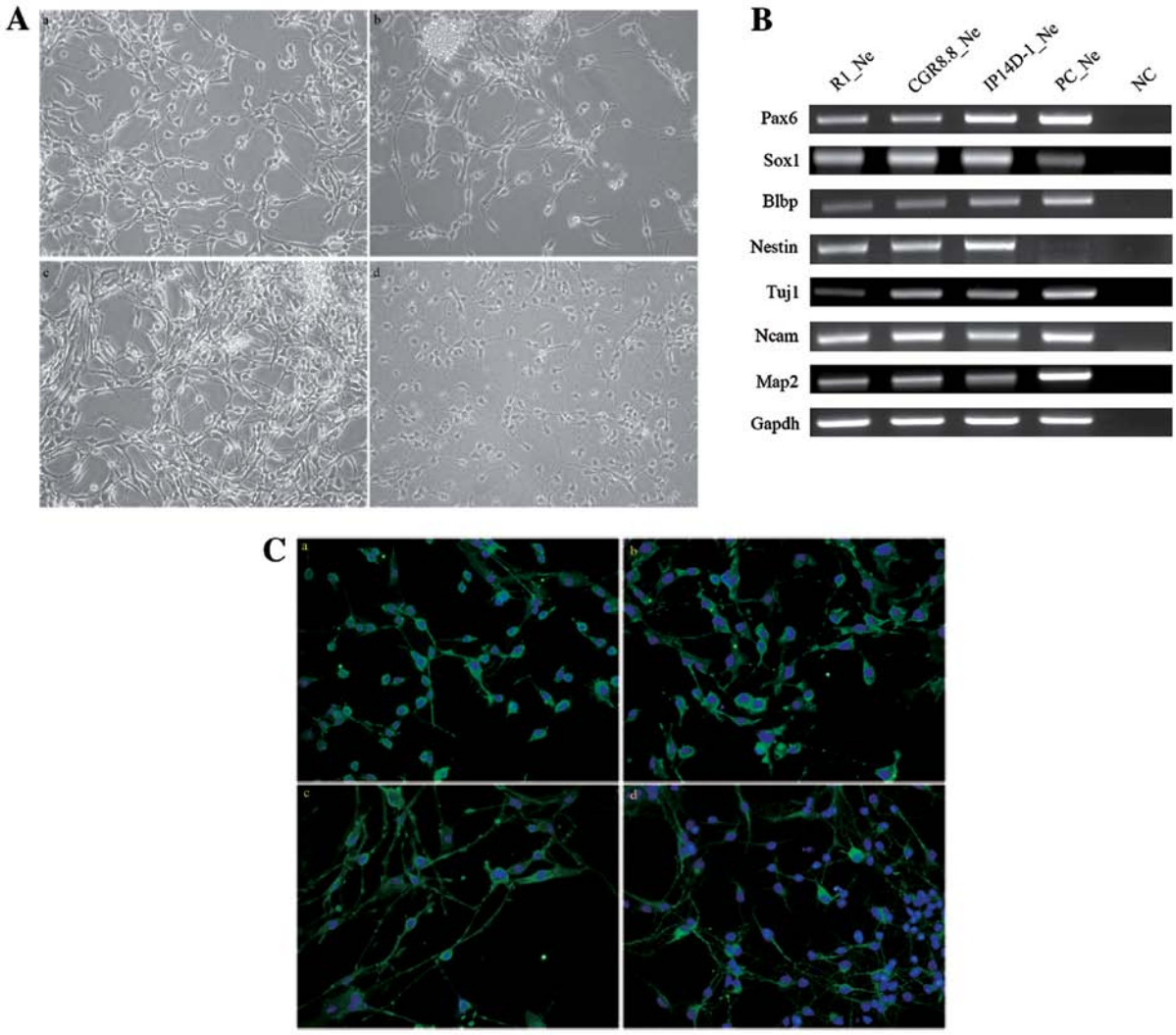

Figure 2. Generation of neurons from the three pluripotent stem cells lines. (A) Representative induced neuron cells are shown at 20x magnification derived from R1 (a), CGR8.8 (b), IP14D-1 (c) and primary cultured neurons (d). (B) RNA expression for neural development marker genes in the neuron-induced cell cultures and primary neuron culture (PC_Ne) was assayed by RT-PCR. (C) MAP2 antigen staining (blue) was positive for the majority of the cells in the induced populations. Cell types are presented as in (A).

strain. The induced pluripotent stem cell line IP14D-1 was induced from C57/DBA2F1 MEFs and can produce embryos by tetraploid complementation as previously reported (6). All three pluripotent stem cell lines were cultured using the same conditions before testing the differentiation procedure. Each showed classic stem cell characteristics such as colony growth with rapid proliferation, smooth edges and strong refraction. There were no obvious cell boundaries within stem cell colonies (Fig. 1A-C). Passages 20, 21 and 28 of R1, CGR8.8 and IP14D-1, respectively, were employed for neuronal differentiation.

Conditioned NIM is beneficial for neural induction from EBs. EB formation is the first stage of differentiation and allows pluripotent stem cells to be primed for further lineage specific development. We adapted a standard neural differentiation method with slight modifications to produce neurons via EB formation. EBs formed after 4 days of suspension culture and then 0.5 μM ATRA was added into NIM for another 4 days of suspension culture (Fig. 1D-F). We used a modified '4+4' induction method developed by Bain *et al* (2). In the second 4 days of EB suspension culture, we replaced EB medium with Neurobasal containing some supplements, growth factors and RA without serum, which was more suitable for promoting neuronal differentiation as serum may have a negative effect on neural induction via RA (13).

Pluripotent stem cells are induced into MAP2 positive neurons. Following suspension culture induction, EBs were transferred into PDL and laminin co-coated plates and cultured with EB culture medium. After 12 h of adhesion, numerous EB cells proliferated and extended from EBs. A week later, cells were cultured in serum-free culture conditioned medium (NIM) for neuronal differentiation. Total EB extension occurred after 2 weeks, and cells showed neuron-like morphology (Fig. 2A).

Neurons derived from each of the three pluripotent stem cell lines were harvested to test for neuronal gene expression patterns. Several genes specifically expressed in neurons and during neural development processes were also expressed by

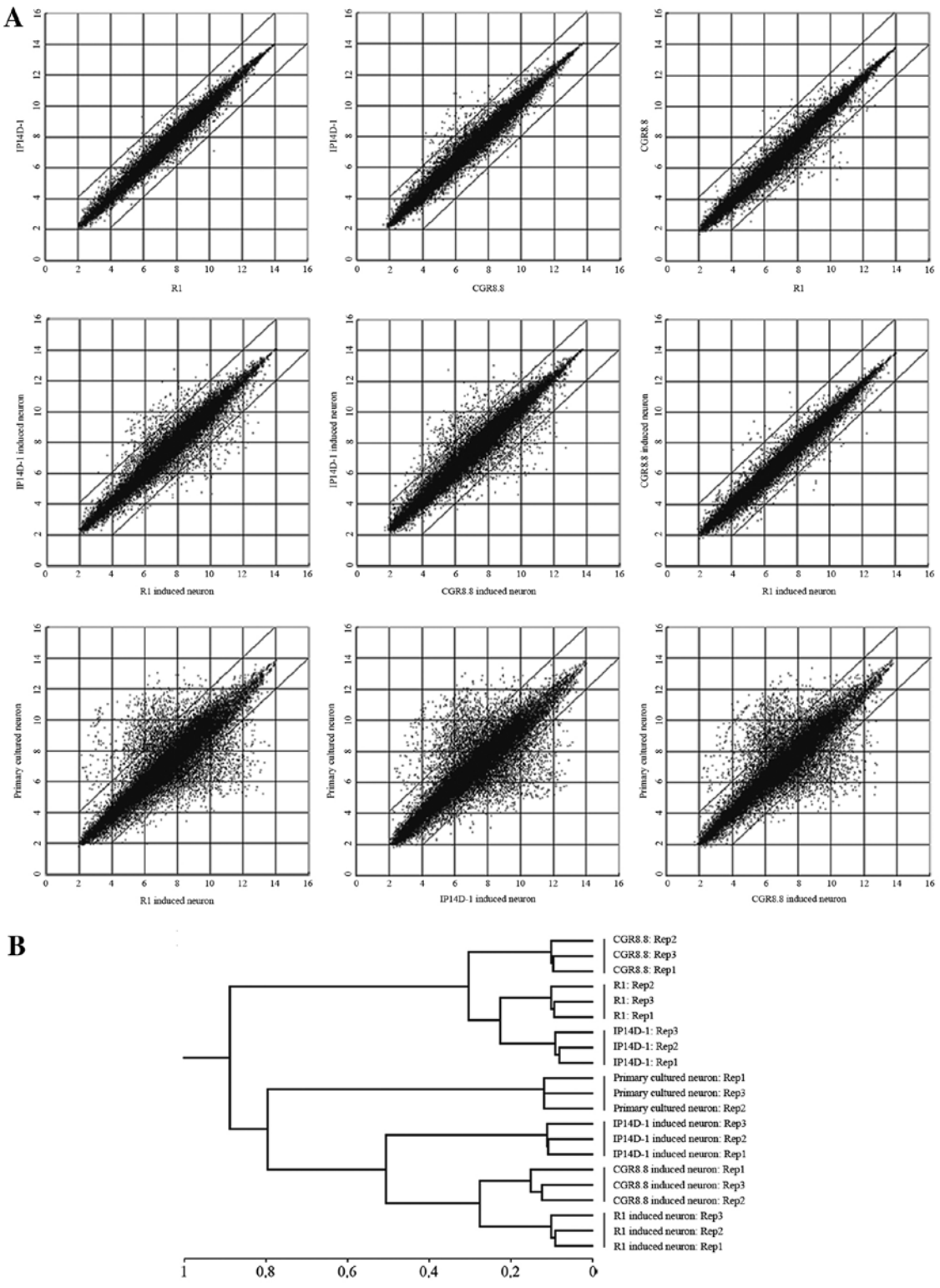

Figure 3. Global gene expression profiles. (A) Pairwise scatter plots were used to compare gene expression levels of all genes targeted on the microarray, normalized across all samples and log2 transformed. (B) Unsupervised hierarchical clustering of microarray data indicates the relative relatedness among replicates and between cell types.

the cell populations differentiated from all three pluripotent cell lines, such as *Blbp* (*Fabp7*), *Nestin*, *Tuj1* and *Map2* (Fig. 2B). MAP2 protein is a marker for mature neurons, and the majority of cells (~75%) from all three populations are positive for MAP2 antigen expression (Fig. 2C).

Global gene expression profiles of cells derived from mouse ESCs and iPSCs are similar. Microarrays were employed to analyze directed neural differentiation of ESCs and iPSCs at a global RNA expression level (Fig. 3A). Gene expression profiles of induced neurons from ES and iPS cells are nearly

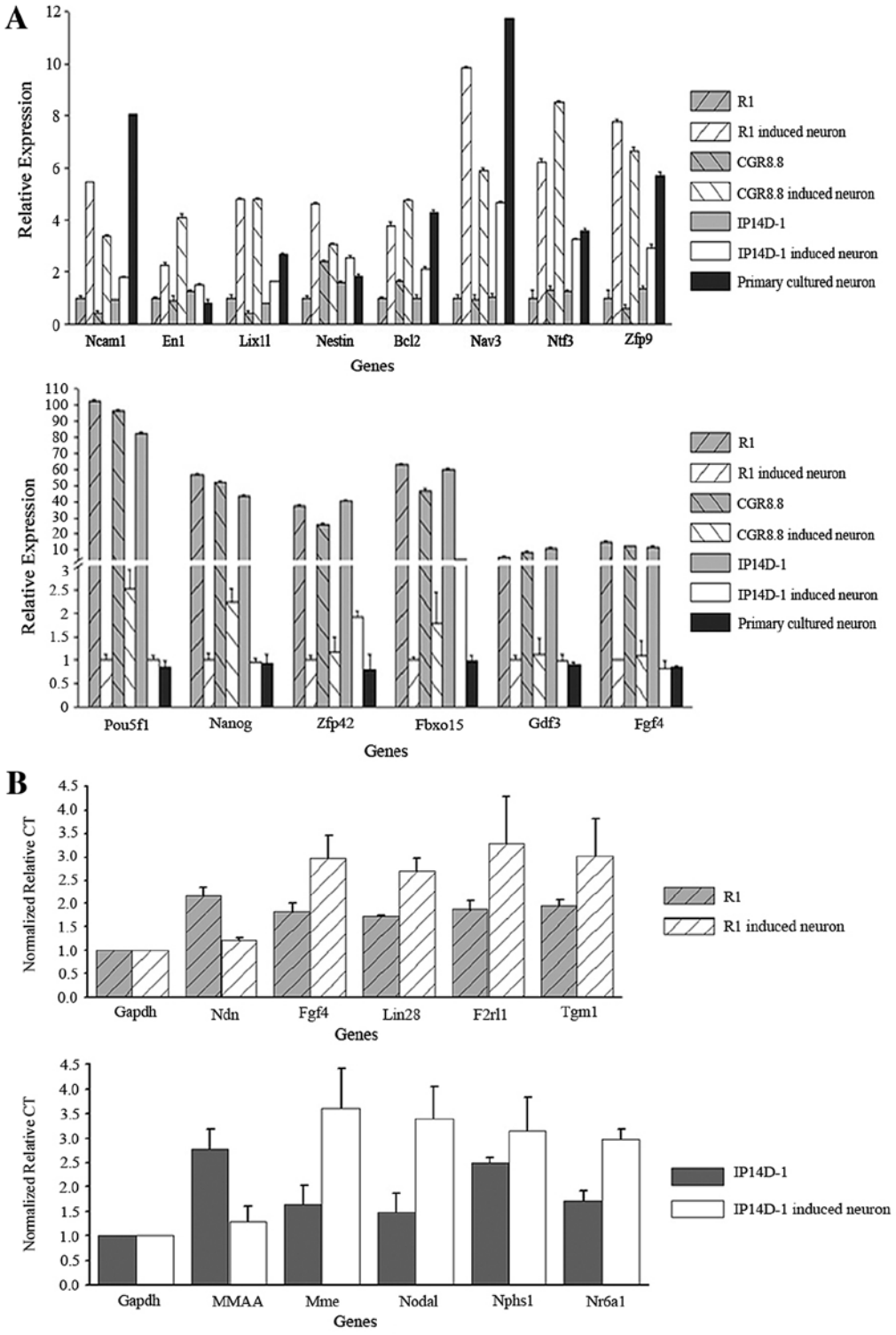

Figure 4. Confirmation of selected gene expression changes during the neural differentiation process. (A) RNA abundance in cell cultures was measured by quantitative PCR for differentially expressed genes from microarray assays. (B) RNA abundance was measured by single cell PCR for selected genes.

identical, particularly between derivatives of the two ES cell populations. However, there are some differences between induced neurons from ESCs and iPSCs, despite the confirmation that the iPS cell line IP14D-1 has a developmental capacity equivalent to ES cells. Moreover, the expression profiles of all these induced neurons are to some extent not consistent with primary cultured neurons. Hierarchical clustering of expression patterns grouped the three induced neuron populations as more related to each other than to primary cultured neurons, but all neurons were distinct from the precursor pluripotent stem cells (Fig. 3B).

Validation of microarray data. We selected differentially expressed candidates from the microarray data for genes related to neurogenesis and pluripotency. *Ncam* (neural cell adhesion molecule), *En1* and *Lix1l* were selected as neuron

Table II. Number of differentially expressed genes detected after induced neural differentiation.

Pairwise comparisons	Upregulated genes (pluripotent > neurons)	Downregulated genes (pluripotent < neurons)
IP14D-1 vs. IP14D-1_neuron	2,278	2,103
IP14D-1 vs. primary neuron	4,574	3,684
Shared set	1,634	613
R1 vs. R1_neuron	2,684	2,707
R1 vs. primary neuron	4,033	3,778
Shared set	1,829	962
CGR8.8 vs. CGR8.8_neuron	2,599	2,378
CGR8.8 vs. primary neuron	4,099	3,680
Shared set	1,791	934
Common among 3 inductions	824	302
Fold-change >2, FDR <0.05		

FDR, false discovery rate.

Table III. Molecular function categories for differentially expressed genes.

Category	No. of mapped genes overexpressed in pluripotent cell vs. neurons	No. of mapped genes underexpressed in pluripotent cell vs. neurons	Sum
Enzyme	1190	35	154
Transcription regulator	67	26	93
Kinase	23	22	45
Transporter	28	13	41
Peptidase	12	10	22
Phosphatase	8	6	14
Growth factor	8	3	11
Transmembrane receptor	5	4	9
Ion channel	1	7	8
Ligand-dependent nuclear receptor	3	4	7
Translation regulator	5	0	5
G-protein coupled receptor	1	3	4
Other	271	113	384
Sum	551	246	797

marker genes, and *Nestin*, *Bcl2*, *Nav3*, *Ntf3* and *Zfp9* were selected as genes related to neural differentiation. Each of the neurogenesis-related genes was upregulated during direct neural differentiation as assayed by microarrays and quantitative PCR. RNA expression differences for the neuron marker genes were also confirmed. Additionally, the three well-known pluripotency genes *Oct4*, *Nanog* and *Klf4* were downregulated during the differentiation process (Fig. 4A).

We also used single cell PCR to validate gene expression levels in individual neurons derived both from R1 and IP14D-1. We randomly chose five genes from the microarray candidate lists for each cell population, as well as selected genes with known relevance in pluripotency or neural differentiation. Expression of genes involved in pluripotency maintenance, such as *Fgf4* and *Lin28*, decreased during neural differentiation. Genes involved in neural lineage development, including *Ndn* and *Nr6a1*, were confirmed to be upregulated (Fig. 4B).

Differential expression between ES/iPS and derived neurons. At an FDR <5% and a difference threshold greater than 2-fold, almost 5,000 differentially expressed genes were identified by pairwise comparisons between pluripotent stem cells and their corresponding induced neurons. There are 1,126 differentially expressed genes common to all three pairwise comparisons (Table II), including 824 upregulated genes and 302 downregulated genes during neural lineage development.

Annotation analysis of regulated genes in ES/iPS cell differentiation. We investigated functional categories within sets of differentially expressed genes using Ingenuity Pathway Analysis

Table IV. Gene ontology analysis of upregulated genes.

Category	Biological process	Count	P-value	Fold enrichment
Transcription related	Positive regulation of transcription, DNA-dependent	19	2.8E-05	3.2
	Positive regulation of transcription from RNA polymerase II promoter	17	5.6E-05	3.3
	Regulation of transcription from RNA polymerase II promoter	22	1.9E-04	2.5
	Regulation of transcription, DNA-dependent	35	1.8E-03	1.7
	Regulation of transcription	48	2.0E-03	1.5
Metabolism related	Positive regulation of macromolecule metabolic process	27	9.8E-07	3.0
	Positive regulation of RNA metabolic process	19	3.1E-05	3.2
	Phosphorus metabolic process	25	1.3E-03	2.0
	Regulation of RNA metabolic process	35	2.3E-03	1.7
	Regulation of cellular protein metabolic process	9	4.4E-02	2.3
Biosynthesis related	Positive regulation of macromolecule biosynthetic process	22	2.1E-05	2.9
	Positive regulation of cellular biosynthetic process	22	3.8E-05	2.8
	Positive regulation of biosynthetic process	22	4.2E-05	2.8
Cell adhesion related	Cell adhesion	22	4.9E-05	2.7
	Homophilic cell adhesion	8	1.5E-03	4.7
	Cell-cell adhesion	11	2.0E-03	3.3
	Regulation of cell-matrix adhesion	3	1.7E-02	14.9
Phosphorylation related	Regulation of phosphorylation	14	2.5E-04	3.4
	Protein amino acid phosphorylation	22	3.1E-04	2.4
	Phosphorylation	22	1.3E-03	2.1
	Regulation of protein amino acid phosphorylation	7	7.0E-03	4.1
Ion homeostasis related	Ion homeostasis	13	9.2E-04	3.1
	Cellular calcium ion homeostasis	6	9.9E-03	4.6
	Homeostatic process	17	8.4E-03	2.1
	Cellular ion homeostasis	11	3.9E-03	3.0
	Cellular chemical homeostasis	11	4.7E-03	2.9
	Cation homeostasis	8	1.5E-02	3.1
	Di-, tri-valent inorganic cation homeostasis	7	1.6E-02	3.4
	Regulation of metal ion transport	4	3.1E-02	5.8
	Regulation of sodium ion transport	3	1.9E-02	13.9
Cell morphogenesis related	Neuron projection morphogenesis	13	7.7E-06	5.2
	Cell morphogenesis	16	3.5E-05	3.6
	Cell morphogenesis involved in neuron differentiation	12	5.7E-05	4.6
	Regulation of neuron projection development	4	3.0E-02	5.9
Cell motion related	Cell motion	20	1.1E-06	3.8
	Cell migration	14	4.0E-05	4.1
	Axon guidance	6	1.3E-02	4.2
	Neural crest cell migration	3	4.2E-02	9.1
Synaptic transmission related	Regulation of membrane potential	7	7.3E-03	4.1
	Synaptic transmission	8	1.4E-02	3.2
	Transmission of nerve impulse	9	1.6E-02	2.8
	Regulation of postsynaptic membrane potential	3	4.9E-02	8.3
Neural development related	Axonogenesis	12	2.0E-05	5.2
	Neuron recognition	4	5.8E-04	23.1
	Regulation of neurogenesis	9	5.9E-04	4.8
	Neuron development	13	9.2E-04	3.1
	Regulation of nervous system development	9	1.3E-03	4.2
	Axonal fasciculation	3	4.1E-03	29.7
	Neural crest cell development	4	1.2E-02	8.4
	Negative regulation of neurogenesis	4	1.6E-02	7.5
	Regulation of axonogenesis	4	1.8E-02	7.1
	Negative regulation of axonogenesis	3	2.1E-02	13.0

tools. Among the 1,126 differentially expressed genes, nearly 700 can be classified into annotated categories that include transcription regulators, growth factors and ion channel formation (Table III). We also tested for statistically significant overrepresentation of Gene Ontology (GO) categories, and classified 302 upregulated genes into biological processes involved in synaptic transmission, regulation of membrane potential, axonogenesis, and neuron recognition, as well as basic functions such as transcription, metabolism and biosynthesis (Table IV). We ranked the GO categories according to overrepresentation P-value, and with the exception of some basic biological processes, the most important categories involved in neural development are focused on neuron projection morphogenesis and axonogenesis.

Discussion

During the last decade, mouse and human ES cells have been induced to differentiate into several cell types to study developmental potential *in vitro* and to develop valuable therapeutics (1,14,15). Diseases of the nervous system have a serious impact on human health, therefore, a number of methods have been adapted to induce ESCs into neurons with the aim of curing disease (1,16). We used a classic induction method with slight modifications to cause mouse ES and iPS cells to differentiate into neurons. RA, an efficient induction factor for neural development, was added to the EB culture medium to promote EBs to differentiate into the neural lineage as suggested by previous *in vitro* research (17,18). With a low RA concentration in culture, pluripotent cells, both ES and EC cells, could differentiate into neurons (19). RA is particularly critical for GABAergic neuron differentiation of ESCs (3,20). To maintain cellular proliferation and promote expansion from EBs, we pretreated cell culture dishes with PDL and laminin at least 2 h (or, optimally, overnight) at 37˚C. Both substances promote efficient cell adhesion and stretching (21). We added growth factors into the neural induction medium to increase cell proliferation and directed differentiation (22,23). These factors were previously shown to protect induced neurons by promoting resistance to apoptosis and necrosis (24), perhaps via regulation of genes such as *Bcl-2* expressed during the neural directed differentiation process (25).

By comparing RNA expression levels among induced neurons from pluripotent stem cells and to primary cultured neurons isolated from ICR E13.5 mice, we found ES and iPS cells follow almost the same neural differentiation process, and the derived neurons have marker gene expression patterns identical to primary cultured neurons.

This level of similarity extended to genomic expression patterns from microarray assays. Neurons derived from all three types of pluripotent cells had mostly overlapping gene expression by cluster analysis, and the differences between these patterns and that of primary cultured neurons is likely due to a heterogeneous mixture of cell types in the induced populations. The number of differentiated cells never exceeded 75%, so samples taken from induced cell populations contained a mixture of neuron and non-neuron RNA.

As an independent validation of the microarray results, we used quantitative RT-PCR of several well-known pluripotency and neuron-relevant genes. As expected, pluripotency genes were downregulated and neural genes were upregulated during differentiation. We also employed single cell PCR in the validation tests, which has become a relatively accurate technology for assessing gene expression (26-28). Primary cultured neurons served as positive control. Genes selected for the PCR assays showed expression patterns consistent with the microarray results. After confirming the concordance of selected microarray and qPCR assays, we conducted a statistical analysis on the full microarray data set to test for gene expression differences between ES/iPS cells and their derived neurons. We identified 824 genes highly expressed in pluripotent cells and 302 genes highly expressed in both induced neurons and primary cultured neurons. The resulting lists of candidate genes were used for pathway and gene ontology analyses that highlighted several biological processes apparently operating during induced neural differentiation. The cadherin superfamily contains calcium-dependent cell adhesion molecules including protocadherins (*Pcdh*) that are important regulators of mouse and human nervous system development (29-32). The *Pcdh* family is involved in maintenance of spinal interneurons, axon convergence and synaptic development, particularly connectivity between neuronal cells (29). In the present study, we detected increased expression of several members of this family including *Cdh2, Cdh10, Pcdh7, Pcdh16, Pcdh18* and *Pcdh22*. The solute carrier gene family (*Slc*) encodes another group of key proteins in neural development for maintaining neuron functions such as ion transport, glutamate transport, and neurotransmitter symporters. Genetic variants of *Slc9a9* affect the development of attention-deficit/hyperactivity disorder, a common behavioral disorder with over-activity and inattentiveness (33-35). This gene, therefore, may play a part in nervous system development, and we observed more than 2-fold upregulation during neuron differentiation.

We also detected differential expression of *Sema* family genes including 3a, 3d, 4c and 6d which have roles in nervous system development (36). The *Sema3a* and *Npn1* genes were upregulated during the directed neuronal differentiation process. Both genes are involved in a neural development regulatory pathway for motor and sensory axon outgrowth (37). microRNA let-7 is known to promote neural lineage differentiation (38,39) and to suppress expression of several pluripotency genes (40). The precursor transcript for let-7 was upregulated 20-fold on average during induced neural differentiation.

Our analysis of *in vitro* directed neuronal differentiation indicates that iPSCs follow almost the same differentiation process as mouse ESCs. Neurons induced from iPSCs and ESCs have similar morphology, neuron marker expression, and global gene expression patterns. Several genes related to known neuronal differentiation processes showed statistically significant changes in expression, and these patterns may be useful for optimizing induction methods, improving the efficiency of neural differentiation from pluripotent stem cells, and understanding neuronal differentiation mechanisms underlying nervous system development.

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