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Transcriptional Profile of NeuroD1 Expression in a Human Fetal Astroglial Cell Line

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NeuroD1, a member of the basic helix–loop–helix (bHLH) protein family, is a transcription factor that plays a pivotal role in terminal differentiation of neural progenitors. The primary objective was to generate an early transcriptional profile triggered by NeuroD1 to guide future studies on mechanisms of neuronal differentiation. The human *NeuroD1* coding region was amplified from human fetal brain RNA using specific primers and cloned into a CMV expression vector (CT-GFP-TOPO/pcDNA3.1). Transfection of a fetal glial cell line with this construct resulted in expression of NeuroD1 in 13–15% of the cells. Markers typical of early neuronal development were observed by immunocytochemical staining in a small proportion of transfected cells. To enrich the population of NeuroD1-expressing cells, fluorescence-activated cell sorting (FACS) was used to purify and collect the NeuroD1/GFP+ cells. Total RNA was extracted from the pair of cultures (NeuroD1/GFP vs. control plasmid/GFP) and processed for gene expression studies. A final gene list was composed from those probe sets that were either increased or decreased in the NeuroD1-expressing cells in three independent experiments ($p < 0.001$). Each gene was investigated further for possible roles in neurogenesis and a subset of 177 genes was chosen based on the following characteristics: a) genes that are potential NeuroD1 dimerization partners, b) genes that modulate other bHLH transcription factors, c) genes related to development, and d) genes associated with neural induction, outgrowth, and terminal differentiation. DNA microarray analysis of NeuroD1 expression in an astroglial cell line produced a "snapshot" transcriptional profile that will be useful in deciphering the complex molecular code that specifies a neuronal fate.

Key words: Transcription factors; Neuronal differentiation; Gene expression

LOSS of neuronal populations with concomitant glial proliferation is a common pathological correlate of many brain diseases. The brain's limited capacity to generate new neurons in adult life is grossly inadequate to replace the significant neuronal loss sustained in neurodegenerative diseases such as Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and in neurologic "accidents" such as head trauma, spinal cord injury, and stroke. One of the major challenges in brain research is to discover therapies to enhance brain repair by promotion of endogenous neurogenesis, neuronal differentiation, followed by appropriate migration and functional integration of new neurons into existing networks. Many different approaches to enhance neurogenesis have been taken. Environmental enrichment has been reported to increase hippocampal neurogenesis (10,21). Administration of growth factors directly into the lateral ventricles of the brain has been shown to increase rate of proliferation of neural progenitors (14,47). A molecular approach utilizes recombinant DNA technology to study the regulation of neurogenesis. Recently, it has been shown that subfamilies of basic helix–loop–helix (bHLH) tran-

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scription factors, such as NeuroD1, Mash1, Math1, and neurogenin, promote neurogenesis (23,24) by forming heterodimers with other ubiquitous bHLH proteins, such as E12 or E47 (9,18). These heterodimers then activate E box gene expression, driving cells into a neuronal lineage (9,18,24). The other set of genes in the bHLH family that keeps cells in undifferentiated form and inhibits neurogenesis is Msx1, Hes1, and Hes5; a balance between these two sets of bHLH proteins is probably pivotal in determining a cell's neuronal fate (20,34,37).

NeuroD1, also known as BETA2, was isolated from an insulin-secreting tumor and can function as a β-cell-specific transactivator of the insulin gene (36). Ectopic expression of NeuroD1 in *Xenopus* embryos causes premature differentiation of neurons and conversion of epidermal cells into neurons (24). It was shown that P19, a mouse embryonal carcinoma cell line, is converted into a neuronal phenotype after transfection with NeuroD1 plasmid (18). An overexpression of NeuroD1 in F11 neuroblastoma cells increased neurite outgrowth in the absence of cAMP and mutant NeuroD1 inhibited neurite outgrowth induced by cAMP (12). Using a human embryonal carcinoma cell line (NTERA-2), a classical model for studying neuronal differentiation, it was shown that expression of nestin was downregulated while expression of NeuroD1 was induced when cells become postmitotic, a characteristic of neuroprogenitors exiting the cell cycle (38). Overexpression of NeuroD1 promotes premature differentiation of retinal neurons (1). NeuroD1 was also found to be a critical regulator of the neuron versus glial cell fate decision (24,35), and retinal explants obtained from NeuroD1-null mice demonstrated increased glial cell formation (35). Additionally, expression of NeuroD1 in interstitial and endovascular invasive cells shows that it is involved in trophoblast differentiation in placental development (45), demonstrating that NeuroD1 expression plays an important regulatory role in differentiation of cells derived from the ectodermal germ layer.

The hypothesis tested in this study postulates that forced expression of NeuroD1 will initiate the conversion of a fetal glial cell line into an early neuronal phenotype in vitro. The fetal astrocyte cell line (SVGp12, from ATCC) was utilized because it does not normally express NeuroD1 and has been shown to be an excellent vehicle for the delivery of genes (31,42,46). The concept of utilizing a glial cell as a neuronal progenitor has been suggested for some time; a subependymal glial cell may be the actual neural progenitor, but this claim remains debatable (3,16). A "ribbon" of astrocytes lining the ventricles in the adult human brain is a source of neural progenitors but, unlike the rodent, the newborn cells in the human brain lack migratory capability in vivo and they do not form new neurons (4).

The first goal of this study was to clone human NeuroD1 into a vector containing CMV promoter and a reporter gene, green fluorescent protein (GFP). The second goal was to demonstrate successful transfection of hNeuroD1 plasmid into an astroglial cell line and to observe early immunophenotypic changes triggered by this transcription factor. The third goal, emphasized in this report, was to assess the transcriptional profile elicited by forced NeuroD1 expression.

MATERIALS AND METHODS

Figure 1 is schematic diagram of procedures used in this article. Using human fetal brain total RNA and specific primers for *NeuroD1*, hND1 cDNA spanning the entire coding region of the gene was amplified. Total fetal brain RNA was from Clontech Inc.'s Human Total RNA panel IV. Restriction enzymes were purchased from Promega (Madison, WI), Qiaex II gel extraction kit and QIA filter Plasmid Maxi Kit were from Qiagen (Valencia, CA), GeneAmp RNA PCR kit was from Applied Biosystems (Foster City, CA). CT-GFP Fusion TOPO Cloning kit was from Invitrogen (Carlsbad, CA). Chemically defined fetal bovine serum (FBS) and penicillin and streptomycin were from Invitrogen (Grand Island, NY). Eagle's minimum essential medium (MEM) and SVGp12, human fetal astrocyte cells, were from ATCC (Manassa, VA). Fugene 6 transfection reagent was from Roche Diagnostics Corporation (Indianapolis, IN).

Cloning and Transformation

Human *NeuroD1* (HNd1) cDNA, generated by RT-PCR, was gel purified using low melting point agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME) and Qiaex II gel extraction kit (Qiagen, Inc.). The concentration of cDNA was approximated by comparison to known standards after gel electrophoresis. The purified cDNA was inserted into pcDNA3.1/CT-GFP-TOPO vector (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. A 2-μl aliquot from the cloning reaction was mixed with 50 μl One Shot TOP10 chemically competent *E. coli* cells and transformed as described by the manufacturer. The cells were plated on agar plates containing carbenicillin (Sigma-Aldrich Inc., St. Louis, MO) and incubated at 37d°C overnight. Several colonies were picked randomly for growth overnight in Luria-Bertani (LB) medium (39) containing carbenicillin at 37°C, shaking at 300 rpm. The plasmid DNA was isolated using the Wizard Plus Miniprep kit (Promega Corp., Madison, WI) as per manufacturer's instructions. The plas-

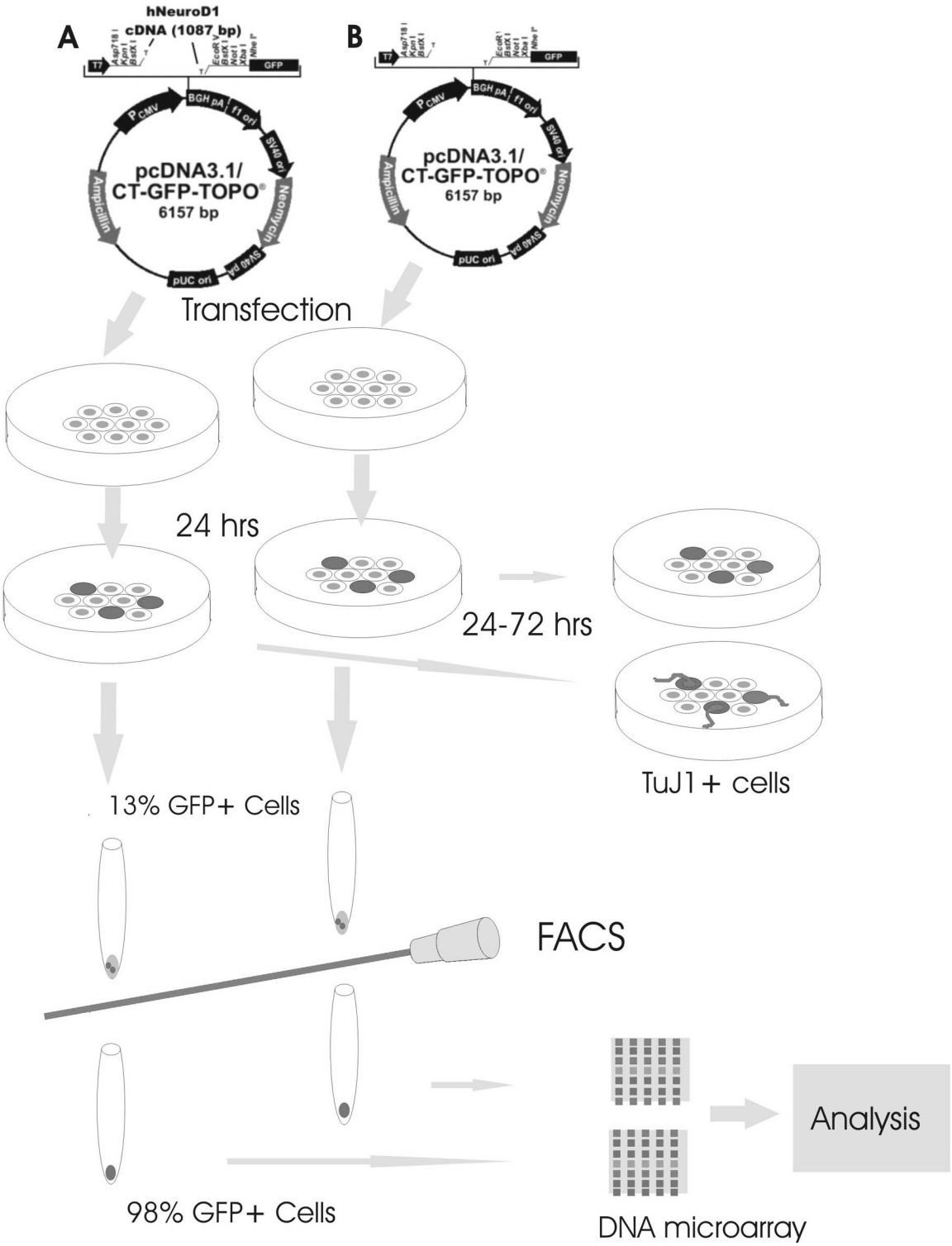


Figure 1. Schematic of research design. (A) Plasmid of cloned human NeuroD1/GFP; (B) control plasmid, X/GFP. Paired sets of human fetal astroglial SV40 transformed (SVG) cell cultures were transfected with (A) or (B) using Fugene 6 reagent. After 24 h, paired cultures were analyzed and processed with fluorescence-activated cell sorting (FACS). GFP-expressing cells were sorted and collected to ~98% purity. Total RNA was extracted from each set of enriched GFP-expressing cells for DNA microarray analysis. In parallel experiments, paired cultures were examined immunocytochemically at 24 and 72 h after transfection for expression of the neuronal protein β-tubulin III (TuJ-1).

mid DNA was then digested with *Sma*I enzyme to check for orientation of the insert. Clones containing the desired orientation were chosen and sequenced at the H. Lee Moffitt Cancer Center Molecular Biology Core, Tampa, FL. The clone of human fetal brain NeuroD1 cDNA selected for use in this study had 100% homology to the reported sequence in Genbank (accession #AF045152). The desired clones were expanded using Qiagen's Highspeed Maxiprep for transfection.

Cell Culture and Transfection

Human fetal astroglial SV40 transformed cells (SVG p12, ATCC) were plated in 75-mm culture flasks (Costar, Corning Inc.) in modified Eagle's MEM (DMEM, ATCC), 10% heat-inactivated FBS (Invitrogen), and penicillin (100 U/ml)/streptomycin (100 µg/ml). The cells were grown in 95% relative humidity, 37°C in 5% CO_2 environment. Twenty-four hours before transfection, cells were trypsinized and plated onto a 75-cm^2 flask in DMEM + 10% FBS. Transfection was done using Fugene 6 reagent kit (Roche Applied Biosystems). To 500 µl DMEM (no FBS) was added 15 µl of Fugene 6 reagent and 5 µg of either pcDNA3.1/CT-GFP (as control) or pcDNA3.1/CT-HNeuroD1-GFP plasmids. The mix was incubated at room temperature for 30–45 min and added to the cells dropwise with swirling to spread the reagent. Cells were incubated at 37°C for 24 h before examining for GFP expression. The cells were harvested by trypsinization and the trypsin was inactivated with 1% FBS in DMEM. The cells were collected by centrifugation and resuspended as a single cell suspension in medium containing 5 mM EDTA in DMEM and no FBS using fire-polished glass pipettes. This manipulation helped us increase fluorescence-activated cell sorting (FACS) efficiency (from approximately 20% to 50–60%).

FACS

Cell sorting was performed on a FACSVantage DiVa (Becton Dickinson) using an argon ion laser at excitation wavelength of 488 nm. GFP emission was detected in the FL1-A (area) parameter using a 530/30 bandpass filter. The instrument was sterilized and sterile technique was used by the operator(s) to reduce/prevent contamination of the cells. The FACS was done at the H. Lee Moffitt Cancer Center Flow Cytometry Core Facility, Tampa, FL.

Immunocytochemistry

The cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min at room temperature. The cultures were then washed three times with phosphate-buffered saline (PBS) and incubated in blocking solution (0.1 M PBS containing 10% goat serum and 0.01% Triton X-100) for 1 h at room temperature followed by incubation at 4°C overnight with the primary antibody, rabbit polyconal anti-nestin (1:200; Chemicon, Inc.), rabbit polyclonal anti-TuJ1 (1:400; Chemicon, Inc.), rabbit polyclonal anti-vimentin (1:400, Chemicon, Inc.), rabbit polyclonal anti-GFAP (1:500; Dako, Carpenteria, CA), mouse monoclonal anti-GALC (1:400; Chemicon, Inc.), and rabbit polyclonal anti-Ki67 (1:200; BD Pharmingen). After washing twice, the cultured cells were incubated with species-appropriate rhodamine-conjugated secondary antibodies for 2 h at room temperature. Controls in which the primary antibody was eliminated resulted in no staining. After rinsing with PBS three times, the cultures were coverslipped with 95% glycerol or Vectashield mounting medium. Cell nuclei were visualized with 4′,6-diamidino-2-phenylindole, dihydrochloride DAPI (2 mg/ml; Roche). "Dead cells" were visualized by counterstaining with propidium idodide (1.5 µM; Molecular Probes). All cultures were examined with the Oympus IX71 fluorescence microscope using appropriate filters for green and red fluorescence. For estimates of cell number in culture, 10 random visual fields (20× objective) in three culture dishes for each marker tested were viewed. The total number of DAPI-labeled cells was counted in each visual field. The mean number of labeled cells was then expressed as a percentage of the total number of cells per field.

Isolation of RNA

Total RNA was isolated using Qiagen's RNeasy Mini kit following manufacturer's protocol. The RNA was treated with RNase-free DNase I (Qiagen Inc.) by incubating on column for 30 min at room temperature. After measurement of OD at 260 nm, the RNA was stored at −80°C. Integrity was tested on 1% nondenaturing Seakem LE agarose gel (Cambrex Bio Science Rockland, Inc.). For microarray analysis the cells were sorted directly into an aliquot of TriZol (Invitrogen, Carlsbad, CA). The RNA was purified following the manufacturer's protocol. The pelleted RNA was immediately resuspended in RNase-free water and further purified using Qiagen RNeasy columns and the RNA clean-up protocol.

Reverse Transcription (RT)

RT was performed using oligo(dT) as primers when hNeuroD1 was used in PCR and for all other transcripts random hexamer was used. These RT con-

ditions resulted in more intense bands. Final volume was 20 µl with 1 µg of total RNA from fetal brain RNA (Clontech Inc.). The reaction mixture contained 1 mM of each deoxynucleoside triphosphate (dNTP), 1 U/µl RNase inhibitor, 5 mM $MgCl_2$, 2.5 U/µl murine leukemia virus (MuLV) reverse transcriptase, 2.5 µM oligo$(dT)_{16}$ in 50 mM KCl and 10 mM Tris-HCl (pH 8.3). It was first incubated at room temperature for 10 min, followed by at 42°C for 15 min. The mixture was then heated at 99°C for 5 min and cooled on ice for 5 min to inactivate the transcriptase.

Polymerase Chain Reaction (PCR)

PCR was performed in the same tubes as RT, in 100 µl total volume. Final concentrations were 2 mM $MgCl_2$, 0.2 mM of each dNTP, and 2.5 U/100 µl Ampli Taq DNA polymerase in the 50 mM KCl and 10 mM Tris-HCl buffer (pH 8.3). To generate a full coding region for human NeuroD1 (accession #NM_002500), a PE 9700 thermocycler (Perkin Elmer, Foster City, CA) was programmed as follows: 1 cycle at 95°C for 105 s, 50 cycles (at 58.9°C for 30 s, at 74°C for 2 min, and at 94°C for 30 s), followed by 1 cycle at 72°C for 7 min. Both RT and PCR were done using GeneAmp RNA PCR kit. The forward primer, nt. 98–121 (GGAAATCGAAACATGACCAAATCG), and reverse primer, nt. (5′T) 1177–1153 (TATCAT GAAATATGGCATTGAGCTGG), were designed to keep in-frame with the vector's GFP sequence; 100 ng of each forward and reverse primers was used in PCR. To confirm its specificity, a 1081-bp-long hNeurod1 cDNA was excised using *Sma*I restriction enzyme to give fragments of sizes 747 and 334 bp (data not shown). The forward primer, nt. 505–523 (TAAGACGCAGAAGCTGTCC) with above reverse primer was used in PCR, in which we had to check the presence of NeuroD1 transcript (674 bp) post-transfection. Primers were selected using SEQWEB (version 1.1) software available on the USF computer network.

Multiplexing PCR was done using β-actin gene as an internal control in the same tube. RT was done as above, but various different PCR cyclings, $MgCl_2$ concentrations, and titration of primers (β-actin and β-tubulin) were tried to get optimal experimental conditions. For this experiment only 22 cycles were used; 5 mM $MgCl_2$, 20 ng β-actin, and 250 ng β-tubulin were used for successful results. For β-tubulin (283 bp, Genbank #AF141349), forward primer, nt. 219–237, and reverse primer, nt. 501–481. For β-actin, primers from human β-actin control amplimer set (Clontech Inc.) were used, which generates 838-bp-long cDNA.

DNA Microarray Analysis

For microarray analysis the poly(A) RNA was specifically converted to cDNA and then amplified and labeled with biotin following the previously described procedure (39). Hybridization with the biotin-labeled RNA, staining, and scanning of the chips followed the prescribed procedure outlined in the Affymetrix technical manual and has been previously described (44). Scanned output files were visually inspected for hybridization artifacts and the signal intensity was scaled to an average intensity of 500 prior to comparison analysis using Affymetrix Microarray 5.0 software. This software identifies the increased and decreased genes between any two samples with a statistical algorithm that assesses the behavior of 11 different oligonucleotide probes designed to detect the same gene (30). The software analyzes the relative transcript abundance and any differences between two conditions independently from the probe level data. The software uses Wilcoxon's signed-rank test to assign a *p*-value for the likelihood of an increase or decrease in the treated sample relative to the control sample used as a reference. All probe sets with a change *p*-value less than 0.1 (increased or decreased) were identified in comparisons between GFP transfected cells as a control and hNeuroD1/GFP transfected cells. Those probe sets that met this criteria in all three experiments were considered to have changed in response to hNeuroD1-GFP (combined value $p < 0.001$).

Probe Arrays

The oligonucleotide probe arrays were the Affymetrix U133A chips. These chips contain 22,215 probe sets (excluding control probe sets), which target known and suspected genes as well as a number of suspected splice variants. The U133 set (A and B chip) represents the most comprehensive survey of human genes as registered in Unigene (build 133), GenBank, and The Institute for Genomic Research (TIGR) databases. Each gene is represented by a series of oligonucleotides that are identical to sequence in the gene and oligonucleotides that contain a mismatch at the central base position of the oligomer used for measuring cross-hybridization.

RESULTS

Construction of the Human NeuroD1/GFP Encoding Plasmid

A reporter plasmid was constructed to express *NeuroD1*. The human *NeuroD1* coding region was

amplified from human fetal brain total RNA using specific primers. The 1081-bp-long PCR product, which contained the entire coding region without a stop codon, was cloned into a CMV expression vector (CT-GFP-TOPO/pcDNA3.1). The PCR primers were designed to allow the cloning of *NeuroD1* directly in-frame with GFP so that a fusion protein would be produced. Successful clones with the correct orientation were sequenced and those with 100% sequence identity with the published sequence for NeuroD1 (GenBank #AF045152) were expanded for transfection.

Transient Transfection of a Human Fetal Astrocyte Cell Line With NeuroD1/GFP

The human fetal astrocyte cell line (SVGp12) was then transfected with the NeuroD1/GFP encoding plasmid. Cells were incubated at 37°C at least 24 h before examining for GFP expression. Coexpresson of GFP with NeuroD was observed in the cultures transfected with the NeuroD/GFP plasmid but not in the control cultures, which expressed only GFP (Fig. 2, panel 1). Twenty-four hours after transfection, approximately 10% (range of 8–14%) of the cells visualized under fluorescence microscopy were GFP+ regardless of the transfection vector. The green fluorescence emitted by the NeuroD1/GFP transfected cells was typically localized in the nucleus, whereas the GFP signal in the control transfected cells was diffusely cytoplasmic (Fig. 2, panel 2, row A). Vimentin was expressed by all cells in both sets of cultures, including the fraction of cells that coexpressed GFP (Fig. 2, panel 2, row B). Unlike the strong vimentin signal typically expressed by these cells, GFAP, a marker of mature astroctyes, was barely detected and GALC, a marker of oligodendrocytes, was not expressed before or after transfection (image not shown). Immunoreactivty for nestin, a marker of neural stem/progenitor cells, was observed in untransfected cells in both sets of cultures (Fig. 2, panel 2, row C). However, double-labeled cells (GFP+ and nestin+) were only detected in the NeuroD1/GFP transfected cultures (approximately in 0.25–0.6% of the total cells), and double-labeled cells were not detected in control cultures. Similarly, TuJ1, a marker of young neurons, was expressed by nontransfected cells in both sets of cultures, but double-labeled cells (GFP and TuJ1+) were only seen in the NeuroD1/GFP transfected cells at a frequency of 1.3% of the total cells at 24 h (Fig. 2, panel 2, row D). Interestingly, many of the cells in the NeuroD1/GFP transfected cultures expressed TuJ1 even when they did not exhibit green fluorescence.

Assessment of Transfection Efficiency With FACS

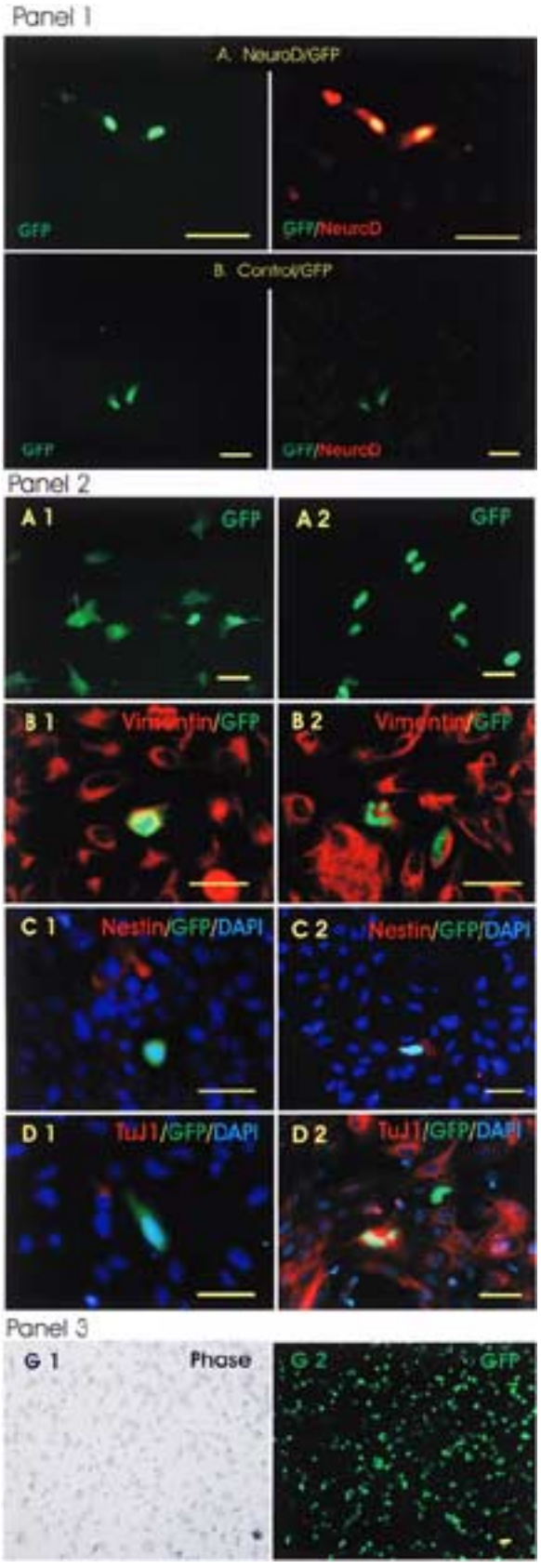
Twenty-four hours after transfection, FACS analysis was performed on cultures transfected with hNeuroD1/GFP and the controls transfected with pcDNA3.1/GFP. Figure 3 shows data from a typical sort, showing 11.37% of the cells were GFP+. The percentage of GFP+ cells in both sets of cultures was similar, ranging from 8.1% to 15.5% in three independent experiments (Table 1). The sorted cells were then examined under fluorescence microscopy, revealing that nearly 100% of the sorted cells expressed GFP (Fig. 2, panel 3). The highly enriched populations of NeuroD1/GFP- and pcDNA3.1/GFP-expressing cells permitted careful assessment of differential gene expression elicited by the transcription factor NeuroD1.

RT-PCR

The NeuroD1/GFP-transfected cultures showed a very strong band for NeuroD1 mRNA, in contrast to cultures transfected with the control plasmid (pcDNA3.1/GFP) (Fig. 4A). Interestingly, both sets of cultures also expressed mRNA of β-tubulin III, a marker of young neurons (Fig. 4B). Other markers associated with neural progenitors and developing neurons were also detected in both sets of cultures (e.g., nestin, glypican 4, pleiotrophin, a neurite outgrowth protein, and Nurr1; data not shown). This result suggests that this glial cell line is competent to develop into an early neuronal phenotype. GFAP mRNA in both untransfected and transfected cells was detected (data not shown), but the signal was of low intensity, a finding that is consistent with the original descriptions of this cell line (31).

Transcriptional Profile Using DNA Microarray Analysis

To assess early molecular consequences of NeuroD1 expression, transcriptional profiles were obtained in paired sets of cultures (hNeuroD1/GFP and control pcDNA3.1/GFP transfected cells). Three independent transfection experiments were performed. The GFP+ cells were sorted to enrich for transfected cells and maximize the potential for differential gene expression. Each experimental pair (control plasmid/GFP vs. NeuroD1/GFP) was independently analyzed and then compared with the results from the other experimental pairs to assess consistency in the gene expression differences. No experiment seemed to be grossly different than the other two (data not shown). A final gene list was composed from those probe sets that were either increased or decreased in the NeuroD1-expressing cells in all three experiments ($p <$



0.001). A list of 985 probe sets, representing 910 independent genes, is available on request (jsramos@hsc.usf.edu). This is 4.4% of the 22,215 probe sets on the array, suggesting that NeuroD1 has initiated significant changes within the transfected cells. The identity of the genes detected by each of the 985 probe sets was determined through the NetAffx Web site hosted by Affymetrix (30).

Each gene was investigated further for possible roles in neurogenesis. This was done initially through links provided by LocusLink, hosted by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/LocusLink/). A subset of 177 genes was chosen based on the following characteristics: a) genes that are potential NeuroD1 dimerization partners, b) genes that modulate other HLH transcription factors, c) genes related to development, and d) genes associated with neural induction, outgrowth, and terminal differentiation. This subset was further trimmed based on literature reports or the gene ontology classification of the genes. The Gene Ontology Consortium is an initiative to describe the biological characteristics of proteins using a controlled vocabulary to make it easier for computer-based uses of large data

FACING COLUMN

Figure 2. (Panel 1) SVG cells were transfected with plasmid encoding hNeuroD1/GFP (upper two panels) and a control plasmid (lower two panels). After 24 h NeuroD1-immunoreactive cells were viewed under fluorescence microscopy. The upper panels show identical fields of an SVG cell culture containing two GFP+ cells coexpressing NeuroD1. The lower panels show cells transfected with the control plasmid. GFP+ cells are seen but they do not express NeuroD1. (Panel 2) A1, B1, C1, and D1 were from cultures transfected with control GFP vector; A2, B2, C2, and D2 were cultures transfected with NeuroD1/GFP. After 24 h in vitro, cultures were immunolabeled with antibodies to vimentin, nestin, and TuJ1 (scale bar = 50 μm in each frame). Row A: Cells expressing green fluorescence (GFP+). No antibodies were utilized. The fluorescence in the NeuroD1/GFP transfected cultures tends to be localized in the nucleus (A2) and in the control GFP transfected cells the fluorescence is more diffuse, involving the cytoplasm (A1). Row B: Vimentin is expressed in every cell regardless of transfection with NeuroD1/GFP (B2) or control GFP (B1). Row C: Nestin immunoreactivity is present in non-GFP-expressing cells in both sets of cultures. A double-labeled (GFP+/nestin+) cell is illustrated in the NeuroD1/GFP transfected cells (C2). In the control GFP transfected cultures (C1), it is rare to find double-labeled (GFP+/nestin+) cells despite the presence of nestin+ cells in non-GFP-expressing cells. Row D: The NeuroD1 transfected cultures (D2) exhibit double-labeled cells (TuJ1+/GFP+). Notice that many of the cells in the NeuroD1/GFP transfected cultures express TuJ1 even when they are not exhibiting green fluorescence. (Panel 3) Row G: Fluorescence-activated cell sorting (FACS) was performed at 24 h after transfection. An aliquot of cells was plated and viewed under phase (G1) and fluorescence microscopy (G2), revealing a highly enriched (95–98% pure) population of GFP+ cells. FACS-purified cells from paired sets of cultures were then processed for DNA microarray analysis in three separate experiments that form the basis for the transcription profiling.

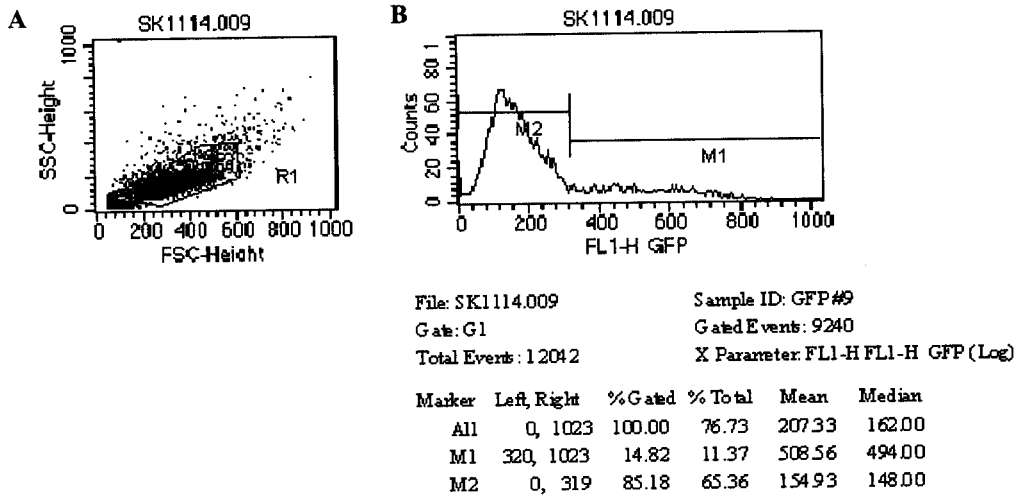


Figure 3. Typical data from FACS sort of SVG cells 24 h after transfection with NeuroD1/GFP. (A) Plot of side scatter (SSC) against forward scatter (FSC). (B) Cell counts plotted against intensity of fluorescence (FL1-H GFP).

sets (7). The description of proteins occurs under three broad categories: biological process, molecular function, and cellular component. Attention was focused on the "biological process" category and genes were chosen that fell into categories that support our hypothesis, such as neurogenesis (nestin, leucine-rich repeat protein-neuronal 1, NTRK3, Ninj1, Zic1, ectodermal-neural cortex, etc.), neuroblast proliferation (novel gene called artemin), axon guidance (SEMA3B and Fez2), CNS and brain development (forkhead box G1B, Down syndrome critical region gene 1, etc.), synaptic transmission and vesicle docking (syntaphilin, cholinergic receptor, nicotinic gamma polypeptide, etc.), neurotransmitter biosynthesis and storage (tyrosine hydroxylase), cell cycle and adhesion (SH3-domain binding protein 4, CHK1 checkpoint homolog), development (Wnt5A, Wnt6, EVX1, HoxD11, ID2, ID3, follistatin, etc.), embryogenesis and morphogenesis (LIM domain only 4, Sox4, Sox 13, etc.), and epidermal differentiation (keratin, hair-acidic 4). The final list contains 53 genes that are modulated by NeuroD1 expression in the astroglial cell, which may indicate a more neuronal phenotype (Table 2). Although fold change values are reported, a new guideline for users of Affymetrix microarray recommends that "fold enrichment value" not be used as an absolute measure of differential expression. In fact, in many instances it might underestimate the changes occurring in these experiments.

DISCUSSION

NeuroD1 has been shown to play a major role in the development of the nervous system and β-cells of the pancreas (13). For the most part, NeuroD1 is detected during brain development at sites where differentiating postmitotoic neurons are distributed (25). Knockout of NeuroD1 in mice results in their death by age 5 days due to severe diabetes, but if rescued by ectopic expression of insulin in the pancreas, the mutant mice do not develop granule cells in either the dentate gyrus of the hippocampus (29) or the cerebellum (33). Mutant NeuroD1 mice also fail to de-

TABLE 1
FACS DATA OF SVGp12 CELLS AFTER 24-h TRANSFECTION WITH hNeuroD1/GFP AND CONTROL (pcDNA3.1/GFP) PLASMID CONSTRUCTS

	hND1/GFP			Control Plasmid: pcDNA3.1/GFP		
Sort No.	Transfection Efficiency (% of Total Cells)	No. of HND1/GFP+ Cells Sorted	% Purity (Postsort)	Transfection Efficiency (% of Total Cells)	No. of GFP+ Cells Sorted	% Purity (Postsort)
1	13.2	102,791	98	9.6	101,714	98
2	15.5	125,000	98	13.7	127,000	94
3	11.9	200,000	95	8.1	189,000	93

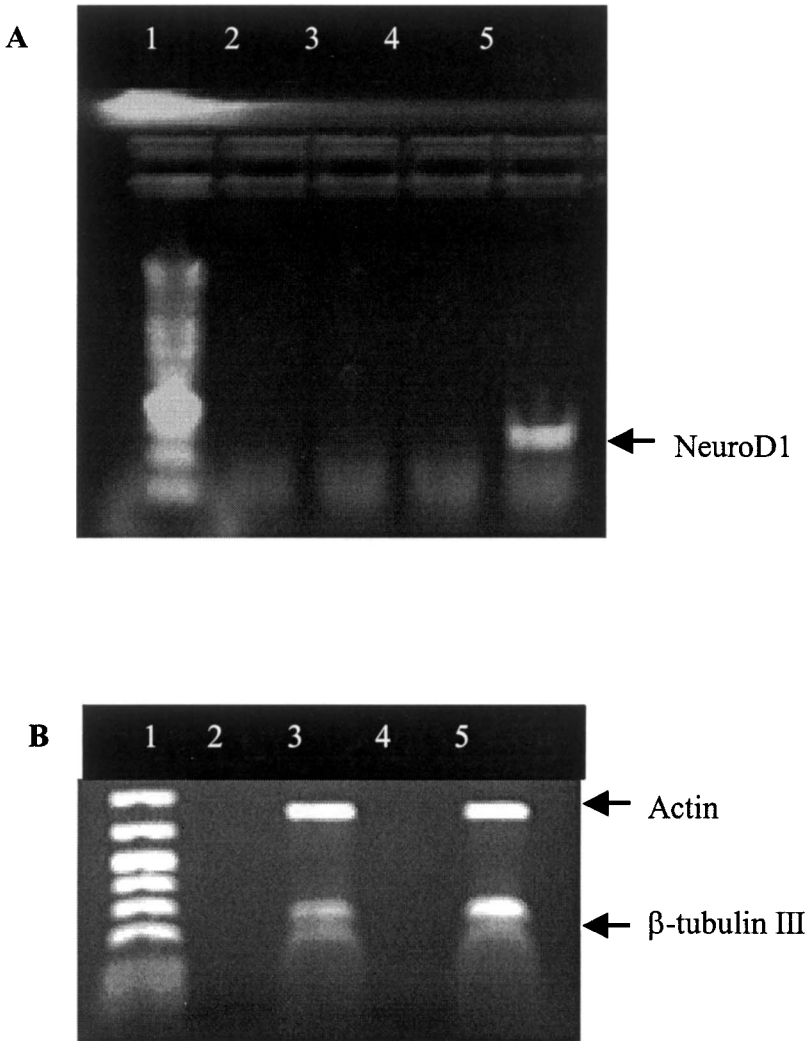


Figure 4. (A) RT-PCR of NeuroD1 mRNA. RT-PCR was performed with human total RNA isolated from 1-day post-hNeuroD1-GFP and X/GFP transfected cells (not FACS sorted). Lane 3: 674 bp cDNA (with total RNA from X/GFP transfected cells). Lane 5: 674 bp cDNA (with total RNA from hNeuroD1-GFP transfected cells). Lanes 2 and 4 were negative controls without reverse transcriptase using RNA under the same conditions as in the next lane. This shows that we could not detect any endogenous NeuroD1 expression and transfection with our construct induces NeuroD1 transcription in this cell type. (B) RT-PCR of β-tubulin III was performed with human total RNA isolated from 3-day posttransfected and untransfected cells in the same tube and modified PCR conditions described in Materials and Methods. The products were separated with electrophoresis on 2% agarose gel. Lane 2: 838 bp for β-actin and 283 bp β-tubulin from untransfected cells. Lane 4: 838 bp for β-actin and 283 bp β-tubulin from hNeuroD1 transfected cells. Lanes 1 and 3 were negative controls without reverse transcriptase in it using RNA under the same conditions as in the next lane.

velop normal sensory neuronal and ganglion cells of the inner ear (28). Ectopic expression of NeuroD1 has been shown to convert both nonneuronal populations of neural crest cells and presumptive epidermal cells into neurons (5,24). NeuroD1 expression has also been detected in a few proliferating neuronal cells during development (25,28,29), Although NeuroD1 appears to contribute to multiple levels of neural development, it has been hypothesized to play a major role in terminal differentiation of postmitotic neurons (13).

In the present study, transfection of the SVG fetal glial cell line with hNeuroD1 resulted in the generation of cells that expressed markers usually observed in neural stem/progenitor cells and cells committed to a neuronal fate. Evaluation of phenotypic markers 24 h after transfection revealed a very small fraction of double-labeled neural progenitor (GFP+/nestin+) cells and double-labeled young neurons (GFP+/TuJ1+) in the NeuroD1 transfected cultures but not in the control cultures. Nestin, an intermediate filament protein, has been the predominant marker used to describe stem and progenitor cells in the mammalian CNS (11,26,41). Nestin also marks neural progenitors

TABLE 2
MICROARRAY DATA BASED ON IMPORTANT BIOLOGICAL PROCESSES MODULATED BY NeuroD1

Affymetrix Probe Set	Accession No.	Gene Name	Gene Symbol		Fold Change
Neurotransmitter biosynthesis and storage (not recorded)					
208291_s_at	NM_000360	tyrosine hydroxylase	TH	1	+4.1
Synaptic vesicle docking (nontraceable author statement)					
215917_at	NM_014723	syntaphilin	SNPH	1	+5.1
Cell cycle (predicted/computed)					
222258_s_at	NM_014521	SH3-domain binding protein 4	SH3BP4	−1	−1.9
203449_s_at	NM_003218	telomeric repeat binding factor (NIMA-interacting) 1	TERF1	−1	−1.2
205394_at	NM_001274	CHK1 checkpoint homolog (*S. pombe*)	CHEK1	−1	−1.4
Cell adhesion (inferred from electronic annotation)					
207093_s_at	NM_002544	oligodendrocyte myelin glycoprotein	OMG	1	+2.2
212713_at	NM_002404	microfibrillar-associated protein 4	MFAP4	1	+9.7
203083_at	NM_003247	thrombospondin 2	THBS2	−1	−1.6
Synaptic transmission (inferred from electronic annotation)					
221355_at	NM_005199	cholinergic receptor, nicotinic, gamma polypeptide	CHRNG	1	+6.7
203999_at	NM_005639	synaptotagmin I	SYT1	−1	−1.6
205280_at	NM_000824	glycine receptor, beta	GLRB	−1	−1.6
Synaptic transmission, cholinergic (traceable author statement)					
220210_at	NM_020402	cholinergic receptor, nicotinic, alpha polypeptide 10	CHRNA10	1	+1.8
210519_s_at	NM_000903	NAD(P)H dehydrogenase, quinone 1	NQO1	1	+1.4
Development (inferred from electronic annotation)					
205990_s_at	NM_003392	wingless-type MMTV integration site family, member 5A	WNT5A	1	+1.5
203222_s_at	NM_005077	transducin-like enhancer of split 1 (E(sp1) homolog, *Drosophila*)	TLE1	1	+1.6
71933_at	NM_006522	wingless-type MMTV integration site family, member 6	WNT6	1	+2.1
207914_x_at	NM_001989	eve, even-skipped homeo box homolog 1 (*Drosophila*)	EVX1	1	+5.9
214604_at	NM_021192	homeo box D11	HOXD11	1	+1.5
214438_at	NM_021958	H2.0-like homeo box 1 (*Drosophila*)	HLX1	1	+1.5
203789_s_at	NM_006379	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	−1	−2.5
201565_s_at	NM_002166	inhibitor of DNA binding 2, dominant negative helix–loop–helix protein	ID2	−1	−1.3
205266_at	NM_002309	leukemia inhibitory factor (cholinergic differentiation factor)	LIF	−1	−1.7
207826_s_at	NM_002167	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	ID3	−1	−1.8
204948_s_at	NM_006350	follistatin	FST	−1	−3.6
Embryogenesis and morphogenesis (traceable author statement)					
201416_at	NM_003107	SRY (sex determining region Y)-box 4	SOX4	1	+1.9
221163_s_at	NM_015977.1	Williams Beuren syndrome chromosome region 14	WBSCR14	1	+1.6
209204_at	NM_006769	LIM domain only 4	LMO4	1	+2.8
38918_at	NM_005686	SRY (sex determining region Y)-box 13	SOX13	−1	−1.8
203636_at	NM_000381	midline 1 (Opitz/BBB syndrome)	MID1	−1	−2.4
Neurogenesis (predicted/computed)					
218678_at	NM_024609	human nestin	NES	1	+15.2
204692_at	NM_002319	leucine-rich repeat protein, neuronal 1	LRRN1	1	+1.6
209870_s_at	NM_005503	amyloid beta (A4) precursor protein-binding, family A, member 2 (X11-like)	APBA2	1	+3.6
206462_s_at	NM_002530	neurotrophic tyrosine kinase, receptor, type 3	NTRK3	1	+2.0
221408_x_at	NM_018932	protocadherin beta 12	PCDHB12	1	+2.2
209465_x_at	NM_002825	pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)	PTN	1	+1.2
203045_at	NM_004148	ninjurin 1	NINJ1		+1.8
201341_at	NM_003633	ectodermal-neural cortex (with BTB-like domain)	ENC1	−1	−2.2
204421_s_at	NM_002006	fibroblast growth factor 2 (basic)	FGF2	−1	−2.2
202668_at	NM_004093	ephrin-B2	EFNB2	−1	−2.1
206038_s_at	NM_003298	nuclear receptor subfamily 2, group C, member 2	NR2C2	−1	−1.5
206373_at	NM_003412	Zic family member 1 (odd-paired homolog, *Drosophila*)	ZIC1	−1	−2.6
206382_s_at	NM_001709	brain-derived neurotrophic factor	BDNF	−1	−1.4
209822_s_at	NM_003383	very low density lipoprotein receptor	VLDLR	−1	−1.5
Neuroblast proliferation (experimental evidence)					
207675_x_at	NM_003976	artemin	ARTN	1	+2.7
Axon guidance (predicted/computed)					
203071_at	NM_004636	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	SEMA3B	1	+2.5
215000_s_at	NM_005102	fasciculation and elongation protein zeta 2 (zygin II)	FEZ2	−1	−1.5
Synaptogenesis (nontraceable author statement)					
202524_s_at	NM_014767	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	SPOCK2	1	+1.8
Central nervous system development (traceable author statement)					
206734_at	NM_003772	jerky homolog-like (mouse)	JRKL	−1	−1.5
203843_at	NM_004586	ribosomal protein S6 kinase, 90kDa, polypeptide 3	RPS6KA3	−1	−1.4
208370_s_at	NM_004414	Down syndrome critical region gene 1	DSCR1	−1	−3.1
Brain development (traceable author statement)					
206018_at	NM_005249	forkhead box G1B	FOXG1B	−1	−2.2
Synaptic vesicle endocytosis (predicted/computed)					
212990_at	NM_003895	synaptojanin 1	SYNJ1	−1	−2.0
Epidermal differentiation (traceable author statement)					
206969_at	NM_021013	keratin, hair, acidic, 4	KRTHA4	−1	−9.0

1 = upregulated; −1 = downregulated.

A list of 985 genes changed consistently in three experiments is available online.

along the early stages of commitment to astroglial and neuronal lineages (32), so it may not be unusual to see nestin expressed at this early stage of forced NeuroD1 expression. However, the presence of TuJ1 immunoreactivity in the NeuroD1 transfected cultures suggests that this transcription factor enhanced neuronal differentiation. Although the percentage of total cells that coexpressed GFP and TuJ1 was at most 1.3%, this represents a significant fraction of the 12–16% of the cells that were successfully transfected.

The SVG cell line has never before been shown to express neuronal markers or assume a neuronal phenotype despite extensive research with the line. This immortalized human fetal glial cell line was established nearly 20 years ago by transfection of human glial cells with a plasmid containing an origin-defective mutant of simian virus 40 (SV40) (31). The SVG cell line was demonstrated to be vimentin, GFAP, Thy 1.1, and MHC class I positive, and negative for neurofilament (200, 140, 68 kDa subunits all tested) and neuron-specific enolase, confirming its glial origin. Vimentin, a type III intermediate filament, was strongly expressed by all the SVG cells in the present experiment and GFAP, another type III intermediate filament, was very lightly expressed in both transfected and untransfected SVG cells, consistent with the original description of this cell line (31). These astroglial cells have in the past been transduced to express tyrosine hydroxylase and grafted into a rat model of Parkinson's disease; the cells were not examined for neuronal characteristics, but were shown to function as a cellular source of dopamine and growth factors when grafted into the striatum (46).

It is important to point out that rare untransfected SVG cells (i.e., GFP–) in the control cultures also exhibited TuJ1+ cells, suggesting that this cell line remains competent to differentiate into neurons even without expression of NeuroD1. In addition, mRNA for β-tubulin III (TuJ1) was present in both transfected and untransfected cultures. This raises the question as to whether NeuroD1 expression stimulated neuronal differentiation in a subpopulation of cells already committed to a neuronal fate or whether there was a reprogramming of cells into a dedifferentiated state followed by differentiation. The marked upregulation of the nestin transcript in the NeuroD1 transfected cells would support this hypothesis. Without further analysis, involving a longer course of study, it is not possible to answer this question. However, the profile of gene expression triggered by expression of NeuroD1 suggests that both mechanisms may be occurring in the same time frame in the population of transfected cells. Of course, it is not possible for differentiation and dedifferentiation to occur in the same cell at the same moment, but it is possible that the freeze-frame of the dynamic sequence of molecular events triggered by NeuroD1 revealed some cells in a dedifferentiated stem cell state and others in the process of neuronal differentiation.

Additional insight into the molecular mechanisms underlying this process was sought in the analysis of the transcriptional profiles. Investigation of the early molecular changes triggered by NeuroD1 overexpression revealed a consistent pattern of gene expression. The results of three separate microarray experiments agreed with respect to 910 genes that might be influenced in fetal glial cells when NeuroD1 is expressed. These data represent the largest transcriptional profile generated by overexpression of NeuroD1. Because only one time point (24 h after transfection) was examined, it is not possible to reconstruct the dynamic changes in gene expression orchestrated by this transcription factor. Nor is it possible to suggest whether these genes are direct targets of NeuroD1 or secondary effects of the transfection. However, many of the genes affected by NeuroD1 are implicated in neurogenesis and neuronal differentiation. The implication is that NeuroD1 has initiated this developmental pathway in the transfected cells and any of the genes could be a downstream consequence of this developmental commitment.

Many of the same genes were identified in other systems studying neuronal differentiation. Embryonic stem (ES) cells induced to differentiate into midbrain and hindbrain neurons (2) revealed that several important transcription factors (Sox4, Sox13, Ptx3, Wnt, TGF, and Klf5) were modulated at a stage when neurogenesis was initiated. In the present study Wnt5a and Wnt6 were both upregulated. SOX4 was upregulated and SOX13 was downregulated. The SOX family members may be particularly important. They play key regulatory roles in embryonic development and neurogenic differentiation in vetrebrates (2). SOX2 can partner with Oct4 (master regulator of the pluripotential state) to interact with several promoters in pluripotent stem cells, but its expression is later restricted to cells of neuronal fate (2). Using SOX2 promoter-based cell sorting, markers for neural and embryonic mouse stem cells have been elucidated (2). Klf5 is a marker for ES cells, Zic1 for both ES and neural stem cells (NSC), and FoxG1 is a marker for NSC. All of these genes were modulated by NeuroD1 overexpression in the present study. Zic1 is known to be a suppressor of Math1, a transcription factor similar to NeuroD1 (17). It appears that NeuroD1 downregulates the neurogenic suppressor Zic1 as well as other inhibitors. For example, inhibitors of differentiation (ID2, ID3), a DNA binding class of HLH proteins, were downregulated by NeuroD1 (Table 2). This observation is consistent with the inverse relationship

reported to exist between the expression of ID and NeuroD1 proteins during neurogenesis (27).

Other markers modulated by NeuroD1 overexpression include nestin, which was upregulated 15-fold consistently in three independent experiments. In contrast, nestin protein expression (indicated by nestin immunoreactivity in GFP+ cells) accounted for only 0.6% of the total cells in NeuroD1 transfected culture. Of course, this discrepancy between the high degree of upregulation of the mRNA for nestin and the extent of protein expressed in the cell is most likely due to differences in the population of cells studied. The transcriptional profile was performed in the FACS sorted, highly purified population of transfected cells, whereas the immunocytochemical analysis was performed in the unsorted cells in vitro where at most 15% of the cells were transfected. During development, nestin expression is initiated in the neuroectoderm of the neural tube (26) and in adult mammalian brain its expression is thought to represent proliferation of progenitor cells (19). Human teratoma-derived cells (NTERA-2 or NT2 cells), when differentiated into a neuronal phenotype with retinoic acid, turn on expression of NeuroD1 as nestin downregulates (38). Forced expression of NeuroD1 into this fetal glial cell line resulted in high levels of nestin transcription. Because nestin is typically turned on in proliferating neural progenitors and disappears as the cells differentiate, the data here suggest that overexpression of NeuroD1 may cause a reprogramming or dedifferentiation to an earlier stage of neural cell development. It remains to be seen whether these cells can eventually develop into mature and functional neurons. Moreover, it will be important to assess the effects of NeuroD1 overexpression in other human cell lines, including primary (nontransformed) human astroglia, neural progenitors derived from fetal neural progenitors, and adult subventricular zone.

Other genes associated with neuronal differentiation are clearly modulated by NeuroD1. Tyrosine hydroxylase, upregulated 4.1-fold in NeuroD1 transfected cells, is associated with differentiation of catecholaminergic neurons. Artemin (Artn) was upregulated by NeuroD1. It is a glial cell line-derived neurotrophic factor ligand for the receptor complex of GFRα3 and RET receptor tyrosine kinase (NTRK3) (GDNF receptor family) (8). Artemin supports the survival and regulates the differentiation of many peripheral neurons including sympathetic, paraympatheric, sensory, and enteric neurons (40). The GFRα3 component provides the ligand specificity and the RET tyrosine kinase is the signaling component. RET was also clearly upregulated (Table 3). GFRα3 appeared to be upregulated in two of the three experiments; however, the expression level was very low and therefore not conclusive. The concomitant upregulation of ligand and receptor suggests that NeuroD1 may be activating a new signal transduction pathway in the transfected cells. Glial cells are known to use receptors of the GFRα–RET complex for signal transduction.

Ninjurin1, which promotes axonal growth, was upregulated. It is a cell adhesion protein that has been shown to play a role in nerve regeneration (6). Ninjurin expression was reported to increase after nerve transaction in dorsal root ganglion neurons. Its localization on the cell surface makes it a good candidate as a marker for isolating specific neuronal phenotypes. LIM domain only 4 (LMO4) is a transcriptional regulator (43) that was increased 2.8-fold by NeuroD1. It has been shown that expression of LM04 in neuroblastoma cells increased cell proliferation and neurite outgrowth (43). The cell adhesion molecule, oligodendrocyte myelin glycoprotein (OMG), was upreguated. It is known to bind with high affinity to gycosyl-phosphytidyl-inositol (GPI)-anchored receptor, Nogo receptor (NR), which is one mechanism responsible for axonal growth (22). Syntaphilin, upregulated 5.1-fold, is a presynaptic membrane protein and is developmentally regulated and expressed in mature rat brain in regions that undergo synaptic plastic change (15). In PC12 cells, syntaphilin expression was induced as neuronal differentiation proceeded (15). Synaptphilin was observed when axonal and dendritic compartmentalization was occurring. These findings suggest a role for NeuroD1 in increasing axonal regrowth and synapse formation. A greater understanding of how NeuroD1 regulates transcription could one day lead to therapeutic applications to promote neuronal differentiation from neural progenitors or from reactive astrocytes, and to stimulate neuronal sprouting following sublethal injury to neurons.

CONCLUSIONS

NeuroD1 is a key transcription factor that regulates neuronal differentiation of neuroepithelial cells during development of the nervous system and continues to be expressed in hippocampus and olfactory bulb of the adult rodent brain. DNA microarray analysis of NeuroD1 expression in astroglial cells produced a "snapshot" profile of gene expression that will be useful as a starting point for deciphering the complex symphony of gene expression that orchestrates neurogenesis and neuronal differentiation.

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