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Stromal Cell–Derived Inducing Activity, Nurr1, and Signaling Molecules Synergistically Induce Dopaminergic Neurons from Mouse Embryonic Stem Cells

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ABSTRACT

To induce differentiation of embryonic stem cells (ESCs) into specialized cell types for therapeutic purposes, it may be desirable to combine genetic manipulation and appropriate differentiation signals. We studied the induction of dopaminergic (DA) neurons from mouse ESCs by overexpressing the transcription factor Nurr1 and coculturing with PA6 stromal cells. Nurr1-expressing ESCs (N2 and N5) differentiated into a higher number of neurons (~twofold) than the naïve ESCs (D3). In addition, N2/N5-derived cells contained a significantly higher proportion (>50%) of tyrosine hydroxylase (TH)$^+$ neurons than D3 (<30%) and an even greater proportion of TH$^+$ neurons (~90%) when treated with the signaling molecules sonic hedgehog, fibroblast growth factor 8, and ascorbic acid. N2/N5-derived cells express much higher levels of DA markers (e.g., TH, dopamine transporter, aromatic amino acid decarboxylase, and G protein–regulated inwardly rectifying K$^+$ channel 2) and produce and release a higher level of dopamine, compared with D3-derived cells. Furthermore, the majority of generated neurons exhibited electrophysiological properties characteristic of midbrain DA neurons. Finally, transplantation experiments showed efficient in vivo integration/generation of TH$^+$ neurons after implantation into mouse striatum. Taken together, our results show that the combination of genetic manipulation(s) and in vitro cell differentiation conditions offers a reliable and effective induction of DA neurons from ESCs and may pave the way for future cell transplantation therapy in Parkinson's disease. STEM CELLS *2006;24:557–567*

INTRODUCTION

Embryonic stem cells (ESCs) are derived from the inner cell mass of mammalian blastocysts [1, 2]. Because of their ability to proliferate indefinitely in vitro while maintaining an undifferentiated state and their developmental potential to differentiate into most cell types [3–8], ESCs are useful not only for analyzing critical steps of cell development but also as a potential source for cell replacement therapy.

A target for such an application is Parkinson's disease (PD) because it primarily involves the degenerative loss of a specific cell type, namely the midbrain dopaminergic (DA) neurons in the substantia nigra [9, 10]. Several laboratories have demonstrated that phenotypes characteristic of midbrain DA neurons can be efficiently induced in vitro and/or in vivo from mouse and primate ESCs. The five-stage method is a successful in vitro induction procedure in which mouse ESCs (mESCs) are first grown to form embryoid bodies (EBs), followed by selection and expansion of Nestin$^+$ neural precursors and differentiation into neural subtypes [11]. This method can generate as much as 34% of tyrosine hydroxylase (TH)$^+$ cells among Tuj1$^+$ neurons in the presence of signaling molecules such as sonic hedgehog (Shh), fibroblast growth factor 8 (FGF8), and ascorbic acid [11].

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Another efficient in vitro method for DA neuron induction is the coculture method with PA6 feeder cells that have stromal cell–derived inducing activity (SDIA) [12]. This procedure also efficiently induces a high proportion of TH^+ neurons (~30% of $Tuj1^+$ neurons) from mESCs. The coculture method offers additional advantages over EB-based methods, such as (a) it does not depend on EB formation and selection of neural precursors, (b) the whole procedure takes approximately half the amount of time as the five-stage method, and (c) it showed reduced variability and generally higher yield of TH^+ neurons when multiple ESCs were tested and compared with each other [13]. Importantly, this stromal cell coculture method was successfully applied for efficient generation of TH^+ neurons from primate ESCs [14], suggesting that ESCs from different species, including human, may be coaxed to differentiate into DA neurons using this procedure. Indeed, several laboratories very recently reported initial results that human ESCs (hESCs) can differentiate into DA neurons in vitro using the stromal cell coculture method [15–17].

It is possible to successfully facilitate the differentiation of ESCs to certain lineages by genetic manipulation consisting of the specific activation of key fate-determining transcription factors [18, 19]. Recent data from this and other groups demonstrated that forced expression of Nurr1, a transcription factor critical for the development of midbrain DA neurons [20–22], can greatly facilitate the induction of DA neurons from mESCs [23, 24]. Our findings showed that Nurr1 transduction in ESCs resulted in an approximately fivefold increase in the proportion of DA neurons [24]. Nurr1 overexpression led to upregulation of midbrain DA markers such as TH, aromatic L-amino acid decarboxylase (AADC), and dopamine transporter (DAT) and showed increased DA release in response to membrane depolarization. It is likely that genetic modification of ESCs combined with optimal culture conditions using extrinsic factors could result in efficient induction of certain cell lineage(s). Indeed, it was demonstrated that Nurr1-overexpressing mESCs, treated with signaling molecules (Shh, FGF8, and ascorbic acid), differentiated into DA neurons with a high efficiency (62%–78% of $TH^+/Tuj1^+$ neurons) [23, 24]. In addition to ESCs, the role of Nurr1 was investigated in neural precursor cells such as adult hippocampal precursor cells [25], cerebellum-derived immortalized cells, C17.2 [26], and in vitro expanded central nervous system presursor cells [27]. Whereas all these studies showed the potent ability of Nurr1 to induce the DA phenotype (typically TH expression), the maturity and function of the resulting DA cells were variable, suggesting that additional transcription factors and/or signaling molecules are necessary.

At present it is not known whether Nurr1 overexpression and PA6 coculture may co-operatively facilitate the differentiation of ESCs into DA neurons. Whereas SDIA is known to accumulate on the surface of PA6 stromal cells, its molecular nature is not known [12, 14]. If SDIA shares a close relationship with the Nurr1 pathway, it may not have a synergistic effect on DA neuron induction using the Nurr1 overexpression and PA6 coculture method. Furthermore, questions remain about the synergistic effect of combining signaling molecules such as Shh and FGF8 with SDIA on the induction of DA neuronal phenotype. Our work centered on two main objectives: (a) to address questions about the combined roles of SDIA, signaling molecules, and Nurr1 on neurogenesis and/or DA neuron differentiation from ESCs; and (b) to establish an effective and reliable protocol for in vitro ESC differentiation to generate DA neurons suitable for transplantation. Toward these goals, we studied the in vitro behavior of Nurr1-overexpressing mESCs in the PA6 coculture method and the effect of signaling molecules on their differentiation. Through systematic analyses, we observed that a very high proportion of midbrain DA neurons (up to 90% of $Tuj1^+$ neurons) can be generated from Nurr1-overexpressing mESCs using the optimized PA6 coculture method within a short period of time (~14 days). Furthermore, transplantation studies using the resulting DA neurons demonstrate that they can stably integrate into the host striatum and express the DA phenotype.

Materials and Methods

In Vitro Differentiation of ESCs

PA6 cells were purchased from Riken (Tsukuba, Japan, http://www.riken.go.jp/engn) and were maintained in PA6 culture medium α–minimum essential medium (Gibco, Rockville, MD, http://www.invitrogen.com) supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin (PEST) (Gibco), and 10% fetal bovine serum (HyClone, Logan, UT, http://www.hyclone.com) [12]. Undifferentiated mESCs (the wild-type D3 and Nurr1-expressing N2 or N5 cells [24]) were maintained on gelatin-coated dishes in Dulbecco's modified minimal essential medium (Gibco) supplemented with 2 mM glutamine (Gibco), 0.001% β-mercaptoethanol (Gibco), 1× nonessential amino acids (Gibco), 10% donor horse serum (Sigma, St. Louis, http://www.sigmaaldrich.com), and 2,000 U/ml human recombinant leukemia inhibitory factor (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). To differentiate ESCs in vitro, PA6 cells were plated on gelatin-coated culture dishes to make a uniform feeder monolayer 1 day before the addition of D3, N2, or N5 cells, and then ESCs were added at a density of 1×10^3 per well of a 24-well plate. Embryonic stem differentiation medium I (Glasgow minimum essential medium [G-MEM] [Gibco] supplemented with 10% knockout serum replacement [Gibco], 0.1 mM nonessential amino acids [Gibco], 1 mM sodium pyruvate [Sigma], 0.1 mM 2-mercaptoethanol [Gibco], and PEST [Gibco]) was used for 8 days and then replaced with embryonic stem differentiation medium II (equivalent to N-2 medium in other paper [12]) (G-MEM [Gibco] supplemented with N-2 supplement [Gibco], 0.1 mM nonessential amino acids [Gibco], 1 mM sodium pyruvate [Sigma], 0.1 mM 2-mercaptoethanol [Gibco], and PEST [Gibco]) for an additional 6 days [12]. The culture medium was changed on day 4 and every other day thereafter.

Analyses of Dopamine

HPLC analyses of dopamine were performed after 24 hours of conditioning at day 14. The proteins from 0.2 ml of media from each well of a 12-well plate were precipitated by adding perchloric acid (PCA) and EDTA at final concentrations of 0.33 M and 0.17 mM, respectively. After aspiration of the residual media (0.6 ml), the cells were treated with 0.2 ml of 50 mM KCl in embryonic stem differentiation medium II for 30 minutes at 37°C. Then the media were collected, and the proteins were precipitated by the addition of PCA and EDTA as described above. The mixture was centrifuged at 4°C for 10 minutes at 14,000*g*, and the supernatant was used for HPLC analysis. Cells were allowed a 1-day recovery in fresh medium after which time

they were washed, scraped, collected, and vortexed in a chilled (4°C) 0.24 ml solution of 0.33 M PCA and 0.17 mM EDTA. After centrifugation at 14,000*g* for 10 minutes, intracellular fraction (supernatant) and cell pellet were separated for intracellular DA and protein analysis (data not shown), respectively. Samples were analyzed as described previously [24]. Briefly, samples were applied to reverse-phase HPLC using a Velosep RP-18 column (100 × 3.2 mm) and a Coulochem II® electrochemical detector equipped with a 5014 analytical cell (ESA Biosciences, Inc., Chelmsford, MA, http://www.esainc.com). The flow rate of the mobile phase (0.1 M sodium phosphate buffer [pH 2.65], 0.1 mM EDTA, 0.4 mM sodium octyl sulphate, and 9% methanol) was 0.8 ml/minute. The potentials of the guard cell and the first and the second electrodes in the analytical cell were set at 330, 0, and 310 mV, respectively. Dopamine was identified by retention time and quantified based on peak height using the EZChrom Chromatography Data System.

Immunostaining

After 14 days of differentiation on PA6 feeder layer, ESCs were fixed with 4% formaldehyde (Electron Microscopy Sciences, Hatfield, PA, http://www.emsdiasum.com) for 30 minutes, rinsed with phosphate-buffered saline (PBS), and then incubated with blocking buffer (PBS, 10% normal donkey serum [NDS] or normal goat serum [NGS], and 0.1% Triton X-100) for 10 minutes. Cells were incubated overnight at 4°C with primary antibodies diluted in PBS containing 2% NDS or NGS. The following primary antibodies were used: rabbit anti–β-tubulin (1:2,000; Covance, Richmond, CA, http://www.covance.com), sheep anti-TH (1:200; Pel-Freez, Rogers, AK, http://www.pelfreez.com), sheep anti-AADC (1:200; Chemicon, Temecula, CA, http://www.chemicon.com), rat anti-DAT (1:1,000; Chemicon), rabbit anti–5-hydroxytryptamine (HT) (1:3,000; DiaSorin, Stillwater, MN, http://www.diasorin.com), and rabbit anti-γ-aminobutyric acid (GABA) (1:5,000; Sigma). The coverslips were washed with PBS and then incubated with fluorescent-labeled secondary antibodies [Alexa Fluor 488 (green) or Alexa Fluor 568 (red)-labeled donkey/goat IgG (1:500; Molecular Probes, Invitrogen, Carlsbad, CA, http://www.invitrogen.com)] in PBS with 2% NDS or NGS for 30 minutes at room temperature. The coverslips were rinsed for 3 × 10 minutes in PBS and mounted onto slides using Gel/Mount (Biomeda Corp., Foster City, CA, http://www.biomeda.com). Cells were examined using a Leica TCS/NT confocal microscope equipped with krypton, krypton/argon, and helium lasers. Cells were counted according to the protocol that our laboratory set up previously [24] with a slight modification. We skipped fields where we could not find any cells.

Semiquantitative Reverse Transcription– and Real-Time Polymerase Chain Reaction Analyses

After differentiation, total RNA from ESC-derived neurons was prepared using TriReagent (Sigma) followed by treatment with DNase I (Ambion, Austin, TX, http://www.ambion.com). cDNA was obtained using 5 μg of RNA with the SuperScript™ first-strand synthesis system for reverse transcription–polymerase chain reaction (RT-PCR) (Invitrogen). The resulting cDNA was used as a template for the PCR reactions. To reduce the nonspecific signals, primers of each gene were designed using the MacVector software (Accelrys, San Diego, CA, http://www.accelrys.com). We selected primer sets that yielded specific products without the presence of nonspecific bands as evaluated by gel electrophoresis. The following primer sets were chosen for semiquantitative RT- and real-time PCR analyses.

Actin: 5′-GGCATTGTGATGGACTCCGG-3′; 5′-TGCCACAGGATTCCATACCC-3′ (358 bp)

TH: 5′-TTGGCTGACCGCACATTTG-3′; 5′-ACGAGAGGCATAGTTCCTGAGC-3′ (336 bp)

AADC: 5′-CCTACTGGCTGCTCGGACTAA-3′; 5′-GCGTACCAGGGACTCAAACTC-3′ (715 bp)

DAT: 5′-CAGAGAGGTGGAGCTCATC-3′; 5′-GGCAGATCTTCCAGACACC-3′ (328 bp)

Pitx3: 5′-CTCTCTGAAGAAGAAGCAGCG-3′; 5′-CCGAGGGCACCATGGAGGCAGC-3′ (491 bp)

Aldehyde dehydrogenase 2 (AHD2): 5′-CATTGAGAGTGGGAAGAAAGAAGG-3′; 5′-AGTGAAGAGTCCTGCTGCTAAACC-3′ (232 bp)

Calbindin: 5′-GCAGTCATCTCTGATCACAGC-3′; 5′-GAGGTCTGTGTACTCTGCTAG-3′ (428 bp)

Girk2: 5′-CCTTGTCTCCCATTTCCTTCTTTTC-3′; 5′-CTGTCTGCTTGTCCCATCTGAAC-3′ (147 bp)

Tryptophan hydroxylase (TPH): 5′-CTACACTCCAGAGCCAGACAC-3′; 5′-GACATCAAGGTCATACCGCAAC-3′ (501 bp)

Glutamic acid decarboxylase (GAD): 5′-GGGTTTGAGGCACACATTGATAAG-3′; 5′-GCGGAAGAAGTTGACCTTGTCC-3′ (279 bp)

Choline acetyltransferase (ChAT): 5′-GCCAATCCATTCCCACTGAC-3′; 5′-CATCCAAGACAAAGAACTGG-3′ (198 bp).

For semiquantitative RT-PCR, PCR reactions were carried out with 1× IN Reaction Buffer (Epicentre Technologies, Madison, WI, http://www.epicentretechnology.com), 1.4 nM of each primer, and 2.5 units of Taq I DNA polymerase (Promega, Madison, WI, http://www.promega.com). Samples were amplified in an Eppendorf Thermocycler (Brinkmann Instruments, Westbury, NY, http://www.brinkmann.com) under the following conditions: denaturing step at 95°C for 40 seconds, annealing step at 60°C for 30 seconds, and amplification step at 72°C for 1 minute for 20–35 cycles. cDNA templates were normalized based on the actin-specific signal, and PCR cycling was adjusted in such a way that each primer set amplified its corresponding gene product at its detection threshold to avoid saturation effects.

We performed real-time PCR to quantify expression levels. The amplifications were performed in 25-μl volumes containing 0.5 μm of each primer, 0.5 X SYBR Green I (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com), and 2 μl of 10-fold diluted cDNA using the DNA engine Opticon (MJ Research, Waltham, MA, http://www.mjr.com). The PCR reactions consisted of 50 cycles using the following temperature profile: 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 79°C for 5 seconds. The melting temperature (T_m) of each PCR product was determined and turned out to be more than 79°C. After each PCR cycle, the fluorescent signals were detected at 79°C to melt primer dimers (T_m of all primer dimers used in this study was <79°C). The purity of each PCR product (defined as the presence of a single, specific band) was confirmed by gel electrophoresis. A standard curve was constructed using GAPDH (glyceraldehyde 3-phosphate dehydrogenase)

plasmid DNA (from 10^2 to 10^7 molecules). The fluorescent signals from specific PCR products were normalized against that of the actin gene, and then relative values for N2 cells were calculated by setting the normalized value of D3 cells as one for each gene. Two independent samples were analyzed for each gene, and all the reactions were repeated more than twice.

Electrophysiological Experiment

Differentiated ESCs were examined using the whole-cell recording configuration of the conventional 'dialyzed' whole-cell patch-clamp technique. Differentiated cells were obtained by seeding ESCs onto 12-mm round coverslips and culturing for 14 days. Patch electrodes were fabricated from a borosilicate glass capillary (Sutter Instrument Company, San Rafael, CA, http://www.sutter.com) by using a vertical micropipette puller (Narishige, Tokyo, http://www.narishige.co.jp). The patch electrodes were fire-polished on a microforge (Narishige) and had resistances of 1–3 MΩ when filled with the internal solution described below. The cell membrane capacitance and series resistance were compensated (typically >80%) electronically using a patch-clamp amplifier (Axopatch-200A; Axon Instruments/Molecular Devices Corp., Union City, CA, http://www.moleculardevices.com). Current protocol generation and data acquisition were performed using pClamp 8.0 software on an IBM computer equipped with an analogue-to-digital converter (Digidata 1322A; Axon Instruments/Molecular Devices Corp.). Voltage traces were filtered at 2 kHz by using the four-pole bessel filter in the clamp amplifier and stored on the computer hard drive for later analysis. All experiments were performed at room temperature (21°C–24°C). For recording of membrane potential in current clamp mode, the patch pipette solution contained (in mM): KCl 134, $MgCl_2$ 1.2, MgATP 1, Na_2GTP 0.1, EGTA 10, glucose 14, and HEPES 10.5 (pH adjusted to 7.2 with KOH). The bath solution contained (in mM): NaCl 126, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1.2, glucose 14, and HEPES 10.5 (pH adjusted to 7.4 with NaOH).

Transplantation Analysis

D3 and N2 ESC colonies were isolated from PA6 feeder layer at differentiation day 9 by incubation with 2.5 ml of papain solution (Papain Dissociation Kit, Worthington Biochemical Corporation, Freehold, NJ, http://www.worthington-biochem.com) for 5 minutes at 37°C. The dissociated colonies were transferred to a 15-ml tube and then incubated for an additional 5 minutes at 37°C followed by gentle trituration using a 1-ml tip and a 5-ml pipette. The cells were suspended at a density of 50,000 cells per μl. One microliter of the cell suspension was grafted into the right striatum (from the bregma: AP +0.05, L −0.18, V −0.30, IB 9) of C57/BL/6 mice ($n = 10$) (Charles River Laboratories, Wilmington, MA, http://www.criver.com). Prior to surgery, mice received an i.p. injection of preanesthesia (acepromazine [3.3 mg/kg, PromAce; Fort Dodge Laboratories, Inc., Fort Dodge, IA]) and atropine sulfate (0.2 mg/kg; Phoenix Pharmaceuticals, Belmont, CA, http://www.phoenixpeptide.com) followed by an i.p. injection of ketamine (60 mg/kg; Fort Dodge Laboratories, Inc.) and xylazine (3 mg/kg; Phoenix Pharmaceuticals). Transplantation was performed using a 22-gauge needle, 10-μl syringe (Hamilton, Reno, NV, http://www.hamiltoncomp.com), and a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA, http://www.kopfinstruments.com). An s.c. injection of buprenorphine (0.032 mg/kg; Sigma) was given as postoperative anesthesia. Four weeks after transplantation, the mice were terminally anesthetized with an i.p. overdose of pentobarbital (150 mg/kg; Sigma). The mice were perfused intracardially with 100 ml of heparin saline (0.1% heparin in 0.9% saline) followed by 200 ml of paraformaldehyde (4% in PBS). The brains were postfixed for 8 hours in the same solution and then equilibrated in sucrose (20% in PBS), sectioned at 40 μm on a freezing microtome, and collected in PBS. For histological analyses, the sections were stained with antibodies against TH and neuronal nuclei (NeuN). Nissl staining was performed as described previously [28]. Cells were counted from blind-coded samples using an integrated Axioskop 2 microscope (Carl Zeiss, Thornwood, NY, http://www.zeiss.com) and StereoInvestigator image capture equipment and software (MBF Bioscience, Williston, VT, http://www.microbrightfield.com).

RESULTS

Nurr1 Overexpression Promotes the Neuronal Differentiation of ESCs When Cocultured with PA6 Feeder Cells

Using the five-stage in vitro differentiation method, it has been shown that exogenous expression of Nurr1 and treatment of signaling molecules (Shh, FGF8, and ascorbic acid) can synergistically increase the induction of DA neurons [23, 24]. To address whether Nurr1 overexpression and/or signaling molecules can induce DA neurons in the presence of SDIA, we differentiated D3 and Nurr1-expressing N2 and N5 ESCs on the PA6 feeder layer for 8 days in embryonic stem differentiation medium I and then for an additional 6 days in embryonic stem differentiation medium II (see 'Materials and Methods'). Consistent with our previous results [24], Nurr1 expression was persistently maintained throughout differentiation. Nurr1 expression was detected only in N2 cells, but not in D3 at the beginning of the experiment, and was approximately sixfold higher in N2-derived cells compared with D3-derived cells at a final stage (Fig. 1B). During this procedure, ESCs were treated with signaling molecules such as Shh, FGF8, and ascorbic acid at different time periods (Fig. 1A). Under all three conditions for D3 cells, the numbers of $Tuj1^+$ cells generated after 14 days in vitro differentiation were not significantly different from each other (Fig. 1C). This is in general agreement with a previous report that treatment with diverse signaling molecules, including Shh and FGF8, did not promote neural differentiation of ESCs on the PA6 feeder layer [12]. In contrast, the number of N2-derived neurons was significantly higher (>twofold; $p < .05$) than that of D3 cells although the same number of ESCs was initially used in each condition (Fig. 1C). N2 cells under condition II [N2+SM (II)] generated a slight, but statistically significant ($p < .05$), increase of $TuJ1^+$ cells, compared with D3+SM (II) or D3+SM (III). In addition, these $TuJ1^+$ cells represented approximately 37%, 58%, and 62% of total cell numbers in D3, N2, and N2+SM (II), respectively (data not shown). Consistent with these results, our real-time PCR analyses showed that the level of β-tubulin mRNA normalized to actin mRNA increased approximately 2.5-fold in N2+SM (II) cells, compared with D3 cells (data not shown). Another Nurr1-expressing ESC clone, N5, showed the same result as the N2 (data not shown). Taken together, Nurr1 overexpression promoted the neuronal differentiation of ESCs. When 4′,6-

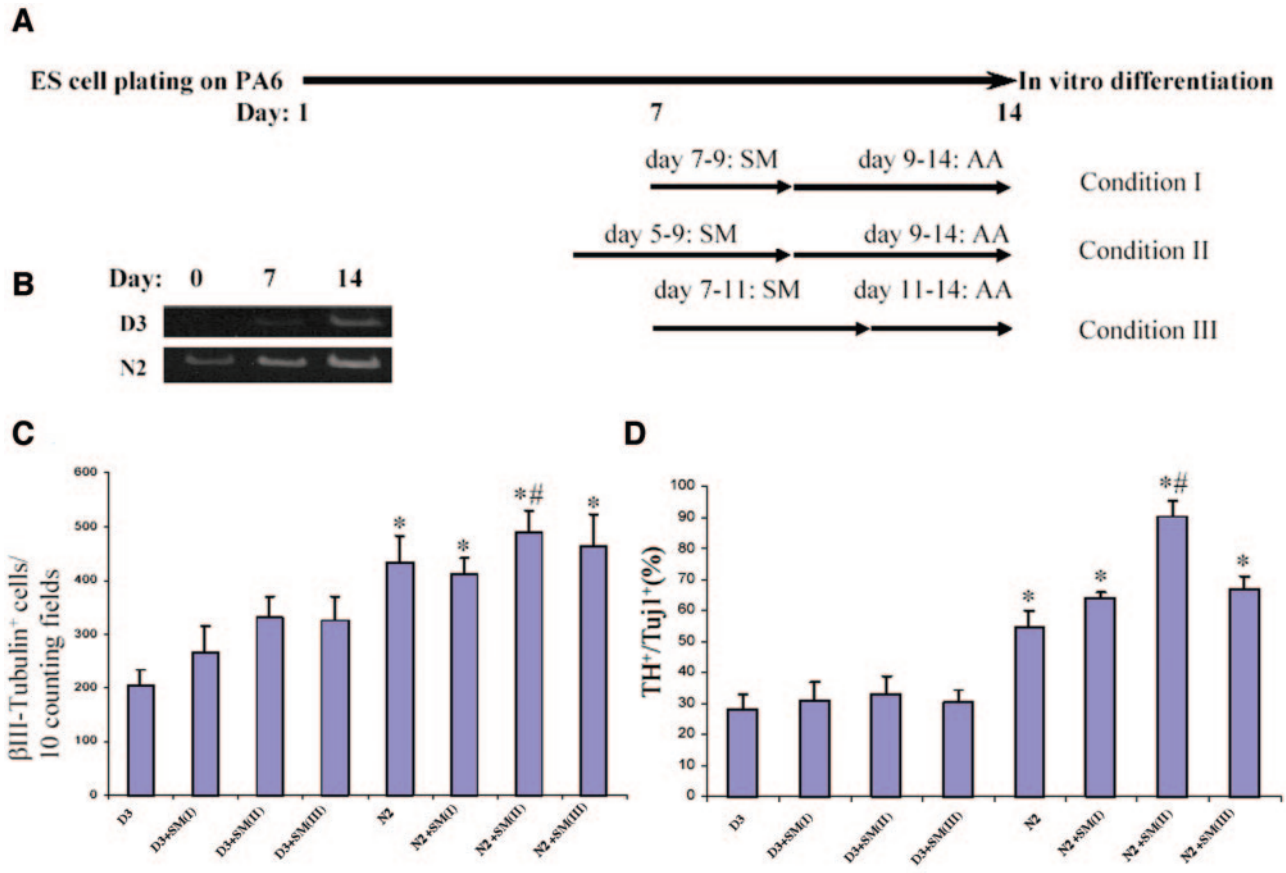

Figure 1. Synergistic effects of Nurr1, signaling molecules, and SDIA on efficient generation of TH^+ neurons. **(A):** A schematic diagram for in vitro differentiation of ESCs on PA6 feeder layer. PA6 cells were plated on gelatin-coated culture dish and incubated overnight to make a confluent uniform feeder monolayer. The next day (day 0), the PA6 feeder monolayer was washed with embryonic stem differentiation medium, and D3 and N2 cells were added to initiate differentiation. ESCs were allowed to differentiate on the PA6 feeder cells for 14 days. During that differentiation period, signaling molecules such as Shh, FGF8, and ascorbic acid were added at different time intervals as follows: condition I, treatment with Shh and FGF8 during days 7–9 and ascorbic acid treatment during days 9–14; condition II, treatment with Shh and FGF8 during days 5–9 and ascorbic acid treatment during days 9–14; condition III, treatment with Shh and FGF8 during days 7–11 and ascorbic acid treatment during days 11–14. **(B):** *Nurr1* expression during in vitro differentiation of D3 and N2 ESCs. *Nurr1* gene expression was examined at day 0, 7, and 14 during in vitro differentiation of D3 and N2 ESCs by semiquantitative reverse transcription–polymerase chain reaction analysis. **(C):** Effect of transgene expression of *Nurr1* on neurogenesis. β-tubulin$^+$ neurons derived from ESCs were detected by staining with anti-Tuj1 antibodies. Positive cells were counted in 10 random fields per sample. Each group represents an average of three samples from independent experiments. Fisher's PLSD post hoc analysis was performed with a significance level of 0.05. $*p < .05$ compared with D3 and D3+SM (I); $\#p < .05$ compared with D3+SM (II) and D3+SM (III). **(D):** Exogenous expression of *Nurr1* under condition II [N2+SM (II)] results in a significantly high proportion (~90%) of TH^+ neurons among total neurons. ESC-derived neurons were stained with anti-TH and anti-Tuj1 antibodies. β-tubulin$^+$ and TH^+ cells were counted in 10 random fields per sample. Cell densities of TH^+ neurons were calculated by dividing the numbers of TH^+ cells by those of β-tubulin$^+$ cells. Each group represents an average of three samples from independent experiments. Fisher's PLSD post hoc analysis was performed with a significance level of 0.05. $*p < .05$ compared with D3; $\#p < .05$ compared with N2. Abbreviations: AA, ascorbic acid; D3, naïve embryonic stem cells; ESC, embryonic stem cell; FGF8, fibroblast growth factor 8; I, condition I; II, condition II; III, condition III; N2, Nurr1-expressing embryonic stem cell line; PLSD, protected least-significant difference; Shh, sonic hedgehog; SM, signaling molecule; TH, tyrosine hydroxylase.

diamidino-2-phenylindole $(DAPI)^+$ cells were counted in 10 fields, the number of $DAPI^+$ cells was slightly higher (by 30%–40%) in N2 cells than D3 cells, suggesting that Nurr1 may also have a modest effect on cell survival in our culture protocol.

Nurr1 Overexpression and Signaling Molecules Synergistically Facilitate the Differentiation of ESCs to the DA Fate When Cocultured with PA6 Feeder Cells

The expression of the DA phenotype amongst $Tuj1^+$ neurons was analyzed next. As shown in Figure 1D, approximately 28% of β-tubulin$^+$ neurons were TH^+ in naïve D3 cells after the whole 14-day procedure. When signaling molecules were added to the D3 cells, the proportions of TH^+ neurons among total neurons were not significantly changed (30%–33% depending on treatment conditions). When N2 ESCs were differentiated on the PA6 feeder cells, a much higher proportion of $Tuj1^+$ neurons was TH^+ (~55%; $p < .05$; Fig. 1D), demonstrating that Nurr1 and SDIA may cooperatively induce the DA phenotype. Furthermore, when treated with the signaling molecules, a much greater effect was observed with statistical significance ($p < .05$), and approximately 90% of $Tuj1^+$ neurons were TH^+ under

the optimal condition II (Figs. 1D, 2A). We did not test the effect of individual signaling molecules in this experiment. However, the previous report indicates that each signaling molecule has a marginal effect on the differentiation into DA neurons and a much greater effect when treated in combination [11]. Another Nurr1-expressing ESC clone, N5, was able to generate a similarly high proportion of $TH^+/Tuj1^+$ neurons under the same optimal condition II (data not shown).

TH^+ Neurons Coexpress the Other Midbrain DA Markers

TH^+ cells exist in many neuronal phenotypes, including DA and noradrenergic (NA) neurons. Therefore, we determined whether TH^+ neurons generated by the optimized in vitro condition II have the midbrain DA neuron phenotype. The in vitro differentiated N2 cells under condition II were costained using antibodies against TH, AADC, DAT, γ-aminobutyric acid (GABA), and 5-HT. AADC is an enzyme that is involved in the last step of dopamine and serotonin biosynthesis [29]. As shown in Figure 2B, all TH^+ neurons were $AADC^+$ but some of the $AADC^+$ cells were TH^-. Thus, these $TH^-/AADC^+$ cells may represent serotonergic neurons. Some of the TH^+ cells were DAT^+, indicating that these neurons have acquired a mature DA phenotype. Notably, these TH^+ cells did not express GABA, suggesting that they have the midbrain, but not olfactory, DA neuronal phenotype. These TH^+ neurons also did not coexpress 5-HT. To investigate mRNA expression of midbrain DA neuron-specific markers, semiquantitative RT- and quantitative real-time PCR analyses were carried out for D3 and N2 clones after 14 days of differentiation on the PA6 feeder layer (Figs. 3A, 3B). The effect of signaling molecules was studied in N2 cells using our optimized condition [SM (II)] and compared with D3 cells in the absence and presence of signaling molecules. We first designed several primer sets for each gene using the MacVector software. Primer sets that can produce a specific band without nonspecific products were screened using cDNA prepared from total RNA. Using these primers, the expression level of each gene was examined and normalized to the housekeeping gene actin. Our results of semiquantitative RT-PCR (Fig. 3A) were very consistent with those of real-time PCR (Figs. 3B, 3C). Expression of TH mRNA was increased approximately sevenfold in N2+SM (II), compared with D3 cells (Figs. 3A, 3B). This is consistent with the increase of the $TH^+/Tuj1^+$ population from 28% to 90% (Fig. 1D). Additional DA markers such as DAT, AADC, and Girk 2 were also dramatically upregulated (>15-fold). High expression of Girk 2, an A9-preferential DA neuronal marker [30–33], indicates that certain feature(s) of the A9 DA phenotype may be induced under these conditions. However, the expression of another midbrain maker, AHD2 [34], was marginally increased. Other DA markers, like calbindin and Pitx3, were also nonsignificantly upregulated (Fig. 3B).

We next examined gene regulation of various other neurotransmitter markers (Fig. 3C). Expression of NA marker, dopamine β-hydroxylase, was not detected in either D3 or N2 clones (data not shown). Therefore, these TH^+ cells exhibit the DA, not the NA, phenotype. In contrast to the upregulation of DA marker genes, the expression of the GABAergic and cholinergic (GAD and ChAT, respectively) was only minimally changed. Thus, Nurr1 overexpression and signaling molecules seem to specifically facilitate the differentiation of ESCs to the DA fate when cocultured with PA6 feeder cells. In support of this, real-time PCR analysis demonstrated that the DA-specific markers are specifically upregulated in the N5 clone as well (data not shown). However, the expression of a serotonergic neuronal marker, TPH, was upregulated by approximately 2.5-fold.

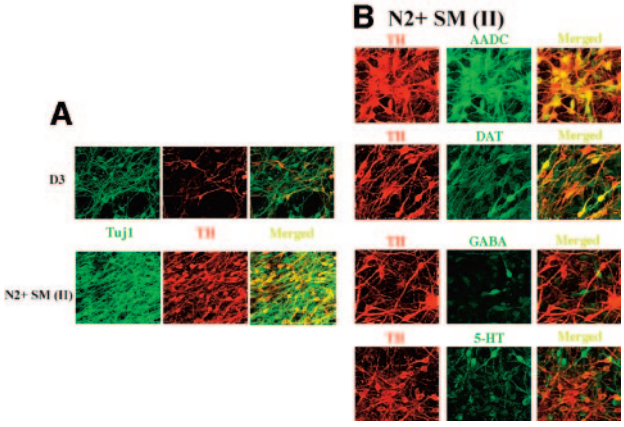

Figure 2. TH^+ neurons generated by N2 cells under condition II coexpress other markers specific for midbrain DA neurons. **(A):** Detection of β-tubulin and TH^+ cells by immunocytochemistry in differentiated D3 and N2 [N2+SM (II)] cells. **(B):** TH^+ neurons coexpress midbrain DA markers such as AADC and DAT, but not GABA and 5-HT. Embryonic stem cell–derived neurons were stained with anti-TH, anti-AADC (dopaminergic, serotonergic), anti-DAT (dopaminergic), anti-GABA (GABAergic), and anti–5-HT (serotonergic) antibodies. Abbreviations: AADC, aromatic L-amino acid decarboxylase; DA, dopaminergic; DAT, dopamine transporter; GABA, γ-aminobutyric acid; HT, hydroxytryptamine; SM, signaling molecule; TH, tyrosine hydroxylase.

In Vitro Differentiated Cells Exhibit Active Membrane Properties Characteristic of DA Neurons

The electrophysiological properties of neurons differentiated from N2+SM (II) cells were investigated using the conventional "dialyzed" whole-cell recording technique. The resting membrane potentials of the differentiated neurons ranged from −48 to −57 mV (average, −55.3 ± 2.3 mV). Recordings in the current-clamp configuration allowed us to determine the active membrane characteristics of these neurons. Prolonged depolarizing current injections (Fig. 4A) demonstrated the capability to fire fast action potentials and action potential series (action potential amplitude ranging from 32 to 47 mV [average, 41 ± 3.5 mV], action potential half width ranging from 1.4 to 3.7 ms [average, 2.2 ± 0.3 ms]). The neurons also exhibited voltage-dependent membrane currents (Fig. 4B). Depolarizing voltage steps elicited both large outward potassium currents (653 ± 134 pA) and fast inward Na^+ current (350 ± 76 pA). These results indicate that the differentiated cells have characteristics of neurons. In addition, the changes of membrane potential were investigated in the neurons subjected to hyperpolarizing current injections (Fig. 4C). As the intensity of the hyperpolarizing current was increased, there was a time-dependent reduction in the membrane deflection, indicating an anomalous rectification that is a characteristic of midbrain DA neurons [35, 36]. These

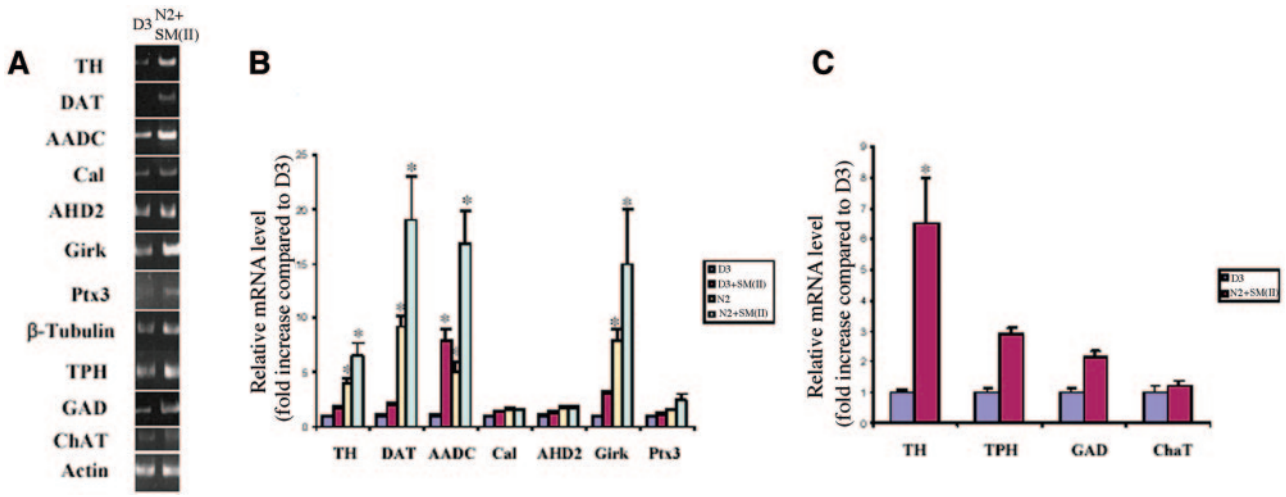

Figure 3. Semiquantitative RT- and real-time PCR analyses for DA neuron markers and other neurotransmitter markers. Semiquantitative RT- **(A)** and real-time PCR analyses **(B, C)** of midbrain DA markers **(A, B)** and neurotransmitter markers **(A, C)** after in vitro differentiation of naïve D3, D3+SM(II), N2, and N2+SM(II) conditions. The expression levels of each gene were normalized to that of actin. Similar patterns were obtained when we used another gene, *GAPDH*, for normalization (data not shown). In **(B, C)**, relative expression levels of each gene were determined in relation to those from naïve D3 cells. Each group represents an average of two samples from independent experiments. Fisher's PLSD post hoc analysis was performed with a significance level of 0.05. $*p < .05$ compared with D3. Abbreviations: AADC, aromatic L-amino acid decarboxylase; AHD2, aldehyde dehydrogenase 2; ChAT, choline acetyltransferase; DA, dopaminergic; DAT, dopamine transporter; GABA, γ-aminobutyric acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HT, hydroxytryptamine; PCR, polymerase chain reaction; PLSD, protected least-significant difference; RT, reverse transcription; SM, signaling molecule; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase.

time-dependent inward rectifications were observed in five out of seven cells tested, indicating that a majority of the neurons have the electrophysiological property of DA neurons.

Nurr1 Overexpression and Signaling Molecules Greatly Facilitate the Production and Release of DA on PA6 Feeder Cells

An important physiological aspect of in vitro differentiated DA neurons is their ability to produce and release DA in response to membrane depolarization. To test this, ESCs were differentiated for 14 days under our optimized conditions and analyzed for the production and release of DA. The 24 hour–conditioned media were prepared at day 14, and then cells were treated with 50 mM KCl. After a 1-day recovery in fresh medium, cells were harvested and intracellular DA levels were measured as described in the Materials and Methods section. For these samples, DA levels were assayed by reverse-phase HPLC and expressed as pg per 20 μl medium/solution. As shown in Figure 5, DA released into conditioned media and in response to membrane depolarization were 15- and four fold higher in N2+SM (II) (48 pg/20 μl and 24 pg/20 μl, respectively) than in D3 cells (3.25 pg/20 μl and 5.75 pg/20 μl, respectively). The intracellular DA level was also 18-fold higher in N2+SM (II) cells (99.5 pg/20 μl) compared with that in D3 cells (5.5 pg/20 μl).

Efficient In Vivo Integration and Generation of TH^+ Neurons after Implantation of N2 Cells Differentiated under Condition II into the Mouse Striatum

The in vitro data above indicate that the combined condition of Nurr1, signaling molecules, and SDIA may provide an optimal method for efficiently generating midbrain DA neurons for the future cell transplantation therapy to treat PD. Toward this long-term goal, we next investigated whether ESCs differentiated under this condition could efficiently integrate or generate TH^+ neurons after implantation. Morizane et al., using naïve ESCs, grafted cells that had been cultured on PA6 for 12 days [37]. When the cells were grafted as individual cell suspensions into mouse striatum on day 12, the number of surviving cells was much lower than when the cells were grafted as nondissociated cell clumps. This may be due to damage to the neurites when the cells are dissociated prior to transplantation. Because cell suspension has the advantage of quantification and reproducibility of grafting conditions, we retested the transplantation condition for individual cell suspension. First, we examined when the ESCs enter the postmitotic phase during differentiation (Fig. 6A). After differentiation for 7–10 days, ESC colonies were all isolated from the feeder layers by treatment with papain for 5 minutes at 37°C, dissociated into a single cell suspension in 5 ml medium, and counted. As shown in Figure 5A, ESCs stopped growing after 8–9 days. N2 cells entered postmitotic phase 1 day sooner than D3 cells, indicating earlier neuronal maturation. Next, we examined cell viability after isolation by papain digestion (Fig. 6B). Cells were isolated from feeder layers after 7–10 days of differentiation, replated onto poly-L-ornithine/fibronectin plates, and then counted for cell viability after 12 hours. The viability of D3 cells decreased after 9 days of induction. This compares favorably with N2 cells whose viability started to decrease at day 7 and even more after the ninth day. Finally, TH^+ cell number was examined during differentiation (Fig. 6C). The number of TH^+ cells in D3 cells showed a mild increase through differentiation, whereas a dramatic increase in N2 cells was observed after 9 days of incubation under condition II. Based on these observations, we transplanted ESCs after 9

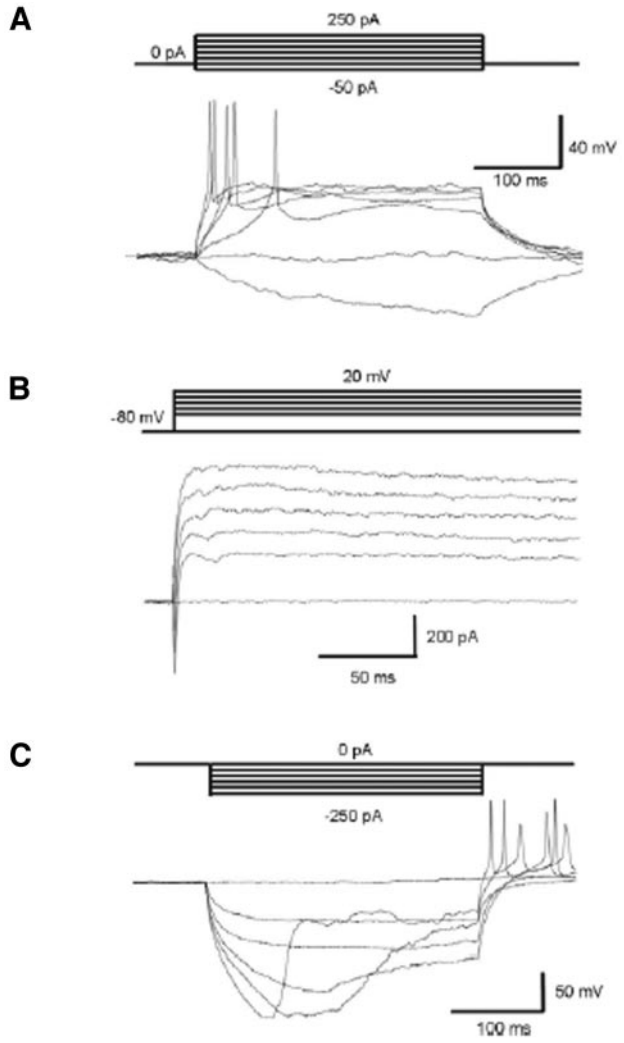

Figure 4. Electrophysiological properties of neurons differentiated from N2+SM (II) cells. **(A):** Current-clamp recordings during prolonged depolarizing current injections. Top traces represent current injections, whereas bottom traces indicate voltage recordings. Depolarizing current injections elicited fast action potentials. **(B):** Voltage-dependent membrane currents. Depolarizing voltage steps (top traces) elicited outward K^+ currents and fast inward Na^+ currents. **(C):** Current-clamp recordings during prolonged hyperpolarizing current injections. The neurons display the time-dependent anomalous rectification characteristic of dopaminergic neurons after a hyperpolarizing pulse. Abbreviation: SM, signaling molecule.

days of differentiation, when those parameters (i.e., total cell number, survival, and TH^+ cell numbers) are suboptimal. Four weeks after transplantation, the recipient mice were sacrificed, fixed by perfusion, and then analyzed for in vivo generation and integration of TH^+ neurons. In a graft of N2 cells differentiated under condition II, we could see many TH^+ cell bodies (Figs. 7C, 7D). In contrast, few TH^+ cell bodies were detected in control grafts of D3 cells (Figs. 7A, 7B). When we counted the total number of TH^+ cells per graft area (mm^3) by staining for TH/Nissl, we found an average of 312 neurons for N2 cells under condition II and 28 for the naïve D3 cells (Fig. 7E). Costaining with antibodies against TH and NeuN (Figs. 7F–7K) was consistent with the TH/Nissl results, showing a much higher number of TH^+ cells with N2 cells (Fig. 7J) than with D3 cells (Fig. 7G). These results indicate that TH^+ cells derived from N2 cells under condition II are more efficiently generated and maintained in vivo.

DISCUSSION

In this study, we showed that coculturing Nurr1-overexpressing ESCs (N2 or N5) with PA6 stromal feeder cells leads to the generation of a high proportion of TH^+ neurons (~55% of $Tuj1^+$ neurons; Fig. 1D). Surprisingly, when N2 cells were treated with Shh and FGF8 during days 5–9 followed by ascorbic acid treatment, the proportion of TH^+ neurons was further enhanced up to ~90% of $Tuj1^+$ neurons, whereas naïve D3 cells did not show any cooperative effect with signaling molecules on the PA6 stromal cell feeder layer. Importantly, these ESC-derived TH^+ neurons were found to coexpress AADC and DAT, but not GABA or 5-HT (Fig. 2B), suggesting that they have the midbrain DA neuronal phenotype.

Lee et al. [11] showed in a five-stage protocol that treatment with the signaling molecules during expansion of neural precursors followed by treatment with ascorbic acid during the differentiation phase (stage 5) is most effective for inducing TH^+ DA neurons from mESCs. Using the same method, it has recently been shown that exogenous expression of Nurr1 in mESCs can significantly enhance the generation of TH^+ neurons among $Tuj1^+$ cells (three- to fivefold depending on ESC clones [23, 24]). Thus, it appears that Nurr1 overexpression similarly enhances the induction of TH^+ neurons in both the five-stage and PA6 coculture procedures. However, the present study reveals several salient features of different in vitro differentiation methods for DA neuron differentiation. First, we found that there was a significant increase (approximately twofold) in the number of β-$tubulin^+$ cells generated from N2 cells in the PA6 coculture method (Fig. 1C), but not in the five-stage method [24]. Given that the PA6 coculture directly induces neuronal cells from ESCs whereas the five-stage method is based on selection of neural precursor cells [11, 14], the twofold increase of β-$tubulin^+$ neuronal cells only in the PA6 method is a significant one. Consistent with this finding, the mRNA level of β-tubulin was upregulated approximately 2.5-fold in the N2+SM (II) condition compared with D3 cell–derived cells (data not shown). Thus, it appears that Nurr1 and unidentified molecule(s) in the PA6 coculture (e.g., SDIA) may cooperatively enhance the generation of neurons. In this context, it is noteworthy that Nurr1 has been shown to induce cell cycle arrest and a highly differentiated cell morphology in dopaminergic MN9D cells [38]. In addition, Nurr1 is known to be essential for the survival of maturing DA neurons by its induction of molecules required for the expression of the DA phenotype [21]. Therefore, Nurr1 may enhance the generation of neurons at the level of neuronal induction ('neurogenesis') and/or survival in the PA6 coculture protocol. Further investigations are needed to clarify this. Secondly, our results demonstrate that SDIA, Nurr1, and signaling molecules can synergistically enhance the differentiation of ESCs to the DA fate. In our optimized condition II, we observed that the majority (up to 90%) of $Tuj1^+$ neurons were TH^+ (and $AADC^+$) after 14 days of in vitro differentiation. When compared side by side with the PA6 coculture protocol in this study, the five-stage method reproducibly results in a significantly

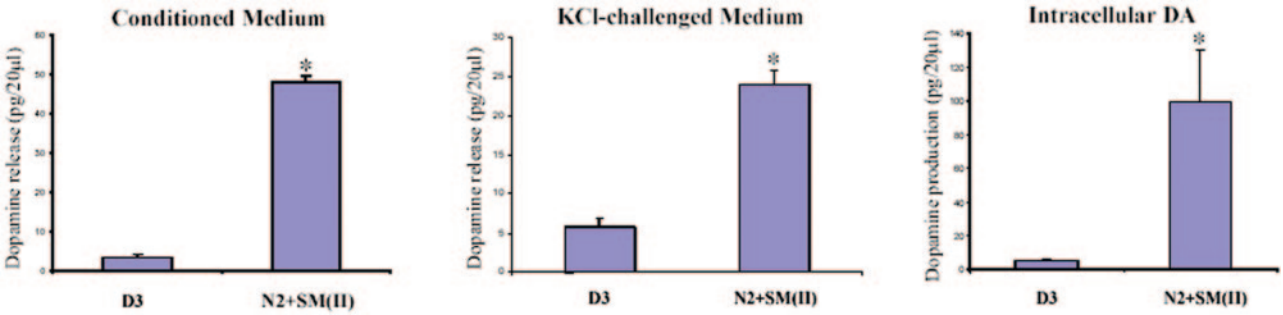

Figure 5. Analyses of dopamine synthesis and release. Results of HPLC analyses of dopaminergic (DA) levels in 24 hour–conditioned media, 50 mM KCl-challenged media, and intracellular fractions of D3 and N2+SM (II) cells are shown. Each group represents an average of six samples. Fisher's PLSD post hoc analysis was performed with a significance level of 0.05. $*p < .05$ compared with D3 cells. Abbreviation: PLSD, protected least-significant difference.

lower population of $TH^+/Tuj1^+$ cells (approximately 60%) and requires twice as long (4 weeks) for the full cycle of in vitro differentiation [24]. Taken together, combined with a higher yield of neurons only in PA6 coculture method, our optimized condition II represents a very effective protocol for efficient generation of DA neurons in a short period of time.

Our coimmunostaining and gene expression analysis indicate that those TH^+ neurons generated from our optimal in vitro differentiation have midbrain DA neuronal properties. First, all TH^+ neurons tested were found to be $AADC^+$, suggesting that they have the ability to synthesize DA. Some of these neurons also coexpressed DAT, whereas none of them express other neurotransmitter markers such as 5-HT and GABA. Absence of GABA is characteristic of midbrain DA neurons, whereas olfactory DA neurons coexpress TH and GABA. Analyses of mRNAs from these neurons by semiquantitative and real-time PCR revealed a robust upregulation of DAT, AADC, and Girk2 mRNAs (Figs. 3A, 3B). However, other midbrain DA markers such as AHD2 and Pitx3 were only minimally upregulated, suggesting that additional factor(s)/signal(s) may be necessary for full maturation of these DA neurons. In this context, it is of great interest that Pitx3-overexpressing ESC lines can generate a significantly higher proportion of $AHD2^+/TH^+$ neurons [39].

An important physiological criterion of a functional DA neuron is the ability to release synthesized DA in response to membrane depolarization. When compared with naïve D3 cells, these N2-derived neurons not only expressed higher levels of intracellular DA (>18-fold) but also efficiently released it into the medium either spontaneously (>14-fold) or in response to depolarization induced by high KCl (>fourfold) (Fig. 5). Thus, N2 cells under our optimized condition II have the ability to efficiently differentiate into functional midbrain DA neurons in vitro. Furthermore, differentiated cells exhibit typical excitable properties of neuronal membrane (e.g., firing of action potentials and existence of fast inward Na^+ current) (Figs. 4A, 4B). In addition, the majority of differentiated cells subjected to hyperpolarizing voltage steps showed anomalous rectification (Fig. 4C), which is the electrophysiological characteristic of mesencephalic DA neurons [35, 36].

Interestingly, a majority of TH^- neurons generated from N2 cells were $5\text{-}HT^+$ (data not shown; Fig. 2B). In addition, our semiquantitative and real-time PCR analyses showed a threefold upregulation of TPH mRNA levels, a key enzyme involved in serotonin synthesis. In agreement with this, in vitro differentiated cells from N2 cells were able to produce and release significantly higher levels of serotonin than D3 ESCs (data not

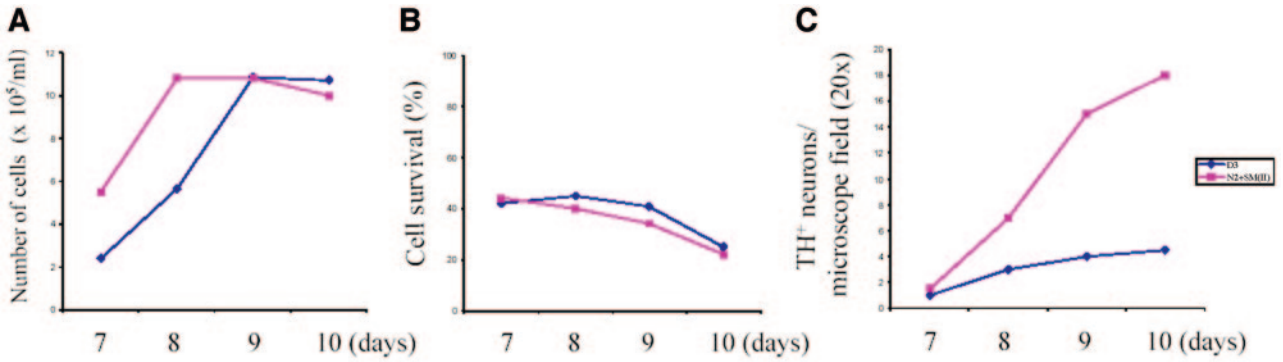

Figure 6. Evaluation of conditions for transplantation. **(A):** Embryonic stem cells (ESCs) stop growing after approximately 8–9 days of differentiation. ESCs (1.05×10^4) were plated on PA6 feeder layer of 6-cm culture dish at differentiation day 0. After differentiation for the indicated times, ESC colonies were isolated from the feeder layer by incubation with papain (1.5 ml/6 cm dish) for 5 minutes, dissociated into single cells, suspended in 5 ml of medium, and counted. **(B):** Cell viability after isolation using papain treatment. At the indicated days, ESC colonies were isolated from the feeder layer by incubation with papain (1.5 ml/6 cm dish) for 5 minutes, dissociated into single cells suspensions, replated onto poly-L-ornithine/fibronectin plates, and counted after 12 hours. Starting cell numbers were expressed as 100%. **(C):** The number of tyrosine hydroxylase $(TH)^+$ cells increased dramatically on induction day 9. At the indicated days, ESC colonies were isolated from the feeder layer by incubation with papain (1.5 ml/6 cm dish) for 5 minutes, dissociated into single cells, replated onto poly-L-ornithine/fibronectin plates, and stained with anti-TH antibodies after 12 hours.

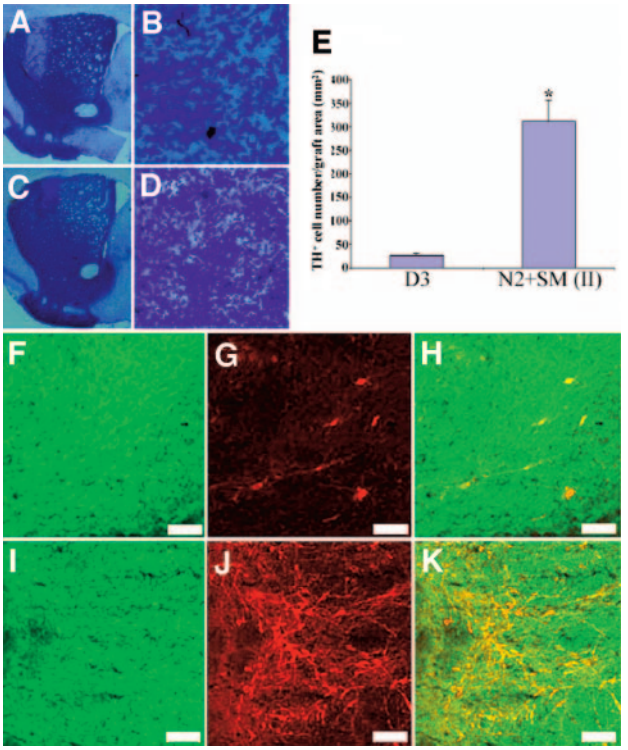

Figure 7. Efficient in vivo generation of TH^+ neurons post transplantation in the mouse striatum from N2 cells subjected to in vitro differentiation condition II. Naïve D3 cells and Nurr1-overexpressing N2 cells [N2+SM (II)] were transplanted into mice striatum at in vitro differentiation day 9, and the animals were sacrificed 4 weeks later for histological analyses. Grafts of D3 **(A, B)** and N2 **(C, D)** cells in naïve mouse striatum were analyzed by TH/Nissl staining. The number of TH^+ cells per graft area (mm^2) is shown **(E)**. Images for panels **(F–H)** and **(I–K)** represent TH/NeuN staining of grafts of D3 and N2 cells, respectively. Each group represents an average of 6–8 animals. Tumor growth was observed in one grafted animal in each group, which was not included in further analysis. Fisher's PLSD post hoc analysis was performed with a significance level of 0.05. $*p < .05$ compared with D3 cells. Scale bar = 50 μm. Abbreviations: NeuN, neuronal nuclei; PLSD, protected least-significant difference; SM, signaling molecule; TH, tyrosine hydroxylase.

shown). Thus, it is likely that some factors/signals regulating the development of DA and 5-HT neurons overlap in our in vitro differentiation system, as is the case in the in vivo situation [40, 41].

Finally, with the long-term goal of translating our in vitro studies to the cell replacement therapy of PD, we transplanted DA neurons generated from our optimized condition. Toward this goal, we first defined the optimal in vitro differentiation period by assessing cell proliferation, viability, and generation of TH^+ neurons under our differentiation condition II. We determined that the optimal time for transplantation is the ninth day of N2 cell differentiation, when the total cell number reaches the maximum with high cell survival rate. At this time, the number of TH^+ neurons is suboptimal and neurites have not been formed extensively (Fig. 6), which may reduce the damage resulting from the dissociation procedure prior to transplantation. When 50,000 cells were harvested at day 9 and transplanted into the striatum of mice, we observed an approximately 10-fold increase in the number of TH^+ neurons of N2 cell grafts compared with D3 cell grafts.

In conclusion, these results suggest that DA neurons generated under in vitro conditions described in the present protocol may be useful as a cell source for in vivo cell transplantation studies. Two research groups have shown that the hESCs can differentiate into neuronal cell types in vitro [42, 43]. More recently some of these hESCs were shown to differentiate into DA neurons in vitro using different procedures [15–17, 44]. However, the maturity and function of hESC–derived DA neurons are to be fully characterized in vitro and in vivo. Importantly, recent reports demonstrated that pluripotent hESCs can be derived from cloned blastocysts through somatic cell nuclear transfer technology [45, 46]. Therefore, it is of great interest to determine whether the optimized protocol we describe here can also be applied to improve the generation of DA neurons from these hESCs.

Summary

The present study sought to optimize the differentiation of ESCs to the DA cell fate by combining genetic manipulation and in vitro culture conditions. When Nurr1-overexpressing ESCs were cocultured with PA6 stromal cells and treated with signaling molecules (i.e., Shh, FGF8, ascorbic acid), we found that ESCs generate a significantly higher number of neurons (~twofold) and that the majority (up to 90%) of them become TH^+. Optimal induction of the DA fate was also confirmed by real-time PCR analysis of mRNA expression, characterization of electrophysiological properties, and the analysis of the production and release of DA. Furthermore, transplantation experiments showed that TH^+ neurons generated by our optimal condition can efficiently integrate into mouse striatum after implantation. Thus, this study shows that combination of genetic manipulation(s) and in vitro cell differentiation conditions offers a reliable and effective induction of DA neurons from ESCs and may serve as a platform for future cell transplantation therapy in PD.

Acknowledgments

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Disclosures

The authors indicate no potential conflicts of interest.

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*The two articles cited in references 45 and 46 have been retracted by *Science*.