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Sonic hedgehog and FGF8 collaborate to induce dopaminergic phenotypes in the Nurr1-overexpressing neural stem cell

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Abstract

Neural stem cells are self-renewing cells capable of differentiating into all neural lineage cells in vivo and in vitro. In the present study, coordinated induction of midbrain dopaminergic phenotypes in an immortalized multipotent neural stem cell line can be achieved by both overexpression of nuclear receptor Nurr1, and fibroblast growth factor-8 (FGF-8), and sonic hedgehog (Shh) signals. Nurr1 overexpression induces neuronal differentiation and confers competence to respond to extrinsic signals such as Shh and FGF-8 that induce dopaminergic fate in a mouse neural stem cell line. Our findings suggest that immortalized NSCs can serve as an excellent model for understanding mechanisms that regulate specification of ventral midbrain DA neurons and as an unlimited source of DA progenitors for treating Parkinson disease patients by cell replacement.

Keywords: Nurr1; Dopaminergic neurogenesis; Tyrosine hydroxylase; Sonic hedgehog; FGF8; Human fetal astrocyte; Parkinson's disease

Dopaminergic (DA) neurons that lie in the ventral midbrain play a key role in voluntary movement, emotional behavior, and cognition. Loss of these neurons is associated with Parkinson's disease [1,2]. Recent advances in neural stem cell (NSC) biology have shown that multipotent neural progenitors can be isolated, expanded, and used as source material for brain transplants [3–5]. However, although multiple studies demonstrate that implanted neural progenitors successfully engraft and assume legitimate neural phenotypes, when transplanted in the intact adult brain, these cells seem biased toward astroglial and oligodendroglial fates [6–8]. Given that most neurodegenerative diseases affect neuronal populations of a specific neurochemical phenotype, an ideal source material for transplantation would be an expandable cell that could be instructed to completely assume the desired neuronal phenotype upon differentiation. However, the complete and coordinated induction of a specific neuronal phenotype in multipotent neural precursors in vitro has proved elusive. Our goal in this study was to define the factors required by NSCs to produce a DA phenotype, the major cell type lost in human Parkinson's disease.

One such factor is Nurr1, a transcription factor of the thyroid hormone/retinoic acid nuclear receptor superfamily [9]. Nurr1 is specifically required for the induction of midbrain DA neurons, which fail to develop in Nurr1-null mutant mice [10–12]. It is unclear, however, whether Nurr1 is also sufficient to specify this neurochemical phenotype. To test this phenotype, we stably overexpressed Nurr1 in an immortalized mouse NSC line termed A3, which does not normally give rise to DA neurons in vitro [13]. These cells possess the ability to differentiate into neurons, astrocytes, and oligodendrocytes in vitro and in vivo, and upon transplantation into the developing brain, adopt regionally appropriate

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neuronal phenotypes. We hypothesized that the NSC cell line, A3, fulfills all necessary criteria to identify the factors sufficient to reconstitute DA neuron development in vitro and to generate renewable source material for transplantation.

Cells in the fetal ventral mesencephalon express neurotrophic factors for DA neurons [14]. In addition, induction of mesencephalic DA neurons is mediated by the concerted action of diffusible and contact-dependent signals from the ventral mesencephalon [15–17]. These findings have suggested that non-neural cells in fetal ventral mesencephalon play an important role in generating and maintaining DA neurons in vivo and have prompted their use to increase the number of DA neurons from various progenitors. Similarly, it has recently been suggested that differentiation of DA neurons in vivo is dependent on critical cooperation of two signals. Implicated in these studies [15] are one member of the fibroblast growth factor (FGF) family, FGF8 [18], and the cell patterning molecule first described in *Drosophila*, sonic hedgehog (Shh) [19]. It is believed that developing cells differentiate into DA neurons when they encounter intersecting signals occurring along the anterior–posterior (FGF8) and dorsal–ventral (Shh) axes [15].

Materials and methods

Culture of mouse NSCs in culture. Mouse NSC line A3 was established as described previously [13], and maintained and passaged on uncoated culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum or in DMEM supplemented with N2 supplement (Gibco-BRL, Gaithersburg, MD) to which was added to bFGF (10 ng/ml) (R&D Systems, Minneapolis, MN) + heparin (10 ng/ml) (Sigma, St. Louis, MO) (N2 plus bFGF medium). Medium was changed every 5 days. Cells were transferred to media containing serum for transduction of retroviral vector carrying Nurr1 gene.

Retrovirus-mediated gene transfer. Amphotropic replication-incompetent retroviral vector was used to infect the mouse NSC line. A vector encoding human Nurr1 gene was generated using the retroviral vector pBabe-puro to infect the PA317 amphotropic packaging cell line. Mouse NSC line was infected with a retrovirus encoding human Nurr1 gene. Mock controls received retrovirus harboring only pBabe-puro vector.

Cloning of NSCs overexpressing human Nurr1 gene. Cells were dissociated, diluted to 1 cell/100 μl, and plated at 100 μl/well of a 96-well plate. Wells with single cells were immediately identified. Single-cell clones were expanded and maintained in bFGF-containing growth medium. A total of 28 stable Nurr1 overexpressing clones were selected for puromycin resistance and isolated. The expression of Nurr1 mRNA was determined by Northern blot analysis in each clone. A 350 bp antisense RNA probe spanning nucleotides 1436–1786 of the human Nurr1 cDNA sequence was transcribed with SP6 and labeled with [^{32}P]CTP (NEN, London, UK).

RT-PCR and semi-quantitative PCR. Total RNA was isolated from cell cultures using RNAsol (Tel-Test, Friendswood, TX) and cDNA was made using the Superscript II reverse transcriptase (Gibco-BRL) from 4 μg of total RNA. First strand cDNA was diluted 3-fold and 2 μl of cDNA was used for each PCR. RT-PCR products were analyzed in an agarose gel containing ethidium bromide. DNA bands were photographed using a Gel Doc 2000 video system (Bio-Rad, Richmond, CA). The image was exported in a TIFF file and DNA bands were quantified using Quantity One 4.0.3 software. The quantification value of the band was designated by the absorbance (Pixels). Measurements of absorbance using the above system were linear up to 120 pixels. For quantitative PCR, cycle number and template quantity were determined to be in the linear range for each gene. Three independent PCRs were done for each experiment. For each condition, at least two independent experiments were carried out.

The following primers were used to amplify target cDNA.

(1) GAPDH	5′-GCAGGGGGGAGCCAAAAGGG-3′,
	5′-TGCCAGCCCCAGCGTCAAAG-3′
(2) TH	5′-AAGGTCCCCTGGTTCCCAAGAAAAGT-3′,
	5′-TCCTCCAGCTGGGGGATATTGTCTTC-3′
(3) AADC	5′-CCTGCTGGCCGCTCGGACCAA-3′,
	5′-GCGCACCAGTGACTCAAACTC-3′
(4) DBH	5′-GTGACCAGAAGGGGCAGATCC-3′,
	5′-GGCCGGCTTCCTCTGGGTAGT-3′

Treatment of neurotrophic factors and co-culture with astrocytes. Mouse NSC line was plated at a final density of 2×10^4 cells/cm^2 on poly-D-lysine- and laminin-coated culture wells in N2 plus bFGF medium to investigate the effects of various neurotrophic factors. After 24 h, all factors were added with fresh medium. Half of medium was replaced every 3 days with fresh medium containing factors.

For co-culture of NSCs and astrocytes, $2.5–5 \times 10^4$ human fetal astrocytes were seeded in laminin-coated insert (Millipore, Bedford, MA) with a membrane with 0.4 μm pores (sufficient to allow passages of proteins but not cells). Purified human fetal astrocytes were obtained from mixed glial cell cultures derived from human fetal brains according to a standard protocol. After 1 day, the membrane inserts was immersed in a well at the bottom of which rested the coverslips. All ages of cultures given use this point as day zero. After 8–10 days, coverslips were fixed and examined for TH gene expression. Half of medium was replaced every 3 days with fresh medium.

NSC cell line overexpressing Shh-N proteins. Mouse NSC line was infected with LPC-Shh-N retrovirus in 4 μg/ml DEAE–dextran for 4 h. Two days after infection, cells were selected for puromycin (5 μg/ml) resistance for 10 days. A pooled population of puromycin-resistant cells was analyzed for expression of Shh-N proteins and used in co-culture to treat mouse NSC line with Shh-N. Intracellular and secreted Shh-N proteins were evaluated by Western blot analysis and Sandwich ELISA technique, respectively. To determine the intracellular Shh protein level, cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% NaDOC, 0.1% SDS, and 50 mM Tris, pH 8.0). Cell lysates were fractionated by SDS–PAGE. The immunoblotting procedure was done with ECL Western blot detection reagents (Amersham, Little Chalfont, UK). To examine secreted Shh-N proteins, 96-well Microtest assay plate (Becton–Dickinson, San Jose, CA) was coated with 5 μg of anti-Shh antibody (Santa Cruz Biotech, CA) overnight at 4 °C per well. Plate was incubated with conditioned media from A3 or A3.Shh-N cells, followed by incubation with 5E1 monoclonal anti-Shh antibody. Bound antibodies were detected by alkaline phosphatase-conjugated anti-mouse IgG antibody. After washing, 100 μl of 0.25 mM *p*-nitrophenyl phosphate disodium salt and 0.5 mM magnesium chloride in 10 mM diethanolamine buffer (pH 9.5) was added. Absorbance was determined at 405 nm and the ELISA cut-off value was defined as two times the optical density (OD) value of the conditioned medium from A3 cells.

Treatment with FGF-8 and Shh-N. Shh-N-overexpressing mouse NSC line ($2.5–5 \times 10^4$) was seeded on laminin-coated side of a membrane with 0.4 μm pores. Target mouse NSCs were plated at a final density of 2×10^4 cells/cm^2 on poly-D-lysine- and laminin-coated culture wells in N2 plus bFGF medium. After 24 h, the membrane inserts were immersed in a well at the bottom of which rested the coverslip in the presence or absence of murine FGF8 isoform b (R&D Systems). All ages of cultures given use this point as day zero. After 7 days, coverslips were processed for RT-PCR or immunocytochemistry for DA neuron related gene expression.

Immunocytochemical analysis. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature before immunocytochemical staining. Fixed cultures were incubated with one of the following antibodies, diluted in PBS containing 1% BSA and 0.3% Triton X-100:mouse anti-tubulin β III (TuJ1, 1:250, Sigma, St. Louis, MO), or sheep polyclonal anti-TH (1:500, Pel-freeze, Rogers, AR). Incubations were carried out at 4 °C overnight. After washing, cultures were incubated with biotinylated horse anti-mouse IgG or goat anti-sheep IgG (1:100, Vector, Burlingame, CA) for 1 h and then reaction products were visualized with AEC substrates (Vector, Burlingame, CA).

Results

Nurr1 expression induces neuronal differentiation in mouse NSCs

Mouse NSC line A3 [13] was stably transduced with retrovirus encoding pBabe-Nurr1, and 28 clones expressing Nurr1 were analyzed for transgene expression by Northern blotting (data not shown). Several clones overexpressed Nurr1 gene, of which the highest expressor was chosen and designated A3.Nurr1 for further experiments. A3.Nurr1 cells behaved similar to the parental and pBabe-transduced clones, with exceptions that they showed slightly slow growth rate and sensitivity to cell–cell contact. To define developmental potential and phenotypic expression of Nurr1 clones, we examined their behavior following growth in serum-free defined medium (SFM) and serum containing medium. In A3.Nurr1 cells, many filopodia around cell bodies were induced compared to parental cell line A3 in serum containing medium (Fig. 1). This morphology could be clearly visualized by staining for actin and paxillin (Figs. 1C–F). Approximately 80% of A3.Nurr1 adopt a neuronal fate, judged by the expression of tubulin β III, a phenomenon not seen in any of the mock clones (data not shown). However, none of A3.Nurr1 cells expressed tyrosine hydroxylase (TH, a marker for DA neurons) under these conditions, suggesting that Nurr1 alone is not sufficient to induce DA phenotypes in mouse NSCs (data not shown).

Characterization of inductive signal

As Nurr1 possessed lineage-restricting capacity in our NSC line but alone is not sufficient to induce DA phenotypes, A3.Nurr1 cell line was treated with agents known to be important in DA neuronal differentiation. Since retinoids and forskolin have been previously implicated in promoting dopamine biosynthesis in primary central nervous system (CNS) progenitor cell cultures [20–22], the effects of 9-*cis*-retinoic acid (RA) and forskolin (FK) on induction of DA phenotypes [expression of TH, AADC, and dopamine-β-hydroxylase (DBH)] in A3.Nurr1 cells were investigated. A small number of Nurr1-overexpressing NSCs were labeled for TH ($4.9 \pm 0.3\%$ in the presence of RA and $3.5 \pm 0.4\%$ in the presence of FK) (Fig. 2B). In addition, some cells were immunoreactive for AADC but none contained detectable DBH (not shown). Consistent with the immunocytochemistry results, RT-PCR showed increased expression of TH and AADC, but absence of detectable DBH mRNA (Fig. 2A). Taken together, these results indicate that although minor in proportion, TH-immunoreactive cells generated in A3.Nurr1 cells are dopaminergic rather than adrenergic or nonadrenergic neurons.

Co-culture with human fetal astrocytes

None of retinoic acid or forskolin, alone or combined (data not shown), induced significant TH expression in any of the Nurr1 clones. To test whether as yet unidentified, regionally specific local factors were required, we co-cultured the Nurr1 clones with primary astrocyte cells derived from human fetal brain using membrane insert. Under these conditions a small, yet significant, percentage of isolated cells ($24.7 \pm 2.3\%$) and mitotic clusters, frequently shown, from the Nurr1 line demonstrated measurable amounts of TH immunoreactivity (Fig. 3C), whereas little or no TH staining was seen in the parental, or any of the mock control lines, indicating that human fetal astrocytes produce a specific activity that acts on Nurr1-overexpressing NSCs. Addition of RA and FK slightly increased TH-immunoreactive cell number in co-culture with human fetal astrocyte cells ($25.5 \pm 1.7\%$ for addition of FK to co-culture; $30.1 \pm 2.3\%$ for addition of FK and RK) (Fig. 3C). This activity from astrocytes might be very labile and diffusible factors, because it could be recovered not from conditioned media, but from co-cultures separated by a microporous insert of human fetal astrocytes (data not shown).

In A3.Nurr1 cells co-cultured with fetal astrocytes, TH mRNA was expressed at 5–10-fold higher levels than in control A3.Nurr1 cells. Expression of AADC mRNA was elevated 55-fold in co-cultured A3.Nurr1 cells and 95-fold in co-cultured A3.Nurr1 cells with FK and RA (Figs. 3A and B). Collectively, these observations suggested that a factor derived from the primary astrocyte cultures interacted directly or indirectly with Nurr1 to induce TH-expressing neurons.

Isolation of immortalized NSCs expressing Shh-N transgene

Since Shh and FGF-8 have been previously shown to promote ventral midbrain fates in neural plate explant [15], we hypothesized that the addition of these factors to NSCs might also increase the proportion of DA neurons in our culture system. To identify if signals that provide positional information for the developing DA neurons do work in mouse NSCs, we have first produced Shh-N

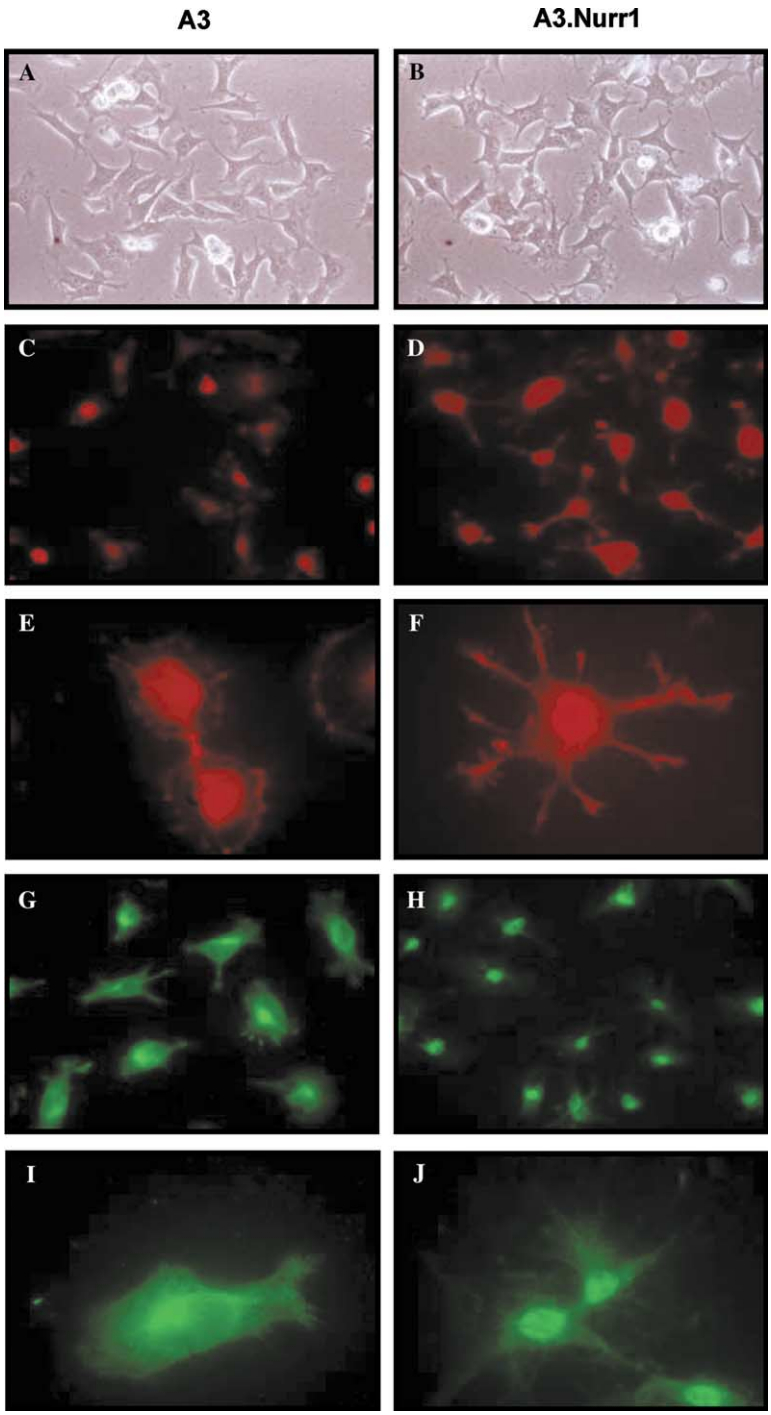

Fig. 1. Nurr1 expression induces morphological differentiation in mouse NSCs. (A,B) Morphological changes are observed in A3 and A3.Nurr1 by phase contrast microscopy. A3 cells showed fibroblast-like shape, bearing occasionally lamellipodia, whereas Nurr1-expressing cells protruded a short pine needle-like protrusion, filopodia. A distinguishable morphology was evident by visualizing the actin cytoskeleton (C,D) and paxillin containing focal adhesion assembly (E,F) in A3 and A3.Nurr1 cells.

overexpressing NSC line. To this end, an amphotropic producer cell line producing LPC-Shh-N retrovirus was selected and expanded. The virus had a titer of 200 CFU/ml for NIH3T3 cells and did not contain helper viruses. Another NSC line C4 was transduced with this retrovirus and selected for stable transfectants with 5 μg/ml puromycin. Colonies were picked after selection for 10 days and tested for the intracellular expression and secretion of Shh-N protein. A clone, C4.Shh, showed intracellular expression of Shh-N proteins (Fig. 4A). On the other hand, endogenous expression of Shh-N proteins could not be detected in parental C4 cells by Western blotting

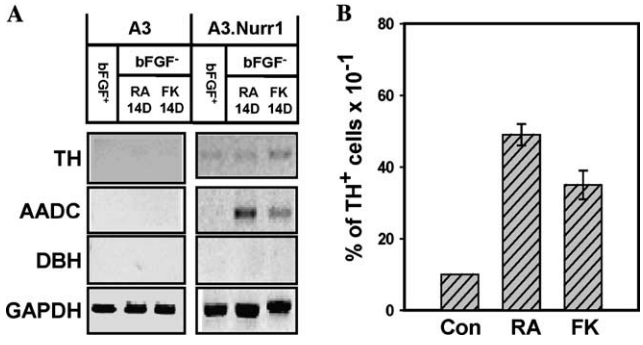

Fig. 2. Effects of retinoic acid and forskolin on Nurr1-dependent TH expression. (A) Expression of genes related to catecholamine-producing neurons in mouse NSCs before and after differentiation. Differentiation was induced by bFGF withdrawal followed by RA or FK treatment and evaluated by semiquantitative RT-PCR. Cells were treated with 0.5 μM RA for 6 days, followed by N2 medium alone for an additional 8 days. In the FK conditions, cells were treated with 5 μM FK for 14 days. The presence of TH transcripts was measured by quantitative RT-PCR analyses and normalized to GAPDH. (B) Histogram demonstrating the percentage of TH^+ cells in cultures of A3.Nurr1 cells in the presence of RA or FK.

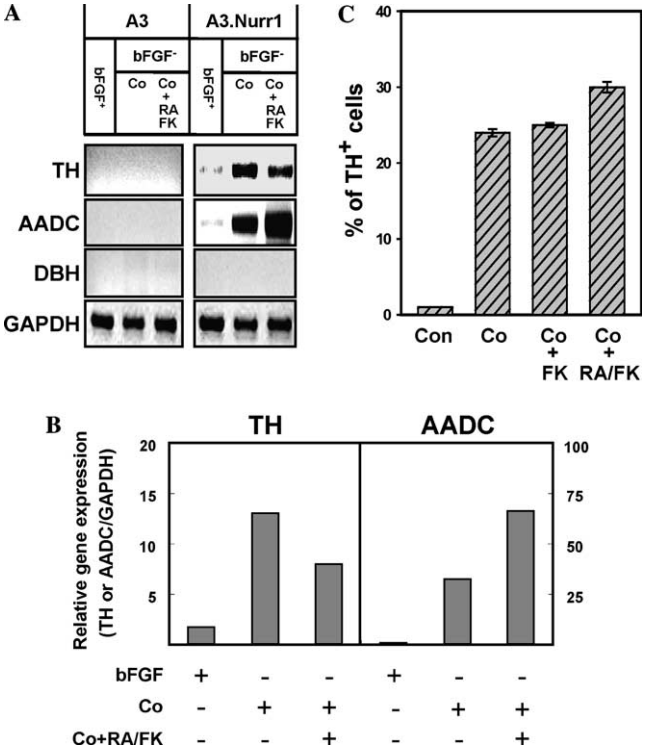

Fig. 3. Nurr1-overexpressing NSCs co-cultured with human fetal astrocytes for TH induction. (A) A3 and A3.Nurr1 cells were co-cultured with human fetal astrocytes and induction of genes related to catecholamine-producing neurons was assessed by RT-PCR. (B) Histogram demonstrating relative expression of TH and AADC mRNA in cultures of A3.Nurr1 cells. The presence of TH transcripts was measured by quantitative RT-PCR analyses and normalized to GAPDH. (C) Nurr1 clones co-cultured with human fetal astrocytes expressed an increase in the number of TH-positive cells. Addition of retinoic acid and/or forskolin slightly enhanced the differentiation of A3.Nurr1 cells into TH-positive cells in co-cultures. The induction of these phenotype was specific to A3.Nurr1 cells and was not observed in parental A3 cells co-cultured with human fetal astrocytes.

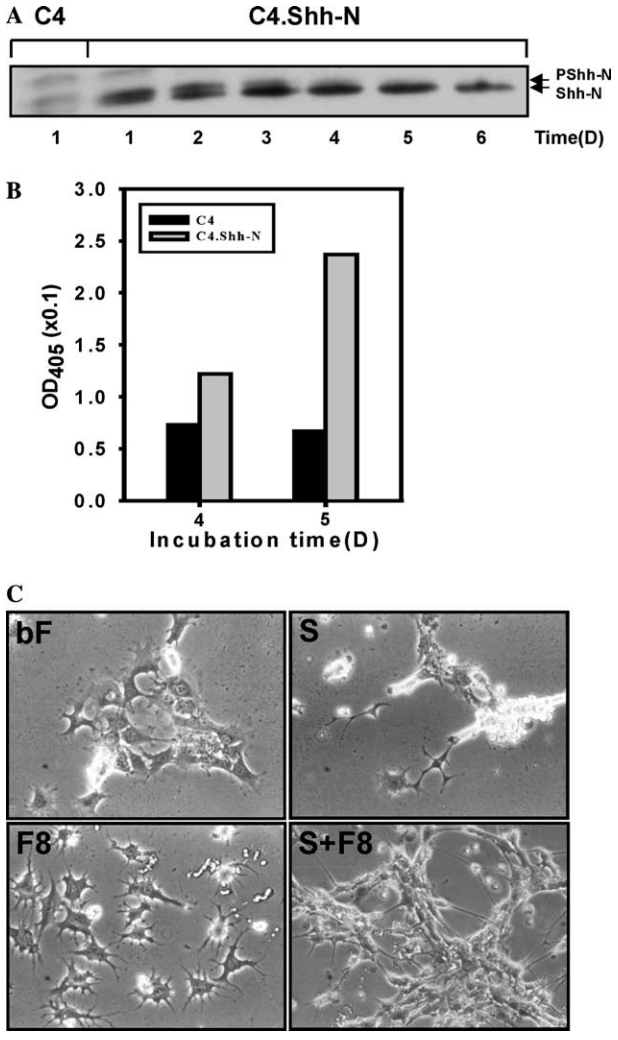

Fig. 4. (A) Western blot analysis with an anti-Shh-N antibody demonstrates intracellular expression of Shh-N proteins (23 kDa) in cell lysates of A3.Nurr1 but not in cell lysates of A3 cells. (B) Secretion of Shh-N proteins. Media conditioned by A3 or A3.Nurr1 cells were assayed for Shh-N proteins by ELISA. (C) Microphotographs showing A3.Nurr1 cells grown in the presence of bFGF, co-culture with C4.Shh cells, or FGF8 (10 ng/ml), and co-culture with C4.Shh cells plus FGF8 (10 ng/ml). Cells maintained a monolayer and extended short neurites around cell periphery when grown in the presence of Shh-N or FGF8 alone for 5 days. However, A3.Nurr1 cells extended many long neurites and arrayed with direction when treated with Shh-N and FGF8 for 5 days. In addition, small clusters of A3.Nurr1 cells were well developed and interconnected by neurites.

(Fig. 4A). ELISA analysis for the medium conditioned by C4.Shh cells demonstrated that Shh-N proteins were secreted by C4.Shh cells (Fig. 4B). In contrast, C4 cells did not secrete a detectable amount of the Shh-N proteins (Fig. 4B).

Shh and FGF8 induces morphological differentiation in NSCs

Mouse NSC line A3 and its Nurr1 clone, A3.Nurr1, were fibroblast-like and polygonal in shape and did not

have long processes (Figs. 1A, B and 4C). Both cells grew rapidly (doubling time of 12 h). However, when NSC lines were grown with FGF8 and insert culture of C4.Shh cells for 3 days, most of the cells stopped dividing, began extending long processes, and developed cell clusters (Fig. 4C). After 5 days, cell clusters increased in size and developed more prominent processes compared with cells grown alone (Fig. 4C). This morphology could be clearly visualized by staining for a neuronal specific marker such as tubulin β III (Figs. 5A and B). Five days after induction of differentiation, almost all the cells displayed a highly mature neuronal morphology, including long, elaborate processes, hypertrophic cell bodies, and developed prominent cell–cell network and cell clusters, and intense levels of tubulin β III immunoreactivity (Figs. 4 and 5). However, A3.Nurr1 cells treated with FGF8 or co-cultured with C4.Shh cells developed long process, hypertrophic cell bodies, but did not form intense network and cell clusters.

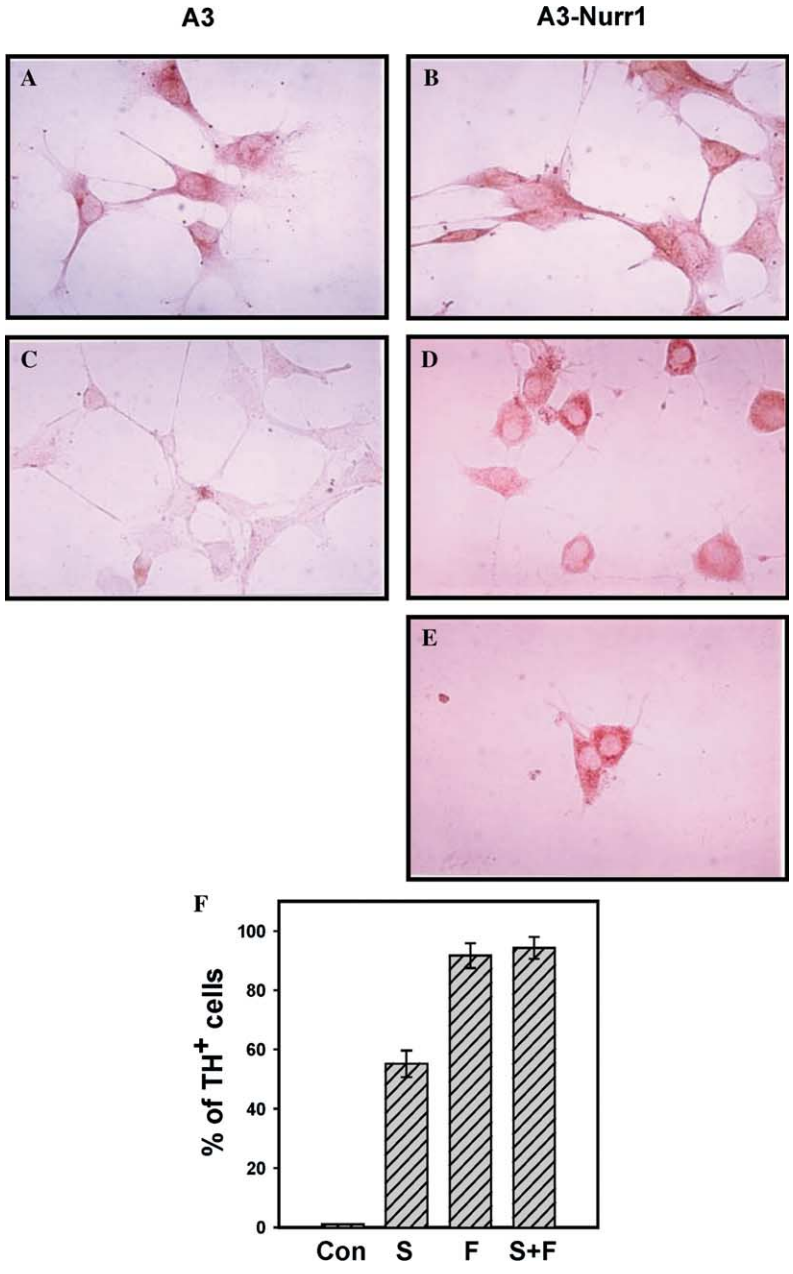

Fig. 5. Immunocytochemical staining for tubulin β III (A,B) and TH (C–E). Treatment with Shh-N and FGF8 induced marked morphological differentiation in both A3 and A3.Nurr1 cells (A,B). However, few TH-labeled cells were observed in parental A3 cells treated with Shh-N and FGF8 for 5 days (C). Compared with parental cells, A3.Nurr1 cells became TH-positive after 5 days when treated with FGF8 alone (D) or with FGF8 (10 ng/ml) plus co-culture of C4.Shh cells (E). (F) Histogram demonstrating the percentage of TH^+ cells in cultures of A3.Nurr1 cells grown alone and grown on a insert culture of C4.Shh cells, FGF8 (10 ng/ml) or FGF8 (10 ng/ml) plus co-culture of C4.Shh cells.

Increase in TH-positive cell numbers by FGF8 and Shh-N

To test whether these changes in morphology were attributed to the differentiation of DA neurons, the cells were examined immunohistochemically. When the cells were grown in the presence of FGF8 and Shh, immunoreactivity of tubulin β III was observed in almost all of the cells in both A3 and A3.Nurr1 cells (Figs. 5A and B). However, immunoreactivity of TH was different in A3 and A3.Nurr1 cells (Figs. 5C–E). The number of TH-positive cells greatly increased with FGF8 and co-culture of C4.Shh-N, as reflected by the increased total number of TH-positive cells ($94.3 \pm 3.7\%$ of total number of cells) in A3.Nurr1 cells. This pronounced effect on the generation of TH-positive cells was specific to Nurr1 overexpressing NSCs (Fig. 5E), but not to parental NSCs (Fig. 5C). Under these conditions, TH-positive cells were occasionally observed but were faintly stained, and the number never exceeded 2% of total cells in A3 cells (Fig. 5C). In addition, while applied as single factor, Shh-N was less effective in inducing DA phenotypes ($55.2 \pm 4.5\%$ of total number of cells) than when added in combination with FGF8, but FGF8 is equally effective ($91.7 \pm 4.2\%$ of total number of cells) when compared to FGF8 and C4.Shh co-cultures (Fig. 5F).

Collectively, the level of TH immunoreactivity increased substantially depending upon the dopaminergic induers used. The percentages of cells stained positive for TH immunoreactivity in the presence of each inducer used were $4.9 \pm 0.3\%$ ($n = 5$) for RA, $3.5 \pm 0.4\%$ ($n = 5$) for FK, $24.7 \pm 2.3\%$ for human fetal astrocyte, and $94.3 \pm 3.7\%$ for co-culture of C4.Shh plus FGF8. These results indicate that Shh-N and FGF8 are specific inducers of DA phenotypes in NSCs.

Discussion

The present study demonstrated that Nurr1 restricts uncommitted multipotent mouse NSCs to neuronal lineage, but it could not induce dopaminergic fate in mouse NSC. The observation that TH gene induction occurs after attainment of a mature neuronal phenotype indicates that Nurr1 alone is not sufficient for activating TH gene expression, but rather requires specific cellular (or neuronal) environments or cofactors. Such cofactors or conditions may be present in Nurr1-overexpressing adult hippocampal precursors [21], embryonic stem cells [23,24], NSC [25,26], mesencephalic cell [17,27], or provided by mesencephalic astrocytes to allow TH induction in mouse neuronal precursors [16]. In addition, it has been demonstrated that Nurr1 can directly activate the TH promoter in a cell line-specific manner [28,29], supporting the view that certain transcriptional conditions are required for TH gene induction by Nurr1.

Various signaling molecules have been extensively tested for their potential to facilitate DA lineage differentiation using ES and progenitor cells [15,30]. We tested if the retinoic acid, forskolin, co-culture with human fetal astrocytes, or the combined treatment with Shh-N and FGF8 can increase induction of TH-positive neurons in the Nurr1-overexpressing NSC clone. For the first time, we tested whether treating A3.Nurr1 cells with combinations of the previously mentioned proliferative, differentiation, and trophic factors could increase the number of TH-positive neurons. Treatments to A3.Nurr1 cells were generally ineffective with bFGF, retinoic acid, and/or forskolin. Thus, these results suggest that bFGF, retinoids, and forskolin do not act as inductive signals in our system. Furthermore, as retinoic acid specifically stimulates RXR receptors [31], which heterodimerize with Nurr1 and/or other nuclear receptors to form transcription-initiating complexes [32], RXR receptor stimulation does not play an important role in enhancing TH-positive neurons, suggesting that TH induction may have acted through distinct mechanism from Nurr1–RXR dimerization pathway.

Previous studies have demonstrated that diffusible and contact-dependent signals from astrocytes [16,17] can increase the number of NSCs differentiating into DA neurons in vitro. In the present study, co-culture via a microporous insert of fetal astrocytes was able to significantly increase the number of TH-positive neurons in A3.Nurr1 cell cultures, suggesting that astrocytes secrete a highly labile diffusible factor that interacts with Nurr1-overexpressing NSC line to generate TH-positive neurons. Collectively, these observations suggested that a factor derived from the primary astrocyte cultures interacted directly or indirectly with Nurr1 to induce TH-expressing neurons. However, because TH expression was limited to a minority subpopulation within a fraction of the Nurr1 clones, some property of the primary cultures or of the Nurr1 clones themselves must have been limiting.

The increase of TH-positive neurons from A3.Nurr1 cells was even more pronounced when Shh-N and FGF8 were applied to the Nurr1-NSC clone during in vitro differentiation. Of signaling molecules treated to NSCs in our system, the combined treatment of Shh-N and FGF8 was the most effective inducer of DA neurons. In this condition, almost all of neuronal clusters contained large numbers of TH-positive neurons and our quantitative analysis showed that approximately $94.3 \pm 0.7\%$ of total A3.Nurr1 cells were TH-positive (Fig. 5). More importantly, induction of TH-positive neurons was evident only in Nurr1 transduced clone, not parental NSCs. These observations therefore do not support the notion that Nurr1 may act downstream of one of these signaling molecules, but rather, suggest that Nurr1 and these molecules may act independently or synergistically to induce the DA lineage differentiation. In addition,

Shh-N and FGF8 simultaneously induced the expression of DA markers and typical neuronal morphology in both NSCs and Nurr1 transduced NSCs, suggesting that these molecules were conductive for both cellular and morphological differentiation of NSCs into DA phenotypes. It will be very interesting in the future to investigate the molecular nature of the signals involved in the generation of DA neurons from NSCs.

In conclusion, our results suggest that Nurr1 induces neuronal differentiation and confers competence to respond to extrinsic signals that induce dopaminergic fate in a mouse NSC line. Moreover, the present study demonstrates that the most potent sources of such signals are Shh-N and FGF8, which induces Nurr1-expressing NSCs to develop into typical DA neurons. Our findings further suggest that Shh-N and FGF8 may be the sources of signals required for the induction of both cellular and morphological differentiation of NSCs into DA phenotypes. Finally, the procedures we describe, taking advantage of the multipotential capacity of NSCs, selector genes such as Nurr1, and in vivo dopaminergic inducers, might be used to engineer neurons with the desired neurochemical phenotypes as a source material for neuronal transplantation in the treatment of neurodegenerative diseases. Taken together, these observations suggest that our immortalized NSCs can serve as an excellent model for understanding mechanisms that regulate specification of ventral midbrain DA neurons and as an unlimited source of DA progenitors for treating Parkinson's disease patients by cell replacement.

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