

Foxa2 and Nurr1 Synergistically Yield A9 Nigral Dopamine Neurons Exhibiting Improved Differentiation, Function, and Cell Survival

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ABSTRACT

Effective dopamine (DA) neuron differentiation from neural precursor cells (NPCs) is prerequisite for precursor/stem cell-based therapy of Parkinson's disease (PD). Nurr1, an orphan nuclear receptor, has been reported as a transcription factor that can drive DA neuron differentiation from non-dopaminergic NPCs in vitro. However, Nurr1 alone neither induces full neuronal maturation nor expression of proteins found specifically in midbrain DA neurons. In addition, Nurr1 expression is inefficient in inducing DA phenotype expression in NPCs derived from certain species such as mouse and human. We show here that Foxa2, a forkhead transcription factor whose role in midbrain DA neuron development was recently revealed, synergistically cooperates with Nurr1 to induce DA phenotype acquisition, midbrain-specific gene expression, and neuronal maturation. Thus, the combinatorial expression

of Nurr1 and Foxa2 in NPCs efficiently yielded fully differentiated nigral (A9)-type midbrain neurons with clearly detectable DA neuronal activities. The effects of Foxa2 in DA neuron generation were observed regardless of the brain regions or species from which NPCs were derived. Furthermore, DA neurons generated by ectopic Foxa2 expression were more resistant to toxins. Importantly, Foxa2 expression resulted in a rapid cell cycle exit and reduced cell proliferation. Consistently, transplantation of NPCs transduced with Nurr1 and Foxa2 generated grafts enriched with midbrain-type DA neurons but reduced number of proliferating cells, and significantly reversed motor deficits in a rat PD model. Our findings can be applied to ongoing attempts to develop an efficient and safe precursor/stem cell-based therapy for PD. *STEM CELLS* 2010;28:501–512

Disclosure of potential conflicts of interest is found at the end of this article.

Additional Supporting Information may be found in the online version of this article.

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INTRODUCTION

Parkinson's disease (PD) is the most common movement disorder and presents with akinesia, rigidity, and tremor. The neuropathology of greatest relevance to the motor symptoms is progressive degeneration of dopamine (DA) neurons located in the substantia nigra of the midbrain. Approximately one decade ago open-label clinical trials demonstrated that intracerebral transplants of human fetal nigral tissue can ameliorate symptoms in PD (for a review [1]). Consequently, PD is considered to be a possible target for stem cell-based therapy in the future [1, 2]. Neural stem/precursor cells, which display self-renewal and plasticity, have been regarded as a possible source of such cells and if the appropriate methodology is developed they could provide a reproducible and standardized supply of functional DA neurons for grafting in PD. However, DA neurons are efficiently generated from cultured neural precursor cells (NPCs) only if the cells are derived from the ventral midbrain at early developmental stages (e.g., rat embryonic day 11.5–12.5) [3, 4]. Studies have aimed at devising culture protocols that would allow stem/precursor cells to differentiate into DA neurons, regardless if they originate from, for example, different brain regions of varying developmental stages, embryonic stem (ES) cells, or induced pluripotent stem (iPS) cells [1].

Midbrain DA neurons arise from the floor plate of the embryonic mesencephalon through an intricate process involving the action of multiple factors. These include cell-type specification factors such as sonic hedgehog (SHH), fibroblast growth factor eight (FGF8), *Lmx1a*, and *Foxa1/2*. In addition, neuron-differentiation factors including *Msx1*, neurogenin2 (*Ngn2*), and *Mash1*, as well as DA phenotype-promoting factors represented by *Nurr1* all play essential roles (for reviews [5, 6]). These developmental factors can be exploited to generate neurons suitable for transplantation in PD. In this regard, *Nurr1* (NR4A2), a transcription factor belonging to the orphan nuclear receptor family, is one of the most potent factors. Induced expression of *Nurr1* in cultured rat NPCs can produce DA neurons that survive after transplantation into a rat model of PD [7]. However, typically only a minority of DA neurons derived from *Nurr1*-overexpressing rat NPCs is functionally viable after grafting [7, 8]. It is assumed that the majority either dies or loses the DA phenotype. Furthermore, the effect of *Nurr1* on NPCs is limited to certain species. Thus, at most a few tyrosine hydroxylase (TH)-positive DA neurons are generated when mouse [9] or human [10] NPCs are transduced with *Nurr1*.

Foxa2 (winged helix/forkhead box A2:HNF3beta) is a transcription factor which, in the central nervous system (CNS), is first expressed in the ventral neural plate. Subsequently, it is expressed in the floor plate of the neural tube and then widely in the ventral midbrain [11–13]. The role of *Foxa2* in midbrain DA neuron development is not fully understood. Two independent loss-of-function studies recently demonstrated lost expression of midbrain-specific developmental genes and reduced numbers of DA midbrain neurons in *Foxa2*-null mice [14, 15].

In this study, we examined the potential use of the *Foxa2* gene in generating transplantable DA neurons. *Foxa2* cooperates with *Nurr1* in promoting the transcription of genes specific for DA phenotypes as well as those for midbrain and nigral (A9) DA neurons. *Foxa2* induced a rapid cell cycle arrest and neuronal differentiation of NPCs, which in turn contributed to enhanced maturation and functions as presynaptic DA neurons. Furthermore, *Foxa2*-expressing DA neurons showed increased cell survival and resistance to toxic stimuli. Consequently, coexpression of

Foxa2 and *Nurr1* in cultured NPCs produced A9 midbrain-type DA neurons exhibiting improved function and survival but with reduced tumorigenic potential after intracerebral transplantation. Finally, we showed that the combinatorial expression of *Nurr1* and *Foxa2* was ubiquitously effective in inducing DA neuronal differentiation from cultured NPCs regardless of brain regions or species (including murine and human) from which they were derived. Taken together, our findings indicate that coexpression of *Foxa2* and *Nurr1* may be a strategy for the generation of functional DA neurons suitable for transplantation in PD.

MATERIALS AND METHODS

NPC Culture and Transduction

We cultured NPCs from diverse brain regions of mouse (ICR) embryos at embryonic day 12 (E12) and cortices of rat (Sprague-Dawley rats) embryos at E14. Human NPCs were derived from human embryonic stem (hES) cells (HSF-6; University of California, San Francisco) by the *in vitro* hES differentiation protocol [16, 17]. We transduced proliferating NPCs with retroviruses expressing *Nurr1* (Flag-tagged), Small Heterodimer Partner-1 (*SHP*), *DAX-1*, estrogen related receptor- γ (*ERR\gamma*), *RXR\alpha*, *Foxa1*, and *Foxa2* (hemagglutinin (HA)-tagged). We induced differentiation 1 day after viral transduction. Protocols for cultures and viral transduction in details are shown in the Supporting Information Materials and Methods section.

Cell Proliferation and Viability

We determined cell proliferation using 5-bromo-2-deoxyuridine (BrdU, Roche, Indianapolis, IN, <http://www.roche.com>)-incorporation, neurosphere formation assays, and cell cycle analysis by flow cytometry as described in the Supporting Information section. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, a tetrazole (MTT; Sigma, St. Louis, MO, <http://www.sigmaaldrich.com>) test and % apoptotic cells in the presence of H₂O₂ (25–200 μ M; Sigma) and by TH+ cell numbers in the cultures treated with 1-methyl-4-phenylpyridinium iodide (MPP+; 5–500 μ M; Sigma).

In Vivo Transplantation

We harvested NPCs on day 2 of post-transduction and transplanted in the striatum of 6-hydroxy-dopamine (6-OHDA)-lesioned PD rats. We assessed amphetamine-induced rotations on four occasions at regular intervals for 8 weeks post-transplantation. Graft volumes and total TH+ cells in the grafts were determined. The detailed methods for transplantation and histological analysis are presented in the Supporting Information section.

Cell Counting and Statistical Analysis

In each cell culture experiment, we counted cells from 8 to 40 microscopic fields randomly chosen (fractionator) across the culture area. The counting data were expressed by means \pm standard errors (SEM) of three to five independent experiments. We performed statistical analyses using the Student's *t*-test or one-way ANOVA with a post hoc analysis (SPSS 16.0; SPSS Inc., Chicago, IL, <http://www.spss.com>) when more than two groups were involved.

DA release and uptake were assessed as previously described [18]. Standard protocols for RT-PCR, immuno-fluorescent and Western-blot analyses were applied (Supporting Information Materials and Methods). The methods for the Luciferase reporter and Chromatin immunoprecipitation (ChIP) assays are described in the Supporting Information.

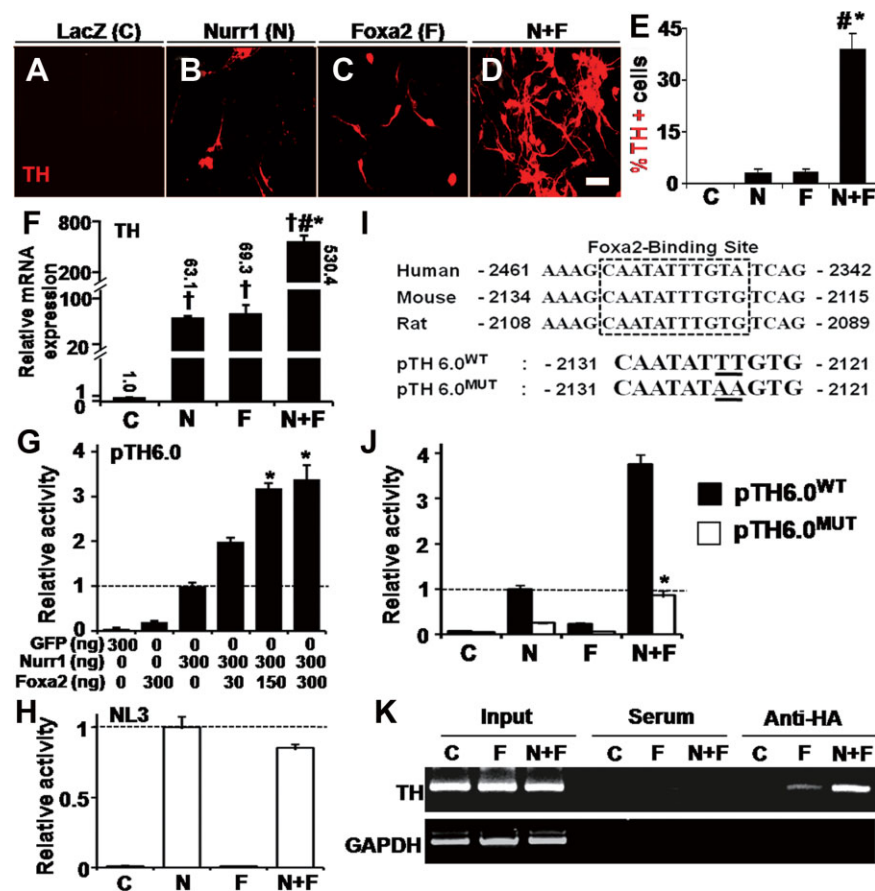


Figure 1. Foxa2 and Nurr1 synergistically promote TH⁺ cell differentiation from cultured NPCs. Precursor cells were cultured from mouse embryonic cortices at E12 and transduced with Nurr1 (N), Foxa2 (F), Nurr1+Foxa2 (N+F) or LacZ (C). TH immunocytochemical (A–E) and real-time PCR (F) analyses were carried out after 3 days of differentiation in vitro. Scale bar, 40 μ m. Graph (E) depicts % TH⁺ cells out of total (DAPI⁺) cells, which significantly increased compared with the values of Nurr1 (#) and Foxa2 (*) at $p < .05$; one-way ANOVA with a post hoc Tukey alpha, $n =$ five experiments. In each experiment, 8–11 microscopic fields were randomly selected for counting. (F): TH mRNA expression estimated by real-time PCR analysis. In the quantitative PCR analyses, mRNA expression was normalized to that of GAPDH and expressed relative to the respective control value ($n = 6$). The mean expression values are shown on each bar of the graph. Significantly increased compared with the control (\dagger), Nurr1 (#), and Foxa2 (*) at $p < .01$. (G, H): Luciferase activities driven by rat TH6.0 promoter (pTH6.0) (G) and Nurr1-binding element (NL3) (H). * $p < .001$ in comparison with Nurr1, one-way ANOVA with a post hoc Dunnett's test, $n = 3$. (I): Sequence alignment of the TH promoters of different species around the consensus Foxa2 binding sequence (boxed with dotted line) and mutation of the binding site in the TH6.0 promoter by transforming "TT" into "AA" (underlined). (J): Luciferase activity of wild-type pTH6.0 (pTH6.0^{WT}) and the Foxa2-binding mutant (pTH6.0^{MUT}). *Significantly different from the respective levels of pTH6.0^{WT} at $p < .001$; one-way ANOVA with a post hoc Dunnett's test, $n = 3$. (K): ChIP assay demonstrating occupancy of Foxa2 (HA-tagged Foxa2) proteins in the consensus Foxa2-binding site of the TH promoter. All the reporter and ChIP assays were carried out in E12 mouse cortical NPCs. Abbreviations: C, control; F, Foxa2; GAPDH, glutaraldehyde dehydrogenase; N, Nurr1; N + F, Nurr1+Foxa2; TH, tyrosine hydroxylase.

RESULTS

Foxa2 and Nurr1 Cooperatively Induce TH Expression in Non-Dopaminergic Precursors Derived from Mouse Fetal Cortices

Nurr1 is known to be a potent inducer of differentiation of rat NPCs into DA neurons [7, 8]. However, Nurr1 is not as effective in this regard when overexpressed in mouse [9] and human NPCs [10]. The apparent inability of Nurr1 to induce human NPCs into DA neurons suggests that Nurr1 overexpression will not have a role in clinical protocols aimed at generating human DA neurons for grafting to patients with PD. At the outset of the present study, we hypothesized that coexpression of Nurr1 coactivators would improve the inductive activity of Nurr1 in mouse and human NPCs. We based this reasoning on knowledge that transcriptional coregulators largely control the activ-

ities of transcription factors belonging to the nuclear receptor family (e.g., Nurr1). To identify the Nurr1 coactivators that could drive DA neuron generation from NPCs regardless of the species of origin, we tested several potential factors in NPCs derived from mouse embryonic cortex. We chose to use mouse cortical precursors because the cortex is a non-dopaminergic brain region. Thus any effects of the coactivators of Nurr1 we observed would not be influenced by other DA neurogenic factors that might have been present had we instead chosen mid-brain-derived NPCs as a model system. Consistent with our previous findings [9], very few of the Nurr1-transduced NPCs expressed the DA neuron marker TH in the mouse NPC cultures (Fig. 1A, 1B, 1E). We tested the effects of coexpressing Dax-1, ERR- γ , Foxa1, Foxa2, SHP-1, and RXR α , along with Nurr1. These have previously been found to exert coregulatory actions in nuclear receptor-mediated transcriptions [19–26] and to be expressed in developing midbrains ([11–15, 26, 27] and Supporting Information Fig. S1a). Among these, Foxa2

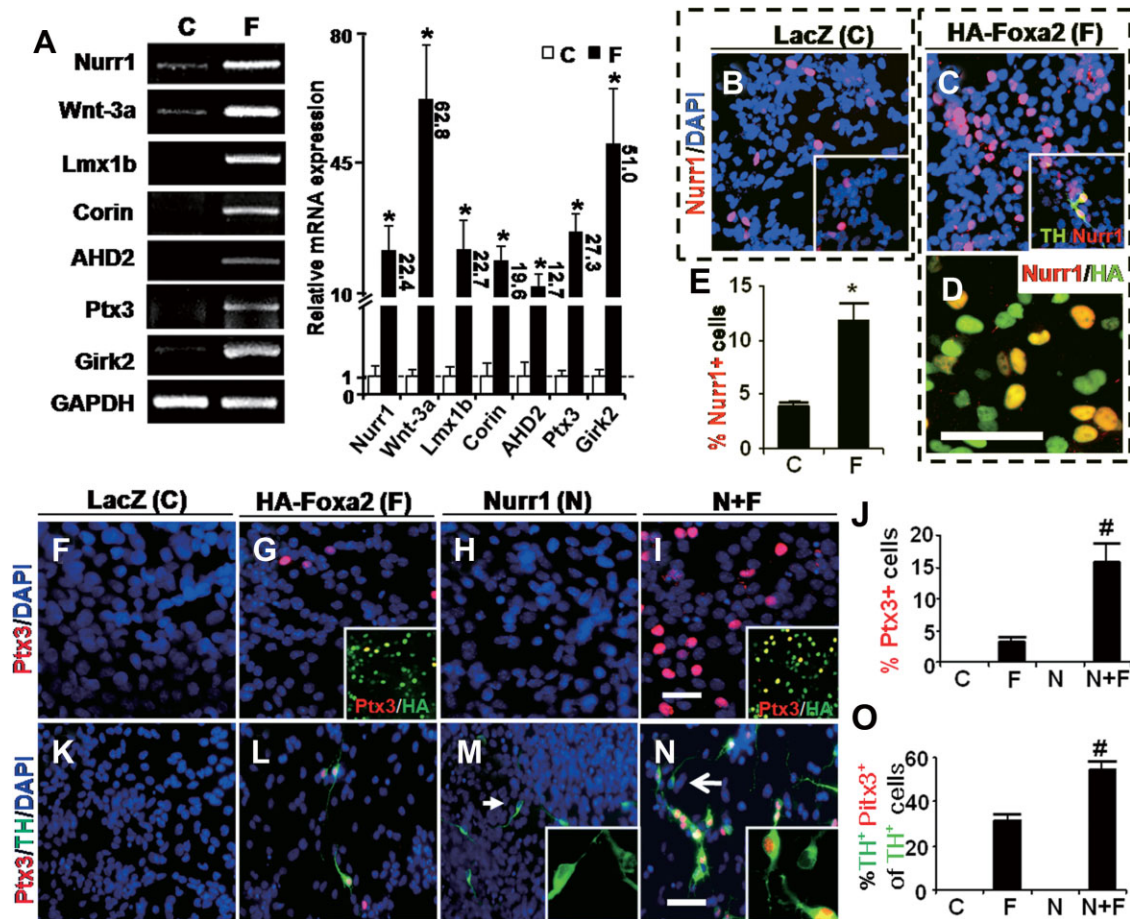


Figure 2. Induction of the midbrain dopamine (DA) neuron-specific genes in Foxa2-expressing precursor cells. (A): mRNA expressions for the midbrain DA neuron-specific markers. RNAs were prepared from E12 mouse cortical cells transduced with LacZ (C) and Foxa2 (F), and mRNA expressions were determined using semi-quantitative (left) and real-time (right) PCR analyses. The graph on the right depicts the mRNA expression values relative to their respective controls estimated by real-time PCR (for each value, $n = 3$). The numbers shown on each bar indicate the mean expression values. *Significantly increased compared with the respective control at $p < .001$. (B–E): Foxa2-mediated induction of Nurr1 expression. Shown in (B) and (C) are the representative images for Nurr1-immunoreactive cells. Graph (E) depicts % Nurr1+ cells out of total cells. Foxa2/Nurr1-colabeled (HA+/Nurr1+) cells in HA-Foxa2-transduced culture are shown in (D). Insets of (B) and (C): TH/Nurr1-stained cells. (F–O): Foxa2-induced Ptx3 expression. Shown in (F–I) and (K–N) are the representative images for Ptx3+ cells and Ptx3+/TH+ cells, respectively, at differentiation day 3 in the cultures as indicated. Insets of (G) and (I): Exogenous Foxa2 (HA)/Ptx3-stained images of the identical microscopic fields. Insets of (M) and (N): High-magnification views of the cells indicated by arrows. Scale bars, 40 μm . Graphs depict % Ptx3+ cells (J) out of total DAPI+ cells, and % Ptx3+, TH+ double positive cells (% TH+ cells expressing Ptx3) out of total TH+ cells (O). All analyses were performed with neural precursor cell cultures derived from mouse embryonic cortices at E12. Statistical significance with respect to LacZ-controls* and Foxa2-transduced cells# at $p < .001$, one-way ANOVA with a post hoc Dunnett's test, $n = 4$, each of which includes duplicate coverslips. Abbreviations: C, control; F, Foxa2; GAPDH, glutaraldehyde dehydrogenase; HA, hemagglutinin; N, Nurr1; N + F, Nurr1+Foxa2; TH, tyrosine hydroxylase.

resulted in a striking enhancement of TH+ cell numbers (% TH+ cells of total cells: $2.0\% \pm 1.4\%$ in Nurr1 alone vs. $37.1\% \pm 5.8\%$ in Nurr1+Foxa2, Supporting Information Fig. S1b–S1j and Fig. 1A–1E). When we transduced non-dopaminergic cortical NPCs with Foxa2 alone, we also obtained a small number of TH+ cells (Fig. 1A–1C, 1E). TH expression was seen only in the cytoplasm of cells transduced with virus expressing HA tagged Foxa2-ires-green fluorescent protein (GFP) (Supporting Information Fig. S2), indicating that Foxa2-mediated TH expression occurs in an intrinsic cell-autonomous manner. The increases in TH+ cell numbers following overexpression of Foxa2 and Nurr1 were dose-dependent, with the greater yields resulting from the higher viral titers. However, the highest TH+ cell yields induced by the Foxa2 virus ($3.1\% \pm 0.9\%$ at 25 multiplicity of infection [MOI]) or by the Nurr1 virus ($2.2\% \pm 1.3\%$ at 25 MOI) were far lower than the yield from the combinatory transduction of the Nurr1 and Foxa2

viruses ($39.1\% \pm 4.2\%$ at 12.5 MOI each, $p < .05$, $n = 5$ independent experiments; Fig. 1A–1E), indicating synergism between these two factors in the generation of TH+ cells. Consistent with the synergistic increase in TH mRNA levels by the coexpression of Foxa2 and Nurr1 (Fig. 1F), Foxa2 enhanced Nurr1-induced transactivation of TH promoter in a dose-dependent manner as estimated by luciferase activity from the TH6.0 promoter (Fig. 1G). Nurr1-induced transcriptional activation of a synthetic reporter containing Nurr1-binding elements (NL3) [28], however, was not altered by Foxa2 coexpression (Fig. 1H). The TH promoter contains a consensus sequence for Foxa2 binding (CAATATTTGT; Fig. 1I, upper panel). Alteration of this cis-element (Fig. 1I, lower panel) resulted in a dramatic reduction of the TH6.0-luciferase activities (Fig. 1J), indicating that Foxa2 acts by directly binding to the TH promoter. We then performed ChIP assays which confirmed the Foxa2 binding to the promoter (Fig. 1K). Notably,

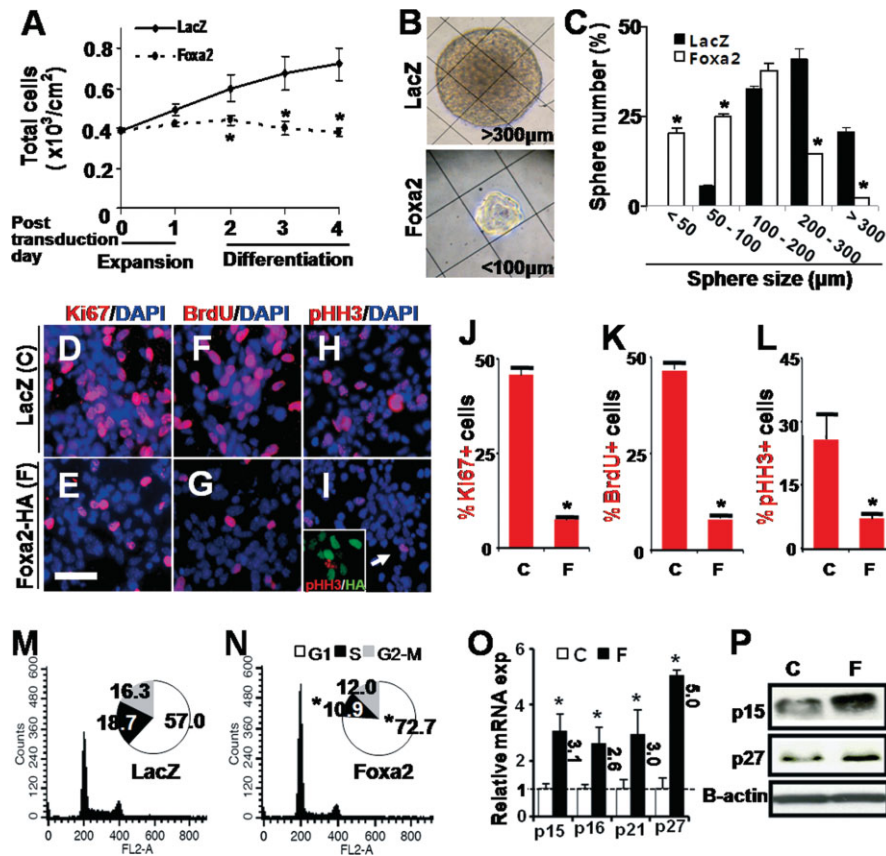


Figure 3. Cell cycle arrest effects of Foxa2. (A): Cell growth curve. E12 mouse cortical NPCs were transduced with Foxa2 (or LacZ). On the following day of transduction, cell differentiation was induced for the subsequent 3 days. Cell growth profiles of LacZ- (control) and Foxa2-transduced cultures were determined by counting total viable cells during the initial 4 days of post-transduction. (B, C): Neurosphere formation assay. Graph (C) depicts the numbers of spheres within the ranges of the neurosphere diameters indicated. The diameters of 1,674 (Foxa2) and 1,530 (control) neurospheres were estimated from three independent experiments. Note that Foxa2 transduction significantly decreased the size of neurospheres, giving rise to unusually small (<100 µm) neurospheres. This is illustrated in (B). (D–L): % of cells positive for Ki67 (D, E, J), BrdU (F, G, K), and pHH3 (H, I, L) at postinfection day 2. Inset of (I) shows that none of HA-positive (Foxa2-expressing) cell is immunoreactive for pHH3 in the region indicated by the arrow. Scale bar, 50 µm. *Significantly different from the control at $p < .001$, Student's *t*-test, $n = 3$ independent cultures. (M, N): Flow cytometric analysis for determination of cell cycle status of the LacZ-transduced control (M) and Foxa2-transduced (N) cultures at post-transduction day 2. The numbers in the graphs represent the means of the % cells accumulated in the cell cycle phases indicated from four independent analyses. (O, P): Real-time PCR (O) and Western-blot (P) analyses demonstrating Foxa2-mediated up-regulation of the cyclin-dependent kinase inhibitors. *Significantly increased compared with the controls at $p < .01$, $n = 3$. Abbreviations: C, control; F, Foxa2.

coexpression of Nurr1 induced a further enrichment of Foxa2 proteins in the TH promoter site, strongly suggesting that Nurr1 and Foxa2 synergize at the level of TH promoter recognition.

Midbrain and Substantia Nigra (A9) DA Phenotype of TH+ Cells Generated by Foxa2 Overexpression

We found that exogenous Foxa2 expression in cortical precursor cells induces not only genes associated with the DA phenotype (TH, DA transporter [DAT], vesicular monoamine transporter two [VMAT2]; Fig. 5F) but also those specific for the midbrain- (Nurr1, Wnt-3a, Lmx1b, and Corin) and for A9 midbrain-type DA neurons (aldehyde dehydrogenase 2 [AHD2], Ptx3, G-protein-coupled inward rectifier potassium [Girk2]; Fig. 2A). A subset of cultured cortical precursor cells expressed endogenous Nurr1 ($4.1\% \pm 0.5\%$) and the proportion increased in Foxa2-transduced cultures ($11.9\% \pm 1.5\%$, $p < .001$, $n = 4$ independent cultures; Fig. 2B, 2C, 2E). Most

of the Nurr1+ cells in the Foxa2-transduced cultures expressed exogenous Foxa2 (Fig. 2D), and virtually all the TH+ cells generated by Foxa2 transduction expressed Nurr1 (Fig. 2C, inset). None of the cortical precursors was immunoreactive for Ptx3, a marker expressed both in A9 and A10 (ventral tegmental area) midbrain DA neurons [29], and which is considered more specific for the cell specification and survival of A9 DA neurons than A10 DA neurons [30–35]. Foxa2 induced Ptx3 expression and yielded TH+ cells expressing the midbrain marker (Fig. 2F–2O). When cultured cells were cotransduced with a combination of Nurr1+Foxa2 (N + F), the Ptx3 expression increased even further. Thus, the percentage of Foxa2-transduced (HA+) cells that expressed Ptx3 was $37.1\% \pm 3.1\%$ in the N + F group, whereas it was only $3.2\% \pm 0.5\%$ in cells transduced with Foxa2 alone. Along the same lines, $55.1\% \pm 2.8\%$ of the TH+ cells expressed Ptx3 in the N + F group compared with $31.8\% \pm 2.3\%$ in cells transduced with Foxa2 alone ($p < .05$, total 14,057–15,701 cells counted from four independent

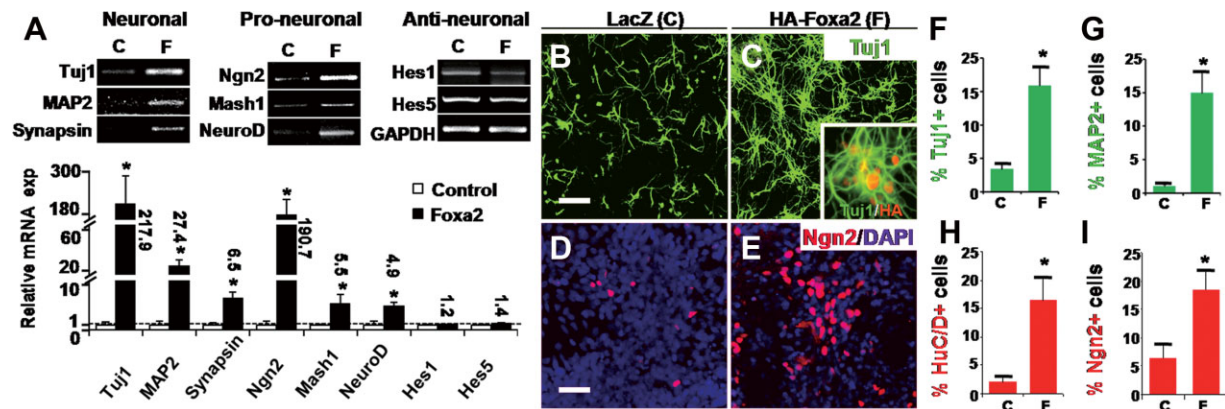


Figure 4. Foxa2 promotes neuron formation from cultured cortical neural precursor cells. (A): Semi-quantitative (upper) and real-time (lower) RT-PCR analyses for the neuron-specific markers, pro-neuronal, and anti-neuronal bHLH transcription factors. The mRNA expression was examined 3 days after differentiation in vitro. The numbers in the graph represent means of the mRNA expression values relative to the respective controls from three real-time PCR analyses. *Significantly increased compared to the control at $p < .01$. (B–I): Immunocytochemical data showing increased neuronal populations positive for neuronal markers TuJ1 (B–F), MAP2 (G), and HuC/D (H) with a significant increase of Ngn2-immunoreactive cells (D, E, I) in the Foxa2-transduced cultures at in vitro differentiation day 3. *Significantly different from the respective LacZ-transduced controls at $p < .001$, Student's *t*-test, $n = 3$ independent cultures. Inset of (C): representative image of TuJ1+/HA+ cells in the cultures transduced with HA-Foxa2. Scale bar, 80 μ m. Graphs depict % immunoreactive cells of total DAPI+ cells. Abbreviations: C, control; F, Foxa2; GAPDH, glutaraldehyde dehydrogenase.

experiments). These findings again demonstrate that Foxa2 and Nurr1 cooperate to promote A9 DA neuronal differentiation.

Cell Cycle Arrest and Neurogenic Role of Foxa2

We induced NPC differentiation by withdrawing the mitogen basic fibroblast growth factor (bFGF). The NPCs typically continue to divide during the initial 3–4 days of differentiation (Fig. 3A and [36]). However, we did not observe any continuation of cell division after withdrawal of bFGF in the Foxa2-transduced cultures. We confirmed the inhibitory action of Foxa2 on cell proliferation in a neurosphere formation assay where we found smaller neurospheres in Foxa2-transduced cultures (Fig. 3B, 3C). Furthermore, we found that cells positive for Ki67 (proliferating cell-specific), BrdU (S-phase), and phospho-Histone H3 (pHH3; M-phase) were drastically reduced in Foxa2-transduced cultures compared with the control cultures transduced with LacZ (Fig. 3D–3L). Consistently, flow cytometry analyses showed that Foxa2-transduced cells arrested in the G0/G1 phases and the proportion of cells in the S-phase was reduced (Fig. 3M, 3N). In the Foxa2-transduced cultures, the cyclin-dependent kinase (CDK) inhibitors p15^{INK4B} and p27^{KIP1} were upregulated both at the mRNA and protein levels (Fig. 3O, 3P). Taken together, our data suggest that Foxa2 induces cell cycle arrest by inhibiting the G1 to S transition of the cell cycle. Our observations are consistent with the cell cycle inhibition reported to take place following expression of the other forkhead family proteins, Foxo4 and Foxo3a [37, 38].

We found that Foxa2 overexpression upregulated the mRNA levels of Ngn2, Mash1, and NeuroD in cortical NPCs (Fig. 4A). We confirmed the effect of Foxa2 on Ngn2 expression by immunocytochemistry (Fig. 4D, 4E, 4I; % Ngn2+ cells of total 4', 6-diamidino-2-phenylindole [DAPI]+ cells: $16.4 \pm 3.9\%$ in Foxa2-transduced vs. $2.1 \pm 0.8\%$ in lacZ-transduced control). On the third day of differentiation, cells positive for the neuronal markers neuron-specific class III β -tubulin (TuJ1), microtubule-associated protein 2(a+b) (MAP2), and human neuron-specific RNA-binding protein (HuC/D) were strikingly enriched in HA-Foxa2-transduced cultures compared with control cultures (Fig. 4B, 4C, 4F–4H; % TuJ1+ cells of total cells: $16.5 \pm 2.3\%$ vs. $4.3 \pm 0.7\%$).

HA immunoreactivity was localized to TuJ1+ cells (inset of Fig. 4C), indicating that the neurogenic effect of Foxa2 is cell autonomous.

Morphologic, Phenotypic, and Functional Maturity of DA Neurons Generated by Nurr1 and Foxa2 Coexpression

When we expressed either Nurr1 or Foxa2 individually in non-dopaminergic cortical NPCs, we found that the VMAT2 and DAT gene expressions were induced (Fig. 5F). Coexpression of Nurr1+Foxa2 resulted in a striking increase in the expressions of those DA homeostasis genes. These two genes are the most specific terminal markers of presynaptic DA neurons, indicating that Foxa2 and Nurr1 cooperate even at the late stage of DA neuronal maturation. Cortical NPCs cotransduced with Nurr1 and Foxa2 viruses differentiated toward morphologically mature TH+ cells. Thus, they exhibited extensive neurite outgrowth and the majority of TH+ cells (>78%) expressed the mature DA neuron-specific marker VMAT2, as well as generic neuronal markers such as TuJ1, HuC/D, and MAP2 (Fig. 5A–5E). Furthermore, we found that cells differentiated with N + F-transduced cultures displayed enhanced DA neuronal function as assessed by DA neurotransmitter release and uptake (Fig. 5G, 5H).

Foxa2-Expressing Cells Are Resistant to Toxic Stimuli

We found that the numbers of TH+ cells in rat NPC cultures transduced with Nurr1 (12.5 MOI) alone and Nurr1+Foxa2 (12.5 MOI each) were similar at early differentiation day (Supporting Information Fig. S4a–S4c). The number of TH+ cells decreased in cultures transduced with Nurr1 alone during the later differentiation period, likely due to cell death or DA phenotype loss. In contrast, the loss of TH+ cells was limited in cultures transduced with both Nurr1 and Foxa2. Consequently, on differentiation day 15, the number of TH+ cells in Foxa2-cotransduced cultures was 2.2-fold higher than that in cultures transduced with Nurr1 alone (Fig. 6A–6E). Notably, the percentage of cells that were apoptotic on differentiation day 15 was far less in the Foxa2-cultures (% cleaved caspase3+ cells of total DAPI+ cells: $10.4 \pm 2.3\%$ in Foxa2-

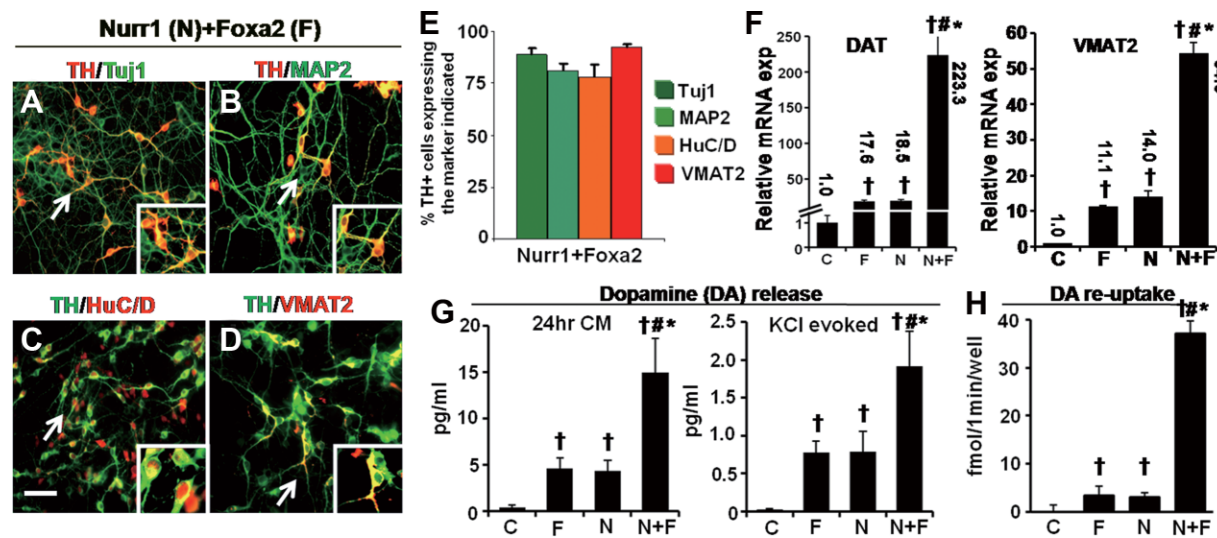


Figure 5. Neuronal maturation and functions of TH⁺ cells derived by co-expression of Nurr1+Foxa2. Colocalization of the proteins specific for neuron-specific TuJ1 (A), MAP2 (B), HuC/D (C), and for mature DA neuron-specific VMAT2 (D) in TH⁺ cells induced by Nurr1+Foxa2 overexpression. Insets: high-magnification views of the colabeled cells indicated by arrows. Scale bar, 50 μ m. Graph (E) shows % TH⁺ cells coexpressing these neuronal marker proteins out of total TH⁺ cells. (F): Real-time PCRs for the DA neuron homeostasis genes DAT (left) and VMAT2 (right) in the cultures transduced with LacZ (C), Nurr1 (N), Foxa2 (F), and N + F. Means of the expression values are indicated on each bar. Significantly increased compared with the control (\dagger), Nurr1 ($\#$), and Foxa2 ($*$) at $p < .01$, $n = 6$. (G): HPLC determination of DA release. The graphs show DA levels in the medium conditioned for 24 hours (24 hours CM, left) and evoked by KCl-induced depolarization for 15 minutes (KCl evoked, right). The levels of KCl-evoked releases were determined by subtracting DA levels in the medium conditioned for 15 minutes from those in the same medium supplemented with 56 mM KCl for the same period of time. (H): DAT-mediated specific DA uptake calculated by subtracting nonspecific uptake (with nomifensine) from total uptake. Significantly increased compared with the control (\dagger), Nurr1 ($\#$), and Foxa2 ($*$) at $p < .01$, Student's t -test, $n = 4$ for DA uptake and $n = 6$ for DA release assays. All the immunocytochemical and functional analyses were carried out in E12 mouse cortical NPC cultures 15 days after differentiation in vitro. Abbreviations: C, control; CM, conditioned medium; F, Foxa2; N, Nurr1; N + F, Nurr1+Foxa2; TH, tyrosine hydroxylase; DA, dopamine; DAT, DA transporter.

transduced vs. $51.4 \pm 1.2\%$ in lacZ-transduced cultures; Fig. 6F–6K). We also found that Foxa2-cotransduced cells were more resistant to the toxic effect of H₂O₂ treatment as assessed by the MTT assay (Fig. 6L) and by counting cells with apoptotic nuclei (Fig. 6M). Furthermore, we observed that Foxa2 coexpression prevented the death of TH⁺ cells when they were exposed to MPP⁺, the toxic metabolite of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which kills DA neurons in vitro and in vivo. In the cultures transduced with Nurr1 alone, MPP⁺ treatment (5–500 μ M, for 24 hours) resulted in a striking dose-dependent reduction in the number of TH⁺ cells (Fig. 6N). The decrease in TH⁺ cell numbers was much smaller in cultures transduced with Nurr1+Foxa2. For example, 79% of TH⁺ cells survived 100 μ M of MPP⁺ treatment in Nurr1+Foxa2-transduced cultures, whereas the TH⁺ cell number at the identical MPP⁺ concentration was only 20% of the untreated control in cultures transduced with Nurr1 alone. These findings together suggest that Foxa2 promotes cell survival and resistance to toxic stimuli in vitro. We found that brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) mRNA levels were upregulated in Foxa2-transduced cultures (Fig. 6O). Furthermore, we monitored a robust induction of mRNA for neurotrophin-3 (NT3) and SHH, which both are known as survival factors in the CNS, suggesting that they may mediate the prosurvival effects of Foxa2.

Cell Transplantation in PD Model Rats

In our next set of experiments, we addressed the effects of Foxa2 on DA neuron yield, survival, and function in hemiparkinsonian rats. We transduced NPCs derived from the rat embryonic cortex with Nurr1 or Nurr1+Foxa2, and transplanted

them into the striatum of adult rats with a unilateral 6-OHDA lesions. We measured amphetamine-induced rotation behavior at different time-points for 8 weeks post-transplantation. Overall, rats grafted with Nurr1+Foxa2-transduced cells demonstrated a significant reduction in rotation asymmetry (Fig. 7A). Consistent with our own published work [8], we found that transplanted precursors transduced with Nurr1 did not cause significant behavioral restoration. When we performed histological analyses at 2 and 8 weeks of post-transplantation, we found that the graft volume and total number of cells in the graft were significantly greater in the rats receiving Nurr1+Foxa2-transduced cells than those grafted with cells transduced with Nurr1 alone (Fig. 7F, 7G). In addition, the numbers of surviving TH⁺ cells were strikingly greater in the transplants of Nurr1+Foxa2 cells than in grafts of cells transduced with Nurr1-only ($2,654 \pm 250$ cells vs. 178 ± 17 cells at 2 weeks; 917 ± 100 cells vs. 56 ± 12 cells per graft at 8 weeks after transplantation, Fig. 7B, 7C, 7H). Consistent with our observations that Foxa2 can cause cell cycle arrest in vitro (Fig. 4), the proportion of cells immunoreactive for proliferating cell nuclear antigen (PCNA) was significantly lower in the grafts generated by Nurr1+Foxa2-transduced cells than Nurr1-cell grafts (Fig. 7D, 7E, 7I). These findings indicate that enhanced donor cell survival supported by Foxa2 is greater in magnitude than the potential reduction in cell number (monitored as graft size and TH⁺ cell number) due to the inhibitory effect of Foxa2 on cell proliferation.

Donor cells were labeled with GFP (see Supporting Information Materials and Methods). Two weeks after transplantation of GFP-labeled cells, 87.2% and 88.9% of cells in Nurr1 and Nurr1+Foxa2-grafts were GFP⁺ donor cells. GFP expression was apparent in virtually all the TH⁺ cells in the grafts (Fig. 7J), confirming that the TH⁺ cells in the grafts were derived from the donor cultures. Nurr1+Foxa2-derived

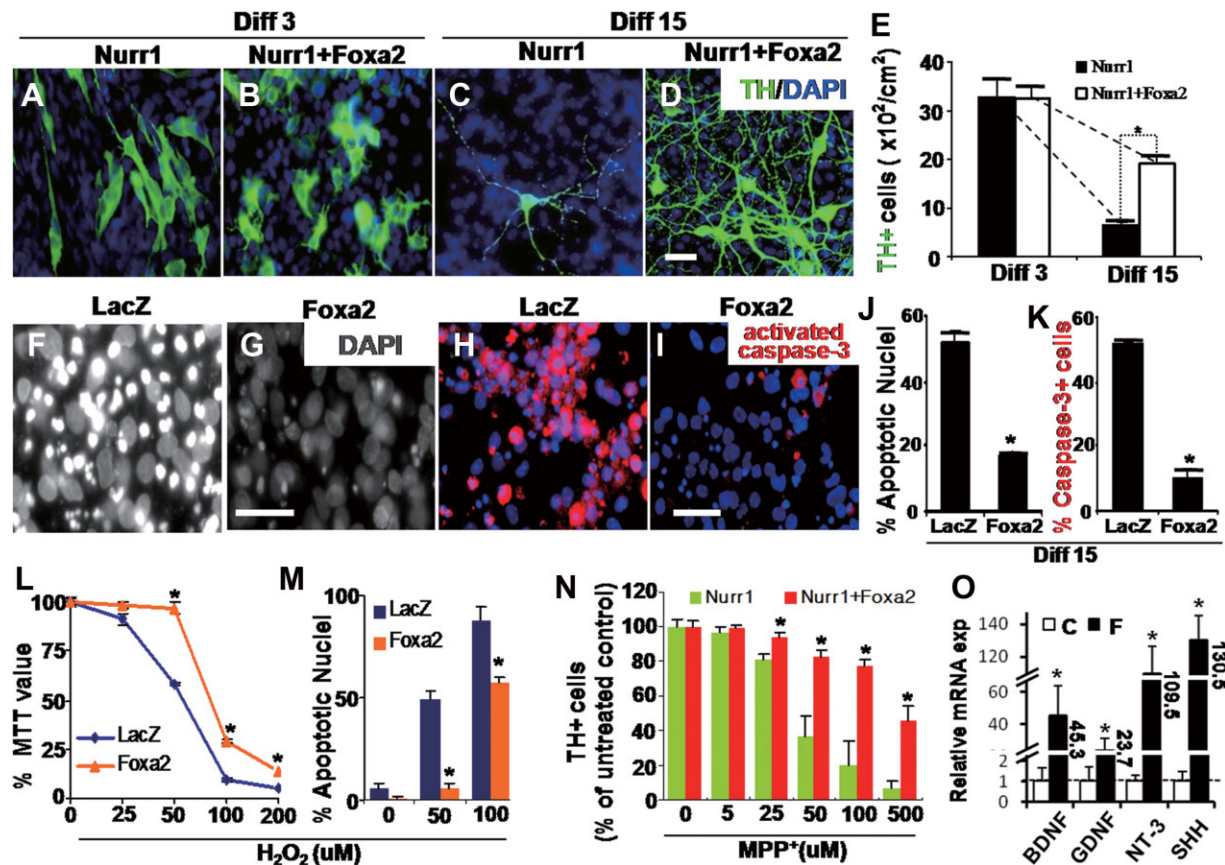


Figure 6. Foxa2-mediated cell survival effects. (A–E): Foxa2-expression prevents decrease in TH+ cell numbers during in vitro differentiation. E14 rat cortical NPCs were transduced with Nurr1 (12.5 MOI) without (A, C) or with Foxa2 (12.5 MOI; B, D) cotransduction. TH+ cell numbers were estimated at 3 and 15 days after differentiation. (F–K): % apoptotic cells estimated by cells with apoptotic nuclei and cleaved caspase3+ cells at differentiation day 15. Scale bar, 20 μ m. * $p < .05$, significantly different from Nurr1 (E) or LacZ (J, K)-transduced controls by Student's *t*-test. (L–N): Foxa2-expressing cells are resistant to toxic stimuli induced by H₂O₂ (25–200 μ M) or MPP⁺ (5–500 μ M) treatments. E14 rat cortical NPCs were transduced as indicated, differentiated for 6 days and treated with the toxins for 24 hours. Cell viability in the H₂O₂-treated cultures was determined by the MTT assay (L; $n = 6$) and direct counting of cells with apoptotic nuclei (M; $n = 6$). Shown in (L) is the % MTT value relative to the respective untreated control. MPP⁺-treated cultures were immunostained for TH, and the surviving TH+ cells were counted. Data shown in graph (N) are expressed as the percentage of untreated culture. * $p < .05$, significantly different from the respective controls at each toxin concentration by one-way ANOVA with a post hoc Dunnett's test. (O): Foxa2-mediated up-regulation of the neurotrophic factor expressions. Real-time RT-PCR analyses were carried out for mRNA levels of the cell survival factors of the cell survival factors BDNF, GDNF, NT3, and SHH. *Significantly increased compared with the controls at $p < .01$, $n = 3$. Abbreviations: BDNF, brain-derived neurotrophic factor; C, control; F, Foxa2; GDNF, glial cell line-derived neurotrophic factor; MPP⁺, 1-methyl-4-phenylpyridinium iodide; SHH, sonic hedgehog; TH, tyrosine hydroxylase.

TH+ cells within the graft exhibited a mature neuronal morphology with multiple elongated processes extending into the host striatum (Fig. 7C'). Moreover, we found that the mid-brain and A9 DA neuron markers Ptx3 and Girk2, respectively, were colocalized in a subpopulation of TH+ cells in the Nurr1+Foxa2-grafts (Fig. 7L, 7M), but not in the TH+ cells in the Nurr1-grafts. Our data collectively indicate that Foxa2 engineering potentiates the survival and function of Nurr1-induced DA cells after grafting. Therefore, manipulation of the Foxa2 gene may be an important component of a strategy to develop sources of donor cells for cell transplantation therapy in PD.

Effect of Foxa2 Expression on the Generation of DA Neurons from NPCs Derived from Diverse Brain Regions and Species

Finally, we wanted to investigate whether viral vector-mediated overexpression of Nurr1 and Foxa2 in NPCs reliably generates DA neurons regardless of the species origin and anatom-

ical region from which the donor cells are derived. Therefore, we examined whether overexpressing both Nurr1 and Foxa2 can induce DA neuron differentiation from rodent NPCs derived from different immature brain regions and human NPCs derived from hES cells. Should this be the case, it would greatly increase the utility of the approach when, for example, trying to generate DA neurons for transplantation in PD.

We found that Foxa2 transduction increased the yield of TH+ cells and TuJ1+ neurons ubiquitously in cultures derived from the ventral and dorsal parts of mouse fetal fore-brains, midbrains, hindbrains, and spinal cords (Supporting Information Fig. S3).

In contrast to earlier findings in mouse NPCs, Nurr1 alone without Foxa2 coexpression efficiently yielded TH+ DA cells in rat NPCs (Supporting Information Fig. S4 and [7–9]). Specifically, 3 days after differentiation, we found that up to 45% of Nurr1-transduced cells derived from the rat fetal cortex were positive for TH. When we coexpressed Foxa2, there was no further increase in the TH+ cell yield at high Nurr1 viral titers (12.5–25 MOI). However, as in the case of mouse

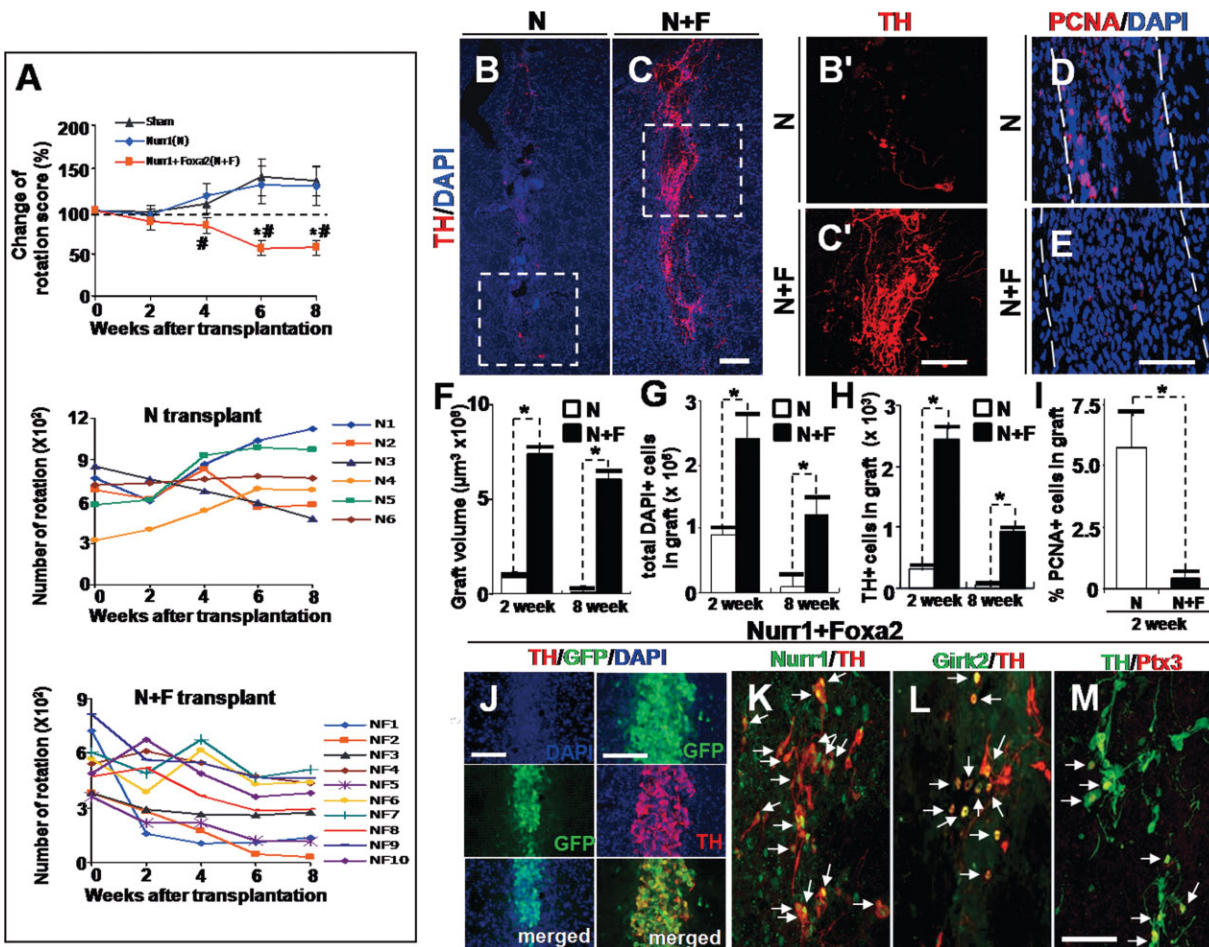


Figure 7. In vivo behavioral and histological analyses after transplantation. Neural precursor cells (NPCs) derived from rat cortices at E14 were transduced with Nurr1 (N) and Nurr1+Foxa2 (N + F) and transplanted into the striatum of hemiparkinsonian adult rat brains. (A): Behavioral recovery was assessed by amphetamine-induced rotation in sham-operated (PBS-injected, $n = 6$), animals grafted with NPCs transduced with N ($n = 6$) and N + F ($n = 10$). Graphs in the middle and lower panels present the absolute rotation numbers of the individual animals grafted with N- and N + F-cells, respectively. The graph in the upper panel depicts mean \pm SEM of % changes in rotation scores as compared with pretransplantation values. Significantly different from the respective sham-controls* at $p < .01$ and from N-grafted# at $p < .05$; one-way ANOVA with a post hoc Dunnett's test. (B, C): Representative confocal image stacks (35 μm in z) of the TH+ cells at 8 weeks after transplantation in the grafts generated by N- (B, B') and N + F (C, C')-transduced cells. The boxed areas in (B) and (C) are magnified in (B') and (C'), respectively. Shown in (D) and (E) are images for the proliferating cell marker PCNA+ cells at 2 weeks of post-transplantation. The borders of the grafts are indicated by dashed lines. Graphs (F–I) depict graft volume (F), total viable cells (G), number of TH+ cells (H), and % PCNA+ cells (I). *Significantly different from N-cell grafts at $p < .001$, Student's t -test, $n = 6$ (N) and 10 (N + F). (J): GFP-labeled donor cells in the grafts. Donor cells were labeled with GFP by transductions with N (Nurr1-IRES-GFP)+F and stained against GFP/DAPI (left) and GFP/TH (right) at 2 weeks of post-transplantation. (K–M): Midbrain and A9 nigral phenotype expressions of the TH+ DA cells in the grafts generated by N + F cell transplantation. N + F grafts were stained against TH/Nurr1 (K), TH/Girk2 (L), and TH/Ptx3 (M). Colabeled cells are indicated by arrows. Scale bars, 70 μm . Abbreviations: GFP, green fluorescent protein; N, Nurr1; N + F, Nurr1+Foxa2; PCNA, proliferating cell nuclear antigen; TH, tyrosine hydroxylase.

NPCs, the yield of TH+ cells increased when we coexpressed Foxa2 in rat NPC cultures transduced with low Nurr1 titers (1–2.5 MOI; Supporting Information Fig. S4a–S4c).

For a stem cell-based therapy to be clinically applicable to patients with PD it will require that human DA neurons can be generated. Consistent with a previous report [10], we found that transduction of Nurr1 alone in NPCs derived from hES cells (hES-NPCs) resulted in a low number of TH positive cells ($1.1 \pm 0.5\%$ of the Nurr1 transduced cells, Supporting Information Fig. S4d, S4g). In contrast, when we cotransduced hES-NPCs with Foxa2 (HA-tagged) and Nurr1 (Flag-tagged) we efficiently generated human TH+ neurons. Specifically, of the Flag+ cells (overexpressing Nurr1) we found that $32.9 \pm 4.5\%$ were TH+ (Supporting Information Fig. S4e, S4g) and of the HA+ cells (overexpressing Foxa2) $29.6 \pm 3.4\%$ were TH+ (Supporting Information Fig. S4f, S4g). Notably, in contrast to our observations in mouse NPC cultures,

Nurr1+Foxa2-mediated stimulation of DA neurons in hES-NPCs was highly dependent on the presence of dibutyryl cyclic AMP (cAMP) and 9-cis-retinoic acid (RA) in the culture medium. Moreover, the resulting TH+ cells did not exhibit the morphologic or phenotypic features of differentiated neurons. Instead they were relatively flat in shape and negative for other neuronal makers. Thus, whether expression of Nurr1+Foxa2 can induce human NPCs to become mature DA neurons needs to be further investigated.

DISCUSSION

We performed gain-of-function studies of Foxa2 in cultured NPCs and gained insight into how Foxa2 participates in the molecular processes that govern the development of DA

neurons. First of all, we found that Foxa2 promotes neuronal differentiation of NPCs. It promotes differentiation by expression of cell cycle arrest genes (p15 and p27) and Ngn2, which is a proneural bHLH gene specifically expressed in the developing ventral midbrain. However, most of the neurons induced by Foxa2 overexpression were nondopaminergic, and a few were immunopositive for TH. This finding is consistent with previous studies reporting that Ngn2 overexpression alone does not induce DA neurons in midbrain cultures [39–41]. We observed that Foxa2 promotes TH expression only in the subset of NPCs that express endogenous Nurr1. Furthermore, we found that Foxa2 and Nurr1 simultaneously overexpressed in NPCs promote TH expression in a synergistic fashion, which suggests that they cooperate during DA neuron differentiation. We further observed that Foxa2 directly binds to the TH promoter and dose-dependently activates TH gene transcription induced by Nurr1. In the NPCs, we also found that the Foxa2 expression induced other genes associated with midbrain DA neurons (even those specific to the A9 nigral subtype) as well as developmental genes specific to the embryonic midbrain. Therefore, the effects exerted by Foxa2 in NPCs differ markedly from what has been reported for Nurr1 previously. Nurr1 has previously been found to induce DA neuron generation in cultured cells, but without inducing a gene expression pattern that is typical of the midbrain [8, 42, 43]. The genes that we found to be controlled by Foxa2 overexpression include a set of developmental genes whose expression was previously found to decrease or disappear in the embryonic midbrain of Foxa2 null mice [14, 15]. In a preliminary web-based analysis (<http://www.cisreg.ca/cgi-bin/oPOSSUM/opossum>), Foxa2 has been shown to have great potential for directly targeting promoters of more than 13 genes associated with midbrain DA neuron development (i.e., TH, Ptx3, AADC, Lmx1b, Corin, VMAT2, Nurr1, Lmx1a, En1, Girk2, DAT, SHH, Ngn2; data not shown). Taken together, Foxa2 appears to be a master regulator of the midbrain DA neuron development responsible for orchestrating the expression of a diverse array of genes. Similarly, Lmx1a has been reported to act as a midbrain DA neuron determinant by inducing the expression of a series of genes, including Ngn2, as well as DA neuron- and midbrain-specific factors [44, 45]. In addition, overexpression of Lmx1a was reported to induce DA neuron generation in vitro and in vivo. However, the competence of Lmx1a to induce DA neurons is limited to the ventral midbrain or in vitro condition supplemented with SHH. This suggests that direct SHH signaling, or an as yet unidentified SHH-induced activity, may act in parallel to Lmx1a to promote DA neuron induction in the cells. Furthermore, we have found that overexpression of Lmx1a alone, or in a combination with Nurr1, does not promote DA neuron differentiation in rat ventral midbrain-derived precursors (data not shown and [39]). In contrast, we found Foxa2 to exhibit a brain region- and species-independent ability to induce differentiation of NPCs into neurons, and in particular DA neurons. Foxa2 strongly induces SHH expression in cortical cultures in which SHH is normally not detected (Fig. 6O). The capacity of Foxa2 to induce SHH is possibly an important reason why the actions of Foxa2 are so ubiquitous in precursors derived from different brain regions.

Interestingly, when we overexpressed Foxa2 in cultured NPCs, it reduced their susceptibility to toxic stimuli, that is, MPP+ and H₂O₂. Kittappa et al. recently reported that mice carrying only one copy of the Foxa2 gene develop a progressive loss of DA neurons and behavioral abnormalities reminiscent of PD in old age [15]. Taken together, the loss of one Foxa2 allele might result in haploinsufficiency, leading to a

reduced capacity to withstand endogenous cell stress with progressive nigral cell loss as the final outcome. There are several possibilities for how Foxa2 might impart enhanced survival. First, SHH, expression of which is induced by Foxa2, could mediate improved cell survival. Previous studies have shown that SHH can promote the survival of NPCs and DA neurons both in vitro and in vivo [36, 46–48]. Second, a neuroprotective pathway involving Ngn2, which is another target of Foxa2, is also implicated. We recently demonstrated that Ngn2 not only enhances neurogenesis but also promotes survival of NPCs when they undergo differentiation in vitro and in vivo [36]. In that study, we observed that there was a gradual increase in NT3 levels during differentiation of NPCs overexpressing Ngn2. We proposed that the NT3 secretion in turn promotes cell viability. In the present study, Foxa2 induced expression of the NT3 gene. Furthermore, the positive effects of Foxa2 on cell survival were particularly robust during late stages of differentiation, when the NT3 expression is likely to have reached its peak. Therefore, we now suggest that a Foxa2-Ngn2-NT3 cascade may at least partially account for the improved survival of differentiated neurons. Because we also found that Foxa2 caused an upregulation of BDNF and GDNF, they too contributed to the improved survival of neurons derived from the genetically modified NPCs. Finally, the Foxa2-induced survival effect may also be due to induction of Nurr1 and Ptx3, two additional genes that we found to be positively regulated by Foxa2 in the NPCs. It is well-established that deletions of Nurr1 and Ptx3 negatively affect midbrain DA neuron survival and maintenance [49, 50]. We should emphasize that the possible explanations for enhanced survival of Foxa2-transduced cells are not mutually exclusive. Indeed, the overall survival effect may represent the combined contribution of all of the aforementioned mechanisms.

The effects of Foxa2 that we observed in vitro indicate that this factor has multiple functions in midbrain DA neurons. We found that Foxa2 increases the yield of midbrain DA neuron yield from culture NPCs. Specifically, it promoted neuronal maturity and cell type-specific functions of DA neurons when it was coexpressed with Nurr1. Furthermore, the neurons generated as a result of Foxa2 overexpression displayed the A9 midbrain phenotype and, for example, expressed both Ptx3 and Girk2. It has been suggested that these are the only type of DA neurons that successfully integrate into the host striatum after transplantation in rat models and patients [51, 52]. The effects of Foxa2 on cell survival that we describe are potentially very important to the field of neural transplantation in PD. Poor survival of grafted DA neurons is probably one of the major roadblocks to the success of clinical trials in PD [1]. Consistent with our in vitro findings, 2 and 8 weeks after grafting we found that NPCs transduced with both Foxa2 and Nurr1 yielded at least 14-fold more DA neurons than those overexpressing Nurr1 alone. The surviving TH-immunoreactive cells resembled morphologically mature neurons, and a subpopulation of them expressed the midbrain and nigral DA markers Ptx3 and Girk2. In agreement with these results, we found that rotational behavior was ameliorated in 6-OHDA-lesioned rats grafted with Foxa2/Nurr1-expressing cells, but not in rats implanted with Nurr1-expressing cells. Furthermore, consistent with our findings that Foxa2 caused cell cycle arrest in vitro, the grafts of Foxa2-expressing cells contained drastically fewer dividing (PCNA-positive) cells than those derived from cells transduced with Nurr1 alone. The risk of tumor formation is a potential hazard of cell replacement therapy, especially if the donor tissue is derived from ES cells [53]. Our results suggest that Foxa2 overexpression is a valid strategy to reduce cell proliferation

in dividing stem/progenitor cells, and could be tested in intracerebral transplants of ES-derived cells.

Another important result of our study is that when we overexpressed *Foxa2* together with *Nurr1* we partially overcame the previously observed species-dependent limitations of *Nurr1* activity to induce DA neuron formation, in which no TH+ cells were generated from *Nurr1*-overexpressed human cells [10]. Similar to rodent cultures, the combined exogene expressions in hES-NPCs also efficiently yielded TH+ cells. The human TH+ cells generated by the exogene expressions, however, were immature neurons, and the yield and maturity of the TH+ cells in the human cultures were highly sensitive to culture conditions. In our further continued experiments, we managed to partially enhance the maturity (judged by morphology) of *Nurr1*+*Foxa2*-induced TH+ cells derived from hES-NPC cultures (data not shown) by adjusting viral titers and increasing the differentiation period. In addition, we observed that overexpression of *Foxa2* in hES-NPC cultures increased cell survival following exposure to the toxins H₂O₂ and MPP+, like those observed in rodent cells (data not shown). These preliminary findings in hES-NPCs indicate that we may eventually be able to complete a full scientific account of the effects of *Nurr1*+*Foxa2* in human-derived neural stem cells. Therefore, our current findings highlight *Foxa2* overexpression as a potentially useful strategy when trying to generate DA neurons from human stem cells for use in transplantation trials in PD. Furthermore, enhancing *Foxa2* gene

expression might be an interesting strategy to inhibit degeneration and promote survival of endogenous DA neurons in PD.

CONCLUSION

In conclusion, our results indicate that a *Foxa2* gene overexpression-based strategy may help increase the efficacy, safety and functionality of future stem cell-based therapy for PD.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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