Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype

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Midbrain dopamine (DA) neurons play a central role in the regulation of voluntary movement, and their degeneration is associated with Parkinson's disease. Cell replacement therapies, and in particular embryonic stem (ES) cell-derived DA neurons, offer a potential therapeutic venue for Parkinson's disease. We sought to identify genes that can potentiate maturation of ES cell cultures to the midbrain DA neuron phenotype. A number of transcription factors have been implicated in the development of midbrain DA neurons by expression analyses and loss-of-function knockout mouse studies, including Nurr1, Pitx3, Lmx1b, Engrailed-1, and Engrailed-2. However, none of these factors appear sufficient alone to induce the mature midbrain DA neuron phenotype in ES cell cultures *in vitro*, suggesting a more complex regulatory network. Here we show that Nurr1 and Pitx3 cooperatively promote terminal maturation to the midbrain DA neuron phenotype in murine and human ES cell cultures.

differentiation | Parkinson | transplantation

Several transcription factors have been implicated in the regulation of mammalian midbrain dopamine (DA) neuron development. Expression of the orphan nuclear receptor transcription factor Nurr1 is initiated in postmitotic midbrain DA neuron precursors at embryonic day 10.5 in the mouse, just preceding expression of tyrosine hydroxylase (TH) (1). Nurr1 is expressed broadly at subsequent stages of development. Nurr1-deficient animals fail to express TH in midbrain DA neurons, but other midbrain DA neuron markers such as Pitx3, Lmx1b, and En1 remain unaltered during development (2–4). Thus, Nurr1 is not required for specification of the early midbrain DA neuron cell fate but does regulate a subset of phenotypic markers. At later stages, Nurr1-deficient mice lack expression of markers for midbrain DA neuron maturation, such as the DA transporter (DAT), which is relatively specific to this cell population.

Expression analyses and loss-of-function knockout mouse studies have revealed roles for additional transcription factors in the specification and maturation of midbrain DA neurons, including the homeodomain proteins Lmx1b (5), Pitx3 (6–9), and En1 (10, 11). None of these factors are uniquely expressed in midbrain DA neurons, nor are they required for TH expression. However, each of these factors is necessary for the complete maturation and survival of the midbrain DA neuron population.

Overexpression of Nurr1 alone in neuronal cell lines (12), primary neuronal precursor cells (13), or embryonic stem (ES) cultures (14, 15) appears to promote the expression of a subset of midbrain DA neurons markers *in vitro*, including TH, but this may reflect a broad proneural activity (16). Pitx3 overexpression alone in undifferentiated ES cultures or in neuron progenitor cells is not sufficient to induce a mature midbrain DA neuron phenotype but may promote expression of a subset of markers (13, 17). Thus, we hypothesized that multiple transcription factors may collaborate within a network to induce late events in midbrain DA neuron maturation. Here we show that Nurr1 and Pitx3 synergistically promote terminal maturation to the midbrain DA neuron phenotype in murine and human ES cell cultures.

Results

Nurr1 and Pitx3 Induce Murine ES Cell Differentiation to the Midbrain DA Neuron Phenotype. We sought to investigate interactions among the transcription factors implicated in midbrain DA neuron development using an ES cell *in vitro* differentiation assay. Murine MM13 ES cell cultures were differentiated *in vitro* by an embryoid body (EB) protocol, as described in ref. 18. Briefly, differentiation is initiated by growth in suspension as EBs, followed by maintenance in defined adherent culture conditions that leads to the expansion of neuronal precursors and subsequent maturation. These culture conditions recapitulate the *in vivo* temporal expression pattern of midbrain DA neuron developmental markers (see the supporting information, which is published on the PNAS web site).

Lentiviral expression vectors (19) that harbor Nurr1, Pitx3, Lmx1b, En1, or control vector alone were introduced at the neural precursor stage, approximating the normal temporal expression of the endogenous genes. Transgene expression with lentiviral transduction was observed in >95% of cells (data not shown). Surprisingly, the combined transduction of Nurr1 and Pitx3 dramatically and synergistically induced expression of the late marker, DAT, but not the earlier marker TH, as quantified by real-time RT-PCR (Fig. 1*A*). When transduced individually, only En1 appeared to significantly induce expression of DAT, but we chose to focus our attention on the more robust and synergistic action of Nurr1 and Pitx3.

Similar findings were obtained with a second independent murine ES cell line, DY-1, that expresses a midbrain DA neuron specific fluorescent marker, enhanced yellow fluorescent protein (*EYFP*), under the regulation of the Cre recombinase gene "knocked-in" to the DAT locus (20). Nurr1 and Pitx3 cotransduction coordinately increased the number of *EYFP*-positive cells in DY-1 cultures (Fig. 1*B*) or of *DAT*-positive cells in MM13 cultures (Fig. 1*C*; see supporting information). In contrast, Nurr1 alone increased expression of the earlier midbrain DA neurons marker, *TH* (Fig. 1 *A–C*), consistent with prior reports of a direct role for Nurr1 in the induction of *TH* expression (13, 14, 21). Most (>90%) of the *EYFP*-positive cells in the Nurr1 and

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Abbreviations: DA, dopamine; DAT, DA transporter; EB, embryoid body; ES, embryonic stem; EYFP, enhanced yellow fluorescent protein; 6-OHDA, 6-hydroxydopamine; SDIA, stromal derived induction activity; TH, tyrosine hydroxylase.

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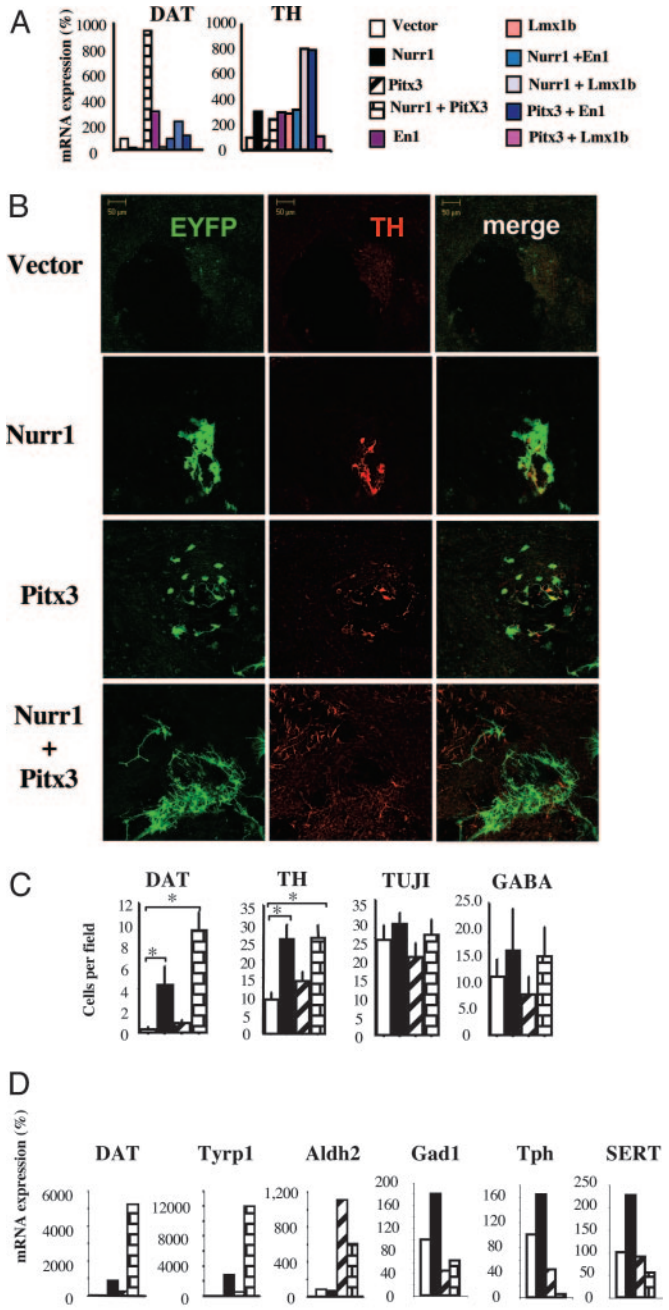

Fig. 1. Nurr1 and Pitx3 coordinately induce the maturation of murine ES cells to the midbrain DA neuron phenotype. (*A*) Quantitative real-time RT-PCR analysis of midbrain DA neurons markers TH and DAT in EB-differentiated MM13 ES cells transduced with lentiviral vectors as indicated. RT-PCR levels are presented as a percentage of the control vector alone after normalization with β-actin levels. (*B*) Immunocytochemical analysis with antibodies specific for EYFP and TH on EB-differentiated DY-1 ES cells transduced with control vector only, Nurr1, Pitx3, or Nurr1 and Pitx3 lentiviral vectors. (Scale bar, 50 μm.) (*C*) Marker expression was quantified in EB-differentiated, virally transduced MM13 ES cultures by immunohistochemistry with specific antibodies and confocal microscopy. Data are presented as mean ± SEM and were analyzed by using Fisher's test ANOVA. *, $P < 0.05$. (*D*) RT-PCR analysis for markers of midbrain DA neurons (DAT, Tyrp1, and Aldh2) and of GABAergic (Gad1) and serotonergic [tryptophan hydroxylase (Tph) and the serotonin transporter (SERT;Slc6a4)] neuron markers.

Pitx3 cotransduced cultures also stained for *TH*, as expected, although the relative intensity of *DAT* staining did not correlate with the intensity of *TH* staining. These data were further corroborated by FACS analysis using a fluorescent ligand specific for the DAT, JHC 1–064 (22). Nurr1 and Pitx3 cotransduction increased the percentage of DAT-positive neurons ≈3-fold over vector-transduced cells, from 15% to 45% (see supporting information).

Analyses of additional markers for midbrain DA neurons in the EB-differentiated cultures, as well as markers for other cell phenotypes, indicated that Nurr1 and Pitx3 specifically instruct midbrain DA neuron maturation. Thus, co-overexpression of Nurr1 and Pitx3 induced late midbrain DA neuron maturation markers such as *DAT* and tyrosinase-related protein 1 (Tyrp1) (Fig. 1*D*) (23). In contrast, earlier phenotypic markers of midbrain DA neurons such as TH and aldehyde dehydrogenase-2 (Aldh2) appear induced by Nurr1 or Pitx3 alone, respectively (Fig. 1 *A* and *D*). Global gene expression profiling of EB-differentiated cultures transduced with Nurr1 and Pitx3 using oligonucleotide microarrays indicated that the maturation markers DAT and Tyrp1 are among the most highly induced genes in cells transduced with both Nurr1 and Pitx3 (within the top 0.1% of annotated genes induced by the combined expression of Nurr1 and Pitx3; see supporting information).

Markers for other neuronal cell types, such as GABAergic neurons (glutamic acid decarboxylase 1, Gad1) and serotonergic neurons [tryptophan hydroxylase, Tph; serotonin transporter, SERT (Slc6a4)], are not induced by Nurr1and Pitx3 overexpression (Fig. 1 *C* and *D*). Similarly, Nurr1 and Pitx3 overexpression did not alter the overall number of neurons, as determined by the neuron marker class III β-tubulin (*TUJI*) immunostaining (Fig. 1*C*) or total cell number (data not shown). En1 and Lmx1b, two transcription factors that are normally expressed early at the midbrain–hindbrain junction and serve as markers for the identity of this region, are expressed in the majority of EB-differentiated cells and appear unaltered by Nurr1 and Pitx3 transduction (see supporting information). Neuronal survival and apoptosis, as quantified by staining with the Annexin V and propidium iodide, is unchanged in the transduced cells (data not shown).

Nurr1 and Pitx3 in Human ES Cell Differentiation to the Midbrain DA Neuron Phenotype. To determine whether the synergistic action of Nurr1 and Pitx3 represents a general mechanism for the induction of midbrain DA neuron maturation, we extended our analyses to human ES cell cultures. Prior studies have described the differentiation of human ES cultures into TH-positive cells *in vitro* (24, 25), but the roles of transcription regulatory factors have not been explored. Human H9 ES cells were differentiated by coculture with bone marrow-derived stromal cells [termed stromal derived induction activity (SDIA)] and subsequently replated in defined media in the absence of stromal cells to induce differentiation as described in refs. 24, 26, and 27. At the neural precursor stage of the SDIA protocol, the cells were transduced with lentiviral vectors that harbor Nurr1 and Pitx3, both together, or control vector alone (GFP). The cells were then cultured for 2 additional weeks and analyzed for the expression of midbrain DA neuron maturation markers by RT-PCR and immunohistochemistry.

The combination of Nurr1 and Pitx3 effectively promoted the maturation of midbrain DA neurons derived from human ES cells, as quantified by RT-PCR analysis of the expression of midbrain DA markers including DAT, TH, the vesicular monoamine transporter 2 (Vmat2), and dopa decarboxylase (Ddc) (Fig. 2*A*). Nurr1 and Pitx3 cotransduction reduced the expression of non-dopaminergic neuronal fate markers, such as glutamic acid decarboxylase (Gad1), a marker for GABAergic neurons, in the human ES cultures.

Nurr1 and Pitx3 cotransduction of SDIA differentiated human ES cultures led to an increase in the number of TH-positive cells relative to control vector transduction, whereas overall neuron

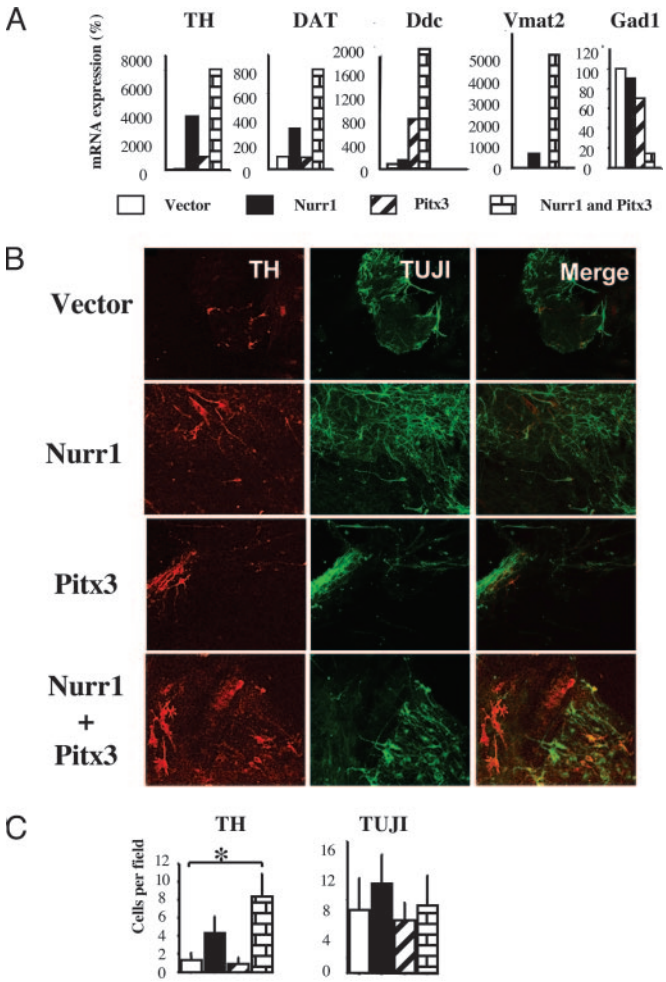

Fig. 2. Synergistic activity of Nurr1 and Pitx3 in the maturation of human H9 ES cultures. (*A*) Human ES cells (H9) were differentiated by using the SDIA method and transduced at the neural precursor stage with control vector (GFP), Nurr1 alone, Pitx3 alone, or Nurr1 plus Pitx3. Nurr1 plus Pitx3 coordinately induce midbrain DA neurons maturation as quantified by the expression of DA neuron markers such as DAT, TH, dopa decarboxylase (Ddc), and vesicular monoamine transporter-2 (Vmat2) using RT-PCR. GABAergic marker (Gad1) expression appears reduced by Nurr1 plus Pitx3 cotransduction. (*B* and *C*) Immunostaining with antibodies for TH and TUJI in virally transduced H9 ES cell cultures. Cell numbers were quantified in 10 random fields for each sample by confocal microscopy. Data are presented as mean ± SEM and were analyzed by using Fisher's test (ANOVA). *, $P < 0.05$. (Scale bar, 50 μm.)

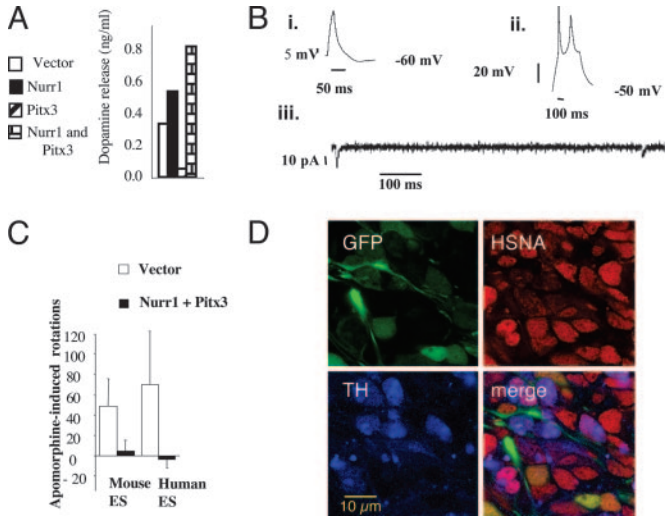

Fig. 3. Functional analysis of Nurr1 plus Pitx3-transduced ES cultures. (*A*) DA release from SDIA-differentiated human ES cells is potentiated by Nurr1 plus Pitx3 overexpression. DA release was quantified by HPLC analysis of extracellular solution after depolarization with elevated potassium media (56 mM) for 15 min. (*B*) SDIA-differentiated, Nurr1 plus Pitx3-transduced cultures display neuronal characteristics including depolarization-induced action potentials (*i*), spiking behavior (*ii*), and miniature excitatory postsynaptic currents (*iii*) (in the presence of 500 μM tetrodotoxin). Whole-cell patch clamp recordings were conducted in current clamp mode. (*C*) Functional rescue of 6-OHDA lesioned mice by transplantation of human or mouse ES-derived cultures transduced with Nurr1 plus Pitx3. Adult mice were lesioned by 6-OHDA injection unilaterally into the striatum and, 2 weeks later, transplanted with either human or mouse ES cells (1×10^5 cells) at the neural precursor stage (see *Materials and Methods*) that have been transduced with Nurr1, Pitx3, and GFP lentiviral vectors ($n = 5$ mouse ES cells; $n = 3$ human ES cells) or control GFP alone (mouse ES cells $n = 4$; human ES cells $n = 3$). Six weeks later, rotatory behavior was quantified in response to apomorphine (0.4 mg/kg) over a 30-min period. Data were statistically analyzed by using Fisher's test (ANOVA). (*D*) Engraftment of the human ES-derived transplants was assessed by immunostaining of striatal sections with antibodies for TH and the human-specific nuclear antigen (HSNA) followed by confocal microscopy.

number appeared unaltered, as quantified by *TUJI* immunostaining (Fig. 2 *B* and *C*). Similar results were observed with Nurr1 and Pitx3 cotransduction in the context of EB differentiation of human H9 cells (see supporting information). Surprisingly, the human ES-derived, *TH*-positive cells appeared morphologically heterogeneous, with the majority of cells coexpressing *TUJI* and exhibiting a typical polarized neuronal morphology, but other cells (≈25%) that appear broad or spindle-shaped and lack *TUJI* staining (Fig. 2*B*). TH-expressing, nonneuronal cells have previously been described in the context of Nurr1 overexpression in murine ES cultures (15), but the significance of these cells is unclear.

We sought to test functional neuronal properties of the human ES-derived cultures. SDIA differentiated H9 human ES cultures transduced with Nurr1 and Pitx3 display potentiated evoked DA release, relative to vector-transduced cells, in the context of elevated extracellular potassium (Fig. 3*A*). Electrophysiological analyses of the differentiated H9 ES cultures were performed by using patch electrodes and current clamp techniques. These studies indicated that the ES-derived cells display basic neuronal characteristics, including depolarization-induced action potentials, burst firing, and miniature spontaneous excitatory postsynaptic currents (Fig. 3*B*). The Nurr1- and Pitx3-transduced cells did not differ from the control vector-transduced cells in terms of basic neuronal excitability properties (data not shown), consistent with a specific role for Nurr1 and Pitx3 in induction of the DA neuron phenotype.

Transplantion of Nurr1 and Pitx3 Overexpressing ES Cells. To investigate further the function of Nurr1- and Pitx3-transduced ES-derived cultures *in vivo*, mouse or human ES cultures were differentiated to the neural precursor stage (see *Materials and Methods*) and transplanted into adult mice that had been lesioned with a unilateral intrastriatal injection of the DA neuron toxin 6-hydroxydopamine (6-OHDA). In the absence of transplanted cells, lesioned animals display a characteristic contralateral turning behavior (away from the side of the lesion) when exposed to the DA receptor agonist apomorphine as a result of dennervation-induced hypersensitivity on the lesioned side (28). Transplantation of human or mouse cells transduced with Pitx3 and Nurr1 led to a significantly greater reduction in this turning behavior than did control vector-transduced cells at 6 weeks after cell grafting (Fig. 3*C*). Lesioned animals that received

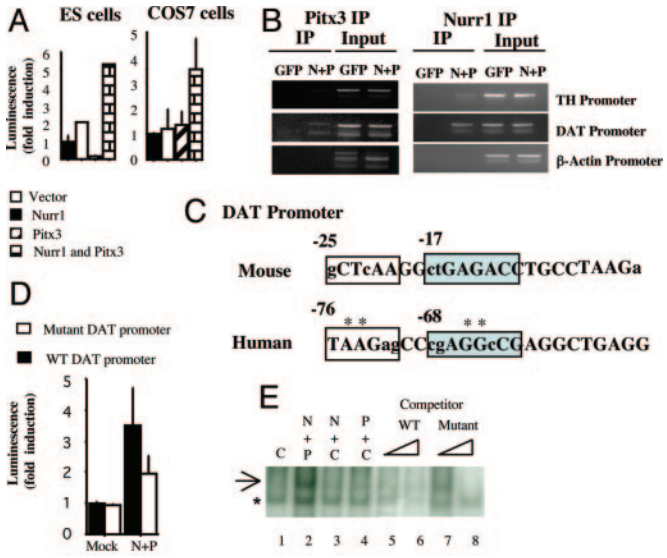

Fig. 4. Nurr1 and Pitx3 cooperatively activate transcription of DAT promoter sequences. (*A*) Nurr1 plus Pitx3 cooperatively induce luciferase expression under the control of DAT gene regulatory sequences. EB-differentiated MM13 ES cells or COS7 cells were transduced with Nurr1 plus Pitx3 vectors and transfected with luciferase test plasmids. (*B*) Chromatin immunoprecipitation (IP) assays were performed on MM13 ES cell lysates by using an antibody to a FLAG epitope tag at the amino terminus of Nurr1 or to native Pitx3 followed by PCR for proximal promoter sequences. Lysates expressing Nurr1 and Pitx3 (N+P) but not control (GFP) bound proximal sequences in the DAT and TH promoters, but not to sequences in the β-actin promoter. (*C* and *D*) Analysis of murine and human DAT promoter sequences identify adjacent Nurr1 (blue box) and Pitx3 (white box) binding sites within the proximal DAT promoter. (*D*) Mutation analysis of DAT promoter activity in the luciferase assay. Induction of luciferase expression by Nurr1 and Pitx3 of wild-type (WT) (8.3 kb) but not mutant (as indicated by asterisks in *C*) DAT promoter in COS7 cells. (*E*) Nurr1 and Pitx3 bind cooperatively to DAT promoter sequences. EMSA analyses were performed by using *in vitro* transcription/translation reticulocyte lysate extracts expressing control vector (lane 1; C), Nurr1 and Pitx3 (lanes 2 and 5–8; N+P), Nurr1 plus control vector (lane 3; N+C), or Pitx3 plus control vector (lane 4; P+C). Nurr1 and Pitx3 extracts bound to complementary oligonucleotide sequences from the human DAT promoter (arrow). DNA–protein complexes were inhibited by competition with unlabeled wild-type (WT) complementary oligonucleotides (lane 5, 3-fold excess unlabeled; lane 6, 10-fold excess) to a greater extent than with mutant oligonucleotides (lane 7, 3-fold excess; lane 8, 10-fold excess). An additional band is observed in all lanes (asterisk).

either mouse or human ES-derived cells performed comparably in the rotatory behavior assay.

Immunohistochemical analysis of brain sections with an antibody to human-specific nuclear antigen (HSNA) confirmed the presence of engrafted human ES-derived cells at the site of transplantation (Fig. 3*D*). However, cells within the core of the graft were tightly packed and appeared to lack normal neuronal process morphology. A low level of TH immunoreactivity could be detected within the soma of transplanted cells transduced with Nurr1 and Pitx3 (45% of HSNA-positive cells) (Fig. 4*D*), but this was not significantly different from transplanted control vector-transduced cells. These data are consistent with prior transplantation studies that indicate limited maturation of engrafted human ES-derived cells (29–31).

Of note, at late time points (8 weeks after cell grafting), three of the mice that received mouse ES cell transplants developed apparent teratoma-like masses at the implantation site (one transplanted with Nurr1 and Pitx3-transduced cells, and two with control virus-transduced cells), consistent with a prior study using murine mouse ES cell cultures (32). This finding likely reflects the presence of undifferentiated cells. No teratomas were observed with the human ES cell transplantation studies.

Evidence for Cooperative Transcription Activation by Nurr1 and Pitx3. We hypothesized that the cooperative activity of Nurr1 and Pitx3 in midbrain DA neuron maturation may be a consequence of cooperative transcriptional activation at midbrain DA neurons-specific genes. Indeed, Nurr1 has previously been implicated in the direct activation of DAT transcription (33). To quantify the activities of Nurr1 and Pitx3 on DAT gene regulatory sequences, a luciferase assay plasmid that harbors an 8.3-kb region upstream of the human DAT gene was transfected into cells overexpressing either Nurr1, Pitx3, both, or neither. Nurr1 and Pitx3 cotransduction led to significantly higher levels of luciferase expression than either factor alone in EB-differentiated mouse ES cultures or COS7 monkey kidney cells (Fig. 4*A*). Chromatin immunoprecipitation with antibodies to Flag epitope-tagged Nurr1 or untagged Pitx3 (Fig. 4*B*) indicated that these factors bind directly to proximal regions of the endogenous DAT and TH upstream regulatory sequences *in vivo*.

Sequence analysis of the promoter region of several midbrain DA neuron-specific genes, including DAT (Fig. 4*C*) as well as Tyrp1 and TH (data not shown) indicated the presence of adjacent putative binding elements for Nurr1 and Pitx3, suggesting a cooperative activation mechanism. Consistent with this observation, mutagenesis of these adjacent sites in the human DAT promoter largely abrogated promoter activation by Nurr1 and Pitx3 in COS7 cells (Fig. 4*D*) and MM13 ES cultures (data not shown). Finally, gel-shift assays with *in vitro*-translated protein extracts show that Nurr1 and Pitx3 bind cooperatively to oligonucleotides that harbor adjacent putative binding sites from the proximal DAT promoter (Fig. 4*E*). Mutagenesis of conserved nucleotides within these sites inhibits competition. Taken together, these data strongly support the notion that cooperative DNA binding by Nurr1 and Pitx3 underlie the induction of midbrain DA neurons maturation genes, and DAT in particular. Of note, cooperativity between an orphan nuclear receptor and a paired-like homeodomain protein has previously been described: Fushi Tarazu factor 1 (Ftz-F1) interacts with Fushi Tarazu (Ftz) in the context of embryonic segmentation in *Drosophila* (34, 35), and this interaction is conserved in vertebrate species (36).

Discussion

Our data, taken together with loss of function gene knockout studies in rodents (2–4, 6–9), indicate that Pitx3 and Nurr1 cooperatively induce the late maturation of midbrain DA neurons. This cooperativity offers a potential mechanism for the relatively cell-type-specific expression of late markers of midbrain DA neurons maturation. Thus, neither Nurr1 nor Pitx3 expression is strictly confined to midbrain DA neurons: Nurr1 is expressed broadly in the brain during late development as well as in other tissues, whereas Pitx3 is also expressed in the eye. The region of expression overlap, however, is confined to midbrain DA neuron.

We describe a network of transcription factors that cooperatively promote the maturation of ES cultures to the midbrain DA neurons phenotype. Prior studies have provided evidence that Nurr1 alone can promote an earlier stage in the differentiation of murine ES cultures (14, 37). We extend this work to show that Nurr1 and Pitx3 together induce the expression of a later maturation step. Also, we demonstrate the feasibility of genetic modification of human ES cultures to promote the generation of midbrain DA neurons. We posit that coexpression of Nurr1 and Pitx3 may significantly enhance the efficacy of cell-replacement therapies for Parkinson's disease, and in particular human ES-derived cell approaches.

It is of particular interest to extend the *in vitro* analysis of Nurr1 and Pitx3 cotransduced human ES cultures to *in vivo* transplantation models. Prior studies have reported limited success with ES-derived grafts (29–31); Ben-Hur *et al.* reported significant functional rescue in a 6-OHDA rat lesion model, but the percentage of TH-positive cells in the grafts were low. We find that Nurr1 and Pitx3 cotransduced H9 ES cells can ameliorate pathological rotatory behavior in 6-OHDA unilaterally lesioned mice, but DA neuron maturation appears incomplete in the grafts, suggesting that additional inductive factors may be necessary *in vivo*. Alternatively, an inhibitory factor for DA neuron maturation or survival may exist in the adult striatum. Additional studies are needed to address the limited engraftment of human ES cultures in transplantation models.

Materials and Methods

Generation of DY-1 ES Cells. Homozygous ''knock-in'' mice that harbor the Cre recombinase gene at the *DAT* locus were bred with mice in which a Cre-inducible EYFP fluorescent marker gene was ''knocked-in'' at the ROSA26 locus (38–40) to obtain double-transgenic blastocysts. An ES cell line (DY-1) was derived from these blastocysts by using standard techniques (41). This cell line was demonstrated to be totipotent by injection into blastocysts and germline transmission (data not shown).

ES Cells Culture and *in Vitro* Differentiation. Mouse ES cells (MM13 or DY-1) were propagated and differentiated as described in refs. 18 and 42. Human ES cell line H9 (passages 30–45) was cultured on mitotically inactivated mouse embryonic fibroblasts (MEF) (Specialty Media). Undifferentiated hES cells were maintained under growth conditions and passaging techniques as described in ref. 24. Differentiation of hES cells into DA neurons was performed by using the SDIA method (24) (see supporting information).

Recombinant Lentiviral Vectors. The recombinant lentiviral vectors are described in refs. 19 and 42–44. Nurr1 and Pitx3 cDNAs were cloned by standard PCR methods from a human cDNA library. Lmx1b was cloned from a chick cDNA library. For the viral transduction, single cell-suspensions (1×10^5 cells) were infected at a multiplicity of infection of 1:1.

Immunocytochemistry and *in Situ* Hybridization. Cultured cells were fixed in 4% paraformaldehyde in PBS and were incubated with the primary antibodies at 4°C for 12 h. The following primary antibodies were used: mouse anti-TUJ1 (Covance; dilution 1/500), rabbit anti-TH (Pel-Freez; dilution 1/1,000), sheep anti-TH (Pel-Freez; dilution 1/1,000), rat anti-DAT (Chemicon; dilution 1/500), rabbit anti-GABA (Chemicon; dilution 1/1,000), rabbit anti-5HT (Sigma; dilution 1/5,000), and rabbit anti-GFP (Molecular Probes; dilution 1/1,000). Appropriate FITC and Cy3- and Cy5-labeled secondary antibodies (Jackson ImmunoResearch) were used. *In situ* hybridization was performed by using Lmx1b and Engrailed-1 probes as described in ref. 45.

Quantitative Real-Time RT-PCR. Total RNA from ES cells differentiated was prepared by using the absolutely RNA Miniprep kit (Stratagene). cDNA was synthesized by using SuperScript (Invitrogen). RT-PCRs were optimized to determine the linear amplification range by using a Stratagene MX3000P system with QuantiTect PCR mix (Qiagen).

HPLC and Electrophysiology Analysis. Extracellular DA levels were measured by HPLC as described in ref. 42.

Human ES cells cultured as described were used for recording under ruptured whole-cell voltage and current clamp modes by using standard techniques. The miniature spontaneous excitatory postsynaptic currents were recorded under voltage clamp (holding potential of −60 mV) in the presence of 0.5 mM tetrodotoxin in the bath solution, and the action potential and spontaneous firing were recorded under current clamp. Action potentials were induced by a brief depolarization step (50–150 pA, 100 ms).

6-OHDA Lesioning, Transplantation, and Behavioral Analysis. Procedures involving animal care were in conformity with the Columbia University Animal Protocols and in compliance with the guidelines of the National Institutes of Health. Adult male CD-1 mice (6–8 weeks; Charles River Breeding Laboratories) were anaesthetized with ketamine and xylazine (60 mg/kg and 10 mg/kg, respectively) and placed in a stereotactic frame (Stoelting). The DA denervation was achieved by stereotactic injection of 6-OHDA (4 μl; 2 mg/ml in normal saline with 0.02% ascorbic acid; Sigma) in the left striatum (anterior 1 mm; lateral 2.2 mm; ventral 3 mm, as determined from the bregma and the skull surface). Turning behavior before the cell transplantation was 187.9 ± 62.55 turns per 30 min.

Stage 3 EB-differentiated human or mouse ES cells were transduced with GFP or Nurr1/Pitx3/GFP lentiviruses and then injected (1×10^5 cells per μl) in the striatum. Apomorphine-induced turning behavior was assessed at 2 weeks after the 6-OHDA injection and before grafting, and again 6 weeks after the cell grafting (28). Mice were placed in hemispheric bowls and left for 20 min to habituate to the new environment. Apomorphine was injected s.c. (0.4 mg/kg). Mice were videotaped, and the number of turns was counted over a 30-min period by an independent observer blinded to the experimental design. One animal that had been transplanted with control vector-transduced mouse ES cells displayed severe contralateral barrel rotations in response to 0.4 mg/kg apomorphine (consistent with the apparent low efficacy of the control cells), but these could not be accurately quantified in this assay, and this animal was therefore not included in the statistical analysis. Additionally, in two animals transplanted with human ES cells (1 GFP and 1 Nurr1/Pitx3), the stereotaxic surgery was not successful and no engraftment was visualized by GFP fluorescence; these were not included in further analyses. Six weeks after transplantation, animals were killed and perfused with 10 ml of saline followed by 35 ml of a 4% solution of paraformaldehyde. Brains were extracted and immunostained as described in ref. 42.

Luciferase Assays, Chromatin Immunoprecipitations, and Gel-Shift Analysis. For luciferase assays, cells were transfected using Lipofectamine (Invitrogen) with a firefly luciferase assay plasmid harboring either DAT regulatory sequences or control along with a Renilla luciferase vector as internal control. Forty-eight hours after transfection, cell extracts were analyzed for firefly luciferase activity and normalized with Renilla luciferase activity (Promega), as per the manufacturer's instructions. Chromatin immunoprecipitation analyses were carried out as described in ref. 46. DNA–protein complexes were immunoprecipitated by using agarose-conjugated anti-Flag antibodies (Sigma) or an antibody to Pitx3 (Chemicon). Electrophoretic mobility-shift assays (EMSA) were performed by using an EMSA kit (Novagen) as per the manufacturer's instructions.

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