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Tyrosine Hydroxylase Gene Regulation in Human Neuronal Progenitor Cells Does not Depend on Nurr1 as in the Murine and Rat Systems

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Abstract

A previous study on the human tyrosine hydroxylase (TH) promoter revealed remarkable differences in the mechanism of TH gene regulation between the human and murine models. Indeed, a low degree of homology was observed in the sequence of TH promoters among human, mouse, and rat systems. Only five short conserved regions (CRs) could be identified among the three species. A human TH minimal promoter was engineered and assembled into a self-inactivating lentiviral vector system. This human TH minimal promoter contained the five CRs plus the first -194 bp from the transcription start of the human TH promoter and the first 35 bp of the untranslated messenger RNA leader of the human *TH* gene. A significant degree of specificity for this human TH minimal promoter was observed only for human neuronal progenitor cells (hNPCs), but not for TH-positive differentiated mouse primary striatal and *substantia nigra* cells, indicating a significant difference in TH gene regulation between the human and mouse systems. Not only is the degree of homology between the human and mouse promoters in the range of only 46%, but also those few elements that share a high degree of homology display totally different functions in human and mouse brain-derived cells. In the rodent system, NR4A2 (Nurr1) is required for the transactivation of TH minimal promoters. Intriguingly, neither the dimeric nor the heterodimeric binding sites for Nurr1 are present in the 13 kb DNA sequence that contains the human TH promoter. Instead, the CRs termed one and four of the human TH promoter encode only for a half palindromic binding site sequence for Nurr1, which failed to bind Nurr1 in an in vitro electrophoretic mobility shift assay (EMSA). Additionally, of the three monomeric NGFI-B response element (NBRE) core sites (AGGTCA) and two NBRE-related sites present in the human TH promoter, only one core and two NBRE-related sites formed protein binding complexes. Interestingly, there was no increase of protein binding complex formation upon TH induction and in no case could antibodies supershift Nurr1 from the complex. These findings, taken together, demonstrate that NBRE-related binding sites for Nurr1 do not play a direct role in mediating an interaction between Nurr1 and the human TH promoter. Likewise, immunohistochemical and Western blot analysis have also confirmed that both endogenous and exogenous Nurr1 expression does not positively correlate with TH gene expression in hNPCs, in contrast to the mouse model. In addition, real-time PCR analysis revealed that the downregulation

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of human Nurr1 gene expression mediated by silencing RNA molecules did not affect human TH gene expression in differentiated hNPCs. A better understanding of human TH gene regulation may have important implications both for the development of novel therapeutic approaches and the study of the pathogenesis of a variety of neurological illnesses, including Parkinson's disease, bipolar disorder, and schizophrenia.

The regulation of tyrosine hydroxylase (TH) gene expression has attracted much attention in the field of neurology. Indeed, the biological function of this enzyme was investigated for four decades (Nagatsu et al., 1964). TH catalyzes the hydroxylation of tyrosine in the production of L-dopa (Nagatsu et al., 1964), which is the rate-limiting step in the synthesis of catecholamine neurotransmitters of the central and peripheral nervous systems (Zigmond et al., 1989). The degeneration of TH-positive dopaminergic neurons is the hallmark of Parkinson's disease (Moore, 2003). Abnormal TH gene expression is also observed in alcoholism and in psychiatric illnesses, such as schizophrenia and bipolar disorder (Ishiguro et al., 1998). On these grounds, a better understanding of TH gene regulation is of extreme interest.

Several studies have been conducted over the years to identify the factors involved in TH gene expression. These studies were carried out mainly on the human (Kessler et al., 2003;Romano et al., 2005), mouse (Kim et al., 2003a), and rats models (Gandelman et al., 1990;Kim et al., 2003b). In a previous report, a 13 Kb DNA fragment containing the human TH promoter was isolated from a genomic DNA library and sequenced (Kessler et al., 2003). Comparative analysis of the sequences of human, rat, and mouse TH promoters revealed only five small regions of high homology (Kessler et al., 2003). In fact, the degree of homology between the human and mouse TH promoters is in the range of 46.6% (determined with a Clustalx program), whereas the human and rat TH promoters share only a 30% degree of homology (Gandelman et al., 1990;Kim et al., 2003b). Such a divergence in sequence raises the question of whether or not TH gene regulation is achieved through the same mechanisms by the different species.

More recently, a study characterizing the functions of the five evolutionarily conserved regions (CRs) was undertaken in order to determine whether or not they played a role in conferring tissue-specificity to the transcriptional activity of the human TH promoter. Towards this end, a human TH minimal promoter was engineered, in which the five CRs were placed upstream of the first -194 bp from the transcription start of the human TH promoter and the first 35 bp of the untranslated messenger RNA leader of the human *TH* gene. This human TH minimal promoter was linked to the green fluorescent protein (GFP) and assembled into a self-inactivating lentiviral vector system (Lois et al., 2002). Lentiviral-mediated gene transfer proved very efficient for the in vitro transduction of human neuronal progenitor cells (hNPCs) (Romano et al., 2005). Interestingly, this construct conferred a significant degree of specific gene expression in hNPCs, following induction by a cocktail of differentiating agents (Du and Iacovitti, 1997;Romano et al., 2003,2005), but failed to do so both in TH-positive differentiated mouse primary striatal cells and in differentiated mouse *substantia nigra* cells (Romano et al., 2005). This finding indicated a possible difference in TH gene regulation between the human and mouse systems.

Therefore, it was of interest to examine the role of NR4A2 (Nurr1) in human cell systems. Nurr1 is a nuclear orphan receptor that binds an NGFI-B response element (NBRE) and is thought to be involved in TH gene regulation (Zetterstrom et al., 1997;Castillo et al., 1998;Saucedo-Cardenas et al., 1998;Kim et al., 2002). In the murine system, Nurr1 is required to transactivate mouse TH minimal promoters (Iwawaki et al., 2000;Kim et al., 2003a), as well as other unrelated promoters, such as the osteocalcin promoter in mouse osteoblasts (Pirih et al., 2004). Importantly, the human TH promoter lacks both the dimeric and heterodimeric binding sites for Nurr1 (Kessler et al., 2003;Romano et al., 2005). In the human promoter, only the CRs termed one and four encode for a half dimeric binding site for Nurr1, which was

initially identified on the human POMC promoter (Powell et al., 1984). In addition, sequence analysis revealed the presence of three monomeric NBRE (AGGTCA) and two NBRE-related sites throughout the human TH promoter. The goal of this study was to establish whether or not any of these binding sequences could allow for a Nurr1-mediated transactivation of the human TH promoter. Using EMSA, EMSA/supershift, and immunohistochemistry, we will show that TH gene expression does not depend on a direct Nurr1-mediated transactivation in the human model, further indicating different mechanisms of TH gene regulation between the human and murine systems.

MATERIALS AND METHODS

Cells and cell lines

Human neuronal progenitor cells (hNPCs) were purchased from Clonexpress, Inc. (Gaithersburg, MD) and grown in DMEM/F12 (Invitrogen, Carlsbad, CA), supplemented with 5% (v/v) fetal bovine serum (FBS) (Invitrogen), 10% (v/v) neuronal cell supplement (Clonexpress, Inc., Gaithersburg, MD), 10 ng/ml hEGF (R&D Systems, Minneapolis, MN), and 10 ng/ml bFGF (B&D Biosciences, Palo Alto, CA). The differentiation of hNPCs was carried out 48 h post-lentiviral vector transduction, for an overnight period, at 37°C, in a CO_2 incubator, in a defined medium (DM) supplemented with a cocktail of differentiating factors. DM is comprised of DMEM/F12 (Invitrogen), 20% (w/v) glucose (Sigma, Saint Louis, MO), 1% (v/v) insulin-transferrin-selenium mix (Invitrogen), and 1.4 mM L-glutamine (Invitrogen). The differentiation cocktail contains: FGFI 10 ng/ml (R&D Systems, Minneapolis, MN), dopamine 10μM (Sigma), TPA 200 nM (Sigma), IBMX 65μM (Sigma), and Forskolin 15μM (Sigma).

Human hNT cells derived from the NT2 teratocarcinoma cell line were purchased from ATCC (Hanasas, VA) and grown in DMEM supplemented with 10% (v/v) FBS (Invitrogen) and 2mM L-glutamine (Invitrogen).

Mouse primary striatal and *substantia nigra* cells were extracted from fetuses 13 days post-coitus as described (Stull et al., 2001). The differentiation of mouse primary striatal cells was carried out 48 h post-lentiviral transduction, for an overnight period, at 37°C, in a CO_2 incubator, in DM supplemented with differentiation cocktail as above.

Fetal human renal carcinoma cell line 293FT was purchased from Invitrogen (Carlsbad, CA) and was grown in Dulbecco's minimum essential medium (DMEM) (Invitrogen) supplemented with 10% (v/v) heat inactivated FBS (Invitrogen), and 2 mM L-glutamine (Invitrogen).

Plasmids

The self-inactivating lentiviral vector system pFUGV was kindly provided by Dr. David Baltimore (California Institute of Technology, USA) and is described elsewhere (Lois et al., 2002). The packaging constructs VSV-G and pCMVΔR9 were kindly provided by Dr. Didier Trono (University of Geneva, Switzerland) (Naldini et al., 1996).

The internal promoter and green fluorescence protein (GFP) were deleted from plasmid pFUGV and replaced with the human cytomegalovirus (CMV) promoter followed by a poly-linker containing a *Bam*HI restriction site. This plasmid was termed pGR330.

The cDNA encoding for human NR4A2 (Nurr1) was purchased from Genecopoeia™ (Germantown, MD). An HA-Tag epitope was fused in frame at the 5′-end of human Nurr1 coding sequence via polymerase chain reaction (PCR). The sequence of the sense primer was: 5′-
ggatccATGTACCCATACGATGTTCCAGATTACGCTCCTTGTGTTCAGGCGCAGTAT

GGGTCCTCGCCT-3′. The sequence of the antisense primer was: 5′-ggatccTTAGAAAGGTAAAGTGTCCAGGAAAAGTTTGTCAATTATTGCTGG-3′. Both primers contained a *Bam*HI restriction site in their overhangs, which is reported in lower case letters. The sequence of the HA-Tag epitope in the sense primer is indicated by upper case and underlined letters. The remaining upper case letters indicate the parts of the primers that anneal to Nurr1 coding sequence. The PCR conditions were as follows: an initial denaturation step of the template at 94°C for 1 min, followed by 1 min interval at 48°C to allow for the annealing of the primers to the template and a 5 min incubation at 72°C for polymerase elongation of the primers. This cycle was repeated 35 times. At the end of the 35 cycles, an additional incubation at 72°C for 20 min was included to allow for the completion of the amplification. These PCR conditions were applied for the generation of all the constructs mentioned in this study. The PCR product was isolated with a kit (Qiagen, Santa Clarita, CA) following manufacturer's instructions. After the isolation, the PCR product was digested with *Bam*HI and electrophoresed on a 1% (w/v) agarose gel. The correct size product was excised from the gel and purified with a gel extraction kit (Qiagen) following manufacturer's instructions. The final digested PCR product was ligated into *Bam*HI digested and dephosphorylated pGR330. The inserted PCR fragment was then sequenced to check for orientation and for possible misincorporations.

Human Nurr1 was also inserted into the *Bam*HI restriction site of the plasmid pEF/V5-His-TOPO vector (Invitrogen), to generate plasmid pGR398. This mammalian expression vector uses the human elongation factor (EF)-1α promoter to express the trangene of interest.

Silencing RNA (siRNA) plasmids specific for human Nurr1 were generated by inserting 21 bp double stranded oligonucleotides into *Bam*HI and *Hind*III restriction sites of plasmid pSilencer-CMV (Ambion, Austin, TX). Three oligonucleotides were used to silence human Nurr1 expression: 5′-AACTCCAGAGTTTGTCAAGTT-3′; 5′-AAGGTAGAAGACATTCAGATG-3′; 5′-AACTTCCACCAGAACTACGTG-3′.

Lentiviral-mediated gene transfer

Lentiviral vector stocks were generated transiently for the gene transduction of target cells as described elsewhere (Soneoka et al., 1995;Naldini et al., 1996;Lois et al., 2002). Transient calcium phosphate DNA transfection of 293FT cell line was carried out as described (Pear et al., 1993;Soneoka et al., 1995). Lentiviral vector stocks were harvested and used for the transduction of target cells as described elsewhere (Soneoka et al., 1995;Naldini et al., 1996;Lois et al., 2002).

DNA transfection

Plasmids encoding either for human Nurr1 (pGR398), or for three types of siRNA specific for human Nurr1 were transfected into hNPCs with the FUGENE-6 kit following the instructions of the manufacturer (Roche, Indianapolis, IN). The three siRNA plasmids were simultaneously co-transfected into hNPCs.

Nuclear protein extraction

Cells were harvested by gentle scraping from 10-cm tissue culture dishes when 80% confluent, after being rinsed twice with 10 ml of ice cold PBS. Cell nuclei were extracted and lysed with a NuCLEAT Extraction kit (Sigma), following the instructions of the manufacturer. Briefly, cells were spun down and resuspended in 1 ml of isotonic lysis buffer containing protease inhibitor mixture and kept on ice for 15 min. Cell membranes were then broken with 10 strokes in a Dounce homogenizer. Nuclei were spun down by centrifugation at 4,000g for 10 min at 4°C. The nuclear pellet was resuspended in 1 ml of nuclear extraction buffer and incubated at 4°C under shaking for 30 min. Nuclear debris and genomic material were removed by

centrifugation at 14,000g for 45 min at 4°C and the supernatant was dialyzed at 4°C for 4 h against 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.1 mM PMSF, 10% (v/v) glycerol, 2μg/ml leupeptin, and 5μg/ml aprotinin. Nuclear proteins were quantified via the Bradford method at 595 nm.

Electrophoretic mobility shift assay (EMSA)

Gel shift, competition, and supershift assays were conducted with several DNA probes, which are listed in Table 1. All the corresponding sense and antisense oligonucleotide strands were annealed and radiolabeled with P^{32}-ATP following standard procedures. Protein-DNA binding reactions were carried out in volumes of 10μl each, with 3μg of nuclear proteins, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM DTT, 4% (v/v) glycerol, and 0.05 mg/ml poly (dI-dC). After 10 min preincubation at room temperature, 1μl (0.07 pmol) of radiolabeled DNA probe was added into each mixture and incubated at room temperature for 20 min. The DNA-protein complexes were then resolved on a 4% non-denaturating polyacrylamide gel in 0.5X TBE running buffer [44.5 mM Tris-HCl, pH 8.0, 44.5 mM boric acid, 1 mM EDTA]. Gels were dried and exposed to X-ray film. For competition experiments, either a 50-fold or a 100-fold molar excess of non-radiolabeled oligonucleotide was added to each reaction, prior to the addition of labeled probe. Super-shift assays were carried out by using antibodies to human Nurr1 at a concentration of 1μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA). Super-shift experiments were carried out as follows: an initial preincubation step for 1 h on ice with 1μl of antibodies to human Nurr1 and 3μl of nuclear extracts, followed by the addition of the various labeled probes.

Antibodies and immunofluorescence

Rabbit antibodies to tyrosine hydroxylase (TH) were purchased from Pel-freez Biological (Rogers, AK) and used at a dilution 1:100 (v/v) in PBS. Rhodamine-conjugated secondary antibody to rabbit immunoglobulins was purchased from Jackson ImmunoResearch (West Grove, PA) and used at a dilution 1:50 (v/v) in PBS.

FITC conjugated rat antibodies to HA-Tag epitope were purchased from Roche and used at a dilution 1:50 (v/v) in PBS. Cells were fixed with 4% (w/v) paraformaldehyde in PBS at room temperature for 10 min. Immunofluorescence assay was carried out as described elsewhere (Du and Iacovitti, 1997;Kessler et al., 2003). Endogenous TH expression was detected with rhodamine-conjugated antibodies. HA-Tag expression was detected with FITC. Slides were analyzed on a Nikon-Scanalytic Image System as described elsewhere (Yang et al., 2004). Numbers of green, red, and overlapped green and red cells were counted manually. Three different fields, at a magnification of 20×, were counted in each well and averaged in order to score green, red, and yellow cells per each sample.

Western blot assay

Cultures at 10^6 cells/dish of uninduced and induced hNPCs were rinsed twice with ice cold PBS, harvested, and lysed with a Dounce homogenizer in 0.2 ml of lysis buffer containing 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.5% (w/v) sodium vanadate, 0.1% (w/v) SDS, 0.15 M NaCl, 10 mM PBS, pH 7.2, 2 mM EDTA, 50 mM NaF, 1 mM DTT, 0.1 mM PMSF, 2μg/ml leupeptin, and 5μg/ml aprotinin. Lysed cells were centrifuged at 11,000g for 10 min at 4°C. The supernatant was collected and protein concentration was determined by Bradford method at 595 nm.

Electrophoresis was carried out by loading 20μg of protein extracts on 15–3% gradient SDS–polyacrylamide gels and transferred with an electroblotting apparatus to an ECL nitrocellulose membrane purchased from Amersham (Arlington Heights, IL). The membranes were blocked overnight under shaking with 5% dry milk in PBS and 0.5% (v/v) Tween 20 (Sigma), washed

twice with 1% dry milk in PBS and 0.5% (v/v) Tween 20, incubated in primary antibodies to human Nurr1 diluted 1:1,000 (stock solution of 1μg/ml) for 2 h under shaking at room temperature, washed three times, incubated for 1 h at room temperature in horseradish peroxidase-conjugated antibodies against rabbit immunoglobulins diluted 1:1,000, and washed three times. Immunoreactivity was detected via chemiluminescence method (Amersham).

Real-time PCR

Real-time PCR was carried out following the instructions provided by the manual of the kit Cells-to-cDNA™ II (Ambion) and by the protocols of SYBR Green PCR Master Mix and reverse transcriptase (RT)-PCR (Applied Biosystems, Pleasanton, CA). Briefly, both undifferentiated and differentiated hNPCs were cultured at 2×10^5/dish, harvested, and counted. Differentiated hNPCs were previously transfected either with a mammalian expression vector encoding for human Nurr1 (pGR398), or with expression vectors encoding for three types of silencing RNA (siRNA) specific for human Nurr1. Cells transfected with siRNA were selected with neomycin (Sigma) at a concentration of 1 mg/ml and then induced. Messenger RNA (mRNA) was extracted from 10^6 cells, which were incubated with 0.5 ml of lysis buffer at 75° C for 10 min. DNAse was added into each sample at a final concentration of 0.06 Uμl to digest genomic DNA at 37°C for 60 min. The RT reaction was prepared by initially incubating 5μl of cell lysate, 4μl of dNTP mixture, 2μl of random decamers, and 5μl of nuclease-free water. This mixture was incubated at 70°C for 3 min. At the end of the incubation, the following reagents were added to the mixture: 2μl of 10× RT buffer, 1μl of M-MLV RT, and 1μl of RNAse inhibitor. At this point RT was conducted at 42°C for 1 h.

Real-time PCR for further analysis of gene expression was carried out with the reagents supplied in the ABI Prism 7700 Sequence Detection System kit (Applied Biosystems, Pleasanton, CA). Each reaction was conducted in a final volume of 25μl, containing 5μl of cDNA template, 50 pmol of each primer and other reagents supplied by the kit. The conditions for real-time PCR were the following: an initial step at 50°C for 2 min, a second step at 95°C for 10 min and 45 cycles at 95°C for 15 sec and 60°C for 1 min. The internal control for real-time PCR was β-actin. Six replicates were run in each real-time PCR reaction, in order to evaluate the mean C_T value for all the samples (TH, Nurr1, and β-actin). The results were analyzed with the Sequence Detection System Software 1.7 (Applied Biosystems, Pleasanton, CA) and computed according to Comparative C_T Method for relative quantization of gene expression. The ΔC_T value was determined by subtracting the C_T value of β-actin from the CT value of the other two samples (human TH and human Nurr1). Undifferentiated hNPCs were used as control to determine $\Delta\Delta C_T$, which was obtained by subtracting ΔC_T of undifferentiated hNPCs either from ΔC_T of pGR398 transfected and differentiated hNPCs, or from ΔC_T of Si-RNA transfected and differentiated hNPCs. The final value was determined by the following formula: $2^{-\Delta\Delta C_T}$.

RESULTS

EMSA of the left palindromic Nurr1 binding site

The function of five evolutionary conserved regions (CRs) in human TH gene regulation was investigated in a previous study on human neuronal progenitor cells (hNPCs) (Romano et al., 2005). The most striking effects on tissue specificity in TH-positive differentiated hNPCs were obtained when CR-I was added to the recombinant human TH minimal promoter assembled into a self-inactivating lentiviral vector system (Romano et al., 2005). Because the CR-I contains a half binding site for Nurr1, which corresponds to the left half of the palindromic sequence initially described on the POMC promoter (Table 1) (Powell et al., 1984;Kessler et al., 2003), we investigated the possible role of Nurr1 in enhancing tissue-specific gene expression of the human TH minimal promoter. The left half Nurr1 binding site was tested by

EMSA for its ability to bind endogenous Nurr1 nuclear proteins in nuclear extracts from uninduced (undifferentiated) and in induced (TH-differentiated) hNPCs. In this assay, no specific interaction was evident between the left half Nurr1 binding site in either cell type (Fig. 1). This is in contrast to an artificially produced full Nurr1 palindromic binding site, which could strongly bind to Nurr1 nuclear proteins. Although binding to this site was specific, as the addition of nonradiolabeled nucleotides was able to compete to some extent with the binding to the radiolabeled probe (Fig. 1), it was not correlated with TH expression. Thus, both undifferentiated and TH-differentiated cells contained proteins that bound the artificial Nurr1 site. However, since no full Nurr1 palindromic binding site is present in the 11 Kb of the human TH promoter (Kessler et al., 2003), this binding has no physiological relevance.

EMSA and supershift analysis of NBRE core and NBRE-related sites

Three upstream monomeric NBRE core sites (AGGTCA) and two NBRE-related sites (Table 1) in the 11 Kb of the human TH promoter were also tested by EMSA/supershift analysis (Fig. 2). Protein binding complexes were found at one core site (NBRE core sequence 2) and at two partial sites (NBRE core-related sequence 1 and 2) (Fig. 2). Their binding was specific, as excess non-labeled oligonucleotides were able to compete with labeled probes. However, in no case could Nurr1 be supershifted from the complex. Interestingly, in both cases the induction of TH expression by incubation of cells with a differentiation cocktail did not significantly increase the formation of protein binding complexes over the uninduced samples. In contrast, AP1, which is a critical mediator in TH gene expression (Guo et al., 1998), showed increased nuclear protein binding after TH induction in cells that could be supershifted with antibodies to AP1 (Fig. 2).

Immunocytochemistry and Western blot analysis of endogenous Nurr1 and TH expression in hNPCs

We next examined whether the endogenous expression of Nurr1 correlated with the induction of TH in differentiated hNPCs. To do so, the phenotype of uninduced (undifferentiated) and induced (differentiated) hNPCs was analyzed by immunocytochemistry and by Western blot assay for endogenous Nurr1 and TH expression (Fig. 3). The expression of endogenous Nurr1 was detected by immunocytochemistry with FITC-conjugated antibodies both for control (uninduced) and induced hNPCs (Fig. 3, parts A–D). The number of Nurr1-positive cells was comparable between the uninduced and induced samples (Fig. 3, parts C and D, respectively). In contrast, TH expression was detectable by rhodamine staining only in the induced sample (Fig. 3, part B), as it has been described in a previous study (Yang et al., 2004).

Moreover, Western blot analysis of endogenous Nurr1 did not reveal any difference of expression between uninduced (Fig. 3, part E, lane CTL) and induced (Fig. 3, part E, lane IND) hNPCs. These findings, taken together, indicate that Nurr1 expression does not correlate positively with TH gene expression in human progenitors.

Analysis of hNPCs transduced with a lentiviral vector system encoding for recombinant HA-Tag fused in frame with human Nurr1

We further tested the possibility that by over-expressing exogenous Nurr1, we could induce TH expression in undifferentiated hNPCs. Thus, recombinant human Nurr1 fused in frame at the 5′-end with HA-Tag epitope was over-expressed via lentiviral-mediated gene transfer in induced hNPCs (Fig. 4). FITC-conjugated antibodies to HA-Tag epitope were used to detect the expression of recombinant HA-Tag-Nurr1 fusion protein, whereas endogenous TH expression was stained by rhodamine-conjugated antibodies (Fig. 4). Recombinant Nurr1 was efficiently over-expressed in induced and transduced hNPCs (Fig. 4, part D), while no recombinant Nurr1 expression could be detected in non-transduced and induced hNPCs (Fi. 4, part A). However, there was no difference in TH gene expression between induced hNPCs

with over-expressed recombinant Nurr1 and induced naïve hNPCs (Fig. 4, parts E and B, respectively), indicating that the over-expression of recombinant Nurr1 did not increase the expression of TH in induced hNPCs. In contrast, immunocytochemical analysis revealed that lentiviral-mediated gene transfer of recombinant HA-Tag-Nurr1 increased remarkably TH expression in mouse neuronal progenitor cells (data not shown). This finding indicates that Nurr1 is involved in the regulation of TH gene expression only in the mouse system and not in the human model.

Effects on the suppression of human Nurr1 expression mediated by siRNA

After the analysis of over-expressed recombinant human Nurr1, our study was concluded by the analysis of the downregulation of human Nurr1 gene expression mediated by siRNA (Table 2). This experiment was conducted on hNPCs, which were simultaneously co-transfected with three plasmids encoding for siRNA specific for human Nurr1, selected for neomycin resistance and then differentiated. A panel of 19 siRNA molecules for human Nurr1 was tested for their ability to downregulate Nurr1 expression in hNPCs, and only three siRNA could achieve that goal. The simultaneous co-transfection of siRNA for Nurr1 synergized in suppressing human Nurr1 expression in hNPCs. Another set of hNPCs were transfected with plasmid pGR398, which encodes for human Nurr1 under the control of the human EF-1α promoter, and then differentiated. Undifferentiated hNPCs were used as a control for real-time PCR analysis of human TH, human Nurr1, and human β-actin gene expression. The siRNA could efficiently suppress endogenous Nurr1 gene expression (Table 2). However, our finding clearly indicates that the downregulation of human Nurr1 gene expression by siRNA does not affect the expression of human TH in differentiated hNPCs.

DISCUSSION

The present report has clearly shown that NR4A2 (Nurr1) has no demonstrable effect on TH gene regulation in human brain-derived cells. This is in contrast to other studies, demonstrating that Nurr1 expression is required for the transactivation of mouse (Iwawaki et al., 2000;Kim et al., 2003a) and, to a less extent, rat (Cazorla et al., 2002) TH promoters. In the case of the rat TH promoter, Nurr1 had a weak effect in terms of transactivation in mouse neuroblastoma Neuro2A cell line (Cazorla et al., 2002). Interestingly, the home-odomain transcription factor Ptx-3 exhibited a considerable interaction on the rat TH promoter, which resulted in an enhancement of transcription in a cell-dependent fashion (Cazorla et al., 2002). Moreover, the co-expression of Ptx-3 and Nurr1 displayed a synergistic effect in stimulating the rat TH promoter (Cazorla et al., 2002).

The importance of Nurr1 in TH expression derives from earlier studies demonstrating that Nurr1 knockout mice lack TH expression in dopaminergic neurons of the *substantia nigra* (Saucedo-Cardenas et al., 1998;Baffi et al., 1999). Importantly, however, our studies (Kessler et al., 2003;Romano et al., 2005) as well as others (Kim et al., 2003b) indicate that the human system is indeed quite different from mouse and rat models. When comparative analysis among the human, mouse, and rat promoters was conducted after isolating and sequencing 11 Kb of the human TH promoter (Kessler et al., 2003), we found that the degree of homology between the human and mouse TH promoters was merely 46.4% (Romano et al., 2005). A similar low degree of homology was also confirmed independently (Kim et al., 2003b). The human and rat TH promoter share even less homology, which was estimated in the range of 30% (Gandelman et al., 1990;Kim et al., 2003b).

More detailed sequence analysis among the human, mouse, and rat TH promoters identified only five small evolutionary conserved regions (CRs), which, however, do not have the same positions among the promoters of the three species (Kessler et al., 2003). Functional characterization of the five CRs revealed a significant role in regulating specificity of

expression only in human brain-derived TH-expressing cells, but not in mouse cells (Romano et al., 2005). Likewise, when the human and rat TH promoters were compared in a previous study conducted by other investigators, species-specific regional differences were observed (Gandelman et al., 1990). Despite the overall low degree of homology and site specific differences, the 11 Kb of human TH promoter driving GFP achieved tissue-specific expression in the "substantia nigra" of transgenic mice (Kessler et al., 2003), indicating that the general machinery for TH promoter regulation is still preserved in the human and mouse systems.

Non-homologous regions encoding for divergent trans-activating sites among TH promoters of various species are likely to be a major factor accounting for diverse mechanisms of TH gene regulation in the human, mouse, and rat models. Our studies suggest that a case in point is Nurr1. As anticipated, Nurr1 could transactivate the mouse TH promoter and, albeit to a lesser extent, the rat TH promoter. However, Nurr1 had no apparent function on the stimulation of the human TH promoter. Consistent with this finding, there were no dimeric or heterodimeric Nurr1 binding sites present in the 11 Kb of the human TH promoter, although conserved regions one and four of the human TH promoter each encode respectively for the left and right half palindromic binding site sequence for Nurr1. In a previous study, the engineering of a series of human TH minimal promoters demonstrated that the presence of the right half palindromic binding site for Nurr1 had no effect on tissue-specific gene expression (Romano et al., 2005). However, the most significant increase in tissue specificity for TH-positive differentiated hNPCs was achieved by adding CR-I to the human TH minimal promoter (Romano et al., 2005), raising the possibility that the left half palindromic Nurr1 binding site in CRI was involved (Kessler et al., 2003). However, the current study showed that the left half palindromic Nurr1 binding site cannot bind Nurr1 or other nuclear proteins present in undifferentiated, or TH-differentiated hNPCs (Fig. 1).

In addition to the half palindromic sites, three upstream monomeric NBRE core sites (AGGTCA) and two NBRE-related sites were also identified in the 11 Kb of the human TH promoter. Interestingly, the human TH promoter lacks the NBRE-like site reported to be of critical importance in the context of the rat TH promoter, at positions −34/−27. When these five potential binding sites were tested by EMSA/supershift assay for their ability to bind Nurr1 in vitro, specific formation of protein complexes were observed only for two partial NBRE-related sites. However, there was no increased binding associated with the induction of TH expression in differentiated hNPCs (NBRE core-related sequence 1 and 2; Fig. 2). Moreover, there was no ability to supershift Nurr1 from the binding complexes, suggesting that Nurr1 may not be among the proteins that bind the NBRE sites. Alternatively, it is possible that Nurr1 works indirectly through interaction with other transcription factors. Such a model has been proposed for NR4A2 regulation of the human dopamine transporter gene, in which Nurr1 acts as a transcriptional coactivator, without direct DNA contact (Sacchetti et al., 2001). The lack of supershift could also be explained by technical reasons. First, the position of Nurr1 in the complex may make it inaccessible to the Nurr1 antibodies. Second, the antibodies to Nurr1 may be suitable for immunocytochemistry, but may not bind epitopes under EMSA in vitro conditions. Thus, while the exact mechanisms remain uncertain, Nurr1 appears to play a major role in murine and rodent TH gene regulation. In contrast, our studies thus far suggest that Nurr1 have minimal impact on human TH gene regulation. Consistent with this notion was the results of immunocytochemical and Western blot analysis of undifferentiated and differentiated hNPCs. These studies established that there was no involvement of either endogenous or over-expressed recombinant Nurr1 in TH gene regulation, although an over-expressed recombinant Nurr1 had a dramatic effect in increasing TH gene expression in mouse neuronal progenitor cells (mNPCs).

Taken together, the findings of our studies indicate that TH gene regulation may be achieved through different mechanisms among various species. Further suggesting this possibility are

previous studies comparing the human and bovine systems, where the functions of three scaffold/matrix attachment regions (S/MAR) in regulating the transcriptional activity of the human TH promoter in a tissue-specific fashion were analyzed (Lenartowski and Goc, 2002;Lenartowski et al., 2003). The first S/MAR is at the 3−-end of the human TH promoter (positions −186/−16), whereas the other two S/MARs are in the first intron of the human *TH* gene (positions +645/+755 and +835/+945) (Lenartowski and Goc, 2002). The association between nuclear matrix of certain cell types and various DNA binding proteins may constitute a subcellular structure in chromatin organization, which, in turn, may result in a particular tissue-specific gene regulation (van Wijnen et al., 1993). Interestingly, the S/MAR at (+645/+755) also encodes for a microsatellite repeat (TCAT) termed HUMTH01, which acts as a silencer for the human TH gene expression by interacting specifically with the following transcription factors: ZNF191, HBP1 and probably AP-1 (Meloni et al., 1998;Albanese et al., 2001). ZNF191 is a zinc finger protein, while HBP1 is an HMG box transcription factor. A number of studies have already reported the intronic colocalization of S/MARs with either enhancer or silencer regulatory elements in many other systems, besides the human TH promoter (Cockerill and Garrard, 1986;Forrester et al., 1994;Boulikas, 1995). Indeed, the S/MARs contained in the human *TH* gene seem to be one of the key factors in the differential TH gene regulation among various species (Lenartowski et al., 2003). A comparative analysis was conducted between bovine and human *TH* genes in binding nuclear matrix obtained from bovine brain and liver tissues (Lenartowski et al., 2003). In contrast to the human *TH* gene (Lenartowski and Goc, 2002), the association between bovine *TH* gene and the nuclear matrix was not tissue specific, although the position of the matrix binding region is conserved in both systems (Lenartowski et al., 2003). This finding indicates that TH gene regulation might indeed be achieved by different mechanisms in the human and bovine models.

In conclusion, our study has revealed substantial evidence for further differences in TH gene regulation in the human cell culture model on one hand, and the mouse and rat systems on the other hand. These findings may have profound basic and clinical implications, considering that a number of laboratories are attempting to genetically engineer human stem/progenitor cells to express TH prior to transplantation into animal models for Parkinson's disease.

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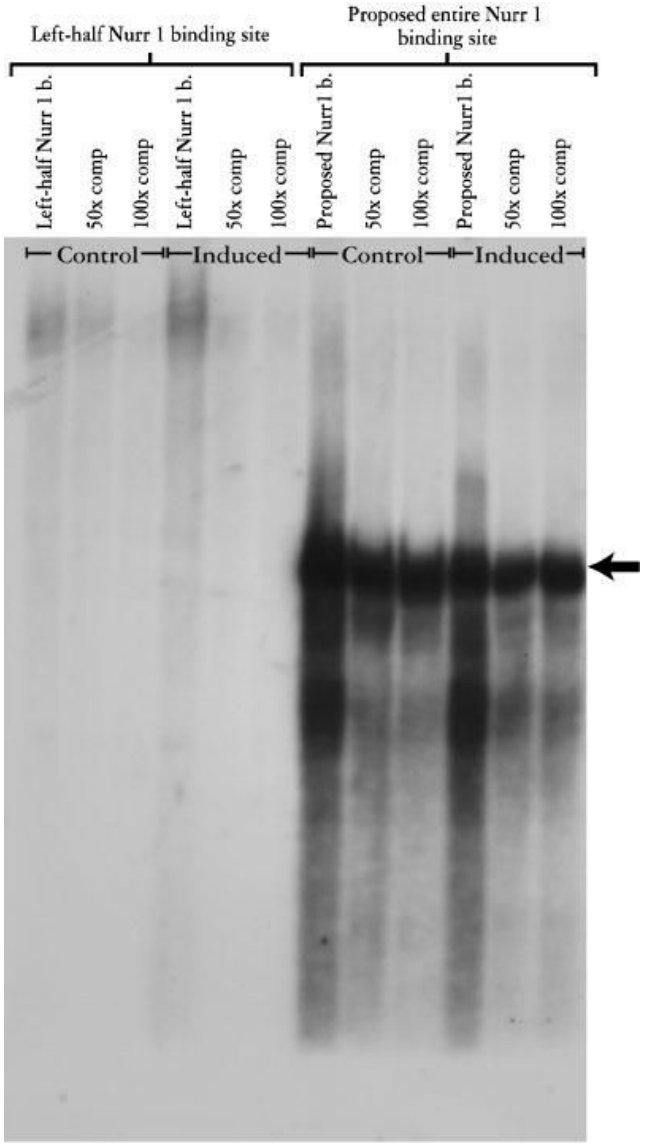

Fig 1.

EMSA of the left palindromic Nurr1 binding site. The arrow on the right hand side shows the formation of the complex between the proposed entire Nurr1 binding sequence and Nurr1 nuclear proteins extracted either from undifferentiated or differentiated hNPCs. Control stands for undifferentiated hNPCs, whereas induced stands for differentiated hNPCs. No complex was observed for the left-half Nurr1 binding site. Abbreviations: b, binding site; comp, competitor.

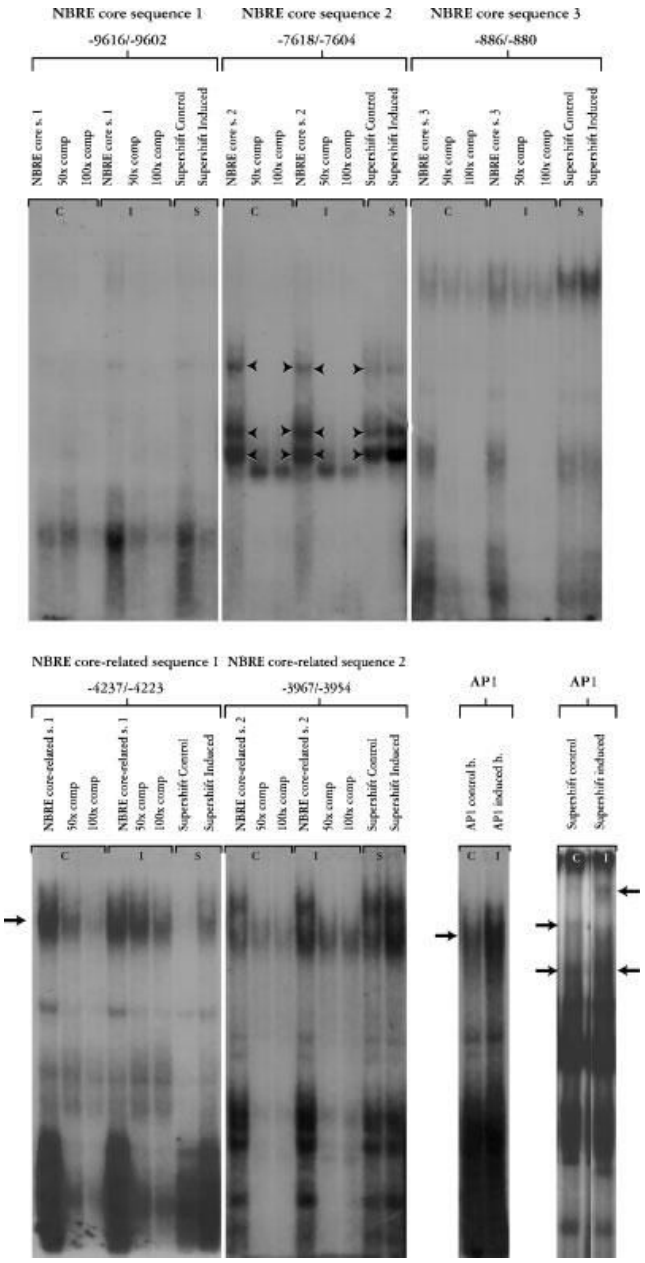

Fig 2.

EMSA and supershift analysis of NBRE core and NBRE core-related sites, conducted on undifferentiated and differentiated hNPCs. The coordinates of each NBRE core sequence and NBRE core-related sequences are reported beneath their corresponding site (see also Table 1). The formation of DNA-protein complexes are depicted either by arrows, or arrowheads. The same applies for AP-1 EMSA and supershift analysis. In the case of AP-1, it was possible to detect a supershift in the induced sample, as indicated by the upper arrow on the right hand side of the lower part of the panel. Abbreviations: s., sequence; comp, competitor; C, control (undifferentiated hNPCs); I, induced (differentiated hNPCs); S, supershift.

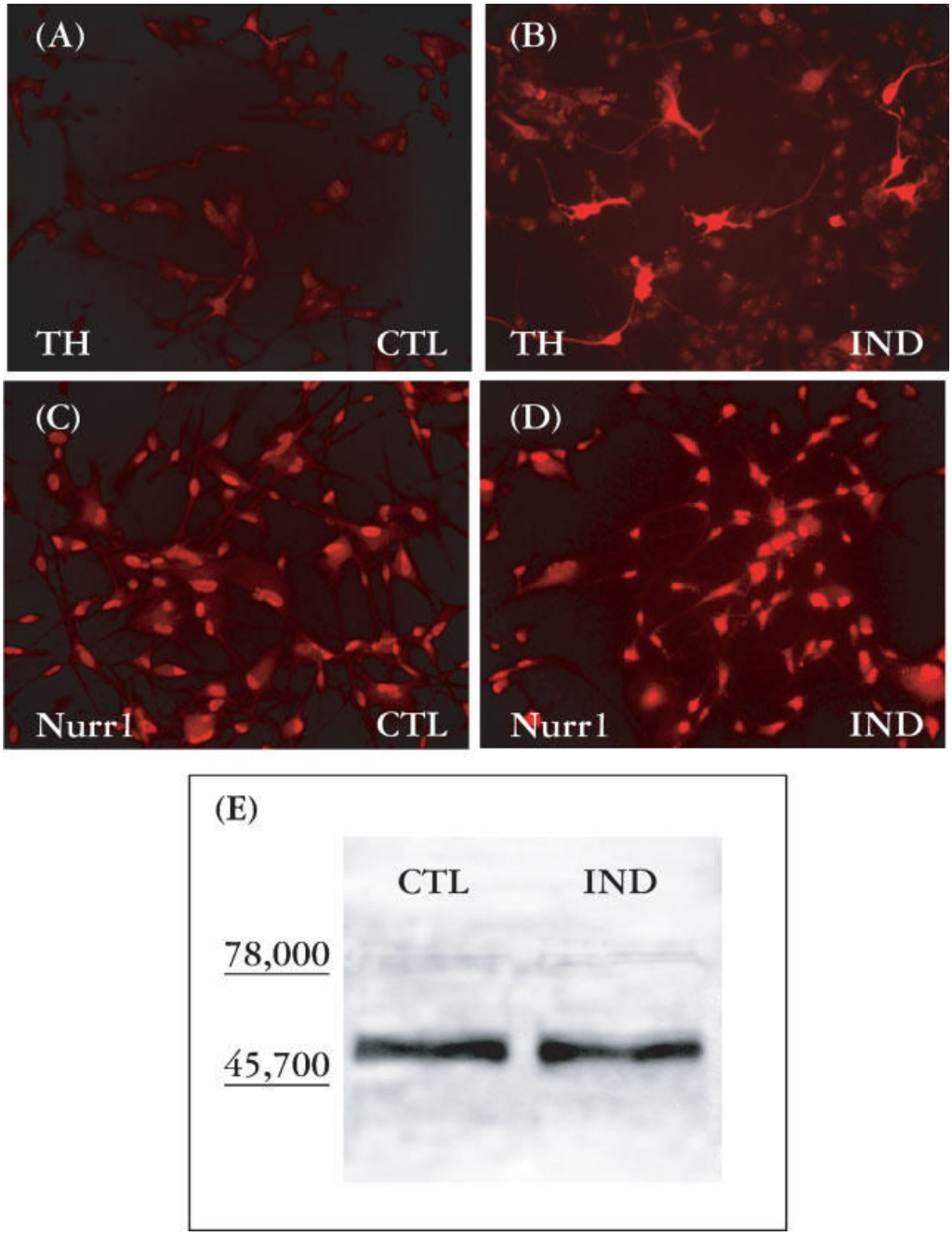

Fig 3.

Immunocytochemistry and Western blot analysis of endogenous Nurr1 and TH expression in hNPCs. **Parts A–D** show the immunocytochemical analysis of endogenous Nurr1 and TH expression in undifferentiated and differentiated hNPCs, whereas **part E** shows the Western blot analysis of endogenous Nurr1 gene expression in undifferentiated and differentiated hNPCs. Abbreviations: CTL, control; IND, induced. Control stands for undifferentiated hNPCs, whereas IND stands for differentiated hNPCs. Immunocytochemical analysis for TH gene expression is indicated by the annotation “TH” (parts A and B), while immunocytochemical analysis for endogenous Nurr1 gene expression is indicated by the annotation “Nurr1” (parts C and D).

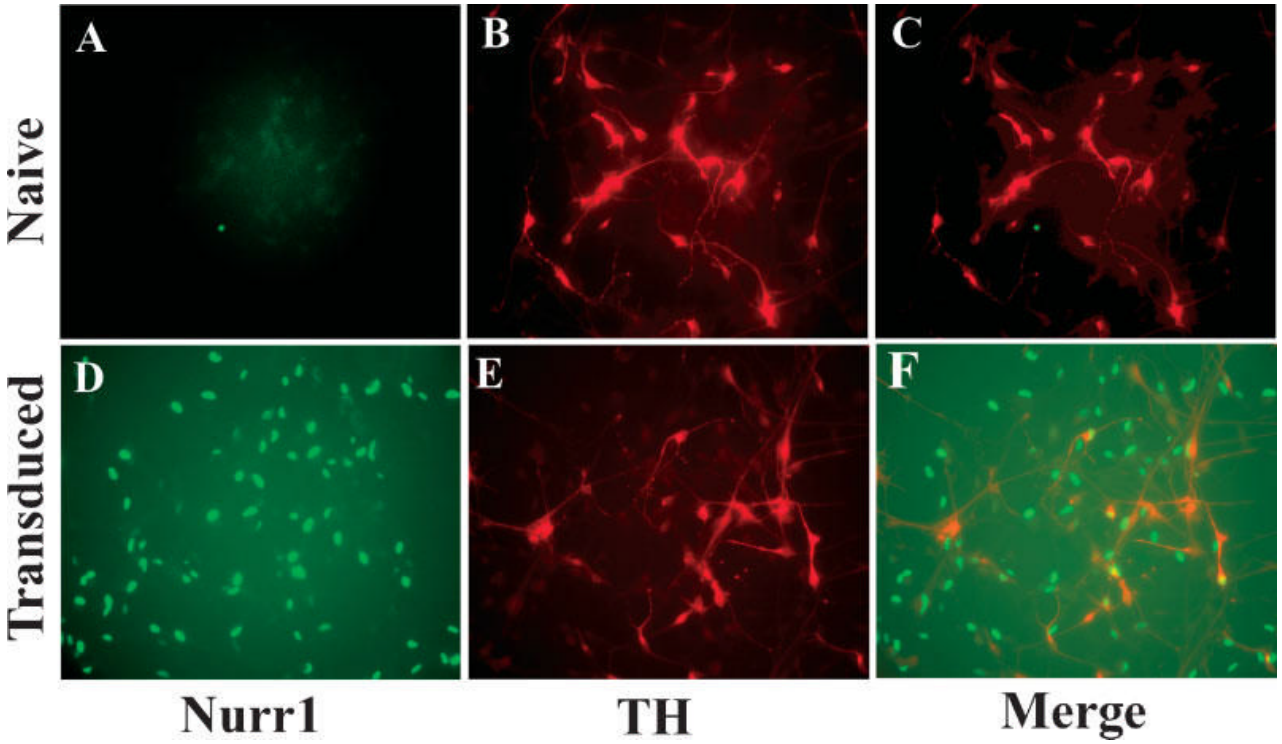

Fig 4.

Analysis of hNPCs transduced with a lentiviral vector system encoding for recombinant HA-Tag fused in frame with human Nurr1. This figure shows the immunocytochemical analysis of lentiviral-encoded Nurr1 and TH gene expression in differentiated hNPCs. Recombinant Nurr1 was stained with FITC-conjugated antibodies to HA-Tag, whereas endogenous TH was stained with rhodamine. Parts A–C show naïve (non-transduced) differentiated hNPCs, while parts D–F show differentiated hNPCs transduced with the lentiviral vector encoding for recombinant Nurr1. Parts A and D show recombinant Nurr1 expression, parts B and E show endogenous TH expression. Part C shows the merged picture between parts A and B, while part F shows the merged picture between parts D and E.

TABLE 1

List of DNA probes used in EMSA

Definition	Sequence and positions in human TH promoter	
Left half Nurr1 site	5′-TAAATATCAC-3′	(−8861/−8851)
Right half Nurr1 site	5′-CTGGCATTTGG-3′	(−5340/−5329)
Proposed dimeric Nurr1 site	5′- TAAATATCACCTGGCATTTGG-3′	(Not present in human TH promoter)
NBRE Core sequence 1	5′-CGGG**AGGTCA**AGGC-3′	(−9616/−9602)
NBRE Core sequence 2	5′-ATTG**AGGTCA**CCTC-3′	(−7618/−7604)
NBRE Core sequence 3	5′-**AGGTCA**-3′	(−886/−880)
NBRE Core-related sequence 1	5′-ACCC**AGAACA**CCTC-3′	(−4237/−4223)
NBRE Core-related sequence 2	5′-AGAG**AGAACA**CGCT-3′	(−3967/−3954)

This table shows only the sense DNA strands. The NBRE core sequences and core-related sequences are reported in bold upper case letters. The coordinates are reported in between parenthesis and are referred to the human TH promoter (Kessler et al., 2003).

TABLE 2

Real-time PCR analysis of human TH and human Nurr1 gene expression in differentiated hNPCs, following suppression of human Nurr1 mediated by siRNA

Cell type	mRNA	C_T +/−SD	ΔC_T +/−SD	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Control: undifferentiated hNPCs	Human TH	30.405±0.01	13.763±0.294	—	—
	Human Nurr1	34.113±0.002	17.619±0.922	—	—
	β-actin	16.494±0.01	—	—	—
Differentiated hNPCs transfected with plasmid pGR398 (human Nurr1)	Human TH	28.585±0.002	10.176±0.639	−3.587	$2^{3.587}$=12.017
	Human Nurr1	30.409±0.002	12.663±0.305	−4.986	$2^{4.986}$=31.690
	β-actin	17.776±0.19	—	—	—
Differentiated hNPCs transfected with plasmids encoding for three siRNA specific for human Nurr1	Human TH	26.236±0.002	9.733±0.470	−4.030	$2^{4.030}$=16.336
	Human Nurr1	42.202±0.003	25.699±4.60	1.622	$2^{-1.622}$=0.378
	β-actin	16.308±0.006	—	—	—

SD, standard deviation; C_T, threshold cycle at which a statistically significant increase in ΔR_n is first detected.

Threshold is the average SD of R_n for the early cycles, multiplied by an adjustable factor. ΔC_T is calculated by subtracting the average β-actin C_T value from the average C_T value of an amplified target gene. $\Delta\Delta C_T$ is determined by subtracting the ΔC_T of undifferentiated hNPCs, which acts as a calibrator, from the ΔC_T value of differentiated hNPCs. $2^{-\Delta\Delta CT}$ expresses the value of the relative amount of target gene expression, which is normalized to an endogenous reference, such as β-actin, and relative to the calibrator, which is the same target gene expression in undifferentiated hNPCs.