

BTK functions as a corepressor of Nurr1  
in human neural stem cells

by

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Major in Neuroscience

Department of Biomedical Sciences

The Graduate School, Aju University

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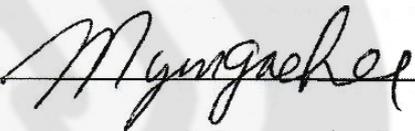
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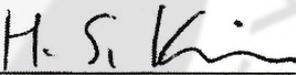
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## ABSTRACT

### **BTK protein functions as a corepressor of Nurr1 in human neural stem cells**

Nurr1, a member of the orphan nuclear receptor superfamily, was demonstrated to be of critical importance in the developing central nervous system, where it is required for the generation of midbrain dopaminergic cell. Although its role during dopaminergic neurogenesis has been extensively studied, its transcriptional regulation of TH, the rate-limiting enzyme of dopamine biosynthesis, is not well understood.

In our previous study, we found that Nurr1 directly mediated transcriptional activation or repression of TH gene through NBRE-A site and a key regulating complex of TH expression, that may be formed in NBRE-A site during dopaminergic neurogenesis. In recent years, a growing number of cofactor has been discovered that participate in the regulation of the transcriptional activity of TH promoter. So, we present the identification of a cofactors binding in and around NBRE-A, which differentially regulates the transcriptional activity of the human TH promoter in human neural stem cell, F3, and human neuroblastoma cell, SH-SY5Y. In order to identify the transcription factors that mediate these contradictory functions in each of the cell types, we conducted biotin-labeled oligonucleotide of Nurr1-binding element (NBRE) and streptavidin-agarose beads pull-down assays. We identified three proteins, bruton tyrosine kinase-associated protein (BTK) and topoisomerase II  $\beta$  (TOPO II  $\beta$ ) in F3 and poly (ADP-ribosyl) transferase (PARP) in SH-SY5Y. To know the interaction of

these candidate proteins with NBRE-A site, we performed by immunoprecipitation and chromatin immunoprecipitation. BTK and TOPOII interact specifically with Nurr1 in the F3 cells, PARP interacts with SH-SY5Y cells. And to know these proteins regulate TH promoter activity, we performed the Luciferase assays. We found BTK repressed TH promoter activities but TOPO and PARP did not. Also when BTK was knockdown, TH promoter activity in F3 cells was activated. These results showed that these Nurr1 binding proteins perform a crucial role expression of the TH gene

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**Keywords:** tyrosine hydroxylase, Nurr1, dopaminergic neuron, neural stem cell, neuroblastoma cell, bruton tyrosine kinase-associated protein (BTK) and topoisomerase II  $\beta$  (TOPO II  $\beta$ ), poly (ADP-ribose) transferase (PARP)

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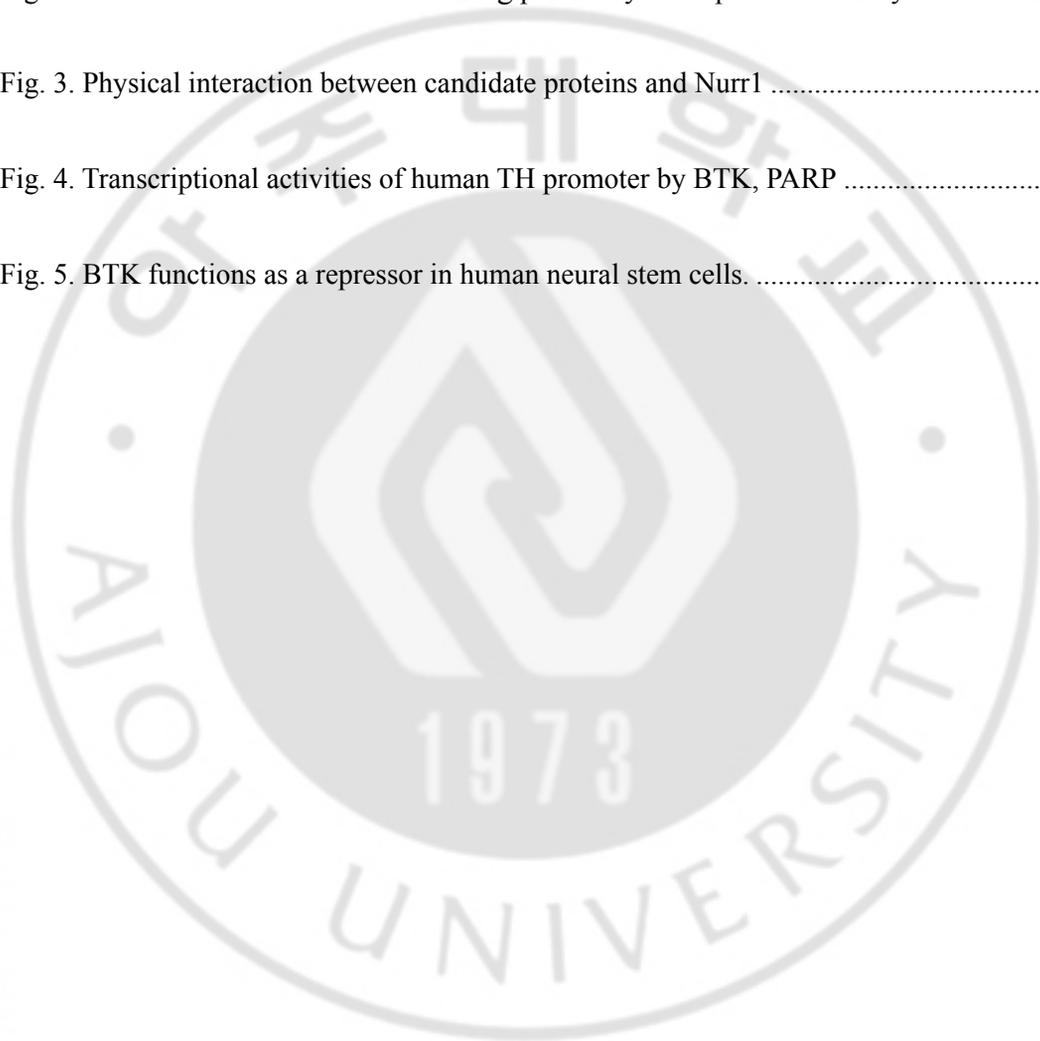
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## I. INTRODUCTION

Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in the synthesis pathway of catecholamine in the central and peripheral nervous system (Mallet et al., 1996). TH is expressed in several cell groups, including the dopaminergic neurons of the substantia nigra, ventral tegmental area, hypothalamus, and olfactory bulb, the noradrenergic neurons of the locus coeruleus and lateral tegmental system, and the adrenergic neuron of the brainstem. Given this wide distribution, it is clear that different transcriptional regulatory mechanisms may account for regulated expression of the TH gene in different cell populations. Some positive and negative cis-regulatory elements within the TH gene have been associated with the control of cell type-specific expression (Yang et al., 1998), but the sequences and positions of these elements are not fully conserved across species (Gandelman et al., 1990). Thus, it is very likely that the TH gene relies on multiple regulatory elements to ensure the plasticity and diversity of its expression.

Nurr1 (NR4A2) is an orphan member of the nuclear receptor superfamily of transcription factors critical for the generation of dopamine (DA) neurons of the substantia nigra (SN) and ventral tegmental area (VTA) (Zetterstrom et al. 1997, Castillo et al. 1998, Saucedo-Cardenas et al. 1998). Precursor cells of DA neurons are born in Nurr1-null mice, however tyrosine hydroxylase (TH), the DA transporter (DAT) are not expressed in these precursor cells (Wallen et al. 2001, Smits et al. 2003, Perlmann & Wallen-Mackenzie 2004). Recently, it was demonstrated that Nurr1 is also essential to maintain midbrain DA neurons in the adult (Kadkhodaei et al. 2009). Indeed, specific ablation of Nurr1 in mature DA

neurons induces TH, VMAT2 and DAT loss, and neuronal degeneration (Kadkhodaei et al. 2009). Nurr1 continues to be expressed in mature DA cells, which are those cells that degenerate in patients with Parkinson's disease. Overexpression of Nurr1 in another immortalized neural stem cell line has provided additional evidence that this approach can result in the expression of dopaminergic markers in cultured stem cells (Kim et al., 2003).

Members of the NR4A subgroup are known to bind to DNA as monomers, homodimers, and heterodimers. As monomers, the NR4A subgroup binds to the NGFI-B response element (NBRE; AAAGGTCA). This sequence consists of the canonical NR-binding motif (AGGTCA) preceded by two adenines (Wilson et al., 1991; Wilson TE and Fahrner TJ, 1993). As homodimers and heterodimers with other NR4A members, all three members of the NR4A subgroup bind to the Nur-responsive element, which consists of everted repeats of the sequence AAAT(G/A)(C/T)CA similar to the monomeric NBRE motif (Philips A and Lesage S, 1997 and Maira M and Martens C, 1999) Nurr1 (but not Nor-1) can also heterodimerize with retinoid X receptor (Perlmann T and Jansson L, 1995; Zetterstrom RH and Solomin L, 1996). NR4A/ retinoid X receptor heterodimers bind to a combination of an NBRE and retinoic acid response element as direct repeats (Perlmann T and Jansson L, 1995).

Here we show that tyrosine hydroxylase (TH) expression is transcriptionally activated by Nurr1 through the NBRE site in dopaminergic neuron-like cells (SH-SY5Y cell) but is repressed by Nurr1 in human neural stem cells (NSCs, F3). We demonstrated this nurr1 dependant regulation of TH gene expression made by recruitment of cofactors and this recruitment is different with cell stage. To identify the novel cofactors, we tried to search for

other component that can bind to the NBRE region in TH gene using DNA pull-down assay. After that we have performed 1D: LC mass and 2D: proteomics. And we identified selected cofactors binding with Nurr1 and regulation of TH gene expression. Our results showed that Nurr1 and differential cofactor complexes may regulate differential transcriptional activity of the TH promoter in neural stem cells and dopaminergic neurons.



## II. MATERIAL AND METHOD

### 1. Cell culture

Immortalized human neural stem cell (NSC) line, F3, was established as described previously (Kim, 2003), and were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10% fetal bovine serum (FBS, Hyclone). SH-SY5Y (human dopaminergic neuroblastoma cell line) were grown in DMEM (sigma) supplemented with 10% FBS (Hyclone).

### 2. Plasmid constructs and mutagenesis.

A 3301-bp fragment containing sequence -3174 to +127 was directly subcloned upstream of the luciferase gene in order to assay TH promoter activity (phTH-3174-Luc). Within this region of the human TH promoter, 3 consensus elements for Nurr1 are located at positions -2413 to -2406, -1440 to -1433 and -833 to -824, and designated as NBRE-A, -B, and -C, respectively.

Site-directed mutagenesis of the Nurr1 binding sites (NBREs) within the human TH promoter was conducted using the QuickChange Mutagenesis kit (Stratagene) in accordance with the manufacturer's instructions. In brief, pGL3-basic-hTH expression vector was utilized as a template. Oligonucleotides with 34-36 nucleotides harboring the desired point mutations were as follows:

mNBRE-A 5'-GACATTTGCTGCTGAAAAACAGAATCCACATCCGGC-3'

mNBRE-B 5'-GAAGCAGTTTTAGGAAAAACAGCAGGGGCTATTGTTG-3'

mNBRE-C 5'-GAGGAGAAACTGCAAAAACAGCTCCAAGGGGAAGGC-3'

The base codings for mutated residues are underlined. The site-directed mutations were confirmed via sequence analysis.

The pLPCX-Nurr1, pLPCX-Nurr1-variant-A, and pLPCX-Nurr1-variant-B contain the coding cDNA sequences of human Nurr1, spliced variant type A and B .

### 3. Transient transfection

Two days prior to transfection, SH-SY5Y cells were cultured in Dulecco modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum (Hyclone). Cell grown in 35mm culture dishes to 60~70% confluence were transiently transfected with 3  $\mu$ g of total plasmid DNA (pGL3-basic-hTH-Luc constructs and pGEM3zf(+)) complexed with 4  $\mu$ l of lipofectAMINE reagent and 6  $\mu$ l of PLUS reagent (Invitrogen), according to protocol provided by the manufacturer. The plasmid DNA pGEM3zf(+) was used as carrier to keep the total DNA amount constant between wells. The DNA, lipofectamine and plus mixture were added to the cells (SH-SY5Y). After incubated for 4 h transfection, 1ml of the medium containing 20% fetal bovine serum was added and then the transfected cells were replaced with fresh medium after a 16h incubation. Cells were also co-transfected with three plasmids mixed in the following amount: pLPCX-Nurr1(1.6  $\mu$ g/well) and various amounts of pGL3-basic-hTH constructs. The total amount of per well was adjust to 3  $\mu$ g by addition of the plasmid pGEM-3zf(+). Cells were treated with TSA (Tricostatin-A, sigma) 48 h after transfection. The cells were harvested approximately 72 h later, after changing fresh medium,

and then the cell extracts were assayed luciferase activities by Single luciferase assay kit (Promega, Madison, WJ, USA). Each experiment normalized three sets of independent triplicates to overcome the variability inherent in transfection experiments.

#### **4. Luciferase Assay**

Promoter activity was determined using the Single-Luciferase™ Reporter Assay System (Promega) in accordance with the manufacturer's recommendations. Luciferase activities were normalized on the basis of pSV-β-galactosidase activity in each well. Statistical analysis of the results was conducted with GraphPAD InStat, version 1.13 (Graph Pad Software, San Diego, CA).

#### **5. Biotinylated Oligonucleotide-Streptavidin Pull-Down**

Binding of NBRE-A and NBRE-mA to the following 5'-biotinylated DNA oligonucleotides was tested using the biotin-streptavidin affinity system:

NBRE - A : AAGACATTTGCTGAAAGTTAAATCCACAT

NBRE - mA : AAGACATTTGCTGAAAcTTAAATCCACAT

The NBRE – A and NBRE – mA sites are underlined, and boldface.

After annealing, biotinylated oligonucleotides(1ug) were incubated with precleared whole cell lysates (0.5-1mg) derived from F3, and SH-SY5Y cell line, transfected Nurr1 or not as above, and 100μl streptavidin – agarose (Pierce, Rockford, IL) in a 2ml of incubation buffer [ Tris 10mM(pH 7.4), NaCl 50mM, glycerol 5%, EDTA 1mM, MgCl<sub>2</sub> 5mM, BSA 1μg, poly-deoxyin-osine-deoxycytosine 20μg, complete mini 1 tablet /10 ml(protease inhibitor cocktail

tablets, Roche) ] Incubation was carried out on a rotating wheel for 2hr. at 4°C. After centrifugation, the pellet containing the streptavidin-agarose was washed four times the washing buffer [ Tris 10mM (pH 7.4), EDTA 1mM, NaCl 100mM, complete mini 1 tablet/10 ml(protease inhibitor cocktail tablets, Roche) ] and the protein eluted from the resin with laemmli sample buffer were resolved on SDS-PAGE and examined by 2-dimensional PAGE with lysis buffer [ 8M urea, 2% CHAPS, 0.8%pharmalyte, 1% DTT ]. The quantitative binding of oligonucleotides to streptavidine-agarose was verified by the analysis of the supernatant in appropriate nondenaturing polyacrylamide gel or IEF strip.

#### **6. Immunoprecipitation assay and Western blotting.**

Cells grown in 100mm culture dishes and transiently transfected with the indicated plasmids were lysed in 1 ml lysis buffer (0.1M Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM MgCl<sub>2</sub>, 1% trioX-100, 10% glycerol, 2 mg/ml phenylethylsulfonyl fluoride, and 1 mM Na<sub>3</sub>VO<sub>4</sub>). 1 mg protein aliquots of the extracts were pre-cleared after dilution with an equal quantity of lysis buffer, soluble proteins were immunoprecipitated at 4°C for 4 h with 2 µg anti-Nurr1, anti-BTK, anti-TOPO II β (Santacruz) and anti-PARP (BD) antibody cross-linked to protein A-rotation. The immunoprecipitated proteins were eluted with SDS sample buffer and resolved via SDS-PAGE. Thus, the proteins were electrophoretically transferred onto PVDF membranes (Millipore). The membranes were incubated with an anti-Nurr 1, anti-BTK, anti-TOPO II β, and anti-PARP with 5% BSA in TBS-T buffer overnight at 4 °C. The membrane was washed three times for 15 min each in TBS-T, and then incubated for 1 h with secondary antibody peroxidase-conjugated anti-

rabbit and anti-mouse (1: 2000, Zymed). Immunoblots were detected with an ECL (Amersham) kit and visualized after exposure to film.

## **7. Chromatin immunoprecipitations (ChIP).**

ChIP assays were conducted with a ChIP kit (Upstate) in accordance with the manufacturer's recommendations. For each ChIP assay,  $2 - 3 \times 10^7$  cells (80 – 90% confluency) were crosslinked with 1% formaldehyde for 20 min at room temperature. The cells were washed twice in ice-cold PBS, scraped from the culture dish and resuspended in SDS lysis buffer (Upstate). Chromatin was fragmented via sonication (Labsonic U, B. Braun) in 20 sec pulses on ice to an average size of 300-400 bp. IP was conducted via the addition of 5  $\mu\text{g}$  anti-BTK, TOPO II  $\beta$ , PARP antibody, or 5  $\mu\text{g}$  unspecific rabbit IgG overnight at 4°C under rotation, and an aliquot was incubated without antibody. Immunocomplexes were precipitated via the addition of protein G plus protein A agarose for 1 h at 4°C. After interaction with protein G plus protein A beads and overnight incubation at 65°C to reverse the cross-links, the DNA was dissolved in Tris-EDTA buffer and PCR analyzed. Site-specific PCR was conducted using primer pairs located at the NBREA binding sites (374 bp). PCR products were run on 2% agarose gel and visualized via ethidium bromide staining. Each ChIP experiment was conducted at least three times with similar results.

## **8. Construction of siBTK Duplexes and Transfection.**

The siRNA duplexes for BTK were designed as described elsewhere (Juhana E, 2002).

The c DNA-targeted region and the sequences of the siRNA duplexes for BTK are as follows:

**Btk siRNA-1** (accession no. NM\_000061 and L29788);

targeted region (cDNA): <sup>1682</sup>-TTGGTAAACGATCAAGGAG-<sup>1700</sup>;

sense siRNA: 5'-UUGGUAAACGAUCAAGGAGUU;

antisense siRNA: UUAACCAUUUGCUAGUCCUC-5';

**Btk siRNA-2**; targeted region (cDNA): <sup>895</sup>-GGGAAAGAAGGAGGTTTCA-<sup>913</sup>;

sense siRNA: 5'-GGGAAAGAAGGAGGUUUCUU;

antisense siRNA: UUCCCUUUCUCCUCCAAAGU-5';

**Btk siRNA-3**; targeted region (cDNA): <sup>518</sup>-GAAGCTTAAAACCTGGGAG-<sup>536</sup>;

sense siRNA: 5'-GAAGCUUAAAACCUUGGAGUU;

antisense siRNA: UUCUUCGAAUUUCCACCCUC-5'.

The siRNA duplexes of BTK were transfected using LiofectAMINEPLUS reagent in accordance with the manufacturer's instructions.

### III. RESULT

#### 1. Cell type-specific regulation of human TH promoter constructs through NBRE-A motif.

In our previous study, deletion analysis indicated that the hTH promoter upstream -3174 to -2164 regions retain the majority of the responsiveness to Nurr1 transcriptional activation or repression in human neural stem and human neuroblastoma cells. We hypothesized that NBRE-A may be crucial cis-regulatory elements for the transcriptional regulation of the TH promoter activity by Nurr1 in differential cell lines. In order to assess these hypotheses, we mutagenized all or each of these three putative cis-elements in the context of the 3,174 kb upstream sequences.

As shown in Fig. 1A, series of luciferase reporter construct containing various fragments of point mutation of the human TH promoter, was cotransfected in both differential cell line such as, human neuroblastoma cells, SH-SY5Y, human neural stem cells, F3, A4, F5 (Fig. 1B) and in the presence or absence of Nurr1 addition. The point mutation of all three sites (i.e. mABC) almost completely not abolished transactivation of hTH promoter by Nurr1 in SH-SY5Y, F3, A4, F5 cell lines. This result thus strongly supports the idea that Nurr1 directly transcriptional regulated hTH transcription by interacting with not only some or all of these NBREs motif, but also 447 regions (k. s. kim et al. 2003). When the NBRE-A site or NBRE-B site or NBRE-C site mutated (mA, mB, mC), the hTH promoter was transcription repressed as efficiently by Nurr1 as the wild-type promoter in SH-SY5Y and F3 cell lines. Moreover, mutation of the NBRE-A (mA) site, caused an approximately 60% decreased in

hTH reporter gene expression by Nurr1 in SH-SY5Y cell (Fig. 1B). These results suggested that, The NBRE-A region important for mediated transactivation of the hTH by Nurr1 in SH-SY5Y cell line. In contrast, mutation of the NBRE-A (mA) site, bring out an approximately 130% increase in hTH reporter gene expression by Nurr1 in F3 cell (Fig. 1B). Therefore, NBRE-A was critical region for mediated transcriptional repression of the hTH in F3 cell line. also, in A4, F5 cell line, the similarly properties of F3 cell in human neural stem cell line, NBRE-A very critical region for transcriptional repression in hTH promoter. Based on previous TH promoter studies indicated that, Neuron-like cell-specific of the human TH gene promoter (Kim et al. 2003). To determine cell- specific regulation of human TH promoter activity by Nurr1, we performed cotransfection of these constructs with Nurr1 or without Nurr1. As shown in Fig. 1C, in SH-SY5Y cell, Neuron-like cell forced expression of Nurr1 caused an approximately 11-fold increase in each reporter expression. In contrast, human neural stem cell, F3, A4, F5 (Fig. 1C) activated only 0.7-fold increase in hTH reporter gene expression. According to results that, Neuron-like cell-specific of the human TH gene promoter by Nurr1.

## **2. Purification of Nurr1 and recruitment cofactor complex in NBRE-A region.**

We have demonstrated that Nurr1 directly mediates the transcriptional activation or repression of TH gene expression through the NBRE-A site and is a key regulating complex of TH expression, which may be formed in the NBRE-A site during dopaminergic neurogenesis. We also determined that Nurr1 transcriptional activity is cell-type-specific, thereby suggesting that the transcriptional cofactors of Nurr1-interacted might participate in

the regulation of promoter transcriptional activity. In order to evaluate Nurr1 and potential recruitment cofactor by Nurr1 in the NBRE-A region, we utilized the 5'-biotinylated oligonucleotide / streptavidin - agarose bead system in transfected Nurr1 or none in a differential cell line. Thus, we established a 30bp biotinylated oligonucleotide probe DNA affinity pull-down assay in order to identify NBRE-A motif-interacting proteins in the identification of cofactors that binding in and around the area (Fig. 3). The eluted sample from the pull-down assay (in Nurr1 transfected or non-transfected cells) was analyzed via SDS-PAGE followed by silver staining. As is shown in Fig. 3B, the eluted sample contained several protein bands, which were not present in the control sample (without the addition of biotinylated oligonucleotide NBRE-A probe in the DNA pull-down assay procedure, lanes 1, 2 and lanes 5, 6). Several different protein-Nurr1 or NBRE-A region complexes evidenced differently-sized patterns in each cell line, F3 and SH-SY5Y cells. Western blot analysis confirmed a high level of Nurr1 protein in the lanes 3, 4 in the F3 cell line, and lanes 7, 8 in the SH-SY5Y cell line, as compared with lanes 1, 2, 5, 6 in the controls (Fig. 3B). The results of the analysis showed that a total of 24 specific binding proteins evidenced significant alterations in their expression in F3 cells. The proteins were then categorized according to their functions, including transcription factors and metabolism-related proteins. These involved burton tyrosine kinase (BTK) associated protein, heterogeneous nuclear ribonucleoprotein family (A, H, K), topoisomerase II  $\alpha$ ,  $\beta$  (TOPO II  $\beta$ ), DNA-3-methyladenine glycosylase, Ruv B-like1, RCC1 like protein, ATP-dependent RNA helicase A, DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, U5 snRNP-specific protein, PRP9 protein, and many others. However, 5 proteins that interact specifically with the NBRE site

were identified in SH-SY5Y cells, including single-stranded DNA-binding protein, protease serine 4 isoform, replication protein, core 2 beta-1,6-N- acetylglucosaminyltransferase and HSPC 255 protein(data was not show). We selected 3 candidate proteins which are BTK, TOPO II  $\beta$  and PARP.

These proteins were identified as new potential transcriptional partners of Nurr1. In order to confirm the specificity of the NBRE-A region-binding proteins, 3 proteins were selected randomly for quantitative Western blot analysis (2C). Thus, the specific proteins of BTK, TOPO II $\beta$  and PARP in the NBRE-A region binding site were identified either in the respective cells or in the SH-SY5Y and F3 cells transfected transiently with Nurr1. The expression level of BTK increased to a greater degree in the F3 cells than in the SH-SY5Y cells, whereas the expression of TOPO II  $\beta$  was not significantly altered in SH-SY5Y cell. PARP expression increased in the Nurr1-overexpressed SH-SY5Y cells as compared to the control cells, whereas the results of Western blotting demonstrated that their expressions were not significantly altered in the Nurr1-overexpressed cells as compared to the respective controls group.

### **3. Conformation BTK, TOPO II $\beta$ and PARP co-immunoprecipitate with Nurr1 in F3 and SH-SY5Y cells.**

To corroborate the above-mentioned observations, we conducted immunoprecipitation analysis for each of the above-mentioned proteins or Nurr1, and conducted Western blotting for specific antigens in order to determine whether they associated in a complex in F3 and SH-SY5Y cells (Fig 3A). The results showed that Nurr1 associated with PARP, TOPO II  $\beta$ ,

and BTK using a specific antibody for Nurr1 and Western blotting for each of the three candidate proteins. Importantly, in the reverse immunoprecipitation using specific antibodies against candidate proteins, Western blot analysis demonstrated that Nurr1 was detected. Thus, BTK is profoundly associated with Nurr1 in F3 cells, as compared to SH-SY5Y cells. Additionally, the interaction of PARP and Nurr1 was found to be increased in SH-SY5Y cells, but similar patterns were noted between the wild-type and Nurr1-transfected cell lines. By way of contrast, TOPO II  $\beta$  associated only with Nurr1 from F3 cells in the reverse immunoprecipitation analysis.

#### **4. Physical interaction both identified proteins and NERE-A motif of Nurr1.**

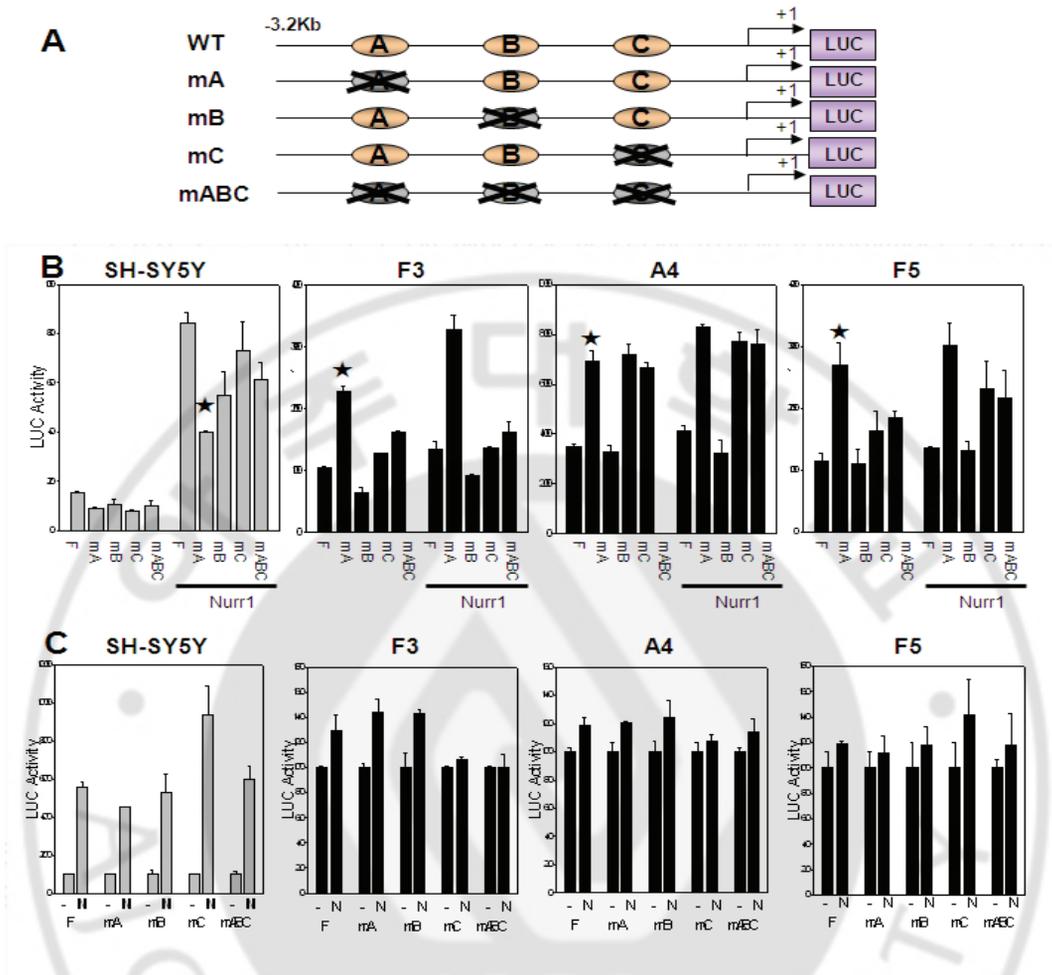
In order to determine whether the candidate proteins are able to bind to the NBRE region, we assessed the interaction between candidate proteins and Nurr1 in F3 and SH-SY5Y cells via ChIP analysis (Fig 3B). The cross-linked chromatin from  $5 \times 10^5$  cells was immunoprecipitated with specific antibodies against BTK, TOPO II  $\beta$ , and PARP, and was amplified with primers that amplified a 374-bp amplicon within the NBRE-A elements. ChIP analysis demonstrated that three candidate proteins, PARP, TOPO II  $\beta$ , and BTK, were all associated with the NBRE-A element, with no detectable signals observed with the match antibody control. In particular, BTK was strongly bound at 210% at the NBRE-A element in F3 cells, whereas PARP was increased by 140% in the SH-SY5Y cells(3C). These results indicate that these proteins are associated with the NBRE elements, and their signals may also include association with Nurr1.

## **5. BTK, TOPO II and PARP are co-regulators of Nurr1 in the F3 and SH-SY5Y cells.**

Our results identified proteins as a potential modulator of Nurr1-induced TH transcriptional activity. Co-immunoprecipitation and streptavidin pull-down experiments established an interaction between Nurr1 and PARP, TOPO II  $\beta$ , and BTK. In order to assess the functionality of the TH promoter and to test the cell specificity of TH gene expression, we introduced a series of TH promoter/luciferase reporter gene constructs into the F3 and SH-SY5Y cells (Fig. 4). The cells were cotransfected with TH and different combinations of Nurr1, PARP, TOPO II  $\beta$ , and BTK expression vectors. Importantly, the co-transfection of Nurr1-PARP and Nurr1-TOPO II  $\beta$  was enhanced significantly in TH gene luciferase levels in both cell lines. By way of contrast, BTK of hTH promoter activity repressed irrelevant Nurr1. These data show clearly that BTK may function as a repressor of the TH promoters in F3 cells. Also, these results show that Nurr1 directly activates the TH promoter in a cell-specific manner, and demonstrated that Nurr1 interacted directly to BTK, TOPO II  $\beta$ , and PARP, may regulate TH transcription activity via the identified proteins. In F3 cells, BTK repressed Nurr1-induced hTH promoter activity in a dose-dependent manner (Fig. 5A). Additionally, BTK profoundly repressed the transcriptional effects, inducing up to an 80% inhibition in F3 cells. In order to determine whether BTK influences Nurr1-induced transactivity, F3 cells that express neither Nurr1 nor BTK were co-transfected with mutant BTK and siBTK. Cotransfection of Nurr1 with BTK caused a significant repression of luciferase activity, whereas double tyrosine-mutant BTK significantly enhanced TH promoter activity (Fig. 5B). Surprisingly, siBTK was still found to be capable of enhancing

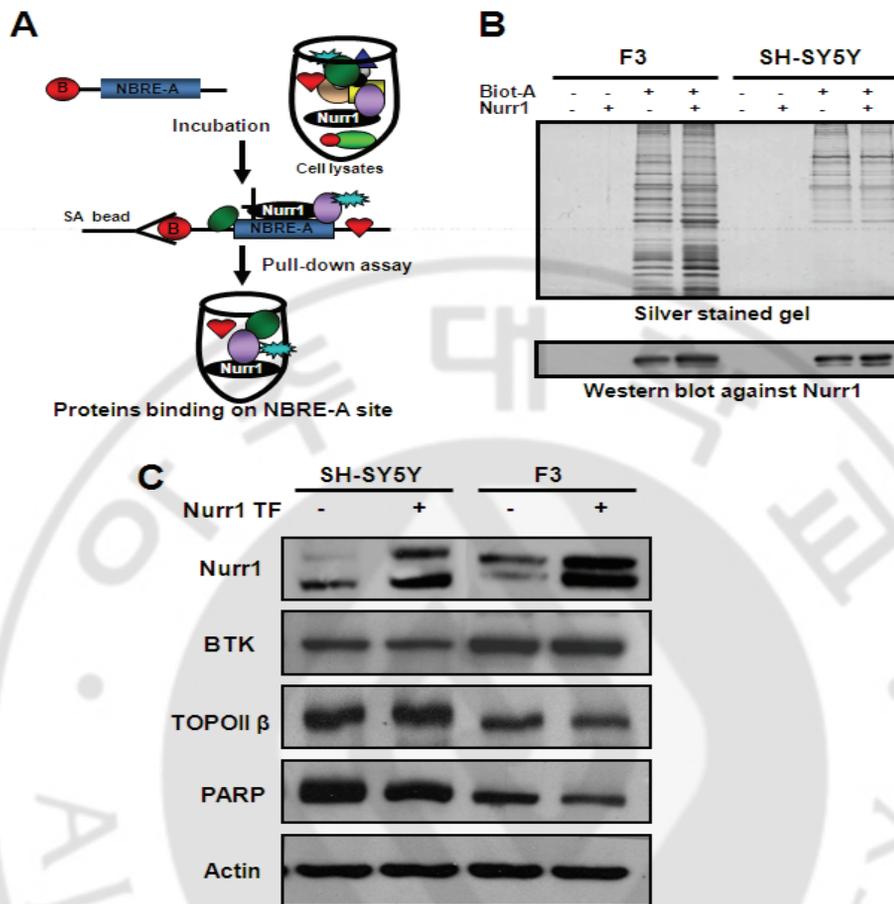
Nurr1-induced TH transcriptional activity (Fig 5C). As shown, the transfection of BTK siRNA duplexes to neural stem cells was quite effective, as BTK expression was strongly downregulated (Fig 5C). These findings show that Nurr1-dependent transcriptional repressor activity in neural stem cells depends upon the coexpression of the BTK. Our present data clearly demonstrate the requirement for BTK kinase at the repressor activity in TH function.





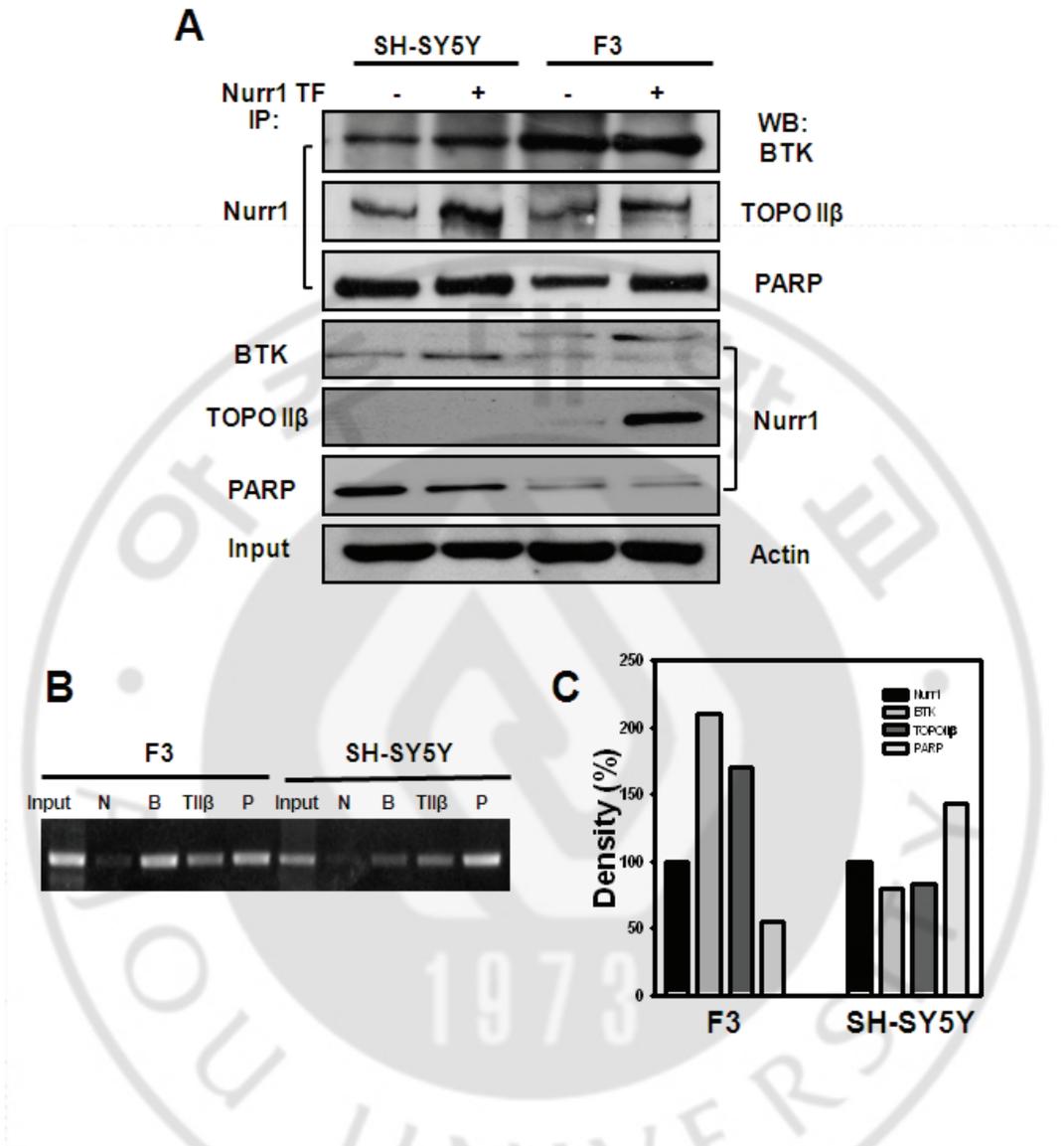
**Figure 1. Cell type-specific regulation of human TH promoter through NBRE - A site.**

(A) Schematic diagram of the TH promoter encompassing three putative NBREs (NBRE-A, -B, -C). (B) Three of mutated TH constructs with or without Nurr1 were influenced in the activity of TH in SH-SY5Y in human NSCs (Neural stem cells), F3 (C), A4 (D), and F5 (E) cells. Effect of mutations on transactivation of the TH promoter activity by Nurr1 in SH-SY5Y (B'), and F3 (C'), A4 (D'), F5 (E') cells.



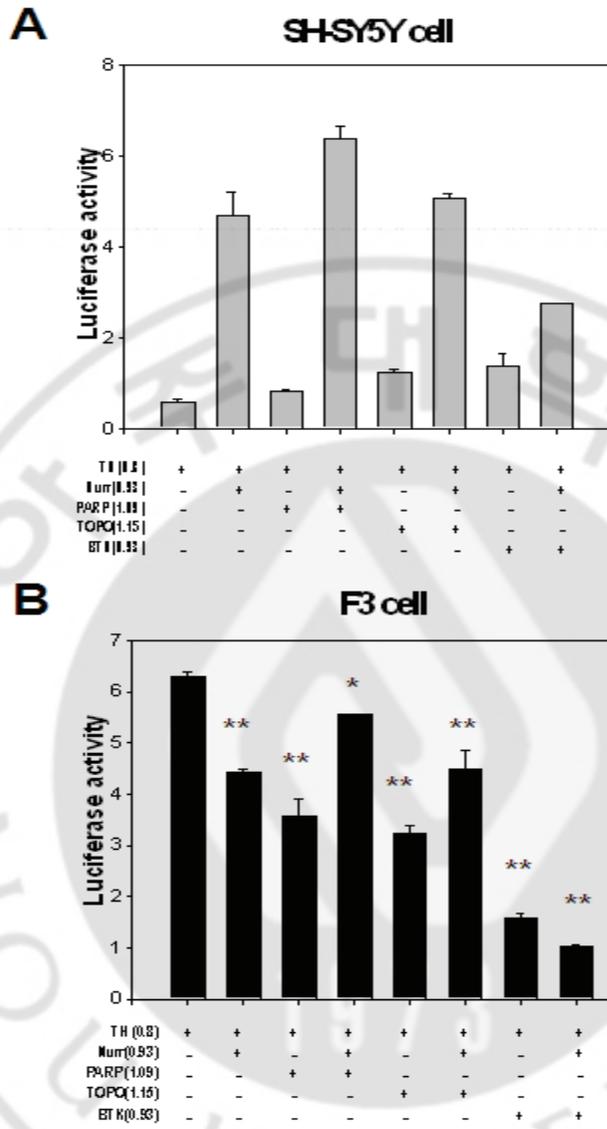
**Figure. 2. Purification of NBRE- A binding protein by DNA pull-down assay.**

(A) Flow diagram for purification of the NBRE-A site binding protein. (B) SDS-PAGE analysis of purified NBRE-A binding, in and around protein eluted from streptavidin agarose bead affinity resin. (C) Western blotting of the purified NBRE-A binding, in and around protein.



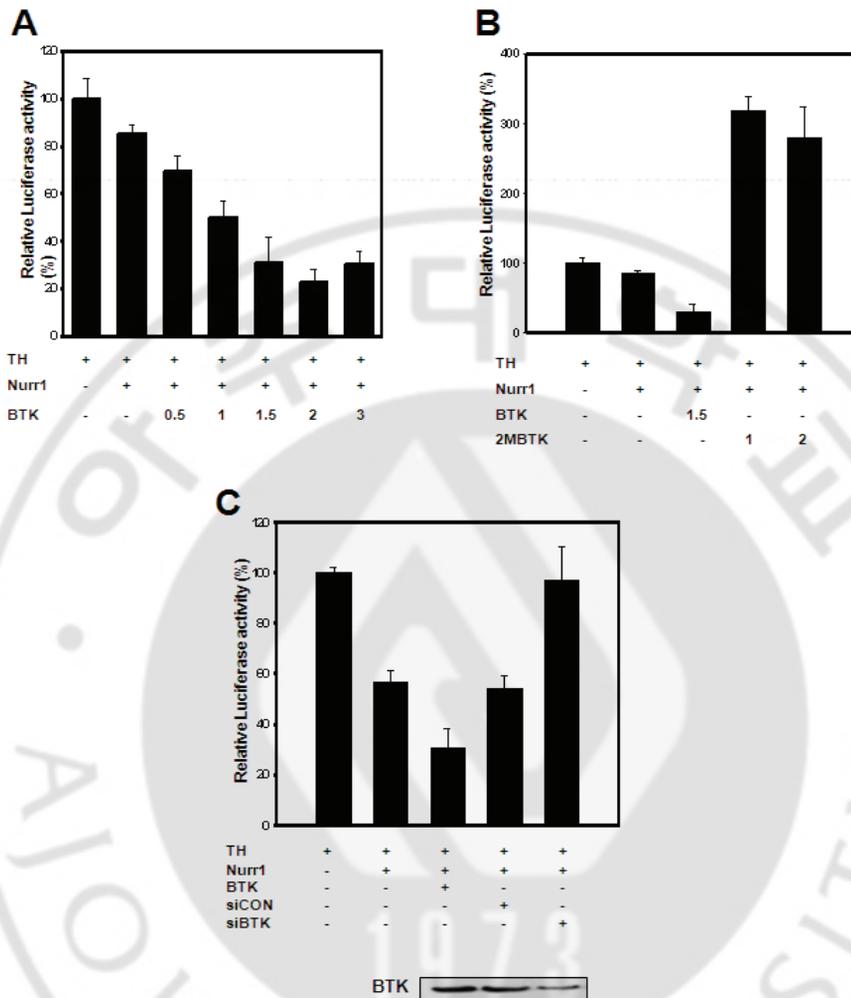
**Fig. 3. Physical interaction between candidate proteins and Nurr1**

(A) The interaction between BTK, TOPO II  $\beta$  or PARP and Nurr1 in F3 and SH-SY5Y cells were confirmed by coimmunoprecipitation analysis. (B) ChIP assay. (C) Quantification of the immunoprecipitation based on the quantification of the specific bands



**Fig. 4. Transcriptional activities of human TH promoter by BTK, PARP and TOPO.**

(A) SH-SY5Y cell and (B) human Neural stem cell F3 \*,  $p < 0.01$  compared with TH promoter group. \*\*,  $p < 0.001$ .



**Fig. 5. BTK functions as a repressor in human neural stem cells.**

(A) TH promoter activity by Nurr1 with increasing amount of BTK expression. (B) Expression of kinase-deficient BTK indicated that BTK functions as a transcriptional co-repressor of Nurr1 on TH expression. (C) Transcriptional activities of human TH promoter in BTK knockdown F3 cells.

## IV. Discussion

Our previously report, we studied human TH sequence analysis to identify transcriptional regulatory element binding sites, which perform essential functions in dopaminergic neurogenesis (Kim et al., 2003). Also to check the cell type-specific and differentiation-related expression of TH, we performed many experiment. And we focus on Nurr1 and revealed Nurr1 direct regulation of TH gene expression. When Nurr1 regulate the TH gene expression, it is different with cell stage that repression in undifferentiated cell stage(neural stem cells) and activation in differentiated cell stage(neuroblastoma cells). We demonstrated this regulation of TH gene expression by Nurr1 may affect other cofactors. So we identified the protein that binds to Nurr1. Our human TH promoter has three Nurr1 binding sites which was mutated one amino acid with NBRE sequence, so we called NBRE-A,B,and C. We made variety structure with or without NBRE-A,B and C. After that check the TH promoter activities with or without Nurr1.In the results NBRE-A site is critical to TH promoter regulation(Fig.1) which repress in F3 cells and activate in SH-SY5Y cells. Therefore, we performed DNA pull-down assay to find cofactors binds to the NBRE-A site. In fact, in our assays we detected an interaction with the TH repressor Nurr1, which was further confirmed by streptavidin-agarose beads pull-down assays. Also LC/mass spectrophotometry analysis identifies new potential dimmer partners of nurr1. We identified three candidate proteins including BTK, TOPO II beta, and PARP.

BTK is a type of kinase enzyme implicated in the primary immunodeficiency disease X-linked agammaglobulinemia and plays a crucial role in B cell maturation. Also BTK

translocate to the plasma membrane upon stimulation with growth factor and expressed in a variety of mammalian neural cells. And TOPO II beta is critical acts for the cell proliferation and transaction including DNA replication, transcription, and recombination. Last, PARP is a protein involved in an number of cellular processes involving mainly DNA repair and programmed cell death.

Each protein expressed in experimental cells(Fig.2) and interacted with Nurr1(Fig3). To check the interaction we performed co-immunoprecipitation and ChIP assay, BTK was strongly interacts with Nurr1 in F3 cells and PARP interacts in SH-SY5Y cells. Also BTK strongly repressed TH promoter activities with Nurr1 in F3 cells, PARP activated TH promoter activities in SH-SY5Y cells(Fig.4). At this result, we pointed BTK in F3 cells and so, performed further experiments to check BTK's functions and effects in F3 cells. In the result, BTK repressed the TH promoter activities as dose dependent manner and when BTK was mutation or knockdown, this activation was disappeared. (Fig.5)

Our results indicate when Nurr1 repress the TH gene expression in immature cell stage(neural stem cell), BTK strongly repress by recruitment of Nurr1. After cell maturation(dopaminergic cell) Nurr1 may recruit PARP, the TH gene expression may restart.

## V. CONCLUSION

The findings of our study demonstrate that Nurr1 directly activates the TH promoter in a cell line-specific manner and cofactors are required for Nurr1-mediated TH gene induction. Additionally, these data indicate that these specifically interacting cofactors may perform a crucial role in the neuron cell-specific expression of the TH gene. Thus, these identified Nurr1 binding proteins provide a number of clues and potential links in understanding the mechanisms of the transcription of TH genes. Finally, these study of TH gene may offer Elucidation of etiology of neuronal disorders such as Parkinson's disease.

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## 미분화 신경줄기세포에서 BTK와 전사인자 Nurr1의

### 상호작용에 관한 연구

아주대학교 대학원 의생명과학과

박 명 선

(지도교수: 이 명 애)

파킨슨 병은 주로 중뇌의 substantia nigra에서 도파민 신경세포의 소멸로 인해 발병된다. 카테콜아민 생합성 과정에서 속도 조절 효소인 tyrosine hydroxylase (TH) 유전자의 발현은 신경세포의 발달과정동안 전사과정과 다양한 환경적인 자극에 의해 조절된다. 또한 Orphan nuclear receptor 중 하나인 Nurr1은 발달 과정 중 중뇌의 substantia nigra와 ventral tegmental 부위에서 발현되고, 도파민 신경 세포 전구체가 완전한 도파민 신경세포로 분화하는데 중요한 역할을 한다. Nurr1은 발달 과정 동안 TH 유전자의 발현을 유도하는데 중요한 전사인자라고 생각된다.

우리는 TH 유전자의 발현에서 세포특이적인 전사 조절을 하고, Nurr1에 의한 활성화에 가장 크게 관여하는 NBRE-A 부분에서 Nurr1과 함께 복합체를 이루어 TH 발현을 위해 전사 조절에 관여하는 인자들을 찾아 동정하고자 하였다. 이

에 DNA pull-down assay를 이용하여 NBRE-A 부분에 결합하여 전사 조절 인자들을 살펴보았다. 이 결과 Nurr1과 결합하는 후보 단백질인 PARP, TOPO, BTK를 선택할 수 있었고, 이 각각의 단백질이 Nurr1과 결합하여 TH 유전자의 발현을 조절하는지를 확인하였다. 이 중 BTK는 Nurr1과 결합하여 사람 줄기세포주인 F3 세포에서 TH 유전자의 발현을 억제시키는 것을 확인 할 수 있었다. 이 결과는 향후 TH 유전자의 발현 정도에 따라 나타나는 퇴행성 뇌질환의 연구 분야에 큰 공헌을 할 것으로 기대된다.

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**핵심어** : 파킨슨병, TH 유전자, Nurr1, NBRE-A, BTK, 퇴행성 뇌질환