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Inverse Expression Levels of EphrinA3 and EphrinA5 Contribute to Dopaminergic Differentiation of Human SH-SY5Y Cells

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Abstract Two key principles underlying successful cellular therapies for Parkinson's disease (PD) are appropriate differentiation of dopaminergic (DA) neurons from transplanted cells and precise axon growth. EphrinAs, a subclass of ephrins, act as axon guidance molecules and are highly expressed in DA brain regions. Existing evidences indicate that they act as either repulsion or attraction signals to guide axon growth. This study investigated whether ephrinAs are involved in DA neuron differentiation. Data from miRCURY™ LNA mRNAs/microRNAs microarrays and quantitative real-time polymerase chain reaction (qRT-PCR) showed upregulated ephrinA3 mRNA (EFNA3) and downregulated ephrinA5 mRNA (EFNA5) during DA neuron differentiation. In addition, hsa-miR-4271 was downregulated, which could influence EFNA3 translation. Furthermore, immunofluorescence (IF) and western blotting confirmed the mRNA results and showed increased ephrinA3 and decreased ephrinA5 protein levels in differentiating DA neurons. Taken together, our results indicate that inverse expression levels of ephrinA3 and ephrinA5, which are possibly influenced by microRNAs, contribute to DA neuron differentiation by guiding axon growth.

Keywords EphrinAs · Parkinson's disease · Cellular therapy · Axon guidance

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Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic (DA) neurons in the nigro-striatal region of the brain. Cellular therapies for PD have become a popular research topic. Because the goal is to replace degenerating neurons with stem cell-derived DA neurons, accurate differentiation of DA neurons from stem cells is an important step (Müller et al. 2015; Remy 2014; Shin et al. 2014; Bu et al. 2014; Rhee et al. 2011; Kiessling 2014; Heuer et al. 2013). Secondly, it is important that replacement DA neuron develop appropriate projections from the substantia nigra (SN) to the striatum (Heuer et al. 2013; Bové et al. 2005; Deumens et al. 2002; Grealish et al. 2008).

Appropriate neuron projection requires axon guidance molecules. These molecules can be nondiffusible or diffusible. Nondiffusible molecules include cell adhesion molecules (CAMs), extracellular matrix (ECM) molecules, and ephrins. Diffusible molecules are the netrins, Slits, and semaphorins. Among these, ephrins are membrane-bound protein ligands for a family of receptor tyrosine kinases (Eph) and are widely distributed in the developing central nervous system (CNS) including the retina, optic tectum/superior colliculus, thalamus, and neocortex (Flanagan and Vanderhaeghen 1998; McLaughlin et al. 2003). Ephrin/Eph are involved in the topography of innervation during neuronal development (Wilkinson 2001); they can modulate either repulsion or attraction during axon projection guidance depending on several cues (e.g., expression sites, expression level) (Holmberg and Frisén 2002; Knoll and Drescher 2002; Hattori et al. 2000; Holmberg et al. 2000; Pittman and Chien 2002). In addition to guiding axon projections, Ephrin/Eph can also regulate axon growth, possibly executed by modulating actin cytoskeletal organization and axon growth cone mobility (Martinez and Soriano 2005; Cowan and Henkemeyer 2002; Davy and Soriano 2005; Drescher et al. 1995; Wang and Anderson 1997). For

example, ephrin/Eph gene expression has been shown to control thalamocortical projections (Dufour et al. 2003).

EphrinAs and ephrinBs preferentially bind to EphAs and EphBs receptors, respectively (Himanen et al. 2001; Bush and Soriano 2012). EphrinAs are attached to the cell surface via a glycosylphosphatidylinositol (GPI) anchor and are highly expressed in DA brain regions. Along with their receptors EphAs, EphrinAs were identified as Sperry-type labels for the anteroposterior (A–P) axis of the projection from the retina to the tectum of the midbrain, and they also act as positional labels for topographic targeting (Cheng et al. 1995; Flanagan 2006; Cang et al. 2008; Knoll et al. 2001). Visual topographic maps suggested that ephrinAs/EphAs guide retinal ganglion cell (RGC) axons to their appropriate termination zones (Feldheim et al. 2000). In the olfactory system, ephrinAs target axons to their precise locations in the olfactory bulb (Cutforth et al. 2003). Another confirmed that ephrinAs independently direct axon growth by chick motor neurons (Marquardt et al. 2005). EphrinAs are also strongly implicated in neuronal plasticity and axonal growth in adult CNS (Xu et al. 2003).

Despite abundance of evidence supporting their involvement in several neuronal populations, whether ephrinAs direct axon growth in differentiating DA neurons remains unknown. We tested the hypothesis that ephrinAs expression levels are altered in differentiating human neuroblastoma SH-SY5Y cells following exposure to glial cell-line derived neurotrophic factor (GDNF).

Materials and Methods

Cell Culture

Human dopaminergic neuroblastoma SH-SY5Y cells (The Center Cell Resources of Shanghai Institute of Life Science Chinese Academy of Sciences, Shanghai, China) were cultured in a 1:1 ratio of minimum essential medium (MEM)-F12 (Thermo Fisher Scientific, Waltham, MA) supplemented with 12–15 % fetal bovine serum (FBS; Thermo Fisher Scientific) in 37 °C, 5 % CO_2. The medium was changed twice a week, and cells were divided at about 80 % confluence. Subsequently, some cells were treated with human Glial Cell Line-derived Neurotrophic Factor (50 ng/ml media; Sigma) (GDNF groups), and the remaining cells were treated with medium (Control groups). Cells were harvested after 24-h incubation. We have confirmed that GDNF can induce SH-SY5Y cells to differentiate into DA neurons (manuscript in preparation). In this study, light microscopy showed that GDNF-treated cells had more neurites than control cells (Fig. 1).

Immunofluorescence (IF)

The cells were fixed using paraformaldehyde and permeabilized using 0.1 % Triton (both from Thermo Fisher Scientific). The cells were blocked using 1 % bovine serum albumin (BSA; Solarbio, Beijing, China) for 30 min at 20 °C. The cells were then incubated with an anti-Ephrin A3 antibody (5 μg/ml; Abcam, Cambridge, UK) and anti-Ephrin A5 antibody (Abcam, 1:500) overnight at 4 °C. The anti-Ephrin A3 and A5 antibodies were detected using goat polyclonal to rabbit IgG conjugated to Cy3 (1:100; BOSTER, Wuhan, China) and goat polyclonal to mouse IgG conjugated to fluorescein isothiocyanate (FITC, 1:100; ZSBG-BIO, Beijing, China) for 30 min. DAPI (4′,6-diamidino-2-phenylindole) applied at a concentration of 50 μg/ml was used to stain the cell nuclei blue. Morphology was observed using an inverted fluorescent microscope (Carl Zeiss, Oberkochen, Germany). ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantify IF images.

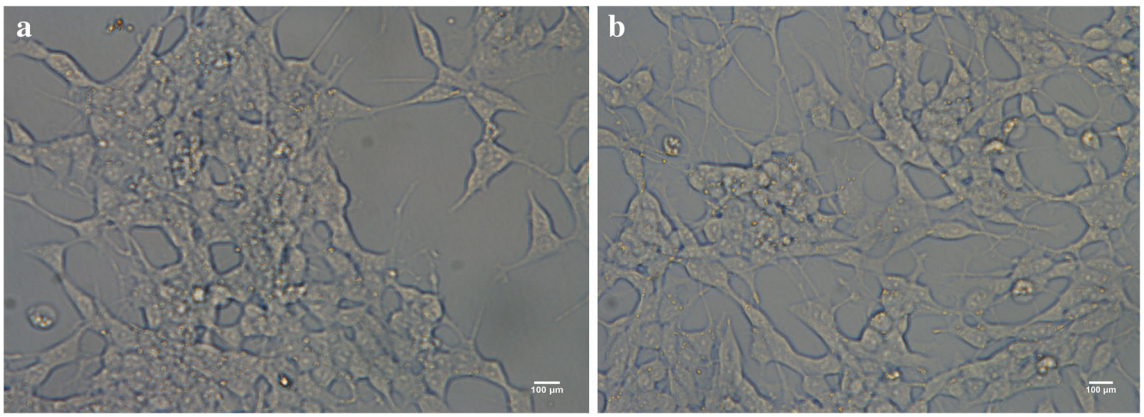

Fig. 1 Light microscopy images. Cells cultured **a** without GDNF or **b** with GDNF (50 ng/ml). Cellular neurite outgrowth is clear in (**b**). *Scale bars* represent 100 μm

Western Blot

SH-SY5Y cells were lysed in cell lysis buffer and spun at 1000×*g* for 3 min. Cell pellets were resuspended in 2× Laemmli sample buffer, separated by SDS–PAGE, and probed with the same anti-EFNA3 and EFNA5 antibodies described above (Abcam). The nitrocellulose membranes were stripped and reprobed with an anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to assess protein loading. Each blot is representative of at least three separate experiments. Immunoblot band densities were quantified using ImageJ software.

RNA Extraction

Total RNA was isolated from both groups using TRIzol (Invitrogen, Carlsbad, CA). RNA quality and quantity were measured with a nanodrop spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, DE), and RNA integrity was determined by gel electrophoresis.

MicroRNA/mRNA Microarray

The sixth generation of miRCURY™ LNA Array (v.16.0) (Exiqon, Vedbæk, Denmark) contains more than 1891 capture probes, covering all human, mouse, and rat miRNAs annotated in miRBase 16.0, as well as all viral miRNAs related to these species. After RNA isolation, the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon) was used for miRNA labeling according to the manufacturer's guidelines. One microgram of each sample was 3′-end-labeled with a Hy3TM fluorescent label using T4 RNA ligase and the following procedure: RNA in 2.0 μl water was combined with 1.0 μl CIP buffer and CIP (Exiqon). The mixture was incubated for 30 min at 37 °C and terminated by incubation for 5 min at 95 °C. Then, 3.0 μl labeling buffer, 1.5 μl fluorescent label (Hy3TM), 2.0 μl dimethyl sulfoxide (DMSO), and 2.0 μl labeling enzyme were added to the mixture. The labeling reaction was incubated for 1 h at 16 °C and terminated by incubation for 15 min at 65 °C. After terminating the labeling procedure, the Hy3TM-labeled samples were hybridized on a miRCURY™ LNA Array (v.16.0) (Exiqon) according to the manufacturer's directions. The mixture of 25 μl Hy3TM-labeled samples and 25 μl hybridization buffer was first denatured for 2 min at 95 °C, incubated on ice for 2 min, and then hybridized to the microarray for 16–20 h at 56 °C in a 12-Bay Hybridization Systems (Hybridization System—Nimblegen Systems, Madison, WI). This provided active mixing action and a constant incubation temperature to improve hybridization uniformity and yield an enhanced signal. Following hybridization, the slides were washed several times using a wash buffer kit (Exiqon) and finally dried by centrifugation for 5 min at 1000×*g*. The slides were scanned for miRNAs using an Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). All experiments were repeated three times to increase miChip accuracy.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Differentially expressed ephrinAs mRNAs and microRNAs obtained from the microRNA/mRNA microarray were confirmed by performing qRT-PCR. Total RNA was isolated according to the method described above. A single-stranded cDNA for a specific mRNA/microRNA was generated by reverse transcription in mixtures containing 1 μg RNA, 1 μl 0.5 μg/μl Oligo$(dT)_{18}$, 4 μl 5× RT buffer (Invitrogen), 1 μl 0.1 M DTT, 1.6 μl 2.5 mM dNTPs (mixture of dATP, dGTP, dCTP, and dTTP) (HyTest Ltd., Turku, Finland), 0.2 μl SuperScript™ III Reverse Transcriptase (Invitrogen), and 0.3 μl RNase Inhibitor (Epicenter, Madison, WI). Nuclease-free water was used to adjust the final volume to 20 μl. The Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA) was used with the following thermal cycling parameters: 30 min at 16 °C, 42 min at 42 °C, 5 min at 85 °C, and then held at 4 °C. After reverse transcription, qRT-PCR was performed using a ViiA 7 Real-time PCR System (Applied Biosystems). The 10-μl qRT-PCR reaction mixture contained 5 μl 2× PCR Master Mix (Promega, Madison, WI), 1 μl 10 μM qRT-PCR primers, 2 μl cDNA, and 2 μl nuclease-free water. The reactions were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 60 s (to collect fluorescence). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal standards. The results are presented as fold change for each mRNA/microRNA by comparing the GDNF and control groups. All reactions were run in triplicate. The real-time RT-PCR primers are listed in Table 1.

MicroRNA/mRNA Microarray Data Analysis

Scanned images were imported into GenePix Pro 6.0 software (Axon Instruments/Molecular Devices, Sunnyvale, CA) for grid alignment and data extraction. After low-intensity miRNA filtering, miRNAs with raw signal intensities ≥50 in all samples were selected to calculate normalization factors. Data were normalized using median normalization, and significantly differentially expressed microRNAs/mRNAs were identified with Volcano Plot filtering.

Gene Prediction Methodology

Differentially expressed microRNAs/mRNAs with >1.5- or <0.6-fold changes between the control and GDNF groups were selected for target prediction. We used three different computational programs to predict putative targets for the

Table 1 qRT-PCR primers

mRNA/microRNA category	Primer sequence	°C	bp
GAPDH (HUMAN)	F: 5′GGGAAACTGTGGCGTGAT3′ R: 5′GAGTGGGTGTCGCTGTTGA3′	60	299
U6	F: 5′GCTTCGGCAGCACATATACTAAAAT3′ R: 5′CGCTTCACGAATTTGCGTGTCAT3′	60	89
EFNA3	F: 5′TGGAGACCGTAAAACAACAAC3′ R: 5′ATGAAAGTCACAGCCAAAGC3′	60	139
EFNA5	F: 5′AACCAGCAGATGACACCGTA3′ R: 5′AATGTCAAAAGCATCGCCAG3′	60	127
hsa-miR-4271	GSP: 5′GTTGTCGGGGGAAGAAAAG3′ R: 5′GTGCGTGTCGTGGAGTCG3′	60	63

microRNAs/mRNAs: the Microcosm (miRBase) database version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5), miRanda database (http://www.microrna.org/microrna/getMirnaForm.do), and Targetscan version 6.0 (http://www.targetscan.org/cgi-bin/targetscan/mmu_60/). Targets that were predicted by two of the computational programs were selected for further analysis using the enrichment of Gene Ontology (GO, http://www.geneontology.org/) biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) pathways. Fisher's exact tests were performed to determine whether the observed counts could have been due to chance.

Array Association Analysis

The target genes of different microRNA and mRNAs were integrated through negative correlations of expression to generate an associated gene list. The enrichment of GO biological processes and KEGG pathways (GenMAPP v2.1) was studied

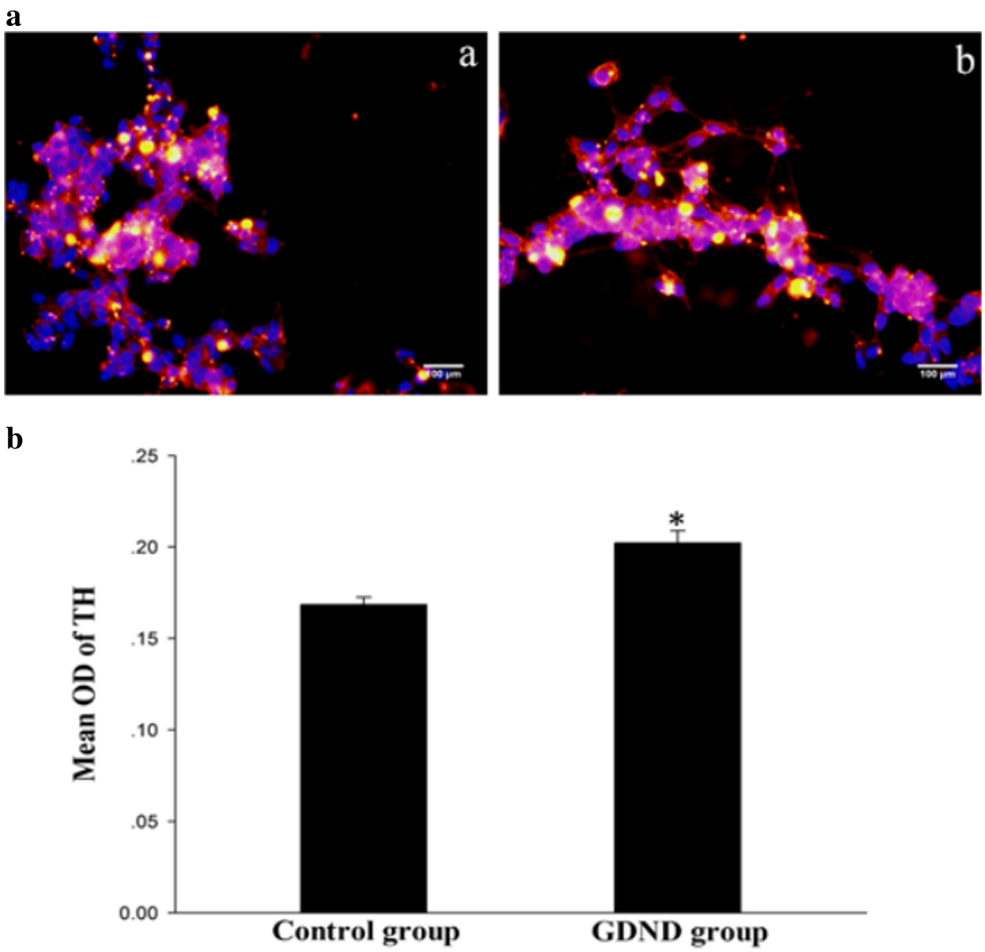

Fig. 2 IF results of TH. **a** IF images of TH in control (*a*) and GDNF (*b*) groups (*blue*, nuclei; *red*, TH). *Scale bars* represent 100 μm. **b** Statistical histogram according to IF images. Means (±SDM) of mean OD, *$P < 0.05$, *vs. Control group, *error bars* represent SEM

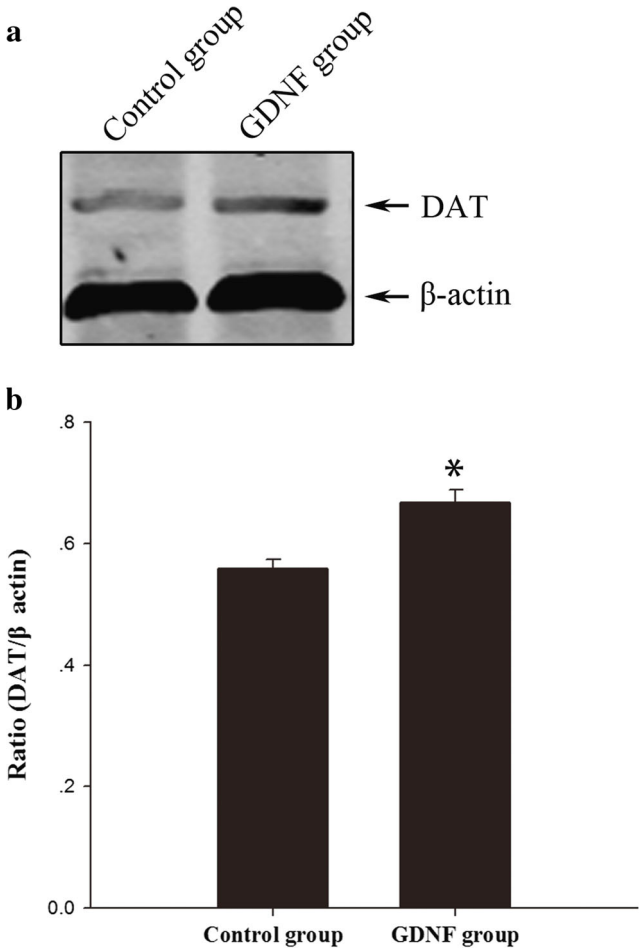

Fig. 3 WB results of DAT. **a** WB bands of DAT and β-actin in the Control and GDNF groups. **b** Statistical histogram according to WB bands. Means (±SDM) of ratio (TH/β-actin), *$P < 0.05$, *vs. Control group, *error bars* represent SEM

among these associated genes to construct a microRNA/gene network.

Statistical Analysis

The data depicted in the graphs are the means ± standard deviation of the mean (SDM). Histograms were generated with SigmaPlot 10.0 (Systat Inc., San Jose, CA). Comparisons between two groups were carried out with independent sample *t* tests. All *P* values <0.05 were considered significant. Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL).

Results

Before this research, we had examined tyrosine hydroxylase (TH) and dopamine transporter (DAT), as marker proteins of DA neurons, during the process of differentiation of SH-SY5Y cells by IF and western blotting. Compared with control groups, IF results showed that the expression of TH increased significantly in GDNF group by optical density (OD) analysis (Fig. 2, $P < 0.05$), and WB evaluated high expression of DAT in GDNF group (Fig. 3, $P < 0.05$). These previous experiments had proved that SH-SY5Y cells indeed differentiated into dopaminergic neurons and the differentiation of GDNF group was more obvious. On this basis, we carried out subsequent studies and obtain the following conclusion.

Identification of EphrinAs mRNAs During DA Neuron Differentiation

During DA neuron differentiation, the mRNA microarray analysis identified significant dysregulation of ephrinA3 and ephrinA5 mRNAs (EFNA3 and EFNA5) between the GDNF and control groups. EFNA3 was upregulated, whereas EFNA5 was downregulated (FC >1.5 or <0.7, $P < 0.05$, Table 2). In addition, microRNA microarray analysis revealed downregulation of has-miR-4271, which could potentially influence EFNA3 translation via array association analysis of mRNA and microRNA, but there was no statistically significant difference (Table 3).

qRT-PCR was subsequently performed to validate the microRNA/mRNA microarray data. The results showed that EFNA3 was upregulated, while EFNA5 and has-miR-4271 were downregulated in GDNF groups (FC >1.5 or <0.7, $P < 0.05$, Table 4), confirming the microRNA/mRNA microarray results.

Increased EphrinA3 and Decreased EphrinA5 Protein Levels During DA Neuron Differentiation

We subsequently performed IF and western blotting to examine ephrinA3 and ephrinA5 protein levels. The results from IF showed increased ephrinA3 and less ephrinA5 in GDNF groups compared to the Control groups. Additionally, many neurites in the GDNF group were positive for ephrinA3 (Figs. 4 and 5). Western blot analysis showed that expression of ephrinA3 protein was increased in the GDNF groups ($P < 0.05$, Fig. 6). Conversely, ephrinA5 expression was decreased ($P < 0.05$, Fig. 7). These results are also consistent with the mRNA data.

Table 2 Results of ephrinAs mRNAs from mRNA microarray analysis

CCDS ID	Gene	Probe name	Fold change (GDNF/control group)	*P* value	Regulation
CCDS1090.1	EFNA3	ASHGA5P041610	2.2551312	0.000626186	Up
CCDS4097.1	EFNA5	ASHGA5P052353	0.392965833	0.005753674	Down

Table 3 Results of EFNA3-related microRNA from microarray analysis

ID	Name	Target gene	Fold change (GDNF/control group)	*P* value	Regulation
147570	hsa-miR-4271	EFNA3	0.655937165	0.193168	Down

Table 4 qRT-PCR findings of EFNA3, EFNA5, and hsa-miR-4271

Name	Fold change (GDNF/control group)	*P* value	Regulation
EFNA3	3.765945763	0.000888343	Up
EFNA5	0.37	0.013209	Down
hsa-miR-4271	0.5633	0.009	Down

Discussion

Accurate DA neuron differentiation requires that neurites terminate at the correct target, and this process requires a variety of attractive and repulsive signals that guide axonal processes. Ephrin and Eph expressed on neighboring cells mediate contacts between the two. Numerous studies have revealed that ephrins and Ephs play important nervous system roles including axon guidance and synaptic plasticity (Grunwald et al. 2004). Although ephrinAs have no intracellular domain, they can participate in reverse signaling, by recruiting molecules such as Src kinase family members (Knoll et al. 2001; Marquardt et al. 2005; Davy and Robbins 2000). Our results showed that the mRNA levels of two ephrinAs (EFNA3 and EFNA5) were altered during DA neuron differentiation.

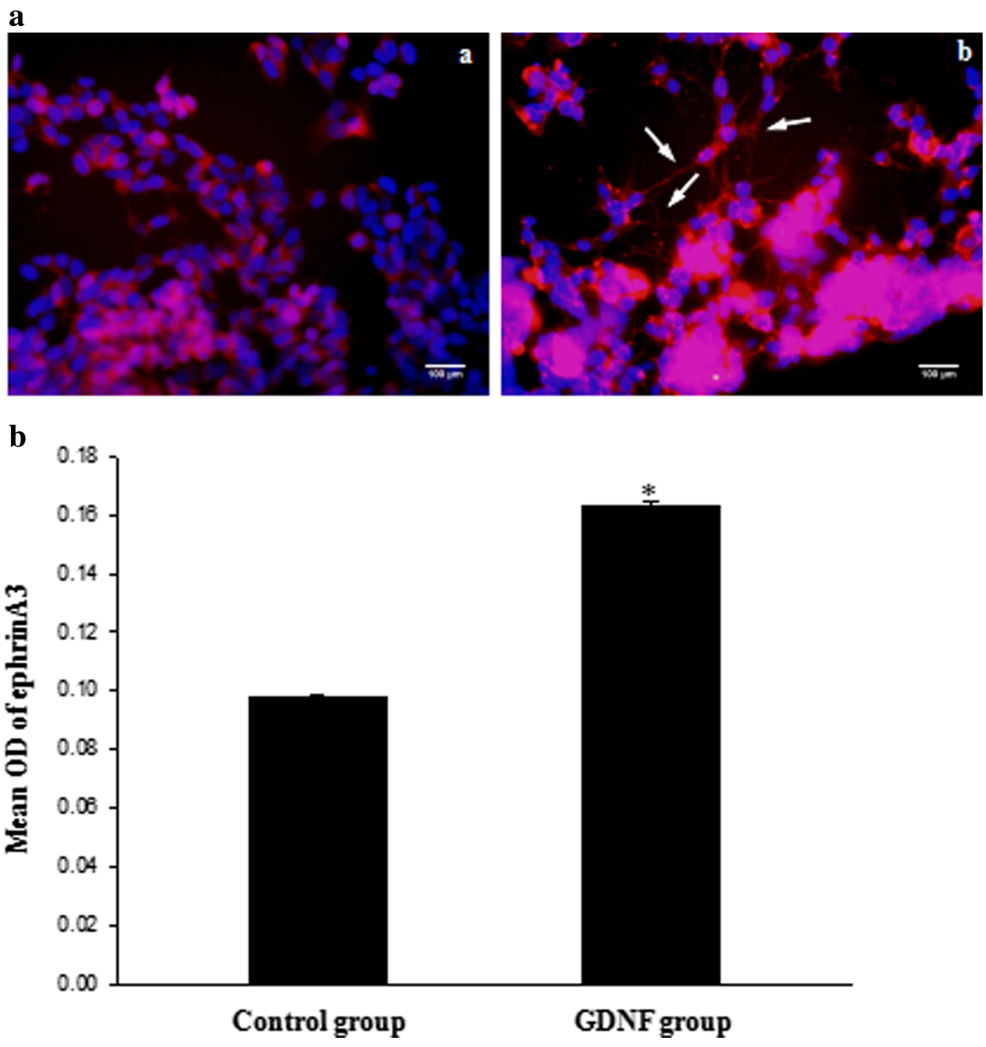

Fig. 4 IF results of ephrinA3. **a** IF images of ephrinA3 in Control (*a*) and GDNF (*b*) groups (*blue*, nuclei; *red*, ephrinA3). EphrinA3 can be obviously observed in neurites (e.g., location pointed by *white arrow*) in (**b**). *Scale bars* represent 100 μm. **b** Statistical histogram according to IF images. Means (±SDM) of mean OD, **P* < 0.05, *vs. Control group, *bars* represent SDM

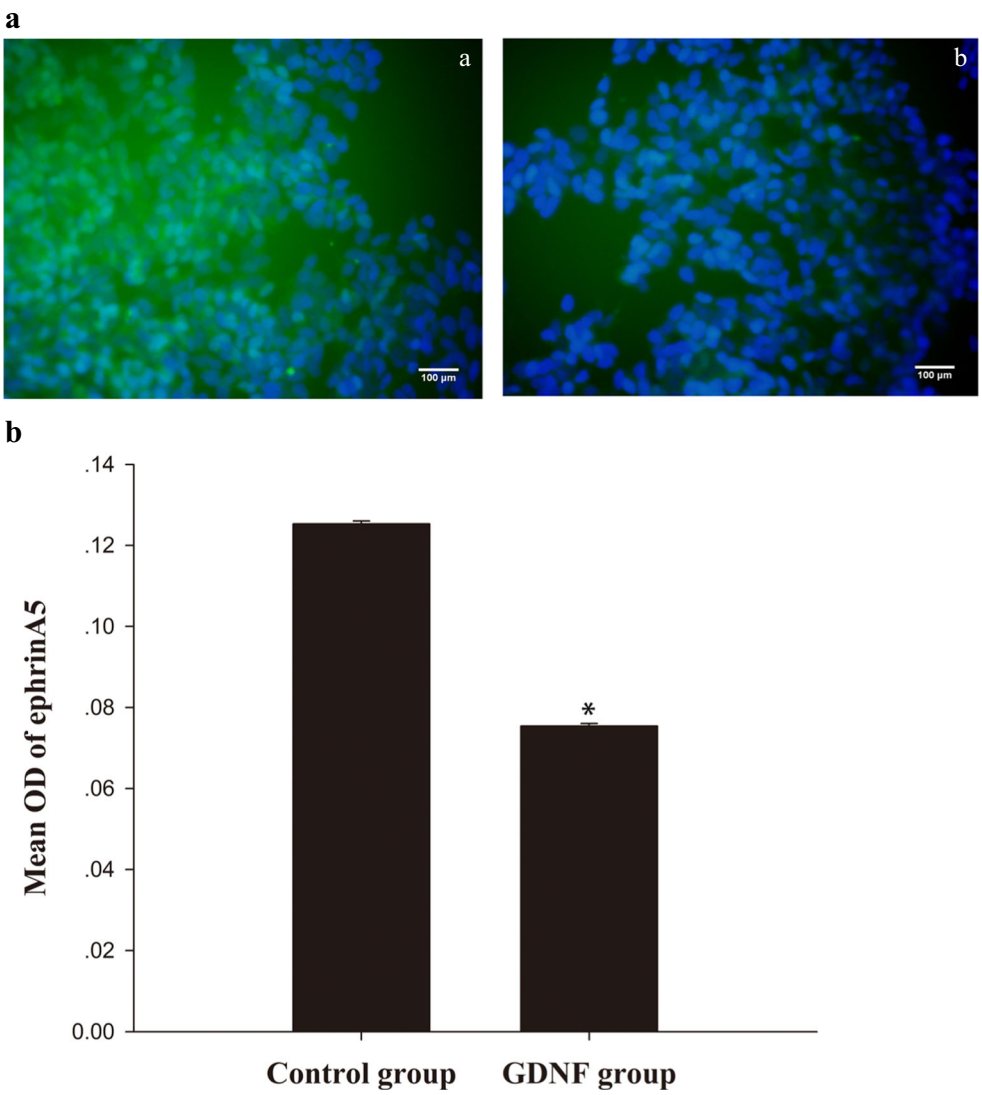

Fig. 5 IF results of ephrinA5. **a** IF images of ephrinA5 in Control (*a*) and GDNF (*b*) groups (*blue*, nuclei; *green*, ephrinA5). *Scale bars* represent 100 μm. **b** Statistical histogram according to IF images. Means (±SDM) of mean OD, *$P < 0.05$, *vs. Control group, *bars* represent SDM

Seiradake et al. demonstrated that ephrinA5 induced EphA4 and collapses growth cones (Seiradake et al. 2013). Moreover, ephrinA5 can induce growth cone collapse by activating Rho and Rho kinase (Wahl et al. 2000), thus terminating axon growth (Yue et al. 2008). We found that expression levels of ephrinA5 mRNA (EFNA5) and protein were decreased in differentiating DA neurons. Transient overshooting into the inferior colliculus in ephrinA5$^{-/-}$ mice revealed that ephrinA5 mediates retinal cells axon growth (Frisén et al. 1998). Research on thalamocortical projections demonstrated that ephrinA5 can also act as a repulsive cue for limbic thalamic axons; ephrinA5 and its receptor EphA5 are expressed in the somatosensory cortex and the medial group of thalamic nuclei, respectively (Vanderhaeghen et al. 2000). Some other studies on topographic retinotectal and hippocampal projections also showed the repulsive ability of ephrinA5 on axonal growth in different types of neurons (Feldheim et al. 2000; Brownlee et al. 2000). Therefore, the change in ephrinA5 expression during DA neuron differentiation is likely involved in axon growth.

EphrinA3 is reported to localize in astrocytic processes via crosstalk with its receptor EphA4 expressed in dendritic spines of hippocampal pyramidal neurons. This interaction is thought to play roles in hippocampal dendritic spine morphology and glutamate transport (Carmona et al. 2009; Murai et al. 2003). Both ephrinA3 and EphA4 are highly expressed in the hippocampus; they participate in compartmentalizing adjacent layers of the hippocampus, showing the potential ability of ephrinA3 to control axonal projection (Murai et al. 2003). In this study, we found that ephrinA3 mRNA (EFNA3) and protein levels were increased, while those of ephrinA5 were decreased in differentiating DA neurons. EphrinA5 is reported to be weakly expressed in regions where EphA4 is abundant (Frisén et al. 1998). In the developing entorhinohippocampal system, EFNA3 and EFNA5 are expressed in different regions on horizontal sections (Stein et al. 1999). Additionally, the topographic retinotectal projection map also

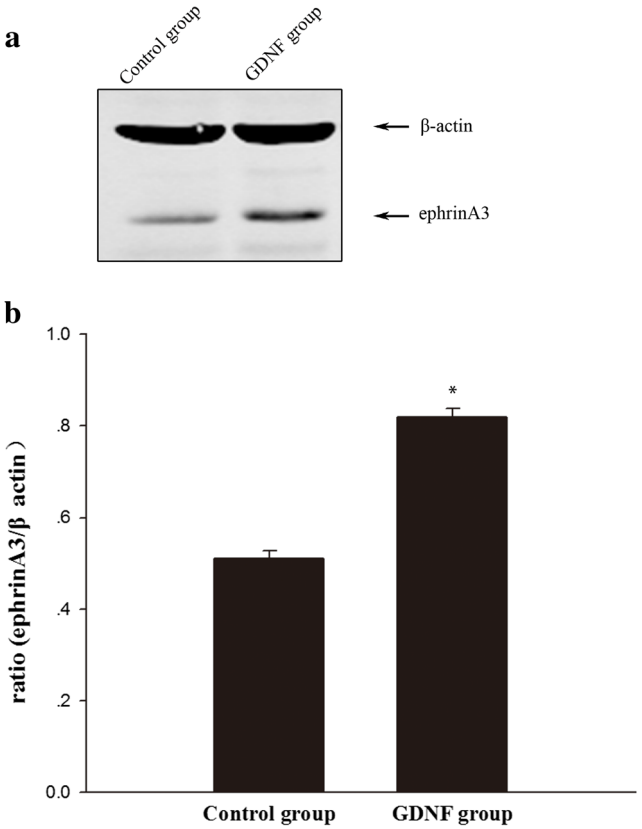

Fig. 6 WB results of ephrinA3. **a** WB bands of ephrinA3 and β-actin in the Control and GDNF groups. **b** Statistical histogram according to WB bands. Means (±SDM) of ratio (ephrinA3/β-actin), *$P < 0.05$, *vs. Control group, *bars* represent SDM

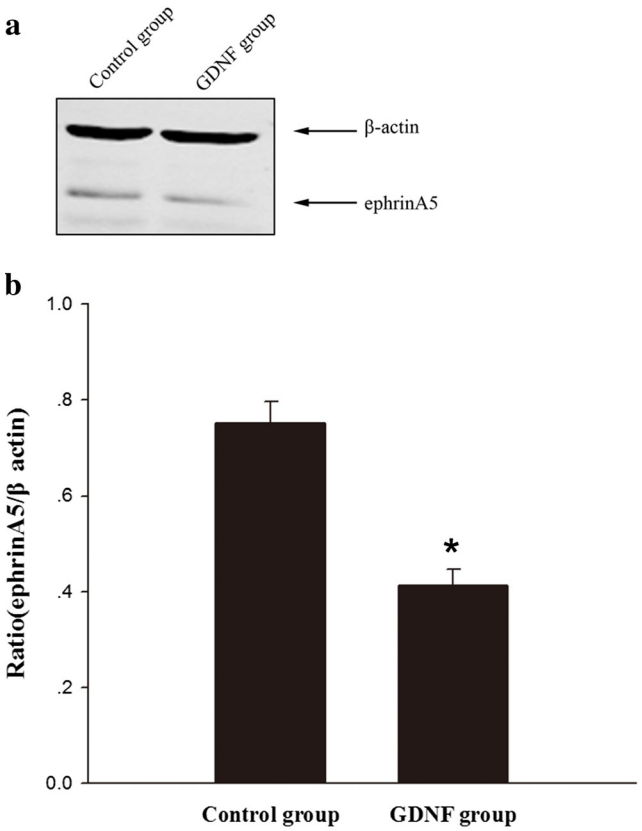

Fig. 7 WB results of ephrinA5. **a** WB bands of ephrinA5 and β-actin in the Control and GDNF groups. **b** Statistical histogram according to WB bands. Means (±SDM) of ratio (ephrinA5/β-actin), *$P < 0.05$, *vs. Control group, *bars* represent SDM

showed different distributions of ephrinA3 and ephrinA5 (Cheng et al. 1995; Feldheim et al. 2000). Our findings suggest that ephrinA3 and ephrinA5 levels are involved in axon guidance in differentiating DA neurons.

microRNAs findings are also meaningful during differentiation. A class of short non-coding RNAs, microRNAs act as post-transcriptional regulators in most eukaryotic cells. They regulate gene expression through binding to mRNA and inhibiting its translation (Bartel 2009; Krol et al. 2010; Kim 2005). Recent studies indicated that neuronal axons can synthesize microRNAs that further participate in axon growth (Kim 2005; Vo et al. 2005; Yu et al. 2008; Schratt 2009). Emerging data suggest that microRNAs usually regulate axon growth by regulating the location of targeted proteins to control the response of the growth cone to axon guidance (Hengst et al. 2006; Aschrafi et al. 2008; Natera-Naranjo et al. 2010; Dajas-Bailador et al. 2012; Liu et al. 2010). Our microRNAs findings and array association analysis results show that hsa-miR-4271 was downregulated, which could potentially regulate EFNA3 translation. This microRNA may influence the expression of ephrinA3 protein, thus affecting axon guidance during DA neuron differentiation.

In conclusion, increased and decreased expression levels of ephrinA3 and ephrinA5, respectively, contribute to DA neuron differentiation, presumably via guiding axon growth. Their levels may be affected by specific microRNAs. These findings will be helpful to guide future studies on cellular therapies for PD. Additional investigations are needed to identify the relevant mechanism(s).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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