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RESEARCH ARTICLE

Impact of *Pitx3* gene knockdown on glial cell line-derived neurotrophic factor transcriptional activity in dopaminergic neurons

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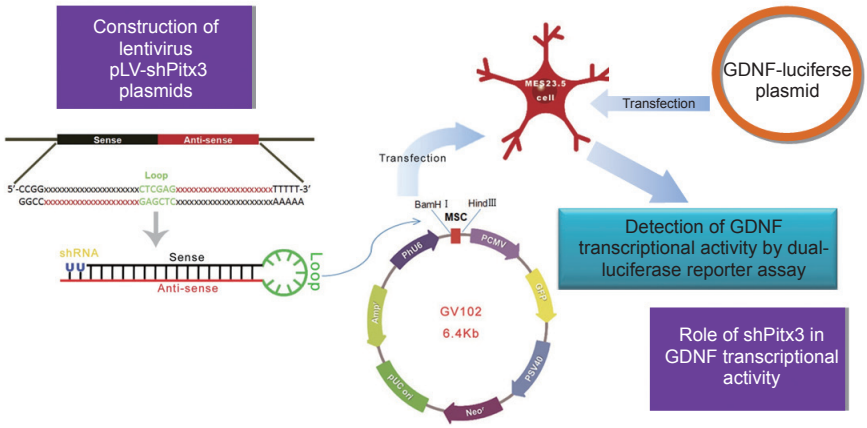
Graphical Abstract

Relationship between Pitx3 and glial cell line-derived neurotrophic factor (GDNF) in dopaminergic neurons



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Abstract

Pitx3 is strongly associated with the phenotype, differentiation, and survival of dopaminergic neurons. The relationship between Pitx3 and glial cell line-derived neurotrophic factor (GDNF) in dopaminergic neurons remains poorly understood. The present investigation sought to construct and screen a lentivirus expression plasmid carrying a rat Pitx3 short hairpin (sh)RNA and to assess the impact of *Pitx3* gene knockdown on GDNF transcriptional activity in MES23.5 dopaminergic neurons. Three pairs of interference sequences were designed and separately ligated into GV102 expression vectors. These recombinant plasmids were transfected into MES23.5 cells and western blot assays were performed to detect Pitx3 protein expression. Finally, the most effective Pitx3 shRNA and a dual-luciferase reporter gene plasmid carrying the GDNF promoter region (GDNF-luciferase) were cotransfected into MES23.5 cells. Sequencing showed that the synthesized sequences were identical to the three Pitx3 interference sequences. Inverted fluorescence microscopy revealed that the lentivirus expression plasmids carrying Pitx3-shRNA had 40–50% transfection efficiency. Western blot assay confirmed that the corresponding Pitx3 of the third knockdown sequence had the lowest expression level. Dual-luciferase reporter gene results showed that the GDNF transcriptional activity in dopaminergic cells cotransfected with both plasmids was decreased compared with those transfected with GDNF-luciferase alone. Together, the results showed that the designed Pitx3-shRNA interference sequence decreased Pitx3 protein expression, which decreased GDNF transcriptional activity.

Key Words: *nerve regeneration; neurodegeneration; Parkinson's disease; glial cell line-derived neurotrophic factor; Pitx3; MES23.5 cells; short hairpin RNA; gene knockdown; plasmid; dual-luciferase reporter gene; neural regeneration*

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative condition after Alzheimer's disease. Its major pathological feature is the chronic and progressive loss of dopaminergic (DA) neurons in the substantia nigra of the midbrain, which leads to clinical symptoms including high muscular tension, gait abnormality, resting tremor, and dyskinesia (Spillantini et al., 1997; Damier et al., 1999).

Glial cell line-derived neurotrophic factor (GDNF), a member of the transforming growth factor-β superfamily, was originally cloned and purified as a protective factor for damaged DA neurons (Lin et al., 1993; He and Yan, 2015). In the nigrostriatal system, GDNF provides specific trophic support and exerts survival-promoting and

post-damage repair effects on DA neurons (Roussa et al., 2004; Yin et al., 2015). GDNF increases the expression of the transcription factors Nurr1 and Pitx3 to sustain DA neuron survival (Lei et al., 2011). After GDNF and GDNF-family receptor alpha-1 (GFRα1) form a complex, the recruited RET receptor protein can also increase the expression of transcription factors such as Pitx3 and Nurr1 (Olanow et al., 2015).

Transcription factors, such as Pitx3, Nurr1, and Msx1, regulate gene expression in DA neurons (Smidt et al., 2000, 2004; Kim et al., 2003; Andersson et al., 2006). Among these transcription factors, expression of Pitx3 is highest in DA neurons in the embryonic midbrain (Semina et al., 1997; Smidt et al., 1997; Messmer et al., 2007). Pitx3 knockout mice have decreased numbers of DA neurons in the substantia nigra (Maxwell et al., 2005), indicating that Pitx3 is a specific factor that regulates DA neuron development and is crucial for establishment and maintenance of the nigrostriatal pathway (Smidt et al., 2004). Interference of Pitx3 expression leads to decreased DA levels and DA neuron loss in the substantia nigra (Le et al., 2015), while Pitx3 overexpression promotes the differentiation of DA neuron precursors from stem cells (Chung et al., 2005; Martinat et al., 2006) and significantly increases GDNF levels in SY5Y cells (Peng et al., 2007).

In this study, we employed genetic engineering to construct a lentiviral plasmid for interference with Pitx3 expression to explore how Pitx3 regulates GDNF expression.

Materials and Methods

Plasmids, bacterial strains, cell strains, and reagents

The three interference sequences designed to target the *Pitx3* gene and the negative control sequence are shown in **Table 1**.

Construction of lentiviral vector pLV-shPitx3 carrying interference sequences

The synthesized *Pitx3* interference sequences (Nanjing Jikai Co., Ltd., Nanjing, China) are shown in **Table 2**. These were ligated into the plasmid pLV-H1-EF1a-Bsd (containing a terminal sequence TTTT) using T_4 DNA ligase, and then DH5α competent bacteria were transformed. The next day, a single colony was picked and inoculated into 10 mL of Luria-Bertani (LB) medium with 100 μg/mL ampicillin. The mixture was centrifuged at 200 r/min and cultured in a shaker at 37°C overnight. A plasmid mini preparation kit was used to extract plasmids, which were then digested with *Bam*HI and *Hind*III enzymes. Recombinant clones with correct enzyme digestions were sent to the Jikai Company (Shanghai, China) for sequencing validation.

Screening Pitx3-short hairpin (sh)RNA interference plasmids

High-glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco) was used to culture MES23.5 cells (Shanghai Cell Institute, Chinese Academy of Sciences, China) in a 6-well plate. Lipofectamine™ 2000 (Gibco) was used to transiently transfect the empty vector and the 3568-1 (1,072 bp), 3569-1 (1,133 bp), and 3570-1 (1,196 bp) shRNA plasmids into MES23.5 cells, which subsequently became the four experimental groups (Control, Sh-3568, Sh-3569 and Sh-3570; $n \geq 3$). All four plasmids contained green fluorescent protein and fluorescence peaked 48 hours after transient transfection. Cell samples were collected, and western blot assays were performed to determine the comparative Pitx3 protein expression level. Based on comparison with the control group, the plasmid with the best interference effect was used in subsequent experiments.

Table 1 Interference and control target sequence

Name ID	Gene ID	Target sequence (5′–3′)	GC (%)
Pitx3-RNAi (3568-1)	NM_019247	ATT GTC AGA TGC AGG CAC T	47.37
Pitx3-RNAi (3569-1)	NM_019247	AGG ATG GCT CCC TGA AGA A	52.63
Pitx3-RNAi (3570-1)	NM_019247	TCG CCT TCA ACT CGG TCA A	52.63
Control		TTC TCC GAA CGT GTC ACG T	52.60

Table 2 shRNA sequences of *Pitx3*

Name	5′–	Stem	Loop	Stem	–3′
Pitx3-RNAi (3568-1)-a	GAT CCC	tcA TTG TCA GAT GCA GGC ACT	CTC GAG	AGT GCC TGC ATC TGA CAA TGA	TTT TTG GAT
Pitx3-RNAi (3568-1)-b	AGC TAT CCA AAA A	tcA TTG TCA GAT GCA GGC ACT	CTC GAG	AGT GCC TGC ATC TGA CAA TGA	GG
Pitx3-RNAi (3569-1)-a	GAT CCC	tgA GGA TGG CTC CCT GAA GAA	CTC GAG	TTC TTC AGG GAG CCA TCC TCA	TTT TTG GAT
Pitx3-RNAi (3569-1)-b	AGC TAT CCA AAA A	tgA GGA TGG CTC CCT GAA GAA	CTC GAG	TTC TTC AGG GAG CCA TCC TCA	GG
Pitx3-RNAi (3570-1)-a	GAT CCC	gtT CGC CTT CAA CTC GGT CAA	CTC GAG	TTG ACC GAG TTG AAG GCG AAC	TTT TTG GAT
Pitx3-RNAi (3570-1)-b	AGC TAT CCA AAA A	gtT CGC CTT CAA CTC GGT CAA	CTC GAG	TTG ACC GAG TTG AAG GCG AAC	GG

Lowercase bases represent introns.

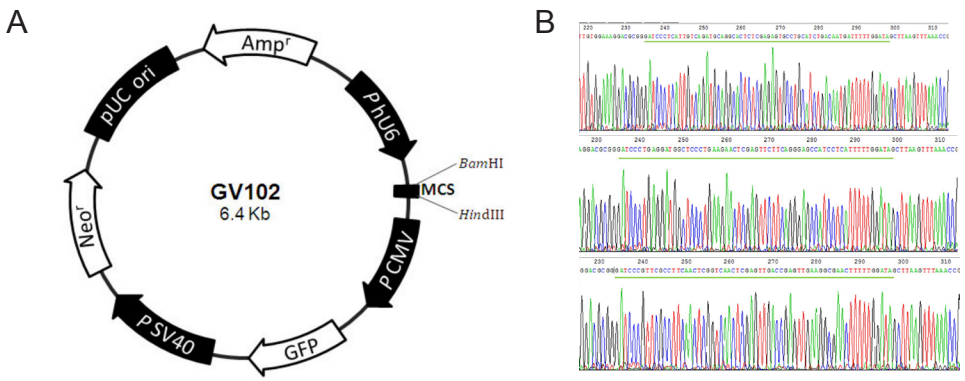

Figure 1 Vector map and sequencing results of the interference plasmid.
(A) Vector GV102 map for constructing a plasmid carrying a sequence for Pitx3 interference. (B) Sequencing peak maps of sequences for *Pitx3* interference. The upper, middle, and lower maps represent Sh-3568, Sh-3569, and Sh-3570 sequences, respectively.

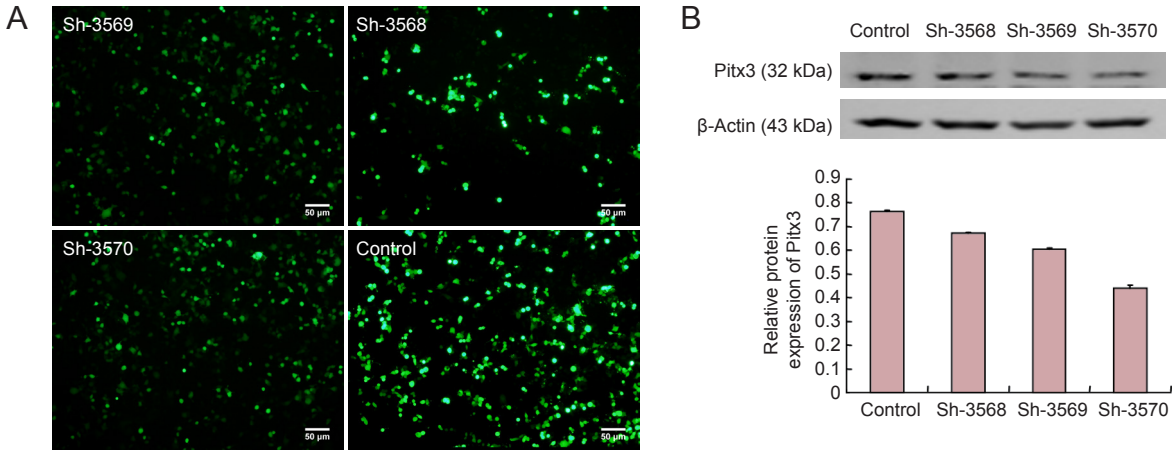

Figure 2 Screening interference plasmids.
(A) Cellular fluorescence 48 hours after transient transfection. Green cells indicate successful transfection of the plasmids. Scale bars: 50 μm. (B) Western blots for knockdown validation. The bars represent the ratio of optical density values of Pitx3 to β-actin. There was significant difference between the two groups ($P < 0.01$, mean ± SD, $n = 3$, one-way analysis of variance and the least significant difference test). Control indicates the empty plasmid; Sh-3568, the Pitx3-Sh-3568 plasmid; Sh-3569, the Pitx3-Sh-3569 plasmid; and Sh-3570, the Pitx3-Sh-3570 plasmid, which were all transiently transfected into MES23.5 cells.

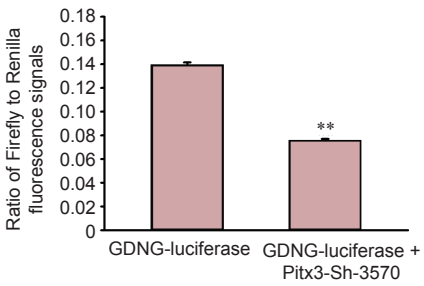

Figure 3 Effects of *Pitx3* gene interference on glial cell line-derived neurotrophic factor (GDNF) transcriptional activity.
GDNF-luciferase represents the cells transfected with the GDNF-luciferase plasmid alone. GDNF-luciferase + Pitx3-Sh-3570 represents the cells cotransfected with Pitx3-Sh-3570 and GDNF-luciferase plasmids. Transcriptional activity of GDNF decreased in the cells cotransfected with Pitx3-Sh-3570 and GDNF-luciferase plasmids; $^{**}P < 0.01$, *vs.* the GDNF-luciferase group (mean ± SD, $n \geq 3$, independent sample *t*-test).

Western blot assay

The cells were collected and lysed with a protein extraction kit (Beyotime Company, Jiangsu Province, China). The bicinchoninic acid assay was used to measure protein concentrations before the samples were denatured at 100°C for 10 minutes, followed by centrifugation. The prepared samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and membrane transfer. After blocking, membranes were incubated with the primary antibodies anti-Pitx3 (rabbit polyclonal antibody; Sigma, St. Louis, MO, USA) and anti-β-actin (mouse monoclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA), then secondary antibodies (IRDye 680RD goat anti-rabbit and IRDye 800CW goat anti-mouse; LI-COR Biosciences, USA). Protein bands were visualized using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE, USA). ImageJ software (Wayne Rasband, National Institutes of Health, USA) was used for analysis. Each blot was representative of at least three experiments.

Detection of GDNF with the luciferase reporter gene

The upstream 1,449 bp sequence of the GDNF gene promoter region was designed (synthesized and validated by

Shanghai Jikai Genchem Co., Ltd.) and ligated into a GV306 dual-luciferase reporter gene vector. Cultured MES23.5 DA neurons were seeded into a 12-well plate and when they reached 80% confluency, they were cotransfected with Pitx3-shRNA and GDNF-luciferase plasmids or with the GDNF-luciferase plasmid alone. A dual-luciferase reporter assay system kit (Promega, Madison, WI, USA) was used to detect GDNF transcriptional activity, which was measured as the ratio of Firefly to Renilla fluorescence signals.

Statistical analysis

All analyses were performed with SPSS 13.0 software (SPSS, Chicago, IL, USA). Histograms were generated with SigmaPlot 13.0 software (Systat Inc., San Jose, CA, USA). Data are presented as the mean ± SD. Independent sample *t*-test, one-way analysis of variance and the least significant difference test were used for statistical analysis, and $P < 0.05$ was considered statistically significant.

Results

Construction of lentivirus pLV-shPitx3 plasmids

Three interference sequences (shPitx3-3568, 3569, and 3570) were designed for the target *Pitx3* gene. The lentivirus RNAi plasmid GV102 was used to synthesize the corresponding upstream and downstream shRNA sequences, which were annealed to generate double-stranded DNA with *Bam*HI and *Hind*III sites. The sequences were ligated to GV102 vectors that were digested with the restriction enzymes, the resulting products were transformed into bacteria and then extracted with a plasmid mini preparation kit. The sequencing results were consistent with the design specifications (**Figure 1**).

Plasmid sh-3570 had the best knockdown effect

Using Lipofectamine™ 2000, the three expression plasmids were transiently transfected into MES23.5 cells. After 48 hours, fluorescence was detectable, indicating that the transfection was successful. Western blot assay was used to determine the knockdown efficiency of the plasmids, which showed that sh-3570 exerted the strongest knockdown effect (**Figure 2**).

Detecting the impact of Pitx3 knockdown on GDNF transcriptional activity

After three stable passages, MES 23.5 DA neurons were transfected with GDNF-luciferase plasmid with or without Pitx3-shRNA. After 48 hours, the cells were collected and subjected to luciferase measurement. The results showed decreased transcriptional activity of GDNF in the cells cotransfected with Pitx3-sh-3570 and *GDNF*-luciferase plasmids compared with those transfected with the GDNF-luciferase plasmid alone (**Figure 3**; $P < 0.01$).

Discussion

PD is characterized by the loss of DA neurons. Neurotrophic factors can exert protective effects on neurons (Kuhlmann et al., 2006; Wang et al., 2014; Duan et al., 2016); therefore, application of neurotrophic factors in PD can slow chronic degeneration and enhance the functional activity of residual DA neurons. GDNF is an important factor for sustaining central nervous system development (Carnicella et al., 2009) and is also a critical neurotrophic factor for treating PD (Pascual et al., 2008; Xing et al., 2016). Studies have shown that GDNF can slow DA neuron degeneration, enhance the functional activity of residual DA neurons, and promote differentiation of new DA neurons (Sun et al., 2004; Yang et al., 2011; Wang et al., 2016).

The *Pitx3* gene encodes a very important transcription factor involved in protecting the nervous system (Blaudin et al., 2016). Pitx3 is expressed in mature DA neurons and regulates the expression of tyrosine hydroxylase, brain-derived neurotrophic factor, GDNF, and other factors (Kim et al., 2014), while Pitx3 overexpression enhances GDNF levels in SY5Y cells (Peng et al., 2007). It also participates in DA neuroblast differentiation into mature DA neurons. Collectively, the evidence suggests that Pitx3 is an important regulator of GDNF expression that exerts protective effects on DA neurons.

In this study, three Pitx3 interference sequences were designed and synthesized. The recombinant plasmids containing interference sequences of Pitx3 were transfected into MES23.5 cells and western blot assays showed that sh-3570 exerted the strongest knockdown effect on Pitx3. Through luciferase reporter gene assays, we further explored whether Pitx3 exerts an effect on GDNF expression. The reporter gene is part of a reporter system that uses fluorescein as a substrate to detect firefly luciferase activity, allowing it to detect an interaction between a transcription factor and the promoter region of a target gene. We designed a luciferase reporter gene plasmid carrying the GDNF promoter region and a plasmid carrying a sequence to decrease *Pitx3* gene expression and transfected them into MES23.5 cells. The results showed that GDNF transcriptional activity was significantly lower in cells with Pitx3 knockdown, indicating that this intervention decreased GDNF transcription. These data imply that Pitx3 may participate in PD protection by regulating GDNF transcription, in agreement with previous studies. Moreover, in light of the complex relationship between GDNF and Nurr1 in DA neurons (Galleguillos et al., 2010; Decressac et al., 2013; Volakakis et al., 2015), the mechanism by which Pitx3 participates in GDNF protection of DA neurons may be very complex. This will be a subject of our further research.

In summary, we successfully constructed a plasmid carrying a sequence to knockdown Pitx3 expression and a luciferase reporter plasmid expressing a GDNF promoter. We transfected them into MES23.5DA neurons and demonstrated that interfering with Pitx3 expression decreased GDNF transcription. Our results lay a foundation to further explore mechanisms to increase GDNF levels to protect DA neurons in PD. It is of importance to identify changes in the GDNF promoter region that alter GDNF expression, and other possible factors, by interfering with *Pitx3* gene expression.

Author contributions: *JC designed this study, performed experiments, collected data, analyzed data, interpreted the data and wrote the paper. XYK performed experiments, collected and analyzed data. CXT collected,*

analyzed and interpreted the data. DSG designed this study, obtained funding, administrated and supported technology. All authors approved the final version of the paper.

Conflicts of interest: *None declared.*

Data sharing statement: *The datasets analyzed during the current study are available from the corresponding author on reasonable request.*

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