Transcription factor Six2 mediates the protection of GDNF on 6-OHDA lesioned dopaminergic neurons by regulating Smurf1 expression

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Glial cell line-derived neurotrophic factor (GDNF) has strong neuroprotective and neurorestorative effects on dopaminergic (DA) neurons in the substantia nigra (SN); however, the underlying molecular mechanisms remain to be fully elucidated. In this study, we found that the expression level of transcription factor Six2 was increased in damaged DA neurons after GDNF rescue in vitro and in vivo. Knockdown of Six2 resulted in decreased cell viability and increased the apoptosis of damaged DA neurons after GDNF treatment in vitro. In contrast, Six2 overexpression increased cell viability and decreased cell apoptosis. Furthermore, genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) indicated that Six2 directly bound to the promoter CAGCTG sequence of smad ubiquitylation regulatory factor 1 (Smurf1). ChIP-quantitative polymerase chain reaction (qPCR) analysis showed that Smurf1 expression was significantly upregulated after GDNF rescue. Moreover, knockdown of Six2 decreased Smurf1 expression, whereas overexpression of Six2 increased Smurf1 expression in damaged DA neurons after GDNF rescue. Meanwhile, knockdown and overexpression of Smurf1 increased and decreased p53 expression, respectively. Taken together, our results from in vitro and in vivo analysis indicate that Six2 mediates the protective effects of GDNF on damaged DA neurons by regulating Smurf1 expression, which could be useful in identifying potential drug targets for injured DA neurons.

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Neurotrophic factors have recently emerged as promising therapeutic drugs for neurodegenerative diseases. Among these, glial cell line-derived neurotrophic factor (GDNF) has attracted special attention for its protective effects on injured dopaminergic (DA) neurons in Parkinson’s disease (PD).1–3 Although enhancing GDNF signalling protects damaged DA neurons, little is known about its protective mechanisms.

Apoptosis has been implicated as one of the important mechanisms leading to DA neuronal death in the substantia nigra (SN) of PD patients.4,5 Previous reports indicated that exogenous GDNF could improve the survival and reduce apoptosis of embryonic DA neurons.6,7 Similarly, the combined application of GDNF and gspase inhibitor enhances the survival of grafted DA neurons.8 In lactacystin-induced DA neurodegeneration, GDNF significantly inhibits DA neuron apoptosis and reduces caspase-3 activation.9 We previously demonstrated that GDNF protects 6-hydroxydopamine (6-OHDA)-injured MN9D cells by upregulating Bcl-2 and Bcl-w expression.10 Those studies suggested that an anti-apoptotic effect was likely the primary protective mechanism of GDNF on damaged DA neurons. However, the precise mechanisms remain to be fully elucidated.

The transcription factor Six2 is a member of the sine oculis homeobox (SIX) family. It was previously identified as a critical regulator in normal kidney development.11,12 Several recent lines of evidence have indicated that Six2 is also involved in organ development and some diseases.13–15 Meanwhile, studies have also indicated that Six2 has the function of anti-apoptosis, for example, downregulated Six2 induces metanephric mesenchyme cell apoptosis,16 and miR181 could also promote apoptosis by targeting Six2 in vitro.17 We previously observed aberrant Six2 expression in damaged DA neurons after GDNF rescue, which led us to hypothesise that GDNF upregulates Six2 to prevent damaged DA neurons from undergoing apoptosis.

In the present study, we provide evidence that Six2 is pivotal for GDNF protection in injured DA neurons. Knockdown of Six2 by lentiviral shRNA and Six2 overexpression decreased and increased the protection afforded by GDNF, respectively. Further screening of Six2 targets by genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) identified the smad ubiquitylation regulatory factor 1 (Smurf1) as a specific target of Six2, and this inhibited the apoptosis of damaged DA neurons. This study may be of significant value to identify potential drug targets for injured DA neurons.

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Abbreviations: GDNF, Glial cell line-derived neurotrophic factor; DA neurons, dopaminergic neurons; PD, Parkinson’s disease; SN, substantia nigra; 6-OHDA, 6-hydroxydopamine; SIX, sine oculis homeobox; Smurf1, smad ubiquitylation regulatory factor 1; CCK8 assay, cell counting kit-8 assay; qPCR, real-time polymerase chain reaction; ChIP, chromatin immunoprecipitation

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Results

GDNF rescues 6-OHDA-damaged DA neurons. To verify that GDNF could protect damaged DA neurons, we generate a rat PD model using 6-OHDA. The posture asymmetry experiment indicated that the frequency of head turning to the lesioned side improved after GDNF rescue (Figure 1A), in addition, apomorphine-induced rotation test confirmed that the motor abilities of PD rats were significantly improved, particularly in the 8 μg GDNF groups (Figure 1B). To further
verify the protection of GDNF on damaged DA neurons, the numbers of TH⁺ DA neurons in the SN were counted. The numbers of TH⁺ neurons were significantly increased in the 8 μg GDNF group compared with the 6-OHDA group; however, cell numbers were only slightly increased in the 4 μg GDNF group and almost unchanged in the 2 μg GDNF group (Figures 1Ca–f and D), indicating a dose-dependent effect. We also examined TH expression in the SN, and found that TH dose-dependently increased after GDNF rescue (Figures 1E and F). These results suggest that GDNF significantly protects damaged DA neurons in the SN of PD rats.

Moreover, we observed protective effects of GDNF on damaged MES23.5 DA cell line in vitro. The CCK8 assay results showed that cell viability decreased to approximately 50% after 6 h of 6-OHDA treatment and then increased after 12 h of GDNF rescue (Figures 2a and b). Similarly, cell counting with trypan blue staining and flow cytometry analyses both indicated that the cell apoptosis rate was about 50% at 6 h after 6-OHDA treatment and decreased after 12 h GDNF treatment (Figures 2c and d). The in vitro results (6-OHDA 6 h followed by GDNF 12 h) were similar with that for the in vivo experiments (6-OHDA 2 w followed by GDNF 1 w). Moreover, we also investigated that TH expression significantly increased after GDNF rescue in damaged MES23.5 DA neurons (Figure 2f). Collectively, these results suggest that GDNF efficiently protects DA neurons damaged by 6-OHDA in vivo and in vitro.

GDNF upregulates Six2 expression in 6-OHDA-damaged DA neurons. In order to detect whether Six2 expression was regulated by GDNF in damaged DA neurons, we assessed
Six2 mRNA and protein expressions in the SN of PD rats after GDNF rescue. Increased Six2 levels were highest in the 8 μg GDNF group (Figures 3a and b). We also analysed Six2 expression changes in damaged MES23.5 DA neuron models after GDNF rescue. The results revealed that Six2 was obviously increased (Figures 3c and d), which was consistent with the in vivo findings. We further detected whether GDNF could increase the Six2 protein level even without 6-OHDA challenge, the results also increased after GDNF treatment both in vivo and in vitro (Figures 3e and f). Taken together, these results suggest that Six2 are involved in the GDNF-mediated protection of damaged DA neurons.

Six2 knockdown attenuates the protective effects of GDNF in damaged DA neurons. To test whether Six2 mediates the protection of GDNF in injured DA neurons, a lentivirus-mediated knockdown strategy was utilised to diminish Six2 expression. An shRNA (shSix2-2-pLV) targeting Six2 decreased Six2 expression in MES23.5 DA neurons by approximately 90% (Figure 4a). We further found that the viability of cells infected with shSix2-pLV was reduced compared with pLV-infected MES23.5 DA neurons after GDNF rescue (Figure 4b). Cell counting with trypan blue staining showed that the dead rate of cells infected with shSix2-pLV was higher than that of control cells (Figure 4c). Similarly, flow cytometry analysis also showed that the apoptosis rate of cells infected with shSix2-pLV was higher than that of control cells (Figure 4d). In addition, the mRNA and protein expression levels of TH were also reduced in the shSix2-pLV group (Figures 4e and f). These results suggest that the transcription factor Six2 mediates the protective effects of GDNF on damaged DA neurons.

Six2 overexpression enhances the protective effects of GDNF in damaged DA neurons. To further demonstrate that Six2 mediates the protective effects of GDNF, we...
generated stably Six2 overexpression MES23.5 cells. Real-time PCR and western blot analysis demonstrated that Six2 was obviously increased in the Six2 overexpression group (Figures 5A and B). Importantly, damaged MES23.5 cell viability increased after GDNF rescue in the Six2 overexpression group compared with the control group (Figure 5C). Cell counting with trypan blue staining indicated that the dead rate of cells infected Six2-pLV was lower than that of control cells (Figure 5D). In accordance with the above findings, flow cytometry analyses indicated that the apoptosis rate of cells infected Six2-pLV decreased than that of control cells (Figure 5E). In addition, TH expression was significantly increased (Figures 5F and G). We further detected whether Six2 overexpression alone could reduce 6-OHDA toxicity in the absence of GDNF, results showed that MES23.5 cell viability increased in the Six2 overexpression group compared with the control group (Figure 5H), the dead rate of cells decreased in Six2 overexpression group (Figure 5I), and the apoptosis rate of cells also decreased in Six2 overexpression group (Figure 5J). In addition, the in vivo experiments indicated that the numbers of TH+ neurons were significantly increased in the Six2 overexpression group in SN of PD rats (Figures 5K and L). Thus, our findings strongly indicate that Six2 is involved in the protective effects of GDNF on DA neurons.

Six2 mediates GDNF protection by upregulating Smurf1 expression. Our results clearly demonstrate that Six2 is critical for regulating GDNF protection. For an unbiased assessment of the plausible mechanisms of Six2, we performed a genome-wide ChIP-seq analysis for Six2 in MES23.5 DA neurons. The peaks of Six2 occupancy were often broad, with some around 1 kb (Figure 6a). We successfully detected 384 differential genes between DA neurons treated with 6-OHDA followed by GDNF group and DA neurons treated with 6-OHDA group (Supplementary Table 1). Of the Six2-binding sites, 7.2% were located in the region of 2000 bp upstream from transcription start site of the 384 differential genes (Figure 6b). We also performed de novo motif discovery to identify specific sequences bound
by Six2 (Figure 6c), among these differentially expressed genes, Six2 directly bound to the promoter CAGCTG sequence of smad ubiquitylation regulatory factor 1 (Smurf1). Smurf1 was significantly upregulated after GDNF rescue by ChIP-qPCR analysis (Figure 6d), the protein expression of Smurf1 also increased after GDNF rescue both in vivo and in vitro by western blot analysis (Figures 6e and f). We further found that Smurf1 expression decreased in cells infected with shSix2-pLV and increased in Six2 overexpression cells after GDNF rescue (Figures 6g and h). We also found that Smurf1 overexpression in 6-OHDA- and GDNF-treated MES23.5 cells with downregulated Six2 could increase cell viability by CCK8 analysis (Figure 7A), decrease the dead rate of cells by cell counting with trypan blue staining (Figure 7B).
and decrease the apoptosis rate of cells by flow cytometry analyses (Figure 7C). In addition, we further detected whether Smurf1 overexpression alone could reduce 6-OHDA toxicity in the absence of GDNF, results showed that MES23.5 cell viability increased in the Smurf1 overexpression group (Figure 7D), the dead rate of cells decreased in Smurf1 overexpression group (Figure 7E) and the apoptosis rate of cells also decreased in Smurf1 overexpression group (Figure 7F). In addition, the in vivo experiments indicated that the numbers of TH+ neurons were significantly increased in the Smurf1 overexpression group in SN of PD rats (Figures 7G and H). These results suggest that Six2 mediates the protective effects of GDNF on damaged DA neurons by upregulating Smurf1.

Smurf1 mediates GDNF protection by degrading p53. Previous study showed that Smurf1 could promote the degradation of p53.18 We first detected the protein expression of p53 after GDNF rescue, the results showed that the p53 protein levels decreased after GDNF rescue in SN of PD rats (Figure 8a). To further test whether Smurf1 mediates the protection of GDNF by degrading p53 in injured DA neurons, a Smurf1 shRNA plasmid (shSmurf1-plasmid) decreased Smurf1 expression in MES23.5 cells by approximately 50%. We further found that the protein expression levels of p53 were increased in the shSmurf1-plasmid group after GDNF rescue (Figure 8b). In addition, we also generated stably Smurf1 overexpression MES23.5 cells. Western blot analysis demonstrated that p53 obviously reduced in the Smurf1 overexpression (Smurf1-pLV) group after GDNF rescue (Figure 8c). These results suggest that the Smurf1 mediates the protective effects of GDNF on damaged DA neurons by degrading the p53 expression.

Six2 knockdown and overexpression attenuates and increases the protective effects of GDNF in SN of PD rats, respectively. To further demonstrate whether Six2 mediates the protective effects of GDNF on PD rats, we injected shSix2-pLV or Six2-pLV encoded EGFP into SN of PD rats, and then investigated the motor ability after GDNF rescue. Immunofluorescence staining analysis showed these viral-encoded EGFP was, respectively, expressed in TH+ neurons in SN of PD rats after GDNF rescue (Figure 9A). Western blot analyses demonstrated that Six2 was obviously decreased in the Six2 knockdown group and increased in the Six2 overexpression group, while GDNF expression were consistent among different groups in SN of PD rats after GDNF rescue (Figures 9B and C). The numbers of TH+ neurons were significantly increased in the Six2 overexpression group, and decreased in the Six2 knockdown group in SN of PD rats after GDNF rescue (Figures 9D and E). The posture asymmetry experiment indicated that the frequency of head turning into the lesioned side decreased after Six2 knockdown, and apomorphine-induced rotation increased after Six2 knockdown (Figures 9F and G). Although overexpression of Six2 could promote a little motor ability, but there was no statistical significance (Figures 9H and I). These results indicated that Six2 is involved in the protective effects of GDNF on PD rat models.

Discussion
GDNF has been shown to ameliorate neurodegeneration in neurotoxin-induced rats and nonhuman primate models of PD;19-21 however, the underlying molecular mechanisms remain largely unknown. Our results are the first evidence that Six2 is at least partially responsible for the protective effects of GDNF in damaged DA neurons. Furthermore, we showed that Smurf1 was directly regulated by Six2 after GDNF rescue, and this inhibited the apoptosis of damaged DA neurons.

GDNF has strong protective effects on DA neurons in the SN of PD models. Previous reports showed that injection of single high doses of GDNF 1 or 3 months after the toxic insult induced upregulation of TH expression and promoted the motor ability of PD models obviously.22,23 Our findings provided additional evidence that single doses of GDNF 2 weeks after 6-OHDA lesion rescued the damaged DA neurons and improved the motor ability of PD rats. This hypothesis is reinforced by the fact that GDNF has been shown to reverse toxin-induced injury to DA neurons.24,25 In this study, we also showed the TH mRNA and protein expression increased 1 day after GDNF injection. A previous study showed that injection of 125I-labelled GDNF into striatum, the obvious fluorescence in SN could be detected 6 h after injection, and lasted for several days.26 Long-time surveillance showed that the numbers of TH+ DA neurons increased from 2 to 21 days in cultured rat midbrain cells treated with GDNF than in control groups.2 In addition, the motor activity of rats increased from 2 to 10 days after GDNF injection, the apomorphine-induced rotation behaviour increased most obviously 1 week after GDNF injection.24,27

![Figure 5](https://example.com/figure5.png)
According to the above studies, we detected the TH mRNA and protein expression after 1 day of GDNF injection, and detected the numbers of TH+ DA neurons and behavioural tests after 1 week of GDNF injection in this study. These findings provided additional evidence that GDNF has strong protective effects on damaged DA neurons.
Figure 6 Six2 mediated the protection of GDNF on DA neurons by upregulating Smurf1 expression. (a) Peaks of Six2 occupancy in genomic fragments in MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h) followed by GDNF (100 ng/ml, 12 h). (b) Loci distribution of Six2-bound differential expressed target genes in MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h) followed by GDNF (100 ng/ml, 12 h). The word ‘down2k’ means 2000 bp downstream from the transcription termination site of the genes, ‘up2k’ means 2000 bp upstream from the transcription start site of the genes, ‘exon’ means exon region of the genes, ‘intron’ means intron region of the genes. (c) Potential candidate motifs prediction of Six2 in MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h). (d) Chromatin immunoprecipitation assay with anti-Six2 antibody for Smurf1 in MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h) followed by GDNF (100 ng/ml, 12 h), ChIP DNA was quantified by qPCR and normalised to input. (e) Cell viability analysis of Six2-knockdown MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h) followed by GDNF (100 ng/ml, 12 h). (f) Cell viability analysis of Six2-overexpressed MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h) followed by GDNF (100 ng/ml, 12 h). *P < 0.05 versus shSix2-pLV group. Bar graphs are shown as means ± S.E.M. (n = 3). The statistical analysis was carried out using unpaired t-tests.

Figure 7 Smurf1 overexpression could rescue the injured DA neurons. (A) Cell viability analysis of Smurf1-overexpressed MES23.5 DA neuron models with downregulated Six2 after treatment with 6-OHDA (100 mM, 6 h) followed by GDNF (100 ng/ml, 12 h). (B) Cell counting after trypan blue staining of Smurf1-overexpressed MES23.5 DA neuron models with downregulated Six2 after treatment with 6-OHDA (100 mM, 6 h) followed by GDNF (100 ng/ml, 12 h). (C) Flow cytometry apoptosis rate analysis of Smurf1-overexpressed MES23.5 DA neuron models with downregulated Six2 after treatment with 6-OHDA (100 mM, 6 h) followed by GDNF (100 ng/ml, 12 h). *P < 0.05 versus shSix2-pLV group. Bar graphs are shown as means ± S.E.M. (n = 3). The statistical analysis was carried out using unpaired t-tests. (D) Cell viability analysis of Smurf1-overexpressed MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h). (E) Immunohistochemistry analysis of TH+ DA neuron after Smurf1 overexpression in SN of PD rats in vivo models (16 μg 6-OHDA for 2 w), (a) pLV group, (b) Smurf1-pLV group; scale bars (in a and b) equals 200 μm. a’ and b’ are higher magnification images in a and b, scale bars equals 20μm. (F) Flow cytometry apoptosis rate analysis of Smurf1-overexpressed MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h). (G) Flow cytometry apoptosis rate analysis of Smurf1-overexpressed MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h). (H) Flow cytometry apoptosis rate analysis of Smurf1-overexpressed MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h). (I) Flow cytometry apoptosis rate analysis of Smurf1-overexpressed MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h). (J) Flow cytometry apoptosis rate analysis of Smurf1-overexpressed MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h). (K) Flow cytometry apoptosis rate analysis of Smurf1-overexpressed MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h). (L) Flow cytometry apoptosis rate analysis of Smurf1-overexpressed MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h). Bar graphs are shown as means ± S.E.M. (n = 3). The statistical analysis was carried out using unpaired t-tests.
The transcription factor Six2 has been suggested to be involved in kidney development, and Six2 downregulation results in renal mesenchymal stem cell apoptosis. Our in vivo and in vitro analysis showed that Six2 mRNA and protein expression was upregulated in damaged DA neurons after GDNF rescue. These results imply that Six2 mediates GDNF protection. Indeed, knockdown and overexpression experiments in vitro confirmed that Six2 mediates the protective anti-apoptotic effect of GDNF, and our in vivo analysis also demonstrated that Six2 could protect damaged DA neurons and improve the motor ability of PD rats. Other studies showed that Six1, another member of the SIX family, played critical roles in protecting neurons from apoptosis. Six1 may also have potential protective functions in DA neurons similar to Six2 because of its similar conserved sequence. Now, the related experiments about Six1 are under way in our laboratory and we wish it would be finished as quickly as possible.

Cell apoptosis occurs via the activation or inhibition of apoptosis-associated factors. Several factors including caspases, p53, and the ubiquitin proteasome system...

**Figure 8** Smurf1 mediated the protection of GDNF on DA neurons by degrading p53. (a) The protein expression and quantitative analysis of p53 in the SN of PD rats in vivo models after GDNF rescue (16 μg 6-OHDA for 2 w followed by 8 μg GDNF for 1 d). (b) The protein expression and quantitative analysis of Smurf1 and p53 in Smurf1-knockdown MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h) followed by GDNF (100 ng/ml, 12 h). (c) Expression and quantification of Smurf1 and p53 protein in Smurf1-overexpressed MES23.5 DA neuron models after treatment with 6-OHDA (100 mM, 6 h) followed by GDNF (100 ng/ml, 12 h). The data were from at least three independent experiments in each case. *P < 0.05 versus control group of Smurf1, #P < 0.05 versus control group of p53. Bar graphs are shown as means ± S.E.M. The statistical analysis was carried out using one-way ANOVA followed by post hoc Dunnett’s tests.
modulate cell apoptosis in damaged DA neurons.\textsuperscript{5,32–34} A previous study showed that overexpression of dominant-negative mutants of caspase-3, -7, and -9 could block the death of embryonic DA neurons induced by deprivation of GDNF.\textsuperscript{35} Other studies showed that GDNF/RET signalling were required for normal mitochondrial function and morphology, and blocking caspase-6 could inhibit the death of GDNF-deprived DA neurons.\textsuperscript{36,37} In addition, overexpression of Nurr1 could reverse the blockade of GDNF signalling, which was induced by deprivation of α-synuclein in SN DA neurons.\textsuperscript{28} In this study, although we have shown that Six2 could mediate the protective anti-apoptotic effect of GDNF, the factors that are directly modulated by Six2 after GDNF rescue have not been identified. In this study, ChIP-seq enabled us to investigate the underlying molecular mechanisms governing Six2-mediated protection by GDNF in damaged DA neurons at the genome level. Our results indicated that Smurf1 was targeted by Six2. Smurf1 belongs to the Nedd4 (neuronal precursor cell-expressed developmentally downregulated 4) family of HECT-type E3 ligases and has critical roles in regulating cell growth, migration, and autophagy.\textsuperscript{39} A previous study showed that Smurf1 could promote the degradation of p53,\textsuperscript{18} which is one of the key effectors promoting apoptosis.\textsuperscript{40,41} In this study, our results also indicated that p53 was degraded by Smurf1 in damaged DA neurons after GDNF treatment. Our results provided additional evidence that the apoptosis of damaged DA neurons could be rescued by GDNF. Taken together, these findings demonstrate that the Six2 is a critical factor that mediates GDNF protection of damaged DA neurons by directly activating Smurf1, which could further degrade p53.

In summary, our results demonstrate that Six2 is a critical regulator of GDNF’s ability to inhibit apoptosis of damaged DA neurons by upregulating the expression of Smurf1, which could further degrade p53. These findings provide new important evidence for the protective mechanism of GDNF, which could be useful in identifying potential drug targets for injured DA neurons.

### Materials and Methods

**Animals and cell culture.** Adult male (230–250 g) Sprague Dawley rats were provided by Xuzhou Medical College. They were housed under controlled conditions (temperature 23 ± 2°C and illumination 12:12 h light–dark cycle) with standard diet and water \textit{ad libitum}. Animal housing and treatment of the rats were performed in accordance with the Guidelines of the Ethical Committee for Use of Laboratory Animals.

MES23.5 DA neurons and 293T cells were obtained from the Shanghai Institutes for Cell Resource Center at the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% foetal bovine serum (Hyclone, Logan, UT, USA), 100 units/ml penicillin, and 1% (v/v) streptomycin (Invitrogen, Burlington, CA, USA) in a 5% CO\textsubscript{2} atmosphere at 37°C.

**Reagents and antibodies.** The 6-OHDA (H116) and GDNF (G1401) were purchased from Sigma (St. Louis, MO, USA). Rabbit anti-Six2 (SAB210306), rabbit anti-GDNF antibody (SAB410150), mouse anti-tyrosine hydroxylase (TH) (T2928), and rabbit anti-SmurF1 antibody (S8449) were also from Sigma. The rabbit anti-Six2 antibody (sc-152724) was from Santa Cruz (St. Louis, MO, USA). The rabbit anti-p53 antibody (BS3736) was from BioWorld Technology (Shanghai, China). The plasmid mini kit (74104) and midi kit (12143) were from Qiagen (Venlo, The Netherlands).

### Constructing of PD rat models using 6-OHDA

The Sprague Dawley rats were anaesthetised with intraperitoneal sodium pentobarbital (50 mg/kg) and placed in the stereotoxic apparatus (bit bar at -3.3 mm). Damage was induced by unilateral stereotaxic injections of 6-OHDA (16 μg total, 4 μg/μl with 2 μl per site) into the left striatum at two coordinates: (i) 0.7 mm anterior and 3 mm lateral to the bregma and 7 mm ventral to the cranial surface and (ii) 0.7 mm anterior and 3 mm lateral to bregma and 5 mm ventral to the cranial surface. The sham-operated rats received injections containing vehicle (0.1% ascorbate in 0.9% saline) at the same coordinates. All injections were performed using a 26-gauge Hamilton syringe connected to an infusion minipump (Hamilton Co., Reno, NV, USA), at a rate of 1 μl/min. After the injection, the syringe was left in place for 5 min and slowly retracted. Two weeks after 6-OHDA injection, the PD rats were screened using behavioural tests. Then, the GDNF was injected into the left striatum of the PD rat models.

**Injection of GDNF into left striatum of PD rat models.** This experiment was divided into six groups: (i) Control group: the normal rats (n=20); (ii) 6-OHDA group: the rats received left striatum injection of 6-OHDA (n=20); (iii) 6-OHDA+ phosphate buffered saline (PBS) group: the PD rats received left striatum injection of PBS (n=20); (iv) 6-OHDA+2 μg GDNF: the PD rats received left striatum injection of 2 μg GDNF (n=20); (v) 6-OHDA+4 μg GDNF: the PD rats received left striatum injection of 4 μg GDNF (n=20); (vi) 6-OHDA+8 μg GDNF: the PD rats received left striatum injection of 8 μg GDNF (n=20). One day after GDNF injection, 5 rats in every group were killed and the left sides of SN were processed for real-time polymerase chain reaction (RT-PCR) analysis, and 5 rats in every group were killed and the left sides of SN were processed for western blot analyses. One week after GDNF injection, the other 10 rats in every group were subjected to behavioural tests, and then, these rats were anaesthetised with sodium pentobarbital and transcardially perfused for immunohistochemistry analyses.

**Behavioural analysis.** The following behavioural tests were carried out 2 weeks after 6-OHDA injection for screening the PD rats, and 1 week after GDNF injection for detecting the effect of GDNF on PD rats.

### Immunohistochemistry

As described previously\textsuperscript{42} after behavioural tests, 10 rats in every group were anaesthetised and perfused through the left ventricle via an ascending aortic cannulation. According to Paxinos & Watson’s The Rat Brain in Stereotaxic Coordinates, we used a stereotaxic apparatus to identify the brain segments containing the SN, which were consecutively sectioned on a microtome in the coronal plane at 6 μm thickness. After blocking for nonspecific binding with 10% donkey serum albumin, these sections were incubated, respectively, with anti-TH antibody and biotinylated horse anti-mouse IgG, followed by the Elite avidin-biotin complex. The reaction was completed with 3-diaminobenzidine, nickel II sulphate, and H2O2. Then, these sections were mounted on gelatin-coated slides, dehydrated through graded alcohol, cleared in xylene, and cover-slipped with cytoseal for cell counting.

**Stereology.** The numbers of TH\textsuperscript{+} DA neurons in the SNpc (–4.7 to –6.3 mm from bregma) on the injured side were counted under a light microscope.\textsuperscript{44} The first sampling item was taken at random from the frontal part of the SNpc, every sixth section (6 μm thickness) was selected. Sampling was carried out using the Olympus C.A.S.T.-Grid system (Olympus Albertslund, Denmark A/S, Denmark). The SNpc was carefully outlined to exclude other subdivisions of the substantia nigra and the ventral tegmental area by using a x 4 objective. The total number of TH\textsuperscript{+} neurons was analysed using a x100 Planapo oil immersion objective with a 1.4 numerical aperture. Counting frame (60 μm x 60 μm) was superimposed on the
image of tissue sections. TH+ neurons were counted only when present completely or partially inside the frame and when they did not touch any of the red exclusion lines, neurons which touched green inclusion lines were counted. Neurons counted in all sections of the SNpc were summed to give a total number of TH+ neurons.

MES23.5 DA neurons treated with 6-OHDA and GDNF. The MES23.5 cell lines treated with 6-OHDA (100 mM) for different times (30 min, 1 h, 3 h, 6 h, 12 h, and 24 h) were selected for cell counting kit-8 assay (CCK8), trypan blue staining cell viability assay, real-time PCR, and western blot analysis.
The MES23.5 DA neurons treated with 6-OHDA (100 mM) for 6 h followed by GDNF (100 ng/ml) for different times (0 h, 3 h, 6 h, 12 h, and 24 h) were also selected for CCK8 assays, cell counting with trypan blue staining, real-time PCR, and western blot analysis.

**Real-time PCR analysis.** Total RNA from the tissue samples and cell lines was extracted using high pure RNA isolation kits (Roche Applied Science, Indianapolis, IN, USA). RNA concentration and quality were determined with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and gel analysis. Then, the RNA was reversely transcribed to cDNA using transcriptor first strand cDNA synthesis kit (Roche Applied Science). The expression of Six2 and TH genes was assayed using the SYBR green PCR master mix (Roche Applied Science). The mRNA data were normalised to β-actin.

The primers for Six2, TH, and β-actin were as follows: Six2-forward, 5′-GAGGGACACGAGCTCCTCA-3′; Six2-reverse, 5′-TTTGCTGACTGGAAATTGCTTCT-3′; TH-forward, 5′-CTGTGACCCCATGGCTGCCC-3′; TH-reverse, 5′-AAATCCAGCGGGCATGAGAC-3′; β-actin-forward, 5′-CCACCCGAGTACCACTC-3′; β-actin-reverse, 5′-CCCATACCCACCATCACACC-3′.

**Western blot analysis.** As described previously,45 total protein from the tissue samples and cell lines was extracted. Equal amounts of protein samples were separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). After blocking with 3% (w/v) bovine serum albumin for 3 h, the membranes were incubated with primary antibodies and then probed with goat anti-rabbit IR-Dye 670 or 800 cw-labelled secondary antibody. Membranes were imaged using a LiCor Odyssey scanner (Pleasanton, CA, USA). The western blot data were normalised to β-actin.

**Establishment of the MES23.5 DA neurons stably expressing shRNA-Six2.** Five different targeted sequences homologous to Six2 were designed using the lentiviral expression vector (pLV-H1-EF1a-Bsd). Target sites were as follows: shSix2-1 sense strand, 5′-CCAGGACAAAAACGAGAACCC-3′; shSix2-2 sense strand, 5′-GGACTCTCCTCTAAAAGCAAA-3′; shSix2-3 sense strand, 5′-AGAAATGGAAACGGCTCTCA-3′; shSix2-4 sense strand, 5′-ACAGCAACCGCTGACCTCA-3′; and shSix2-5 sense strand, 5′-GCACTTGGCAGGACCTCA-3′. These plasmid DNAs transcribed shRNA with loop sequences of 5′-CTCTGGTCCAGA-3′. The negative control sequence was shScramble sense strand, 5′-ACAAGTTCAACTGCTAGAC-3′.

**ChIP-seq and ChIP-qPCR.** MES23.5 DA neurons were treated with 6-OHDA (100 mM) for 6 h followed by GDNF (100 ng/ml) for 12 h; 12 h after GDNF treatment, cells were fixed in 1% formaldehyde for 10 min, followed by quenching with 2 μl propidium iodide and 2 μl annexin V-fluorescein isothiocyanate for 15 min. In this study, an annexin-V-FLUOS staining kit (Roche Applied Science) was used to assess cell apoptosis, we used the BD FACS Calibur flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and the MACSQuant flow cytometry (Milenyi Biotechnology, Bergisch Gladbach, Germany) to examine the cell apoptosis.

**Cell viability assay.** Cell viability assay was performed using a cell counting kit-8 assay (CCK8: Dojindo Laboratories, Shanghai, China) and a trypan blue staining cell viability assay kit (Beyotime, Jiangsu, China).

**Evaluation of cell apoptosis by flow cytometry analysis.** Cells were harvested using 0.25% trypsin and then washed with 0.1 M PBS. After centrifugation at 800 x g for 5 min, cells were treated with 10 μl binding buffer and then stained with 2 μl propidium iodide and 2 μl annexin V-fluorescein isothiocyanate for 15 min. In this study, an annexin-V-FLUOS staining kit (Roche Applied Science) was used to assess cell apoptosis, we used the BD FACS Calibur flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and the MACSQuant flow cytometry (Milenyi Biotechnology, Bergisch Gladbach, Germany) to examine the cell apoptosis.

**Substantia nigra lentivirus infection to measure Six2 expression and the motor ability of PD rats after GDNF treatment.** The adult adult male (230–250 g) Sprague Dawley rats were randomly divided into three groups: (A) Sham, (B) GDNF treatment, and (C) control. Substantia nigra infection was performed bilaterally with 3 μl lentivirus containing Six2-pLV or shSix2-pLV per injection. The lentivirus infection was confirmed by immuno-staining with anti-Six2 antibody. After GDNF treatment, the rats were divided into two groups: (I) Control and (II) Postural asymmetry tests and apomorphine-induced rotation tests in PD rats infected with Six2-pLV or shSix2-pLV. Bar graphs are shown as means ± S.E.M. (n = 6). The statistical analysis was carried out using one-way ANOVA followed by post hoc Dunnett’s tests.
groups: (i) pLV groups (n = 11), in which the rats received a left substantia nigra injection of pLV-EGFP and left striatum injection of 6-OHDA followed by 8 μg GDNF; (ii) Six2-pLV groups (n = 11), in which the rats received a left substantia nigra injection of Six2-pLV-EGFP and left striatum injection of 6-OHDA followed by 8 μg GDNF; (iii) shSix2-pLV groups (n = 11), in which the rats received a left substantia nigra injection of shSix2-pLV-EGFP and left striatum injection of 6-OHDA followed by 8 μg GDNF. The rats were anesthetized with chloralic hydras (10%) and placed in a stereotaxic frame. Burr holes were drilled to permit unilateral stereotactic injection of 4 μl Six2-pLV or shSix2-pLV or pLV (the final dose was 6 × 10^7 infectious units/ml) at a rate of 1 μl/min into the left SN (stereotaxic coordinates from bregma: anteroposterior: −5.3 mm from bregma, mediolateral: −2.3 mm, and dorso-ventral: −8.3 mm below the surface of the dura). The 6-OHDA lesions were induced as above (see Materials and Methods: Constructing of PD rat models using 6-OHDA), which was injected immediately following substantia nigra pLV delivery. Two weeks after 6-OHDA injection, the a morphomrine-induced rotation analysis was used to screen the PD rat models.

Then, GDNF was injected into these PD rats (see Materials and Methods: 2.4 GDNF treatment), 1 day after GDNF treatment, the SN of three rats in every group were quickly removed into liquid nitrogen and used for western blot analysis of Six2 expression. One week after GDNF treatment, the other eight rats in each group were anesthetized with chloralic hydras and perfused with 4% paraformaldehyde. The brain was removed and overview sections were cut and stained with Hoechst 33342. The rats were then observed under a fluorescence microscope.

Statistical analysis. All data were processed with SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Bar graphs are shown as means ± S.E.M. Difference was carried out using t test to compare two independent samples, and one-way analyses of variance (ANOVA) by follow post hoc Newman–Keuls tests to compare all groups of pLV or post hoc Dunnett’s tests for comparing all experimental groups with the control group. P < 0.05 was considered statistically significant for all tests.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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1. Kordower JH, Bjorklund A. Trophic factor gene therapy for Parkinson’s disease. Mov Disord 2013; 28: 96–109.
2. Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. GDNF: a glial cell line-derived neurotrophic factor gene delivery via a polyethylene imine grafted chitosan carrier. J Neurosci Res 2004; 78: 1317–1324.
3. Peng YS, Lai PL, Peng S, Wu HC, Yu S, Tseng TY et al. 3. Protein tyrosine phosphatase inhibitors enhance neuroprotection of grafted neurons with a GDNF/caspase inhibitor cocktail. J Neurosci Res 2004; 78: 98–103.
4. Helt CE, Hoeming GR, Abeck DS, Gerhardt GA, Ickes B, Reyland ME et al. Neuro-protection of grafted neurons with a GDNF/caspase inhibitor cocktail. Exp Neurol 2001; 170: 258–269.
5. Lu X, Peng G, Li L, Ming M, Yang D, Le W. GDNF cell-derived neurotrophic factor protects against proteasome inhibition-induced dopamine neuron degeneration by suppression of endoplasmic reticulum stress and caspase-3 activation. J Gerontol A Biol Sci Med Sci 2007; 62: 943–950.
6. Cao JP, Wang HJ, Yu JK, Liu HM, Gao DS. The involvement of NF-κB/p65/p52 in the effects of GDNF on DA neurons in early PD rats. Brain Res Bull 2008; 76: 505–511.
7. Garoz RC, Le Doughin NM, Creuzet SE. Combinatorial activity of Six1-2-4 genes in cephalic neural crest cells controls cranial and brain development. Cell Mol Life Sci 2014; 1: 2149–2164.
8. Self M, Lagutin OV, Bowling B, Hendrix J, Cai Y, Dressler GR et al. Six2 is required for suppression of nephrogenesis and progenitor renewal in the developing kidney. EMBO J 2005; 25: S214–S228.
9. Senanayake U, Koller K, Pichler M, Leuschner I, Stromhaier M, Hadler U et al. The perpetual renal stem cell regulator Six2 is activated in renal neoplasms and influences cellular proliferation and migration. Hum Pathol 2013; 44: 335–345.
10. Wang CA, Drasin D, Pham C, Jedlicka P, Zaberezhnyy V, Guney M et al. Homeoprotein Six2 promotes breast cancer metastasis via transcriptional and epigenetic control of E-cadherin expression. Cancer Res 2014; 74: 7357–7370.
11. Yamamoto-Shiraishi Y, Kuroiwa A. Wnt and BMP signaling cooperate with Hox in the control of neural crest cell specification. Deve Biol 2013; 377: 363–374.
12. Zhou P, Chen T, Yang G, Wang H, Li M, Pa M et al. Down-regulated Six2 by knockdown of neurofibromin results in apoptosis of metanephric mesenchyme cells in vitro. Mol Cell Biochem 2014; 390: 205–213.
13. Lu X, Mao Z, Lyu Z, Zhang P, Zhan A, Wang J et al. mir181b promotes apoptosis and suppression of nephrogenesis and progenitor renewal in the developing kidney. EMBO J 2005; 25: S214–S228.
14. Wang CA, Drasin D, Pham C, Jedlicka P, Zaberezhnyy V, Guney M et al. Homeoprotein Six2 promotes breast cancer metastasis via transcriptional and epigenetic control of E-cadherin expression. Cancer Res 2014; 74: 7357–7370.
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