

# FOXA1 transcription activates TFF1 to reduce 6-OHDA-induced dopaminergic neuron damage

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**Abstract.** Forkhead box A1 (FOXA1) plays an important role in the central nervous system, and its loss can lead to the downregulation of tyrosine hydroxylase, which directly affects the synthesis of dopamine, thus leading to Parkinson's disease (PD). The present study aimed to explore the specific role of FOXA1 in PD. Blood samples from patients with PD were collected to determine the expression levels of FOXA1 using reverse transcription-quantitative PCR (RT-qPCR). In addition, mouse dopaminergic neuron MES23.5 cells were induced with 6-hydroxydopamine (6-OHDA) to construct an *in vitro* PD model in order to study the effect of FOXA1 overexpression on cell inflammation, oxidative stress and apoptosis with RT-qPCR, assay kits and TUNEL assays, respectively. Subsequently, the expression of FOXA1 was silenced to assess the effect on the downstream mechanism. The results revealed that the expression level of FOXA1 was downregulated in patients with PD, and FOXA1 overexpression attenuated 6-OHDA-induced inflammation, oxidative stress and apoptosis in MES23.5 cells. Furthermore, FOXA1 could bind to the trefoil factor 1 (TFF1) promoter, and the effects of FOXA1 overexpression on cells were reversed by TFF1 silencing, indicating that TFF1 mediated the mechanism of FOXA1 overexpression in MES23.5 cells. In conclusion, following FOXA1 transcription, TFF1 expression was activated, thereby relieving 6-OHDA-induced cell inflammation, oxidative stress and apoptosis. The present findings suggested that FOXA1 may serve as a target for the treatment of PD.

## Instruction

Parkinson's disease (PD) is a neurodegenerative disease that is characterized by the degeneration of dopaminergic neurons in the substantia nigra compacta (SNc) area (1). The primary clinical manifestations of PD are resting tremor, muscle rigidity and physical movement disorders, such as motor retardation, abnormal posture and gait, as well as non-motor symptoms, including depression and cognitive impairment (2). The condition of patients with PD progressively worsens, which seriously affects the daily quality of life and sociability of patients (3). An article indicates that, among the degenerative diseases of the neurodegenerative system, PD is the most common disease in the middle-aged and elderly population worldwide, second to Alzheimer's disease (4). The average incidence rate estimated in developed countries is 14/100,000 people per year (5). Although the exact pathogenesis of PD is not yet fully understood, various possible mechanisms have been proposed, including oxidative stress, neuroinflammation, mitochondrial dysfunction and ubiquitin proteasome system dysfunction (6). At present, the primary treatment for PD is to increase the concentration of dopamine (DA) or directly stimulate drug-dependent DA receptors to improve symptoms (7). However, these treatments cannot prevent the progression of PD (8). Therefore, investigating the pathogenesis and treatment of PD is of importance.

Tyrosine hydroxylase (TH), a type of monooxygenase, is the rate-limiting enzyme that catalyzes the first step of the reaction in the synthesis of DA (9). In the body, L-tyrosine is catalyzed by TH to produce levodopa, which is then catalyzed by aromatic decarboxylase to decarboxylate and finally forms DA. Due to the important position of TH in the synthesis of DA, its absence or reduced expression directly affects the synthesis and secretion of DA, thus leading to PD (10). A previous study revealed that forkhead box A1 (FOXA1) can maintain dopaminergic properties, and the loss of FOXA1/2 can lead to the downregulation of TH (11). FOXA1 is a member of the Fox transcription factor family and is expressed in multiple human tissues, such as the digestive, urinary and reproductive systems (12,13). It is also widely expressed in the hippocampus, the region where the formation of new neurons occurs in the brain (14). A previous study has shown that FOXA1 can control the differentiation of midbrain DA

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neurons by regulating the expression of genes that are pivotal for neural differentiation (15). Furthermore, it was found that FOXA1 expression was significantly reduced in the serum samples of 30 Indian patients with PD (16).

To explore the potential role of FOXA1 in PD, the present study collected blood samples from patients with PD to determine the expression level of FOXA1. Mouse dopaminergic neuron cells (MES23.5) were induced with 6-hydroxydopamine (6-OHDA) to construct an *in vitro* PD model in order to study the effect of FOXA1 on dopamine neuron damage, as well as the underlying mechanism.

## Materials and methods

**Clinical samples.** The present study was approved by the Ethics Committee of Lianyungang Oriental Hospital Affiliated to Xuzhou Medical University (approval no. KY-2020-003-01). All procedures were performed in accordance with the 1964 Declaration of Helsinki and its later amendments. The patients or their guardians (for patients with reduced brain capacity) were informed of the project and provided written informed consent. Blood samples were collected from 15 patients with PD (nine male patients and six female; age, 63.4±7.76 years) and 15 healthy patients (controls; 11 male patients and four female patients; age, 63.07±9.56 years) who were admitted to the Lianyungang Oriental Hospital Affiliated to Xuzhou Medical University (Lianyungang, China) between April 2020 and August 2020. Inclusion criteria for patients with PD were as follows: i) Clinical features of the patient were in line with the clinical diagnostic criteria of the Parkinson's Society of England Brain Bank; and ii) PD was clearly diagnosed by two physicians at or above the deputy director level. The exclusion criteria for patients with PD were as follows: i) Cerebrovascular disease; ii) Parkinson's syndrome; iii) family-related PD; iv) Parkinson's superimposed syndrome; and v) hepatolenticular degeneration caused by external factors, such as drugs, encephalitis, and brain injury. A total of 5 ml fasting venous blood was drawn from the study subjects and placed into tubes containing anticoagulant. Subsequently, total RNA was extracted from these samples, as previously described (16).

**Cell culture and transfection.** MES23.5 cells were purchased from American Type Culture Collection and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% Penicillin-Streptomycin. Cells were grown at 37°C with a 5% CO<sub>2</sub>/95% air atmosphere. 6-OHDA (purity ≥97%; Merck & Co., Inc.) was dissolved in sterile deionized water to prepare a 60 mmol/l stock solution, which was stored at -20°C in the dark. MES23.5 cells pretreated with 6-OHDA for 24 h served as the 6-OHDA group, whereas untreated cells served as the control group.

MES23.5 cells were plated into 10-cm dishes (2×10<sup>6</sup> cells/dish) and then transfected with 25 nM pcDNA3.1-FOXA1 vector plasmid [overexpression (Ov)-FOXA1; Hunan Fenghui Biotechnology Co., Ltd.], empty vector plasmid (Ov-NC; Hunan Fenghui Biotechnology Co., Ltd.), 100 nM small interfering RNA (siRNA) targeted against trefoil factor 1 (TFF1; siRNA-TFF1-1, 5'-GGCCCAGGAAGA

AACATGTAT-3'; siRNA-TFF1-2, 5'-GGCCATCGAGAA CACTCAAGA-3'; Genepharma Biotech Corp.) or scrambled siRNA (siRNA-NC, 5'-GAAGACATCCTGCGGAAG TAA-3'; Genepharma Biotech Corp.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C according to the manufacturer's instructions. Following 48 h of transfection, the expression levels of FOXA1 and TFF1 were determined.

**Reverse transcription-quantitative PCR (RT-qPCR).** MES23.5 cells (2×10<sup>6</sup>) were suspended and homogenized in 0.75 ml TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min on ice. The quantity of total RNA was measured using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a TaqMan™ RT kit (cat. no. N8080234; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed using SYBR Green PCR Master Mix (Beijing Solarbio Science & Technology Co., Ltd.) on an ABI PRISM 7300 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95°C for 5 min; followed by 38 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 90 sec. The relative mRNA expression levels of each specific gene were quantified using the 2<sup>-ΔΔC<sub>q</sub></sup> method (17) and normalized to the internal reference gene GAPDH. The sequences of the primers used for RT-qPCR are listed in Table I.

**Western blotting.** MES23.5 cells (2×10<sup>6</sup>) were lysed using RIPA buffer (cat. no. P0013C; Beyotime Institute of Biotechnology) for 10 min on ice, and total protein was quantified using a BCA protein assay kit (cat. no. P0012; Beyotime Institute of Biotechnology). Protein samples (25 μg/lane) were subjected to 10% SDS-PAGE and then transferred onto PVDF membranes. After blocking in 5% fat-free milk for 2 h at room temperature, the membranes were incubated at 4°C overnight with primary antibodies (all purchased from Abcam) targeted against FOXA1 (cat. no. ab170933; 1:1,000), TFF1 (cat. no. ab92377; 1:1,000), TH (cat. no. ab137869; 1:5,000), Bcl-2 (cat. no. ab32124; 1:1,000), Bax (cat. no. ab32503; 1:1,000), cleaved caspase 9 (cat. no. ab2324; 1:200), caspase 9 (cat. no. ab202068; 1:2,000) and GAPDH (cat. no. ab9485; 1:2,500). Subsequently, the membranes were incubated with an HRP-labelled goat anti-rabbit secondary antibody (cat. no. ab6721; 1:5,000; Abcam) for 2 h at room temperature. Protein bands were visualized using an ECL kit (cat. no. P0018S; Beyotime Institute of Biotechnology). Protein expression was semi-quantified using ImageJ software (version 1.8; National Institutes of Health) with GAPDH as the loading control.

**Cell counting kit-8 (CCK-8) assay.** MES23.5 cells (5×10<sup>3</sup> cells/well) were seeded into a 96-well plate. Before the addition of 10 μl CCK-8 solution (Beyotime Institute of Biotechnology) to each well, the cells were cultured for 24 h at 37°C. Following a further incubation for 2 h at 37°C after the addition of CCK-8, the optical density was measured at a wavelength of 450 nm using a microplate reader (Molecular Devices, LLC).

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Sequence (5'-3')
FOXA1	F: TGTGTATTCCAGACCCGTGC R: AGGGGAAGGAGTGAAAGGGA
TNF- $\alpha$	F: AGGCACTCCCCCAAAGATG R: TGGTGGTTTGTGAGTGTGAGG
IL-1 $\beta$	F: TGCCACCTTTTGACAGTGATG R: ATGTGCTGCTGCGAGATTTG
IL-6	F: GCCTTCTTGGGACTGATGCT R: GTGACTCCAGCTTATCTCTTGGT
TFF1	F: CCATGGCCATCGAGAACACT R: GGGGTTGAACTGTGTCACCA
TH	F: TACTTTGTGCGCTTCGAGGT R: TGGGTAGCATAGAGGCCCTT
GADPH	F: GGGTCCCAGCTTAGGTTTAT R: ATCCGTTACACCGACCTTC

FOXA1, forkhead box A1; TFF1, trefoil factor 1; TH, tyrosine hydroxylase.

**Immunofluorescence.** MES23.5 cells ( $2 \times 10^4$  cells/well) were plated in 24-well plates, fixed with 4% paraformaldehyde (Macklin, Inc.) for 1 h at room temperature, and blocked in 1% BSA (Beijing Solarbio Science & Technology Co., Ltd.) for 0.5 h at room temperature. Next, the cells were incubated in the presence of a primary antibody against TH (cat. no. ab137869; 1:100; Abcam) at 4°C overnight, followed by incubation with the Alexa Fluor® 488-labelled goat anti-rabbit secondary antibody (cat. no. ab150077; 1:200; Abcam) for 1 h at room temperature. The nuclei were counterstained with DAPI for 5 min at room temperature. The results were observed under a fluorescence microscope (magnification, x200; Olympus Corporation).

**Determination of oxidative stress index.** The level of reactive oxygen species (ROS; cat. no. S0033S) and malondialdehyde (MDA; cat. no. S0131S), and the activity of superoxidase dismutase (SOD; cat. no. S0101S) in MES23.5 cells ( $2 \times 10^6$ ) were quantified using commercial ELISA kits (all purchased from Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The optical density was measured at a wavelength of 525 nm for ROS, 550 nm for SOD and 532 nm for MDA using a microplate reader (Molecular Devices, LLC).

**TUNEL assay.** MES23.5 cells ( $2 \times 10^4$  cells/well) were seeded into a 24-well plate. Subsequently, the TUNEL assay was performed using a TUNEL assay kit (cat. no. C1086; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The results were observed under a fluorescence microscope (magnification, x200; Olympus Corporation) at five fields of view.

**Chromatin immunoprecipitation (ChIP) assay.** ChIP assay was performed with a ChIP assay kit (cat. no. P2078; Beyotime Institute of Biotechnology) according to the manufacturer's

instructions. Briefly, MES23.5 cells ( $1 \times 10^6$ ) were cross-linked with 1% formaldehyde followed by centrifugation at  $1,000 \times g$  for 1 min at 4°C. The precipitated cells were lysed with SDS lysis buffer containing 1 mM PMSF for 10 min, and sonicated in an ice bath. Cell lysates (2 ml) were incubated with 70  $\mu$ l Protein A + G agarose for 30 min at 4°C before incubation with 60  $\mu$ l Protein A + G beads coated with 1  $\mu$ g anti-FOXA1 antibody (cat. no. ab170933; Abcam) for 1 h at 4°C. Anti-rabbit IgG (cat. no. 172730; Abcam) served as a NC. After the incubation, the sample was centrifuged at  $1,000 \times g$  for 1 min at 4°C and the precipitate was collected. Cross-linked DNA released from the precipitate was purified using a DNA purification kit (cat. no. D0033; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The eluted DNA was subjected to RT-qPCR according to the aforementioned protocol.

**Luciferase reporter assay.** The TFF1 promoter region containing a FOXA1 putative binding site [wild-type (WT), 5'-AGGTCACGGTGGCCAC-3'] or mutant (MUT) site (5'-TCCAGTGCCACCGGTG-3')] was cloned into the pGL3-based vector (BioVector NTCC, Inc.). MES23.5 cells were seeded ( $1 \times 10^5$  cells/well) into 24-well plates and co-transfected with 0.5  $\mu$ g aforementioned reporter vector and 0.5  $\mu$ g Ov-FOXA1 or Ov-NC vectors using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.) at room temperature. Following 48 h of transfection, the relative luciferase activity was normalized to *Renilla* luciferase and measured using a dual-luciferase reporter assay system (Promega Corporation) according to the manufacturer's instructions.

**Statistical analysis.** Data are presented as the mean  $\pm$  SD from at least three independent experiments. Statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software, Inc.). Statistical differences between two groups were analyzed using an unpaired Student's t-test. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**FOXA1 overexpression enhances 6-OHDA-induced MES23.5 cell viability and decreases TH expression.** The mRNA expression levels of FOXA1 in the samples from patients with PD and healthy subjects were determined using RT-qPCR. The results revealed that the expression levels of FOXA1 in patients with PD were significantly lower compared with those in the controls (Fig. 1A).

Subsequently, the expression levels of FOXA1 in MES23.5 cells and pretreated cells (6-OHDA group) were determined using RT-qPCR and western blotting. FOXA1 expression was significantly downregulated in the 6-OHDA group compared with that in the control group (Fig. 1B and C).

A FOXA1 overexpression plasmid was constructed to explore the role of FOXA1 in MES23.5 cells, and the transfection efficiency was verified by RT-qPCR and western blotting (Fig. 1D and E). The expression level of FOXA1 in the Ov-FOXA1 group was significantly upregulated compared

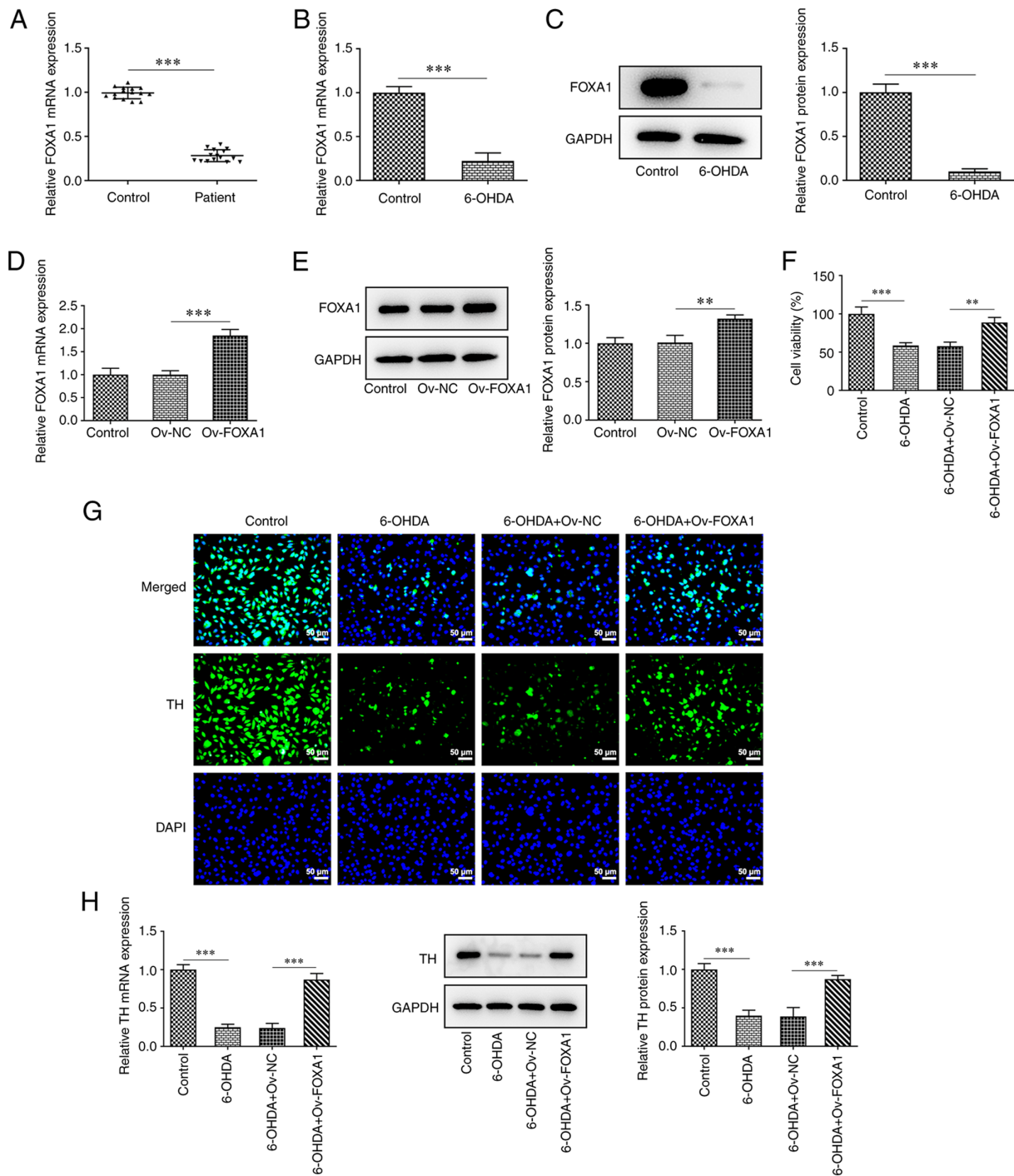


Figure 1. FOXA1 overexpression enhances 6-OHDA-induced MES23.5 cell viability and decreases TH expression. (A) mRNA expression levels of FOXA1 in samples from patients with Parkinson's disease and healthy control subjects were determined using RT-qPCR. Expression levels of FOXA1 in MES23.5 cells were determined using (B) RT-qPCR and (C) western blotting. FOXA1 overexpression was verified by (D) RT-qPCR and (E) western blotting. (F) Cell viability in MES23.5 cells was assessed using Cell Counting Kit-8 assays. Expression of TH in MES23.5 cells was evaluated using (G) immunofluorescence (magnification,  $\times 200$ ), (H) RT-qPCR and western blotting. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . FOXA1, forkhead box A1; RT-qPCR, reverse transcription-quantitative PCR; TH, tyrosine hydroxylase; Ov, overexpression; NC, negative control; 6-OHDA, 6-hydroxydopamine.

with that in the Ov-NC group. Subsequently, MES23.5 cells were divided into four groups: i) Control; ii) 6-OHDA; iii) 6-OHDA + Ov-NC; and iv) 6-OHDA + Ov-FOXA1. Cell viability was assessed using the CCK-8 assay. The results revealed that cell viability in the 6-OHDA group was significantly downregulated compared with that in the control group, whereas FOXA1 overexpression significantly attenuated this decrease (Fig. 1F).

As aforementioned, the absence of TH can affect the formation of DA (10); therefore, the expression of TH in these groups was evaluated using immunofluorescence, RT-qPCR and western blotting. The staining results revealed that the fluorescence intensity of the 6-OHDA group was markedly lower compared with that in the control group (Fig. 1G). FOXA1 overexpression notably increased the fluorescence intensity compared with that in the 6-OHDA + Ov-NC

group, suggesting that FOXA1 overexpression could alleviate the downregulation of TH expression induced by 6-OHDA. In addition, the RT-qPCR and western blotting results also indicated that FOXA1 overexpression could significantly inhibit 6-OHDA-induced downregulation of TH expression (Fig. 1H).

*FOXA1 overexpression attenuates 6-OHDA-induced inflammation, oxidative stress and apoptosis in MES23.5 cells.* Cells were grouped as previously described, and the effect of FOXA1 overexpression on cell inflammation and oxidative stress was subsequently determined. The mRNA expression levels of inflammatory factors, including TNF- $\alpha$ , IL-1 $\beta$  and IL-6, were determined using RT-qPCR (Fig. 2A). The expression of these molecules was significantly increased in the 6-OHDA group compared with that in the control group, whereas FOXA1 overexpression significantly reversed these effects. Furthermore, the levels of SOD, ROS and MDA were determined using ELISA kits. The results demonstrated that the level of SOD was significantly decreased in the 6-OHDA group compared with that in the control group, but significantly increased after FOXA1 overexpression (Fig. 2B). The levels of ROS and MDA were significantly increased in the 6-OHDA group compared with those in the control group, whereas FOXA1 overexpression significantly decreased these levels. Furthermore, cell apoptosis was determined using a TUNEL assay and western blotting. The fluorescence of apoptotic cells was more intense in the 6-OHDA group compared with that in the control group, and FOXA1 overexpression significantly downregulated the fluorescence intensity (Fig. 2C). The western blotting results showed that the protein expression levels of Bcl-2 were significantly decreased in the 6-OHDA group compared with those in the control group, which was accompanied by significantly increased expression levels of Bax and cleaved caspase 9 (Fig. 2D). FOXA1 overexpression significantly alleviated the effects of 6-OHDA on Bcl-2, Bax and cleaved caspase 9 expression.

*FOXA1 overexpression activates TFF1 in 6-OHDA-induced MES23.5 cells.* It has been reported that TFF1 co-localizes with TH in midbrain neurons in rats, and TFF1-positive neurons in a 6-OHDA-induced model were dead (18). Therefore, in the present study, the expression levels of TFF1 in MES23.5 cells were determined using RT-qPCR and western blotting. The results revealed that TFF1 expression was significantly decreased in the 6-OHDA group compared with that in the control group (Fig. 3A and B). FOXA1 overexpression significantly increased the expression levels of TFF1 in 6-OHDA-induced MES23.5 cells (Fig. 3C). In addition, FOXA1 was considered to be able to bind to the site 405-390 bp of the TFF1 promoter (Fig. 3D). Therefore, a ChIP assay was performed to verify the interaction of FOXA1 with TFF1 promoter, and the results indicated that FOXA1 could bind to the promoter of TFF1 (Fig. 3E). Next, the binding site was mutated, and luciferase activities were measured using a luciferase reporter assay. The activity of the WT group was significantly higher compared with that of the MUT group, indicating that FOXA1 did bind to the aforementioned site (Fig. 3F).

*TFF1 mediates the mechanism of FOXA1 overexpression in 6-OHDA-induced MES23.5 cells.* To study the role of TFF1 in MES23.5 cells, TFF1 was silenced via siRNA, and its expression was determined using RT-qPCR and western blotting. The results revealed that the expression levels of the siRNA-TFF1-1 group were lower compared with those of the siRNA-TFF1-2 group; thus, the siRNA-TFF1-1 group was selected for subsequent experiments (Fig. 4A and B). MES23.5 cells were divided into the following five groups: i) Control; ii) 6-OHDA; iii) 6-OHDA + Ov-FOXA1; iv) 6-OHDA + Ov-FOXA1 + siRNA-NC; and v) 6-OHDA + Ov-FOXA1 + siRNA-TFF1. Cell viability of these groups was assessed using a CCK-8 assay, and the results revealed that the cell viability of the siRNA-TFF1 group was significantly reduced compared with that of the siRNA-NC group, indicating that TFF1 silencing reversed the increase in cell viability induced by FOXA1 overexpression (Fig. 4C). Next, the expression of TH in these groups was evaluated using immunofluorescence, RT-qPCR and western blotting. The expression of TH was significantly decreased after TFF1 silencing, which indicated that TFF1 silencing could reverse the effect of FOXA1 overexpression on TH expression (Fig. 4D-F). Furthermore, the effect of TFF1 silencing on cell inflammation was assessed by measuring the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6. The RT-qPCR results suggested that TFF1 silencing could significantly elevate the levels of inflammatory cytokines in 6-OHDA-induced cells overexpressing FOXA1, which is equivalent to blocking the inhibitory effect of FOXA1 overexpression on cell inflammation (Fig. 5A). TFF1 silencing significantly reversed FOXA1 overexpression-induced alterations in the activity of SOD, ROS and MDA, which indicated that TFF1 could mediate oxidative stress (Fig. 5B). The effect of TFF1 silencing on cell apoptosis was determined using a TUNEL assay and western blotting. The fluorescence intensity in the siRNA-TFF1 group was enhanced compared with that of the siRNA-NC group (Fig. 5C). Moreover, the expression levels of Bax and cleaved caspase 9 were significantly increased and that of Bcl2 was decreased in the siRNA-TFF1 group compared with those in the siRNA-NC group (Fig. 5D). These findings suggested that TFF1 silencing reversed the effect of FOXA1 overexpression on cell apoptosis.

## Discussion

Numerous studies on the pathogenesis of PD have confirmed that neuroinflammation and oxidative stress participate in, and promote the occurrence and progression of PD (19-21). In the central nervous system, glial cells can be activated by pathogen- and injury-related molecular patterns, leading to persistent neuroinflammation (22). In addition, since the metabolic activity of neurons is markedly high, the demand for energy and the consumption of oxygen are high, which leads to the generation of large quantities of ROS. By contrast, the content of antioxidants in neurons is relatively low, which increases the risk of oxidative stress (23). In the present study, FOXA1 overexpression decreased the levels of inflammatory factors in 6-OHDA-induced MES23.5 cells. The MES23.5 cell line is a dopaminergic neuroblastoma, which displays the characteristics of dopaminergic neurons, and its cultivation is

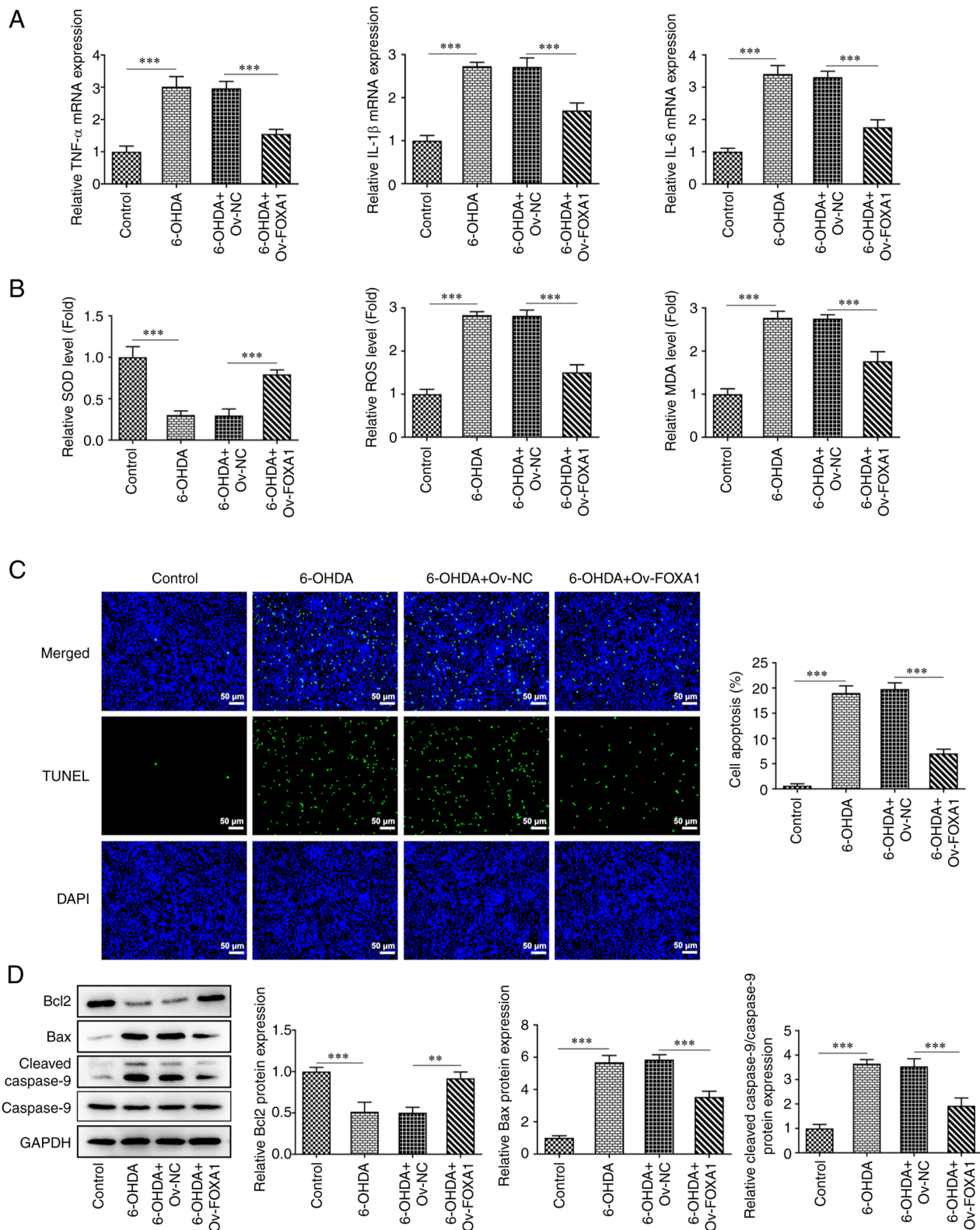


Figure 2. FOXA1 overexpression attenuates 6-OHDA-induced inflammation, oxidative stress and apoptosis in MES23.5 cells. (A) mRNA expression levels of inflammatory factors, including TNF- $\alpha$ , IL-1 $\beta$  and IL-6, were measured using reverse transcription-quantitative PCR. (B) Expression levels of SOD, ROS and MDA were determined using ELISA kits. Cell apoptosis was determined using a (C) TUNEL assay (magnification, x200) and (D) western blotting. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . SOD, superoxidase dismutase; ROS, reactive oxygen species; MDA, malondialdehyde; Ov, overexpression; NC, negative control; FOXA1, forkhead box A1; 6-OHDA, 6-hydroxydopamine.

easier than that of midbrain nerve cells. Therefore, this cell line has been widely used as a tool for studying neurodegenerative diseases (24). Considering that excessive ROS is the

cause of oxidative stress (25) and that MDA is a metabolite of oxygen free radicals (26), the levels of ROS and MDA were assessed in the present study.

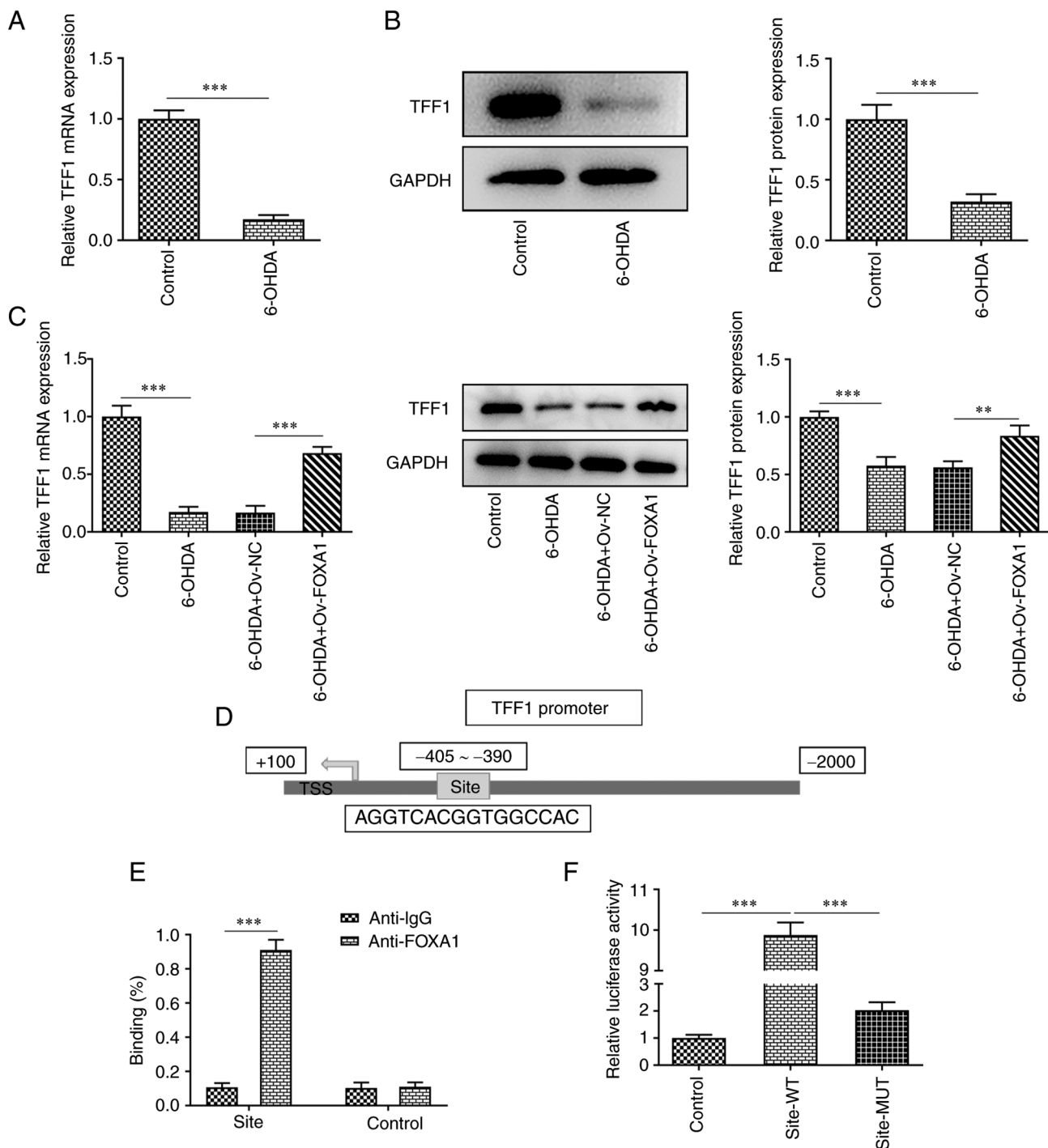


Figure 3. FOXA1 overexpression activates TFF1 in 6-OHDA-induced MES23.5 cells. Expression levels of TFF1 in MES23.5 cells were determined using (A) RT-qPCR and (B) western blotting. (C) Expression levels of TFF1 in different groups of MES23.5 cells were determined using RT-qPCR and western blotting. (D) FOXA1 was able to bind to the site 405-390 bp on the TFF1 promoter. The interaction between FOXA1 and TFF1 was verified using (E) chromatin immunoprecipitation and (F) luciferase reporter assays. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . TFF1, trefoil factor 1; RT-qPCR, reverse transcription-quantitative PCR; FOXA1, forkhead box A1; Ov, overexpression; NC, negative control; WT, wild-type; MUT, mutant; 6-OHDA, 6-hydroxydopamine.

Due to the development of novel experimental techniques, the study of the regulatory role of transcription factors in eukaryotes has attracted considerable attention. Through studying the specific functions and mechanisms of various transcription factors in transcriptional regulation, various drug molecules have been designed, which possess the ability to inhibit or activate transcription factors, thereby altering gene expression patterns (27,28). This kind of strategy may have important

application prospects for the development of treatments. In the present study, the role of the transcription factor FOXA1 in DA nerve cells was evaluated. The results suggested that FOXA1 overexpression may reduce cell inflammation, oxidative stress and apoptosis. Importantly, FOXA1 overexpression could increase the expression of the marker TH, which implies that it can reduce the loss of dopaminergic neurons. In previous studies, FOXA1/2 was also considered to play an important

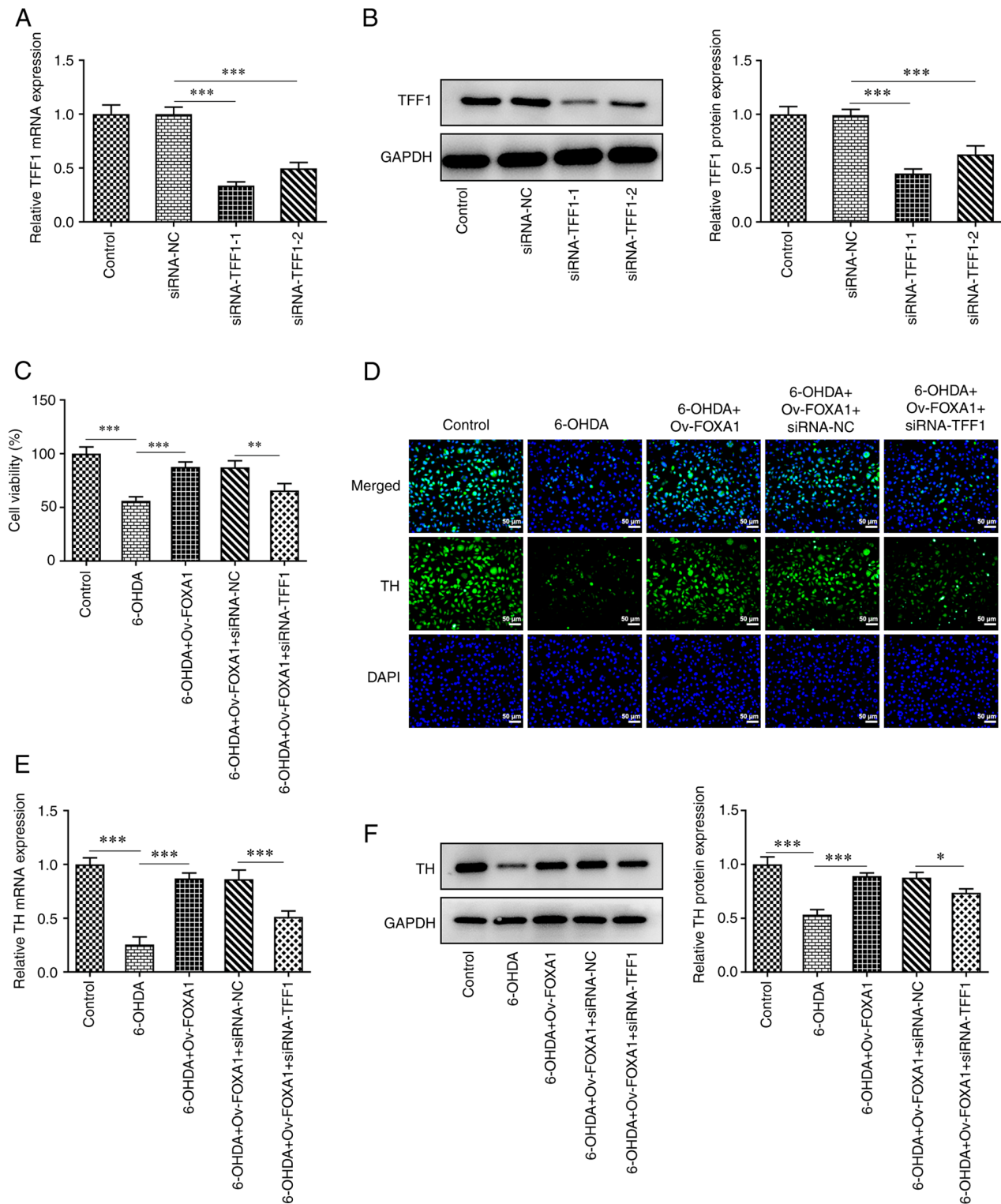


Figure 4. TFF1 mediates the mechanism of FOXA1 overexpression in 6-OHDA-induced MES23.5 cell viability and TH expression. TFF1 silencing was verified using (A) RT-qPCR and (B) western blotting. (C) Cell viability in MES23.5 cells was assessed using a Cell Counting Kit-8 assay. Expression levels of TH in MES23.5 cells was evaluated using (D) immunofluorescence (magnification, x200), (E) RT-qPCR and (F) western blotting. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . TFF1, trefoil factor 1; RT-qPCR, reverse transcription-quantitative PCR; TH, tyrosine hydroxylase; siRNA, small interfering RNA; NC, negative control; FOXA1, forkhead box A1; 6-OHDA, 6-hydroxydopamine.

role in the central nervous system. It has been reported that FOXA1/2 participates in the early formation, late development and function of dopaminergic neurons after maturation, and it is necessary for maintaining the normal neuronal firing activity of dopaminergic neurons in the SNc (29,30).

TFF1 belongs to the trefoil family of proteins and is primarily found to be stably expressed in the gastrointestinal mucosa, which can protect the mucosa from damage, stabilize the mucous layer and promote epithelial healing (31,32). In the present study, it was considered that TFF1 was involved

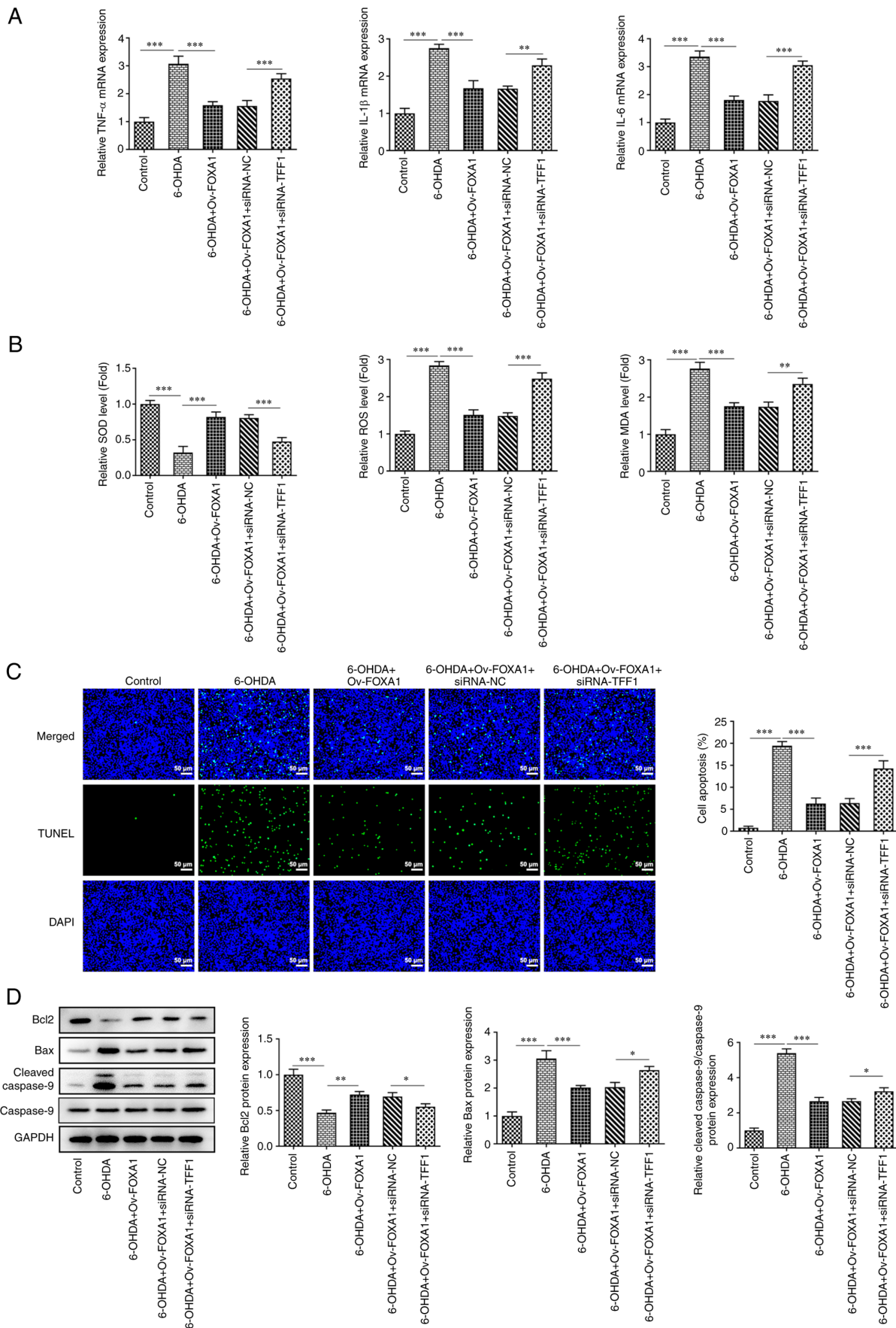


Figure 5. TFF1 mediates the mechanism of FOXA1 overexpression in inflammation, oxidative stress and apoptosis in MES23.5 cells. (A) mRNA expression levels of inflammatory factors, including TNF- $\alpha$ , IL-1 $\beta$  and IL-6, were measured using reverse transcription-quantitative PCR. (B) Expression levels of SOD, ROS and MDA were determined using ELISA kits. Cell apoptosis was determined using a (C) TUNEL assay (magnification, x200) and (D) western blotting. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . SOD, superoxidase dismutase; ROS, reactive oxygen species; MDA, malondialdehyde; Ov, overexpression; FOXA1, forkhead box A1; siRNA, small interfering RNA; NC, negative control; TFF1, trefoil factor 1; 6-OHDA, 6-hydroxydopamine.

in the FOXA1-mediated regulation of dopaminergic neuron cells. Upon FOXA1 transcription, the expression of TFF1 is activated, which reduces the damage of 6-OHDA to neurons. Notably, there are several studies on TFF1 in human tumors; for example, TFF1 is considered to inhibit the function of the IL-6/STAT3 proinflammatory signaling axis in inhibiting gastric tumorigenesis (33). TFF1 is generally upregulated in the serum of patients with breast cancer; it is considered to be relevant to estrogen receptor level and it can serve as a prognostic marker, particularly for non-triple-negative breast cancer (34). However, it is worth noting that TFF1 is reported to be a neuropeptide and is co-expressed with TH in dopaminergic neurons (18). The present results further indicated that TFF1 may play a role in neurons. Notably, the present study exhibits certain limitations, including the fact that experiments were only conducted at the cellular level; thus, future *in vivo* studies in mice should be conducted. In addition, the association between FOXA1 expression and individual disease duration should be investigated in future studies.

In conclusion, the expression level of FOXA1 was down-regulated in patients with PD, and FOXA1 overexpression attenuated 6-OHDA-induced inflammation, oxidative stress and apoptosis in MES23.5 cells. Furthermore, FOXA1 overexpression activated TFF1, indicating that TFF1 mediated the mechanism of FOXA1 overexpression in MES23.5 cells. The present findings suggested that FOXA1 may serve as a target for the treatment of PD and contribute to the development of targeted drugs.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

TL, PZ and HC designed the study. TL, PZ, XZ, XH and LL performed the experiments. LL made considerable contributions to the drafting of the manuscript. HC revised the manuscript for important intellectual content. BH and LK collected the clinical data and analyzed the data. All authors read and approved the final manuscript. TL and LL confirm the authenticity of the raw data.

### Ethics approval and consent to participate

All procedures were performed in accordance with the Declaration of the Institutional Research Committee's Ethical standards, as well as the 1964 Declaration of Helsinki and its later amendments. The present study was approved by the Ethics Committee of Lianyungang Oriental Hospital Affiliated

to Xuzhou Medical University (approval no. KY-2020-003-01). All patients or their parents/guardians provided written informed consent.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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