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Original Article

GATA3 modulates neuronal survival through regulating TRPM2 in Parkinson's disease

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Abstract: In our study, we have found the molecular mechanism of GATA3 in 6-OHDA-treated PC12 cells which mimic to PD. Western blotting and qRT-PCR results suggested that GATA3 was low expression in 6-OHDA-treated PC12 cells, and the ectopic expression of GATA3 remarkably facilitated the cells proliferation and survival rate in 6-OHDA-induced PC12 cells, however, inhibition of GATA3 reversed these effects. Moreover, over-expression or inhibition of GATA3 in 6-OHDA-treated PC12 cells regulated ROS production and caspase-3 activity, respectively. ChIP analysis and luciferase reporter assay all confirmed that GATA3 transcriptional inhibited transient receptor potential melastatin 2 (TRPM2), and over-expression of GATA3 significantly decreased the expression of TRPM2. Subsequently, further analysis demonstrated that GATA3 regulated 6-OHDA-induced PC12 cell proliferation and survival via transcriptional inhibiting TRPM2. In conclusion, our works revealed that GATA3 reversed effects of 6-OHDA-induced PC12 cell growth and apoptosis through transcriptional inhibiting TRPM2, and provided GATA3 as a potential molecular therapy target for PD.

Keywords: GATA3, PD, TRPM2, proliferation

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder. The dopaminergic neurons loss is its character [1, 2]. The incidence of PD is very low before the age of 50, however after 80 years, the morbidity of PD is increase to almost 4% [3]. The dopaminergic neurons was reported to control numerous brain functions, including voluntary movement, and the abnormal degeneration of dopaminergic neurons led to bradykinesia, postural instability, rigidity and resting tremors [4]. Clinical evidence demonstrated that reactive oxygen species (ROS) was obviously increased in specific patient's brain regions who was had PD [5, 6]. High production of ROS resulted to dopaminergic neuron apoptosis in PD [7]. Thus, how to suppress ROS production was a novel therapy strategy in PD. It is known that 6-hydroxydopamine (6-OHDA), a mitochondrial inhibitor, can induce neuron loss similar to PD *in vivo* [1, 8]. So, 6-OHDA-treatment cell could be used to investigate the mechanism of PD.

GATA3 belonged to GATA family, including GATA1-6. GATA3 worked as a transcription factor, it binded conservative sequences which containing GATA motif [9-11]. Furthermore, aberrant GATA3 expression exhibited in multiple carcinomas, such as Hodgkin's lymphoma [12], renal cell carcinoma [13], urothelial carcinoma [13], cervical carcinoma [14], orpancreatic carcinoma [15]. Multiple reports indicated that GATA3 played crucial function in T lymphopoiesis [16, 17], moreover, GATA3 also regulated the differentiation of mammary gland through interacting with the estrogen receptor (ER) [18-24]. In addition, absence of GATA3 expression was closely coordinated with undifferentiated tumors and poor prognosis in breast cancer [25]. However, the detail molecular mechanism of GATA3 in Parkinson's disease was still unknown.

The transient receptor potential melastatin 2 (TRPM2) belonged to the TRP superfamily [26]. Recently work indicated that TRPM2 acted as ROS sensor and played pivotal role in the regu-

lation of ROS production [27, 28]. Moreover, TRPM7, another member of TRP family, was found that it could regulate excitotoxic-independent neuronal death [29]. Thus, we detected whether TRPM2 also regulated excitotoxic-independent neuronal death in PD. In our works, we investigated whether GATA3 regulated 6-OHDA-induced rat pheochromocytoma cell (PC12) death, and explored its downstream target.

Here, we found that GATA3 was low expressed in PD, and GATA3 reversed effects of 6-OHDA-induced PC12 cell growth and apoptosis through transcriptional inhibiting TRPM2.

Materials and methods

Cell culture and transfect

Human neuroblastoma cell line SH-SY5Y and rat pheochromocytoma cell line PC12 were purchased from ATCC (Manassas, VA, USA). Cells were cultured in F-12K medium supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin and 10% FBS at 37°C with 5% CO_2. 100 μM 6-OHDA (Sigma, St. Louis, MO, USA) was added in cells for 24 h to simulate PD-like neuron loss. SH-SY5Y cells and PC12 cells were transfected with vector, FLAG-GATA3, scramble siRNA (SCR) and GATA3 siRNA by using TransIT-X^2 reagent (Mirus, TX, USA) according to the manufacturer's protocol. After transfection 48 h, cells were collected for further experiments.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated by using TRIzol reagent (Invitrogen, Dusseldorf, USA) according to the manufacturer's protocol. cDNA was synthesized by TransScript First-Strand cDNA Synthesis SuperMix (Transgene biotech, Beijing, China). The mRNA level of gene was measured by utilizing a SYBR Green mix (Takara, Japan). The primers used for qRT-PCR were as follows: GATA3 forward: 5'-GCTTCACAATATTAACAGACCC-3'; reverse: 5'-TTAAACGAGCTGTTCTTGGG-3'; TRPM2 forward: 5'-CATGTTCAACTACACCTTCCAG-3'; reverse: 5'-CCTTCAGGTAGATCTCCCAG-3'. β-actin was used as an internal control. Each experiment was repeated at least three times. All data were presented as mean ± SD.

ChIP assay

EZ ChIP Kit (Millipore) was used to perform ChIP assay according to the manufacturer's protocol. In brief, cells were lysed and sonicated chromatin into almost 250 bp to 1×10^3 bp fragments. Subsequently, the chromatin was immunoprecipitated by anti-GATA3 antibody, and next the specific primer was used to amplify the promoter region of target gene. The primers used for ChIP were as follows: GATA3 forward: 5'-GAAATGAGGGTGGCG-3'; reverse: 5'-GAGAAGGCGAATGTG-3'. IgG served as a negative control.

Western blot

The whole protein was extracted by using RIPA lysis buffer (BLKW, Beijing, China). The BCA assay kit (Poerce, USA) was used to measure the protein concentration, and equal amount of protein was resolved by 10% SDS-PAGE gel. Next, protein was transferred to PVDF membranes (Millipore, Billerica, MA, USA) and blocked with 5% skimmed milk at room temperature for 1 h, incubating with indicated antibodies at 4°C overnight. Membranes were washed with PBST solution for three times and incubated with HRP-conjugated secondary antibody, and then membranes were washed with PBST solution for three times. Finally, the immunoblot was visualized by ECL kit (Pierce, Rockford, USA). Each experiment was repeated at least three times. All data were presented as mean ± SD. Each experiment was repeated at least three times.

Cell viability assays

To explore the function of GATA3 over-expression or inhibition against 6-OHDA-induced cytotoxicity, we performed MTT analysis to measure the cell viability [30]. After transfection 48 h, 100 μM 6-OHDA was added in cells for 24 h. Next, we added 20 μl 5 mg/ml MTT in cells and incubated at 37°C for 3 h. Utilizing a microplate reader to measure the absorbance at 490 nm. Each experiment was repeated at least three times. All data were presented as mean ± SD.

CCK-8 assay

CCK-8 analysis was used to determine cell proliferation. After transfection for 48 h, 10 μl CCK-8 solution was added to each well and incubated cells for 2 h at 37°C. A microplate reader was used to measure the absorbance at 450 nm. Each experiment was repeated at

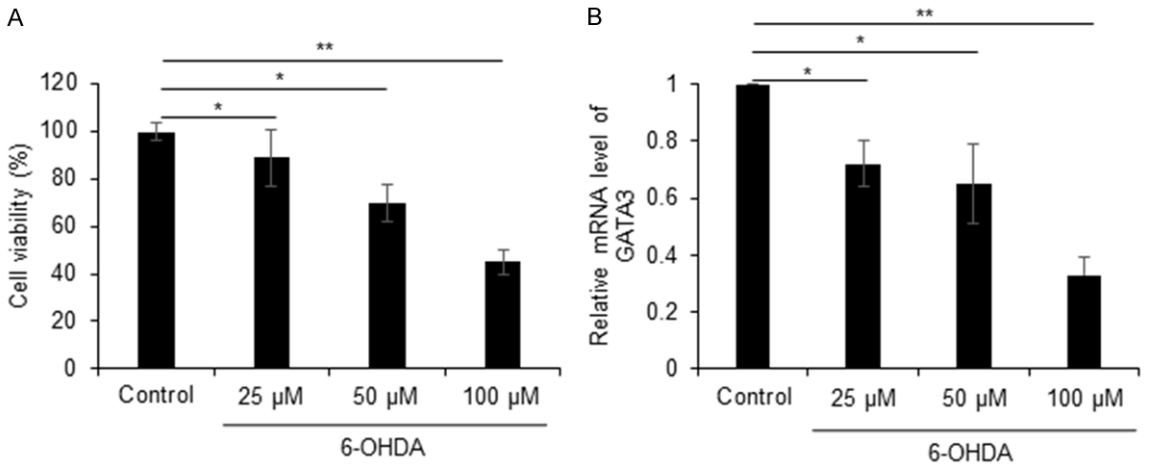

Figure 1. GATA3 is low expression in 6-OHDA-treated PC12 Cells. A: Different concentrations of 6-OHDA (0-100 μM) was added in medium and used to detect PC12 cell viability. After incubation 24 h, the cell viability was detected through MTT assay according to manufacturer's method. Each experiment was repeat at least three times. All data were presented as mean ± SD. *P<0.05, **P<0.01. B: The mRNA level of GATA3 in different concentration of 6-OHDA treatment PC12 cells were measured by qRT-PCR. Each experiment was repeated at least three times. All data were presented as mean ± SD. *P<0.05, **P<0.01.

least three times. All data were presented as mean ± SD.

Caspase-3 activity assay

The caspase-3 activity kit (Beyotime, Haimen, China) was used to detect caspase-3 activity according to the method described previously [31]. In briefly, cells were homogenized in 2 mM caspase-3 substrate (Ac-DEVD-pNA) and reaction buffer (137 mM NAD, 1% NP-40, 20 mM Tris-HCl and 10% glycerol). Next, cells were incubated for 2 h and the absorbance at 405 nm was measured by using an ELISA reader. Each experiment was repeated at least three times. All data were presented as mean ± SD.

Intracellular ROS analysis

ROS production was detected according to manufacturer's method described previously [6]. PC12 cells were placed in 6-well plates (4×10^4 cells/well), next transfected with vector, FLAG-GATA3, scramble siRNA (SCR) or siGATA3. After 6-OHDA treatment, we added 5 μM fluorescent probes CM-H2DCFDA to the cells in the dark and incubated at 37°C for 30 min. Utilizing a fluorimeter to determine fluorescence intensity, with excitation wavelength of 493 nm and emission wavelength of 522 nm, respectively. Each experiment was repeated at least three times. All data were presented as mean ± SD and normalized through utilizing the control group.

Luciferase reporter analysis

Luciferase activity was determined according to previously manufacturer's instructions by using the Firefly Luciferase Reporter Gene Assay Kit (Beyotime, Haimen, China) [6]. The pGL3-TRPM2 promoter region contained the GATA3 binding sequences. PC12 cells were placed in 6-well plates and co-transfected with vector, GATA3, luciferase reporter constructs containing TRPM2 promoter region and renila. Finally, the relative luciferase activity was measured via utilizing the Firefly Luciferase Reporter Gene Assay Kit (Beyotime, Haimen, China) according to the manufacturer's method. Each experiment was repeated at least three times. All data were presented as mean ± SD.

Statistical analysis

All data were analyzed by SDSS.19.0 and presented as mean ± SD. One-way ANOVAs followed by Student's t tests were used to compare with groups. *P<0.05 was regard as statistically significant (*P<0.05, **P<0.01).

Result

GATA3 is low expression in 6-OHDA-treated PC12 cells

GATA family plays key function in several tumors, GATA1, GATA2 and GATA4 were report-

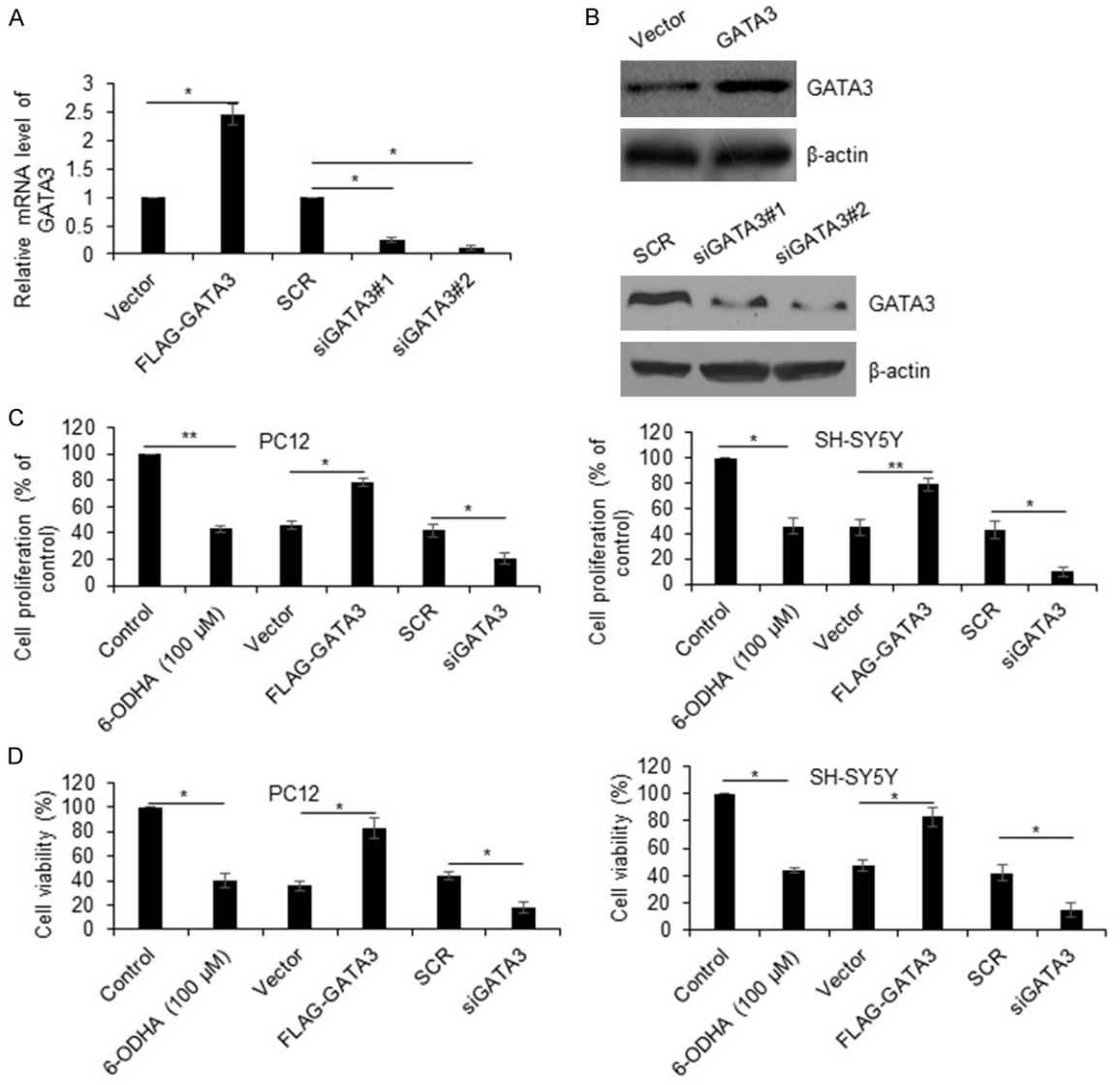

Figure 2. Effects of GATA3 on PC12 proliferation and survival. A: Overexpressed or silenced GATA3 in PC12 cells, after transfection 48 h, the mRNA level of GATA3 was measured by qRT-PCR. Each experiment was repeated at least three times. All data were presented as mean ± SD. *P<0.05. B: Overexpressed or silenced GATA3 in PC12 cells, after transfection 48 h, the protein level of GATA3 was measured by western blotting. C: After transfection 48 h, 100 μM 6-OHDA was added in cells for 24 h. CCK-8 assay was used to measured PC12 and SH-SY5Y cells proliferation. Each experiment was repeated at least three times. All data were presented as mean ± SD. *P<0.05, **P<0.01. D: After transfection 48 h, 100 μM 6-OHDA was added in cells for 24 h. MTT assay was used to measured PC12 and SH-SY5Y cells viability. Each experiment was repeated at least three times. All data were presented as mean ± SD. *P<0.05.

ed to regulate the Parkinson's disease-linked gene α-synuclein [32, 33]. We utilized 6-OHDA in PC12 cells to simulate PD-like neuron loss, and measured the mRNA and protein level of GATA3. Compared with the control group, the expression of GATA3 was significantly down-regulation in 6-OHDA-treated PC12 cells (**Figure 1A** and **1B**).

Effects of GATA3 on PC12 proliferation and survival

To further investigate the function of GATA3 in PC12 cells, we next over-expressed or silenced GATA3 in 6-OHDA-treated PC12 cells. By using western blotting and qRT-PCR, we observed that GATA3 expression was dramatically in-

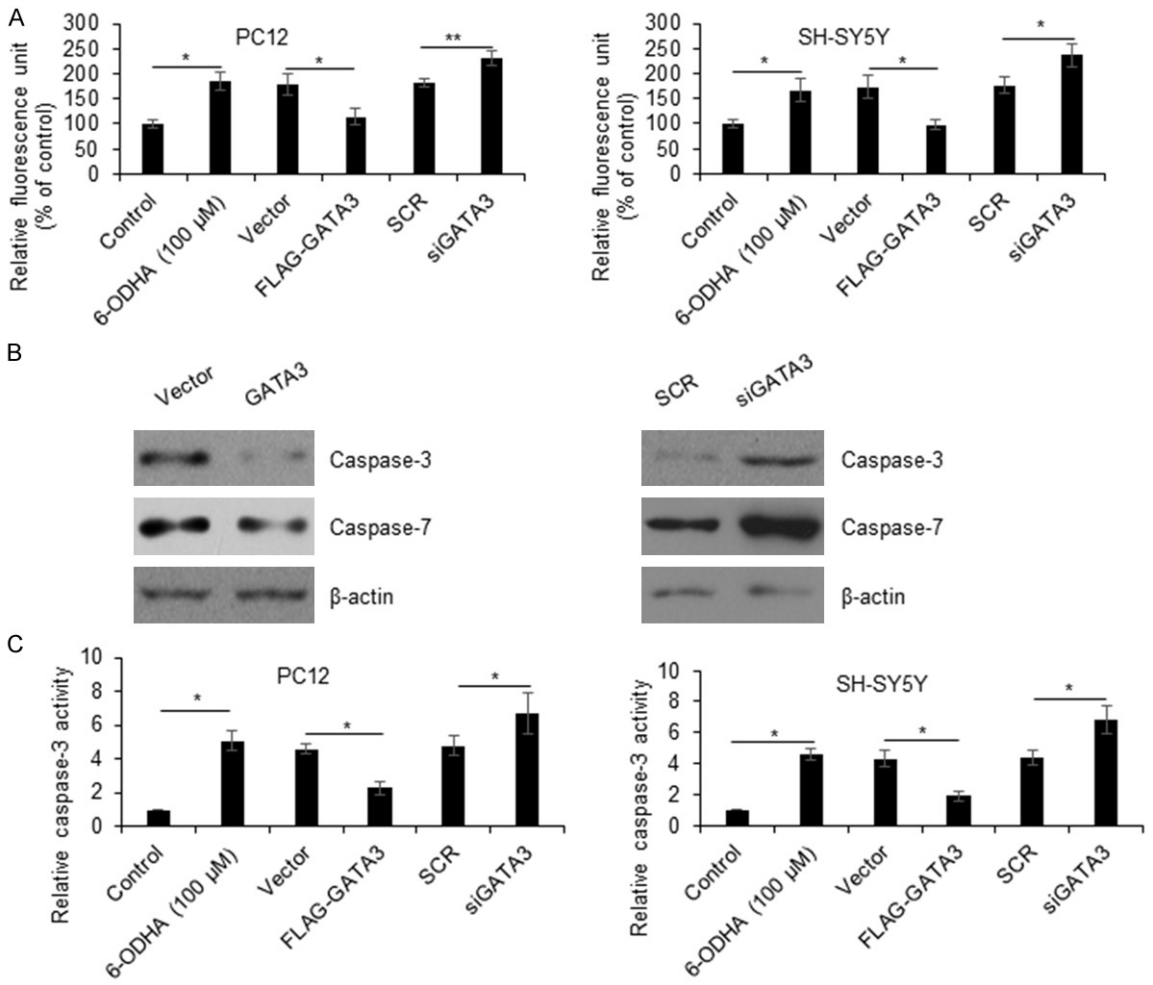

Figure 3. Effects of GATA3 on ROS production and caspase-3 Activity. A: After transfection 48 h, 100 μM 6-OHDA was added in cells for 24 h. The fluorescent probe CM-H2DCFDA was used to detect ROS production in PC12 and SH-SY5Y cells. Each experiment was repeated at least three times. All data were presented as mean ± SD. *p<0.05, **p<0.01. B: The protein level of caspase-3 and capspase-7 were detected by western blotting. C: After transfection 48 h, 100 μM 6-OHDA was added in cells for 24 h. The caspase-3 activity kit was used to measured caspase-3 activity in PC12 and SH-SY5Y cells. Each experiment was repeated at least three times. All data were presented as mean ± SD. *P<0.05.

creased or suppressed in FLAG-GATA3 or GATA3 siRNA transfected cells, respectively (**Figure 2A** and **2B**). To explore the role of GATA3 in responsed to 6-OHDA treatment, cell proliferation assay and cell survival assay were performed. The ectopic expression of GATA3 obviously increased 6-OHDA-treated PC12 cell proliferation and survival (**Figure 2C** and **2D**), whereas GATA3 inhibition strikingly suppressed PC12 cell proliferation and survival (**Figure 2C** and **2D**). In addition, our works suggested that over-expression of GATA3 also increased 6-OHDA-induced SH-SY5Y proliferation and survival (**Figure 2C** and **2D**). These data demonstrated that GATA3 regulated PC12 cells proliferation and survival under 6-OHDA treatment.

Effects of GATA3 on ROS production and caspase-3 activity

Multiple evidence revealed that 6-OHDA-induced cell injury through inducing ROS generation [34]. So, in order to explore whether over-expression of GATA3 suppressed the generation of ROS in PC12 cells under 6-OHDA treatment, we used the fluorescent probe CM-H2DCFDA to measure the accumulation of ROS. We found ROS was significantly accumulated in 6-OHDA-treated PC12 cells, however,

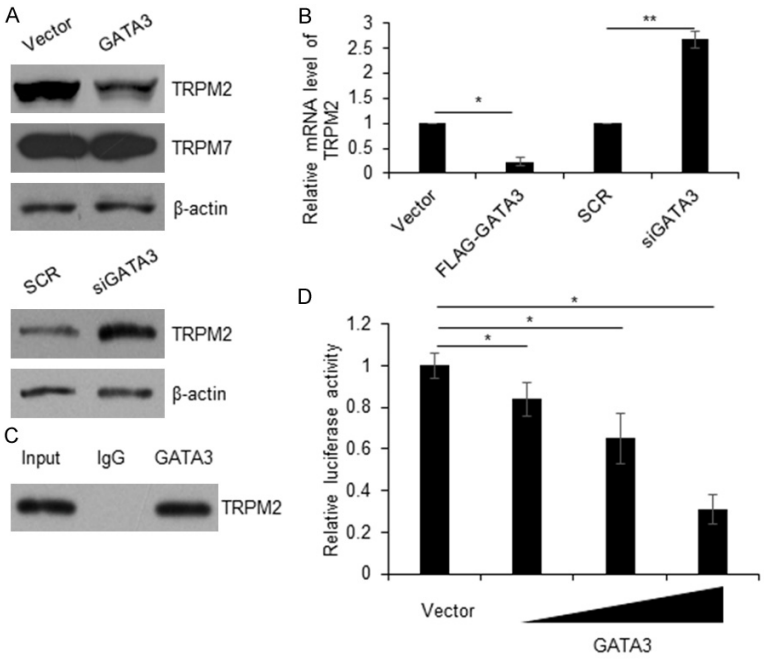

Figure 4. TRPM2 is a target gene of GATA3. A: The protein level of TRPM2 was detected by western blotting. B: The mRNA level of TRPM2 was detected by qRT-PCR. Each experiment was repeated at least three times. All data were presented as mean ± SD. *P<0.05, **P<0.01. C: ChIP analysis was performed to detect the interaction between GATA3 and the promotor region of TRPM2. D: Dual-luciferase reporter assay was performed to determine the interaction between GATA3 and the promotor region of TRPM2. Each experiment was repeated at least three times. All data were presented as mean ± SD. *P<0.05.

ectopic expression of GATA3 strikingly decreased ROS production (**Figure 3A**). Moreover, we also found the level of ROS was obviously increased in 6-OHDA-treated PC12 cells while GATA3 was silenced (**Figure 3A**). The similar results were observed in SH-SY5Y cells (**Figure 3A**). Cell apoptosis was regulated by several protein, including caspase-3, caspase-7. We also detected whether GATA3 regulated these protein in 6-OHDA-treated PC12 cells. As shown in **Figure 3B**, the expression of caspase-3 and caspase-7 were increased in 6-OHDA-treated PC12 cells while GATA3 was silenced. Whereas over-expressed GATA3, the expression of caspase-3 and caspase-7 were decreased. Moreover, inhibition of GATA3 facilitated caspase-3 activity in 6-OHDA-treated PC12 cells (**Figure 3C**), whereas over-expression of GATA3 suppressed caspase-3 activity (**Figure 3C**).

TRPM2 is a target gene of GATA3

It has been demonstrated that TRPM2 and TRPM7 plays some role in neurons [35]. GATA3 usually works as a transcription factor, we assumed whether GATA3 transcriptional regulated TRPM2 and TRPM7. To further confirm whether GATA3 regulated TRPM2 or TRPM7 expression, we detected the expression of TRPM2 and TRPM7 in PC12 cells with over-expressed or silenced GATA3. As shown in **Figure 4A**, we found that TRPM2 was regulated by GATA3. Moreover, the mRNA level of TRPM2 also increased when GATA3 was silenced, however, ectopic expression of GATA3 decreased the mRNA level of TRPM2 (**Figure 4B**). But TRPM7 was not regulated by GATA3 (**Figure 4A**). To assay the direct interaction between GATA3 and TRPM2, we performed ChIP analysis and luciferase reporter analysis. We found that GATA3 interacted with the promoter region of TRPM2 (**Figure 4C**). In addition, luciferase reporter analysis indicated that ectopic expression of GATA3 obviously decreased the luciferase activity in pGL3-TRPM2-promoter transfected cells (**Figure 4D**).

GATA3 mediates PC12 cell proliferation and survival by targeting TRPM2

The above results indicated that GATA3 regulated PC12 cells proliferation and survival under 6-OHDA treatment and significantly down-regulated TRPM2 expression. TRPM2 was reported to play roles in neuron injury. So, we decided to further investigate the function of TRPM2 in the neuroprotective effects of GATA3. We over-expressed TRPM2 by using pcDNA3-TRPM2 (**Figure 5A** and **5B**). As shown in **Figure 5C** and **5D**, over-expression of TRPM2 significantly inhibited the effect of GATA3 over-expression on 6-OHDA-induced PC12 cell proliferation and survival. In addition, ectopic expression of TRPM2 also promoted the ROS production which was down-regulated by GATA3 over-expression (**Figure 5E**). Caspase-3 activity assay revealed that over-expression of TRPM2

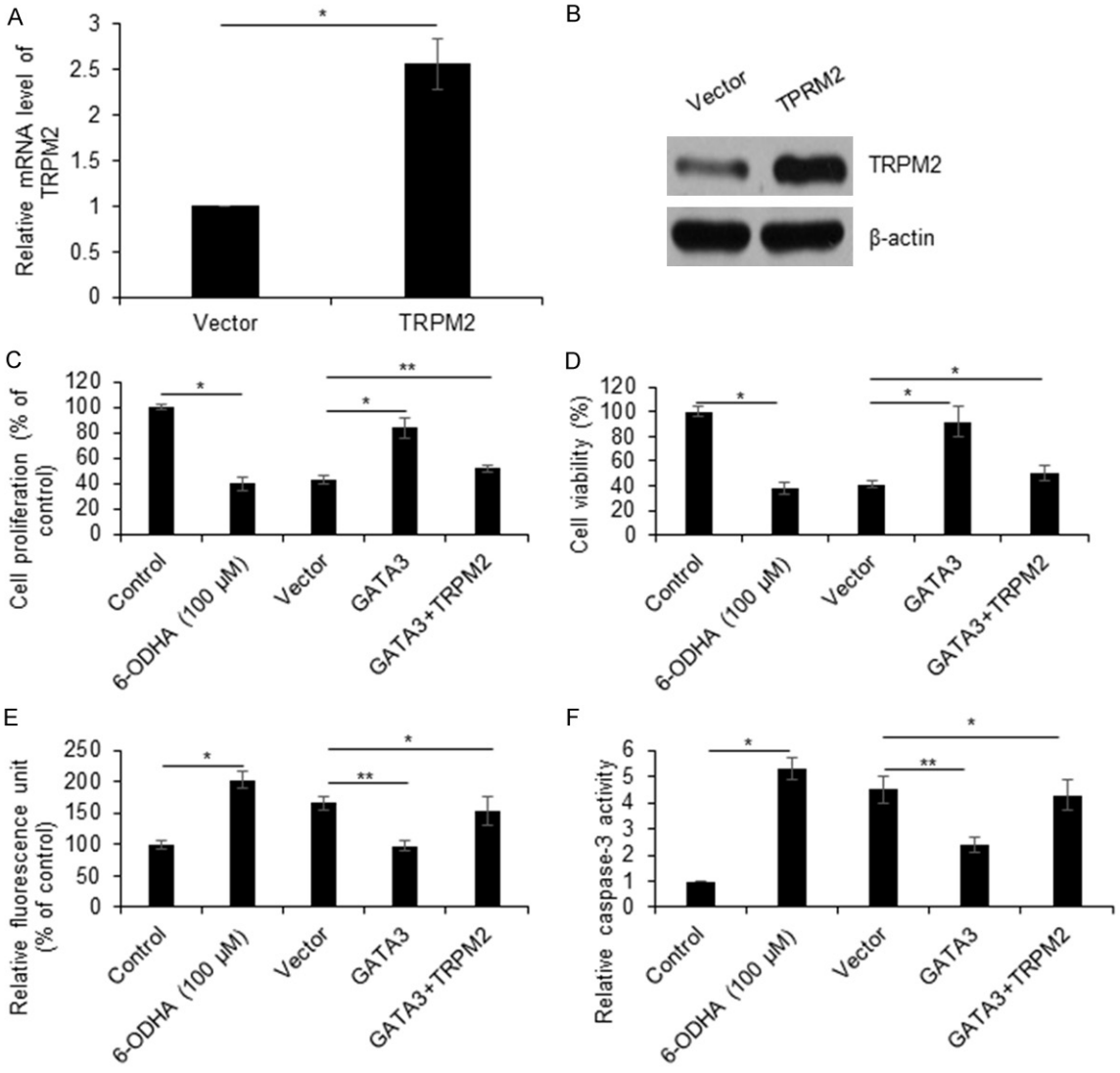

Figure 5. GATA3 mediates PC12 cell proliferation and survival by targeting TRPM2. A: Over-expressed TRPM in PC12 cells, after transcription 48 h, the mRNA level of TRPM2 was assessed by qRT-PCR. *P<0.05. B: Over-expressed TRPM in PC12 cells, after transcription 48 h, the protein level of TRPM2 was assessed by western blotting. C: After transfection 48 h, 100 μM 6-OHDA was added in cells for 24 h. CCK-8 assay was used to measured PC12 cells proliferation. Each experiment was repeated at least three times. All data were presented as mean ± SD. *p<0.05, **p<0.01. D: After transfection 48 h, 100 μM 6-OHDA was added in cells for 24 h. MTT assay was used to measured PC12 cells viability. Each experiment was repeated at least three times. All data were presented as mean ± SD. *P<0.05. E: After transfection 48 h, 100 μM 6-OHDA was added in cells for 24 h. The fluorescent probe CM-H2DCFDA was used to detect ROS production in PC12 and SH-SY5Y cells. Each experiment was repeated at least three times. All data were presented as mean ± SD. *P<0.05, **P<0.01. F: After transfection 48 h, 100 μM 6-OHDA was added in cells for 24 h. The caspase-3 activity kit was used to measured caspase-3 activity in PC12 and SH-SY5Y cells. Each experiment was repeated at least three times. All data were presented as mean ± SD. *P<0.05, **P<0.01.

up-regulated the GATA3-induced caspase-3 activity (**Figure 5F**).

Discussion

Parkinson's disease (PD) is a complex neurodegenerative disorder. The dopaminergic neurons was reported to control numerous brain functions, including voluntary movement, and the abnormal degeneration of dopaminergic neurons led to bradykinesia, postural instability, rigidity and resting tremors [4].

Recently, a report indicated that GATA factors played a key function in the pathogenesis of

PD. GATA2 regulated neuronal development through regulating expression of endogenous neuronal a-synuclein [33]. GATA3, as a transcription factor, binded conservative sequences which containing GATA motif [9-11]. Whether GATA3 also played crucial function in PD is unknown.

Here, our findings suggested that GATA3 was low expression in 6-OHDA-treated PC12 cells which similar to PD cells. Several functional experiments showed ectopic expression of GATA3 not only remarkably facilitated 6-OHDA-treated PC12 cell proliferation and survival, but also suppressed ROS production and cell apoptosis. However, GATA3 inhibition conversed these results. Moreover, we found TRPM2 was a downstream target of GATA3, GATA3 transcriptional inhibited TRPM2, and GATA3 mediated PC12 cell proliferation and survival by targeting TRPM2.

To sum up, our works demonstrated that ectopic expression of GATA3 inhibited TRPM2 expression and low expression of TRPM2 facilitated 6-OHDA-induced PC12 cell survival. We provided that GATA3 worked as a neuroprotective factor on 6-OHDA-treated PC12 cell. GATA3 might become a novel molecular therapy target in PD.

Disclosure of conflict of interest

None.

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