Wnt2 overexpression protects against PINK1 mutant-induced mitochondrial dysfunction and oxidative stress

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Abstract. The PTEN induced putative kinase 1 (*PINK1*) mutation is the second most common cause of autosomal recessive adolescent Parkinson's disease (PD). Furthermore, mitochondrial disorders and oxidative stress are important mechanisms in the pathogenesis of PD. Numerous members of the *Wnt* family have been found to be associated with neurodegenerative diseases. Therefore, the present study investigated the role of the *Wnt2* gene in *PINK1*^{B9} transgenic flies, which is a PD model, and its underlying mechanism. It was identified that overexpression of *Wnt2* reduced the abnormality rate of PD transgenic *Drosophila* and improved their flight ability, while other intervention groups had no significant effect. Furthermore, an increase in ATP concentration normalized mitochondrial morphology, and increased the mRNA expression levels of NADH-ubiquinone oxidoreductase chain 1 (*ND1*), *ND42*, *ND75*, succinate dehydrogenase complex subunits B, Cytochrome b and Cyclooxygenase 1, which are associated with *Wnt2* overexpression. Moreover, overexpression of *Wnt2* in PD transgenic *Drosophila* resulted in the downregulation of reactive oxygen species and malondialdehyde production, and increased manganese superoxide dismutase (MnSOD), while glutathione was not significantly affected. It was found that overexpression of *Wnt2* did not alter the protein expression of β-catenin in *PINK1*^{B9} transgenic *Drosophila*, but did increase the expression levels of PPARG coactivator 1α (*PGC-1α*) and forkhead box sub-group O (*FOXO*). Collectively, the present results indicated that the *Wnt2* gene may have a protective effect on PD *PINK1*^{B9} transgenic *Drosophila*. Thus, it was speculated that the reduction of oxidative stress and the restoration of mitochondrial function via *Wnt2* overexpression may be related to the PGC-1α/FOXO/MnSOD signaling pathway in *PINK1* mutant transgenic *Drosophila*.

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Introduction

PTEN-induced kinase 1 (*PINK1*) is located in the mitochondrial membrane, and helps to regulate mitochondrial morphology and autophagy (1). Mutations in *PINK1* have been recognized to be the second most common cause of autosomal recessive adolescent Parkinson's disease (PD) (2). Loss of *Drosophila PINK1* leads to mitochondrial morphological disorders and mitochondrial complex function damage (3). Furthermore, neurons are dependent on mitochondria, which act as the major energy producers (4). Therefore, when *PINK1* is mutated, neurotoxicity is increased and this may be associated with mitochondrial defects, thus contributing to the loss of dopaminergic (DA) neurons (5). Oxidative stress is a prominent and common feature of all forms of PD, and may have a toxic effect that causes neuronal cell death (6). Moreover, oxidative stress in PD is closely associated with a series of pathogenic factors, including mitochondrial dysfunction, DA metabolism and metal ion dysregulation (7).

The human *Wnt* gene family, known as the wingless-type MMTV integration site family, consists of 19 members, and the interaction between *Wnt1* and *Wnt5a* promotes the development of DA neurons in the midbrain (8). Moreover, the *Wnt2* gene is one of the 19 *Wnt* family members that is highly expressed in the human thalamus, and plays an important role in the late development of human genes and the brain (9,10). A previous functional study has shown that *Wnt2* promotes the migration of primitive neurons and increases the number of

DA neurons (11), thus enhancing DA function in the midbrain, and affecting mRNA and protein expression levels. The Wnt signaling pathway can be cross-linked with a number of signaling pathways (12). The interaction of β-catenin with the forkhead box sub-group O (FOXO) signaling pathway can inhibit Huntington protein toxicity (13). Furthermore, the Wnt signaling pathway also regulates mitochondrial energy, metabolism and oxidative stress (14,15).

The present study selected *Drosophila* as the experiment model, as *Drosophila* genes have been fully sequenced and annotated, and are highly homologous to human genes (16,17). Compared with other models, the *Drosophila* life cycle is very short; therefore, it is easier to observe the whole process of disease development (18). Mature genetic systems, abundant strain resources and powerful genome editing techniques also make *Drosophila* one of the primary choices for genetic research in neurodegenerative diseases (19).

The present study identified that overexpression of *Wnt2* gene had a significant effect on *PINK1*^{B9} transgenic *Drosophila*. While the Wnt pathway may have neuroprotective effects, the role of *Wnt2* in neurodegenerative diseases remains unknown. Therefore, the aims of the present study were to investigate the function of *Wnt2* in *PINK1* mutant transgenic *Drosophila*, and to identify its association with the Wnt/β-catenin and PPARG coactivator 1α (PGC-1α)/FOXO/manganese superoxide dismutase (MnSOD) signaling pathways.

Materials and methods

Drosophila stocks. A total of five fly stocks were used: two stocks of *Drosophila melanogaster* (UAS-*Wnt2*OE and UAS-*Wnt2*RNAi) were purchased from the Bloomington *Drosophila* Stock Center. In total, three stocks (UAS-*PINK1*^{B9}/FM7; MHC-Gal4, W1118 and MHC-GAL4) were provided by Institute of Life Sciences of Fuzhou University. W1118 is a wild-type genotype. UAS-*Wnt2*OE is a stock that overexpresses the *Wnt2* gene. UAS-*Wnt2*RNAi is a *Drosophila* that has lost the function of the *Wnt2* gene by using RNA interference technology. MHC-GAL4 is a *Drosophila* with an indirect flying muscle promoter. UAS-*PINK1*^{B9}/FM7; MHC-Gal4 is a PD *Drosophila* model, in which the *PINK1* mutation gene can be specifically expressed in indirect flying muscles. The classic GAL4/UAS system is divided into two parts: GAL4 and UAS. The GAL4 stock and UAS stock are two independent stocks (20). The fusion of GAL4 and tissue-specific promoter can regulate the expression of GAL4 protein in different tissues of *Drosophila* (21). Furthermore, UAS and target genes are fused to construct a transgenic line with a UAS-target gene (22). Only when the two hybridize, can GAL4 recognize the UAS promoter and induce expression of UAS downstream genes in specific tissues (23). *Drosophila* were placed in *Drosophila* culture tubes containing corn medium and cultured at a constant temperature of 25˚C and 60% relative humidity.

Drosophila construction. The flies were driven via the muscular driver MHC-GAL4. MHC-GAL4 virgin flies were crossed with W1118 male flies, and the F1 generation genes were W1118/+; MHC-GAL4/+, which served as the control group. In the *PINK1*^{B9} disease group, UAS-*PINK1*^{B9}/FM7; MHC-GAL4 virgin flies were crossed with W1118 male flies, which produced the F1 generation with a genotype of UAS-*PINK1*^{B9}/+; MHC-GAL4/+. In the overexpression (OE) intervention group, UAS-*PINK1*^{B9}/FM7; MHC-GAL4 virgin flies were hybridized with male flies of UAS-*Wnt2*OE, and the F1 generation genotype was UAS-*PINK1*^{B9}/y; MHC-GAL4/*Wnt2* OE, UAS-*PINK1*^{B9}/y. In the RNA interference (RNAi) intervention group, UAS-*PINK1*^{B9}/ FM7; MHC-GAL4 virgin flies were crossed with males of UAS-*Wnt2* RNAi *Drosophila*, and the F1 generation obtained had the genotype UAS-*PINK1*^{B9}/y; MHC-GAL4/*Wnt2*RNAi.

Morphological observation of Drosophila (24). Flies carrying the MHC-GAL4/UAS systems were grouped as follows: Normal control group (W1118), disease control group (*PINK1*^{B9}), *Wnt2*OE intervention group (*PINK1*^{B9}; *Wnt2*OE) and Wnt2 knockdown intervention group (*PINK1*^{B9}; *Wnt2* RNAi). On day 5, ~100 male flies were selected from each group. After being anesthetized by CO_2, the 100 flies were divided into transparent glass tubes with five flies per tube. After the flies completely woke up (after ~1 h), the shape of their wings and whether they could fly were observed. The number and the ratio of abnormal wings and flying were calculated. All assays were performed in triplicate and independently repeated three times.

Drosophila mRNA expression detection. The experimental groups were the same as aforementioned. On day 5, the head and abdomen were removed, and the chest was kept from 30 male flies from each group. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract the total RNA from the chest, according to the manufacturer's protocol. The primers were synthesized by Sangon Biotech Co., Ltd. and are presented in Table I. Subsequently, RNA samples were reverse transcribed into cDNA using PrimeScript™ reverse transcription (RT) reagent kit with gDNA Eraser (Takara Bio, Inc.). Following the manufacturer's instructions, this reaction was a two-step process. The first step was to remove the genomic DNA, adding 1 µl gDNA eraser and 2 µl gDNA eraser buffer to the RNA sample, which was then incubated at 42˚C for 2 min. The second step was to synthesize cDNA by adding the 1 µl enzyme, 1 µl primer, 4 µl buffer and 4 µl RNase-free H_2O to the 10 µl gDNA eraser-treated sample. The reaction temperature was 37˚C for 15 min and 85˚C for 5 sec. Quantitative PCR (qPCR) was conducted using an Applied Biosystems ABI 7500 system (Thermo Fisher Scientific, Inc.) using Power SYBR® Green PCR Master mix (Thermo Fisher Scientific, Inc.). The RT PCR conditions were as follows: Initial denaturation at 50˚C for 20 sec and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec; annealing at 60˚C for 1 min and extension at 72˚C for 1 min. Moreover, 18S served as an endogenous control for data normalization. The $2^{-\Delta\Delta Cq}$ method (25) was used to analyze the relative expression.

Western blotting for detection of protein expression. *Drosophila* grouping was the same as aforementioned. The the chest tissue of 20 *Drosophila* was cut on ice. Fresh tissues were lysed with 200 µl ice-cold RIPA buffer (Solarbio Science & Technology Co., Ltd.) containing 1 mM phenylmethylsulfonyl

Table I. Primer sequences used in the reverse transcription-quantitative PCR assay.

Gene	Primer sequence (5'→3')
18S	Forward: TCTAGCAATATGAGATTGAGCAATAAG
	Reverse: AATACACGTTGATACTTTCATTGTAGC
ND1	Forward: GTTATAGTAGCTGGTTGGTCGTC
	Reverse: AAGGAGTCCGATTAGTTTCAGC
ND42	Forward: CAAGAAGATGCTCGACTGGC
	Reverse: TGTCTGCATTGTAGCCAGGA
ND75	Forward: AAGCTCTTCCTTACCGAACTG
	Reverse: ATCGATGCTGCTCACCTTAC
sdhB	Forward: CCATCGCCGAGATCAAGAAG
	Reverse: GGGACGAACTGGGAGTAGA
cytb	Forward: GGATACGTATTACCTTGAGGACAAA
	Reverse: CAACAGCAAATCCACCTCATAATC
COX1	Forward: TGGTGGATTTGGAAATTGATTAGTG
	Reverse: GTAAAGAAAGAGCAGGAGGTAGAA
PGC-1α	Forward: AAGACGTGCCTTCTGTCGTTCATC
	Reverse: ATTCGGTGCTGGTGCTTCCTTG
FOXO	Forward: CTCATCCAATGCCAGTTCCT
	Reverse: TCATCGTTGTGTTCTGGTAGTC

ND, NADH-ubiquinone oxidoreductase chain 1; sdhB, succinate dehydrogenase complex subunits B; cytb, Cytochrome b; COX1, Cyclooxygenase 1; PGC-1α, PPARG coactivator 1α; FOXO, forkhead box sub-group O.

fluoride (Beijing Solarbio Science & Technology Co., Ltd.). Subsequently, tissues were ground to a homogenate, followed by centrifugation at 14,000 x g for 15 min at 4˚C. Then, 120 μl supernatant liquid was collected and added to 40 μl 4Xloading buffer (Beijing Solarbio Science & Technology Co., Ltd.), mixed and boiled at 100˚C for 10 min. The samples were stored at -20˚C prior to further experiments.

For analytical SDS-PAGE, 10 μl protein was loaded per lane to 5% concentrated gel (30% acrylamide, 10% SDS; 10% APS; 1 M Tris-HCl pH 6.8; TEMED) and dissociated in 10% separation gel (30% acrylamide; 10% SDS; 10% APS; 1.5 M Tris-HCl pH 8.8; TEMED). The proteins were then transferred to 0.2-μm PVDF membranes (Beijing Solarbio Science and Technology Co., Ltd.), blocked in 5% milk for 2 h at room temperature and washed in TBS-T (TBS, 3 M NaCl, 200M Tris; pH 7.5, containing 0.1% Tween-20). Subsequently, overnight incubation at 4˚C was performed with the following primary antibodies: Rabbit anti-MnSOD (1:1,000; cat. no. ab13534; Abcam; polyclonal), rabbit anti-α tubulin (1:1,000; cat. no. ab52866; Abcam; monoclonal) and mouse anti-β-catenin (1:1,000; cat. no. AB_528089; Developmental Studies Hybridoma Bank; monoclonal). All the primary antibodies were *Drosophila*-specific. Following washing with TBS-T, the membrane was incubated with the corresponding secondary antibody, horseradish peroxidase-AffiniPure Goat Anti-Rabbit/Mouse IgG (H+L; 1:5,000; cat. nos. EM35111-01 and EM35110-01; Emarbio Science and Technology Co., Ltd.), for 1 h at room temperature. All the resulting immune complexes were visualized with chemiluminescence reagent (Thermo Fisher Scientific, Inc.,), followed by imaging using Image Lab 5.1 (National Institutes of Health). The target protein bands were quantified by scanning densitometry using ImageJ software (v.1.49v; National Institutes of Health).

ATP, malondialdehyde (MDA) and reactive oxygen species (ROS). In total, ten *Drosophila* thoraxes were cut on ice from each group, and 1,000 μl lysate was added to homogenize the chest tissue on ice. Samples were then heated at 100˚C for 2 min and centrifuged at 12,000 x g for 5 min at 4˚C. The thoracic ATP level was measured using a luciferase-based bioluminescence assay (cat. no. S0027; Beyotime Institute of Biotechnology). Then, 40 fly chests from each group were ground by adding 500 μl 1X PBS and centrifuge at 1,425 x g for 10 min at 4˚C. The supernatant was collected and the MDA content was measured by the thiobarbituric acid method (26). Analyses were performed according to the instructions of the reagent kit for MDA (cat. no. A003-1-2; Nanjing Jiancheng Bioengineering Institute). ROS were measured using the CellROX Orange reagent (cat. no. BB-470512; Shanghai Bio-Tech Co., Ltd.). The thoraxes of 30 *Drosophila* were obtained, homogenized, centrifuged at 1,000 x g for 10 min at 4˚C and the supernatant was collected. The supernatant and 20 μM CellROX Orange Reagent were mixed and incubated at 37˚C in the dark for 30 min, and the fluorescence intensity at 510 and 610 nm (maximum excitation light and maximum emission wavelength) was measured using a multi-function microplate reader.

Transmission electron microscopy analysis. Drosophila was grouped as aforementioned, and 10 male flies from each group F1 generation were randomly selected on day 5. Flies were anesthetized by CO_2, and the chest was cut carefully so as not to damage the muscle tissue. Thoraxes were fixed overnight at 4˚C in 2.5% glutaraldehyde, washed several times with 0.1 mol/l phosphate buffer and post-fixed in 1% osmium-tetroxide in distilled water for 2 h at room temperature. The samples were dehydrated in a 50, 70 and 90% graded ethanol series, and embedded in Epoxy resin for 48 h at room temperature. The polymerization conditions in the polymerization tank were 36˚C for 24 h, 45˚C for 12 h and 65˚C for 48 h.

The embedded polymer samples were cut into 1 μm sections using a Leica UC7 ultrathin slicer, stained with 1% toluidine blue for 30 sec at room temperature and observed under an optical microscope (magnification, x100). The samples were then cut into ultra-thin sheets (70 nm), stained with 3% uranyl acetate for 15 min at room temperature and 3% lead citrate for 15 min at room temperature. Sections were observed with a Hitachi H-7650 transmission electron microscope (magnification, x30,000).

Statistical analysis. Statistical analysis of data was performed using SPSS 16.0 (IBM Corp.). Normally distribution continuous variable data were compared by one-way ANOVA followed by Bonferroni's post hoc test. Data are presented as the mean ± SD. $P<0.05$ was considered to indicate a statistically significant difference.

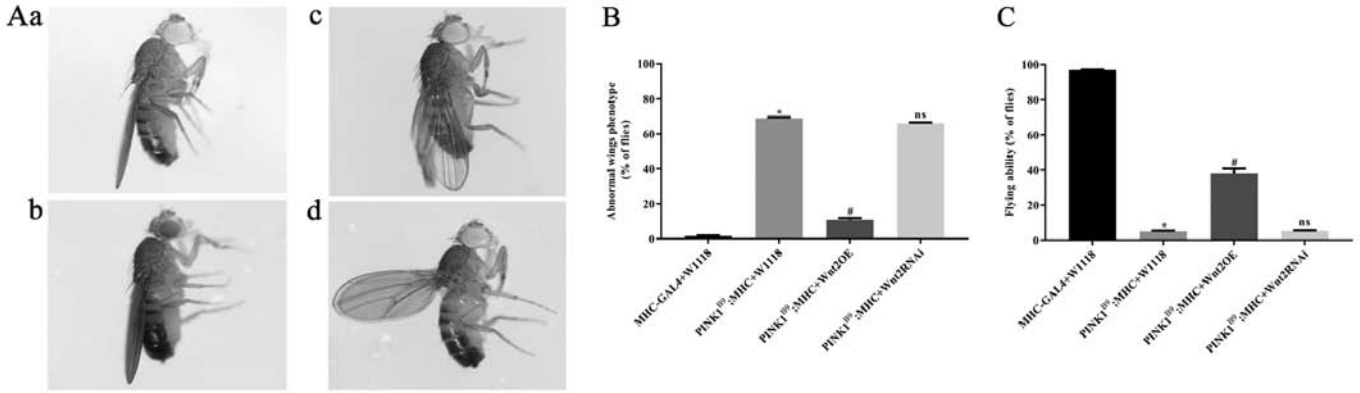

Figure 1. *Wnt2* overexpression suppresses *PINK1*^{B9} mutant phenotypes. (A) Side view of *Drosophila* wings. (Aa) Control flies, (Ab) *PINK1*^{B9} flies, (Ac) *Wnt2*OE flies and (Ad) *Wnt2*RNAi flies. Both (B) wing phenotype and (C) flying ability were abnormal in *PINK1*^{B9} flies. Abnormal wing phenotype and flying rate was restored in *Wnt2*OE flies compared with in *PINK1*^{B9} flies. n=20, 5-day-old males. *P<0.05 vs. the control flies. $^{\#}$P<0.05 vs. the *PINK1*^{B9} flies. ns vs. the *PINK1*^{B9} flies. PINK1, PTEN induced putative kinase 1; *Wnt2*OE, Wnt2 overexpression; *Wnt2*RNAi, *Wnt2* RNA interference; ns, not significant.

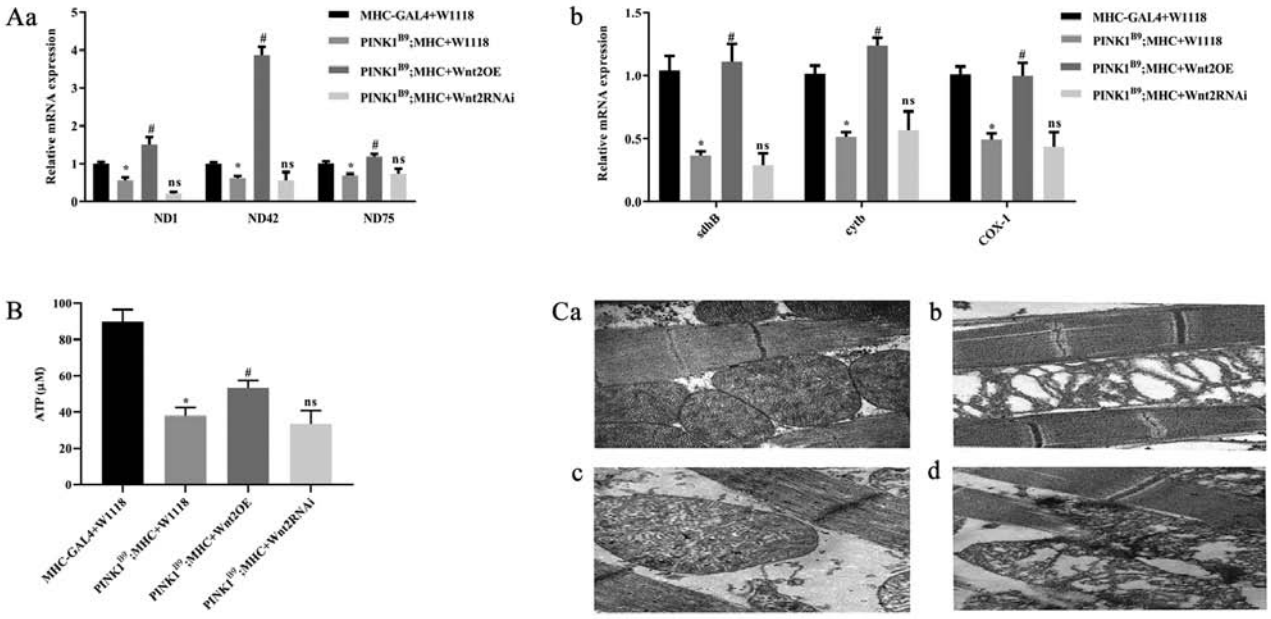

Figure 2. Overexpression of *Wnt2* rescues mitochondrial function. (A) Overexpression of *Wnt2* increased mitochondrial complex subunit-related genes. (Aa) mRNA level of mitochondrial complex subunit ND1, ND42 and ND75, (Ab) mRNA level of mitochondrial complex subunit sdhB, cytb and COX-1. (B) ATP levels in flies. (C) Abnormal mitochondrial morphology of *PINK1*^{B9} flies was restored by *Wnt2* overexpression. (Ca) Control flies, (Cb) PINK1B9 flies, (Cc) *Wnt2*OE flies and (Cd) *Wnt2*RNAi flies. Magnification, x30,000. Scale bar, 1 μm. n=30, 5-day-old males. *P<0.05 vs. the control flies. $^{\#}$P<0.05 vs. the *PINK1*^{B9} flies. ns vs. the *PINK1*^{B9} flies. PINK1, PTEN induced putative kinase 1; *Wnt2*OE, *Wnt2* overexpression; *Wnt2*RNAi, *Wnt2* RNA interference; ns, not significant; ND, NADH-ubiquinone oxidoreductase chain 1; sdhB, succinate dehydrogenase complex subunits B; cytb, Cytochrome b; COX1, Cyclooxygenase 1.

Results

Wnt2 overexpression rescues the abnormal phenotype caused by PINK1^{B9} *mutation*. The *Wnt2* gene was specifically expressed in *Drosophila* flight muscles using the MHC-GAL4 promoter. The phenotypic changes of *Drosophila* were demonstrated by changes in wing morphology and flight ability. In the normal control group, most of the wings completely overlapped and were parallel to the body (Fig. 1A). However, in the transgenic disease model of *PINK1*^{B9} the wings had bifurcations, erections and sagged. Furthermore, compared with the low rate of abnormal wings in the control group, the disease group had a significantly higher rate of abnormal wings (Fig. 1B) and a significant decrease in flight ability (Fig. 1C). In the *Wnt2*OE intervention group, the incidence of wing anomalies was significantly reduced and the flight capabilities were improved, compared with the disease group. Moreover, there were no significant differences between the *Wnt2* RNAi intervention group and the PD disease model group. Therefore, the present results suggested that *Wnt2*OE may have a protective effect on the phenotype of *PINK1*^{B9} transgenic *Drosophila*.

Wnt2 gene overexpression enhances mitochondrial function in PINK1^{B9} *transgenic Drosophila*. RT-qPCR results demonstrated that in *PINK1*^{B9} disease model group, the mRNA expression levels of the mitochondrial complex subunit-related genes, Complex I [NADH-ubiquinone oxidoreductase chain 1 (*ND1*), *ND42* and *ND75*], Complex II (succinate dehydro-

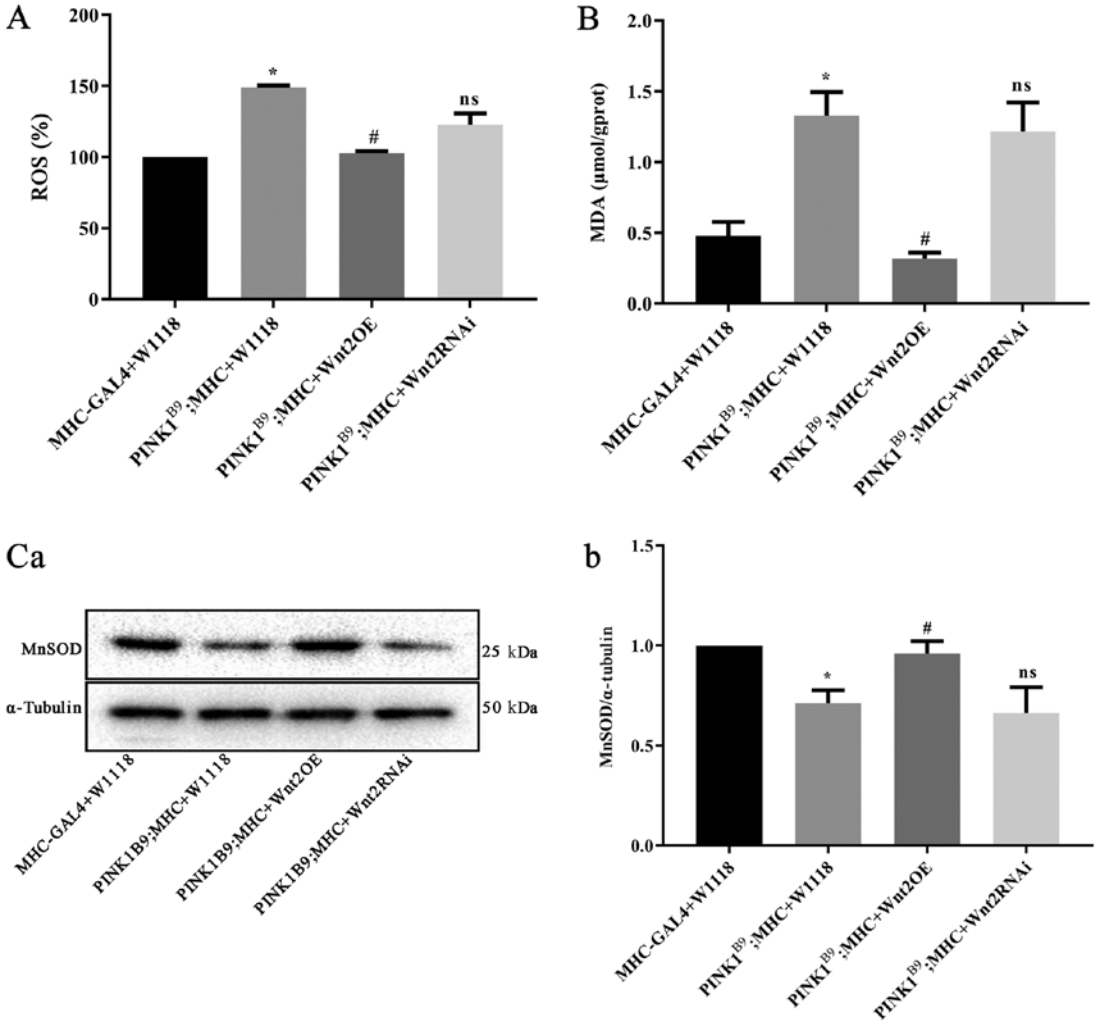

Figure 3. *Wnt2* overexpression alleviates oxidative stress in *PINK1*^{B9} flies. (A) ROS levels. (B) MDA levels. (C) Protein expression levels of MnSOD were quantified by western blotting. (Ca) Western blot analysis of MnSOD protein, (Cb) Relative quantitative analysis of MnSOD protein. n=40, 5-day-old males. *P<0.05 vs. the control flies. $^{\#}$P<0.05 vs. the *PINK1*^{B9} flies. ns vs. the *PINK1*^{B9} flies. ROS, reactive oxygen species; MDA, malondialdehyde; MnSOD, manganese superoxide dismutase; PINK1, PTEN induced putative kinase 1; *Wnt2*OE, *Wnt2* overexpression; *Wnt2*RNAi, *Wnt2* RNA interference; ns, not significant.

genase complex subunits B), Complex III (Cytochrome b) and Complex IV (Cyclooxygenase 1), decreased significantly. While *Wnt2*OE intervention in *PINK1*^{B9} transgenic *Drosophila* increased the mRNA expression levels of these related genes (P<0.05), in the *Wnt2* RNAi intervention group there was no significant difference compared with the disease model group (Fig. 2A). The results of the ATP assay demonstrated that ATP production in the PD model group was decreased. Furthermore, the amount of ATP produced by mitochondria in the *Wnt2*OE intervention group was ~1.5 times higher compared with the disease model group (Fig. 2B). Ultrastructural transmission electron microscopy analysis identified that mitochondria were disrupted in *PINK1*^{B9} transgenic *Drosophila,* and mitochondrial morphology was not recognizable. Moreover, *Wnt2*OE could rescue mitochondrial defects in *PINK1*^{B9} flies (Fig. 2C). Therefore, the present results suggested that overexpression of *Wnt2* can improve the mitochondrial function of *PINK1*^{B9} transgenic *Drosophila*.

Wnt2OE reduces oxidative stress damage in PINK1^{B9} *transgenic Drosophila*. ROS production in the *PINK1*^{B9} disease model group was significantly higher compared with the normal control group (P<0.05; Fig. 3A). Furthermore, following *Wnt2*OE intervention in *PINK*^{B9} transgenic *Drosophila*, ROS production was significantly reduced (P<0.05) and almost returned to normal levels. MDA, a commonly used indicator of lipid peroxidation injury (27), was significantly increased in the *PINK1*^{B9} disease model group compared with the normal control (P<0.05; Fig. 3B). Moreover, after *Wnt2*OE intervention, MDA production was reduced (P<0.05). It was demonstrated that the content of ROS and MDA were not significantly different between the *Wnt2*RNAi intervention groups and the disease model group (Fig. 3A and B).

Western blot analysis results revealed that the protein expression of MnSOD in the PINK1^{B9} disease model group was significantly lower compared with the normal control group (P<0.05; Fig. 3C). However, the expression of MnSOD was significantly increased (P<0.05) following *Wnt2*OE intervention in the *PINK1*^{B9} disease model. Collectively, the present results indicated that *Wnt2* overexpression reduced oxidative damage in *PINK1*^{B9} transgenic *Drosophila*.

Possible mechanism of Wnt2 overexpression-mediated protection. Western blot analysis results revealed that there was no

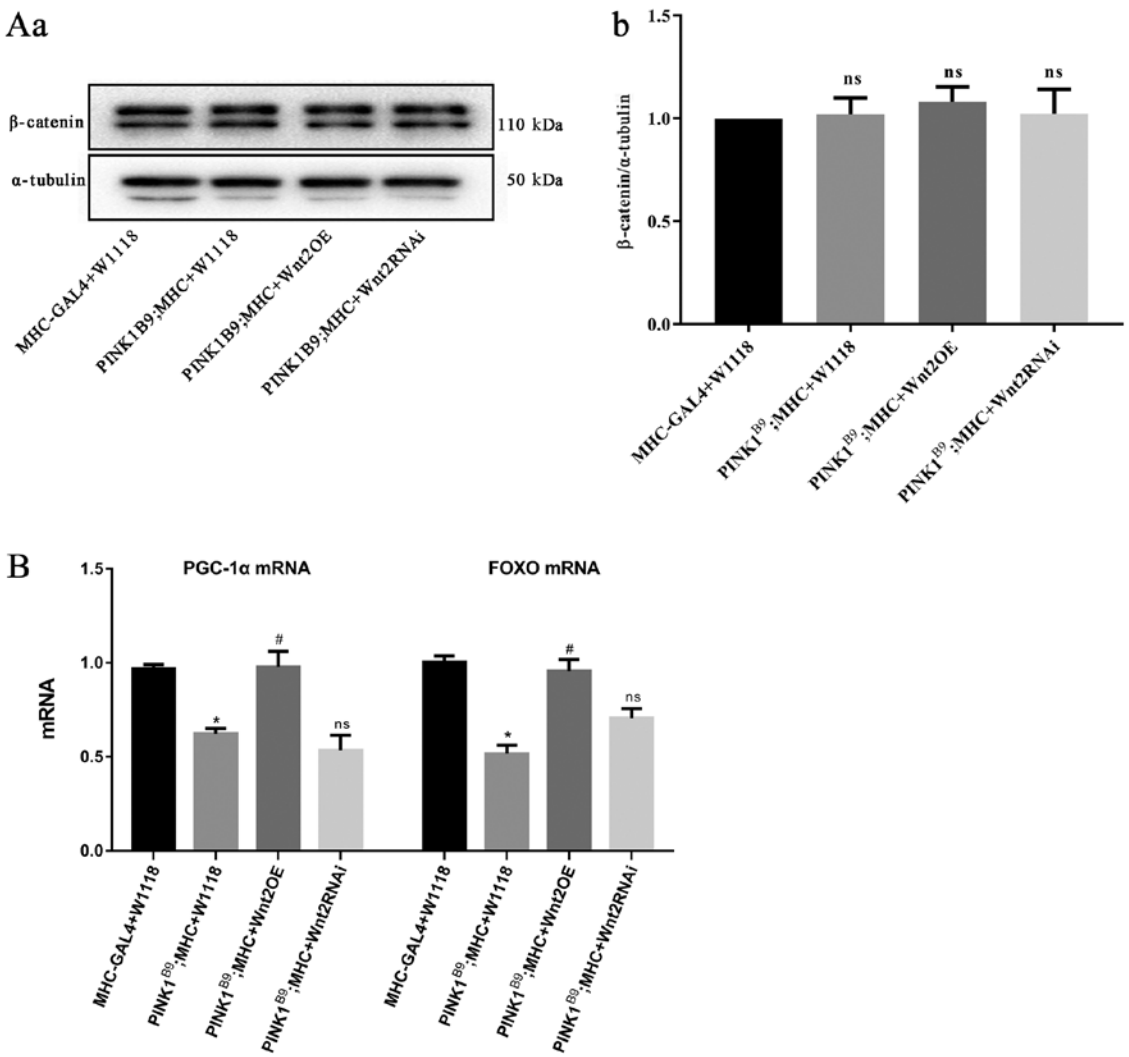

Figure 4. *Wnt2* overexpression activates the FOXO/PGC-1α pathway to protect *PINK1*^{B9} flies. (A) Protein expression of β-catenin was quantified by western blotting. (Aa) Western blot analysis of β-catenin protein, (Ab) Relative quantitative analysis of β-catenin protein. (B) mRNA expression levels of PGC-1α and FOXO were detected by reverse transcription-quantitative PCR. n=30, 5-day-old males. *P<0.05 vs. the control flies. #P<0.05 vs. the *PINK1*^{B9} flies. ns vs. the *PINK1*^{B9} flies. PINK1, PTEN induced putative kinase 1; *Wnt2*OE, *Wnt2* overexpression; *Wnt2*RNAi, *Wnt2* RNA interference; PGC-1α, PPARG coactivator 1α; FOXO, forkhead box sub-group O; ns, not significant.

significant difference in the protein expression of β-catenin, a key molecule of the classic Wnt/β-catenin signaling pathway (28), between each group (Fig. 4A). Moreover, RT-qPCR showed that the mRNA expression levels of *FOXO* and *PGC-1α* were decreased in the *PINK1*^{B9} disease model group, and were increased following *Wnt2*OE intervention in the *PINK1*^{B9} disease model (Fig. 4B). Therefore, it can be speculated that *Wnt2* may activate the FOXO/PGC-1α signaling pathway to regulate mitochondrial function and inhibit anti-oxidative stress-induced damage.

Discussion

In *Drosophila melanogaster*, mutations in *PINK1* causes phenotypic abnormalities and decrease exercise capacity, resulting in dysfunction of mitochondria and decreased ATP levels, which leads to selective degeneration of DA neurons and sensitivity to stress (16,17). In the present study, the disease model demonstrated a pathological phenotype that was consistent with previous experimental results (24,29), including a high abnormal wing rate, low flight rate, lower mRNA expression of mitochondrial complex subunits and lower ATP level, which suggested that *PINK1* mutations could cause serious damage in mitochondria. Furthermore, overexpression of *Wnt2* gene rescued the phenotype of *PINK1*^{B9} transgenic *Drosophila*, increased ATP production level and enhanced the expression of the mitochondrial biosynthesis-related gene *PGC-1α*. PGC-1α is a transcriptional cofactor for numerous mitochondrial proteins, which can regulate mitochondrial function to meet cellular needs and protect from oxidative damage (30). Therefore, it is speculated that *Wnt2*OE inhibited mitochondrial dysfunction caused by the *PINK1* mutation and eventually protects *PINK1*^{B9} transgenic fruit flies.

When mitochondria are damaged, particularly damage to the mitochondrial respiratory chain complex, electron chain leakage may occur and cause the production of superoxide and hydrogen peroxide (31). These products, not only participate in the damage of DNA, proteins and lipids, but also pose a serious threat to cells and tissues (31). Mitochondrial dysfunction and oxidative stress play key roles in the development of

PD, as both lead to excessive ROS production (32). This in turn leads to damage and death of DA neurons (33). A small level of ROS is essential for normal physiological functions; however, the accumulation of large amounts of ROS further destroys mitochondria and exacerbates oxidative stress (34). It has been shown that maintenance of ATP and inhibition of ROS production protects DA neurons (35). Reactive oxygen damages polyunsaturated lipids and forms MDA, which is a marker of lipid damage in oxidative stress (36). Furthermore, the present results indicated that the *PINK1* mutation increased ROS and MDA levels, but reduced MnSOD protein expression.

MnSOD, which is mainly distributed in the mitochondrial matrix, is an important scavenger for the superoxide anion that is produced during mitochondrial oxidative phosphorylation (37). Previous findings have shown that overexpression of *MnSOD* serves an important role in the protection of *PINK1*-mutant PD *Drosophila* (38). In the *PINK1* mutants, the present study identified increased ROS and MDA production, and a reduced MnSOD, thus suggesting that the *PINK1* mutation leads to oxidative stress damage. Moreover, overexpression of *Wnt2* improved mitochondrial function, which also partially reduced ROS production. However, *Wnt2* overexpression also appeared to directly participate in the regulation of oxidative stress levels in $PINK1^{B9}$ transgenic *Drosophila*, as it not only reduced ROS and MDA production levels, but also increased the protein expression levels of MnSOD.

The *Drosophila* gene *Wnt2*, homologous to the human gene *Wnt7a*, is involved in the Wnt/β-catenin signaling pathway (39). However, it remains unknown whether the protective effect of *Wnt2* overexpression on $PINK1^{B9}$ transgenic *Drosophila* is related to the Wnt/β-catenin signaling pathway. The present results indicated that *Wnt2* overexpression did not increase the protein expression of β-catenin, a key protein of the Wnt/β-catenin signaling pathway (28), which suggested that it did not protect $PINK1^{B9}$ transgenic flies via the Wnt/β-catenin signaling pathway. Furthermore, a previous study found that *Wnt2* does not act via the Wnt/β-catenin pathway, but activates the non-canonical pathway (40). Thus, this raises the question of how *Wnt2* improves mitochondrial function, reduces oxidative damage and enhances antioxidant capacity. The present study evaluated the expression of *FOXO*, a gene associated with mitochondrial oxidative stress, which regulates the expression levels of *PGC-1α* and *MnSOD* (38,41). Under conditions of mitochondrial dysfunction and oxidative stress caused by *PINK1* mutation, the present study hypothesized that the overexpression of *Wnt2* directly regulates the expression of PGC-1α/FOXO/MnSOD, improves mitochondrial function and improves antioxidant capacity to rescue $PINK1^{B9}$ transgenic fruit flies.

The *FOXO* family members are key regulators of neuronal processes, such as dendritic structural function and memory consolidation (42,43). *FOXO* is also an important regulator of cellular stress response, which enhances cellular antioxidant defenses (44). A previous study revealed that the expression of genes, such as *MnSOD*, could be controlled by the forkhead transcription factor *FOXO3a* (44). Moreover, *PGC-1α* has been shown to regulate *FOXO* activity in different systems. For example, it has been reported that *PGC-1α* is a positive regulator of fasting-induced hepatic gluconeogenesis, which is mediated by its interaction with *FOXO1a* (45). Similarly, overexpression of *PGC-1α* enhances the stimulatory effect of *FOXO1a* on selenoprotein P promoter activity and insulin attenuation (46). *PGC-1α* has also been shown to interact with *FOXO3a*, which regulates antioxidant gene expression in endothelial cells and skeletal muscle (47). In addition, upregulation of *PGC-1α* and *FOXO3a* protects against oxidative stress injury induced by a high-fat diet and inhibits adipocyte apoptosis (47,48). The Wnt signaling pathway is also involved in the regulation of mitochondrial energy metabolism and oxidative stress (49). When ROS levels exceed the body's ability to scavenge, *Wnt* and *β-catenin* interact with *FOXO* under stimuli of oxidative stress (13,50). Furthermore, *FOXO1* interacts with *PGC-1α* in different systems (45,46). PGC-1α is a mitochondrial energy-metabolizing enzyme. When ROS are in excess, the human body can enhance the detoxification ability of mitochondrial ROS by increasing the expression levels of *FOXO1* and *PGC-1α* to promote the expression of downstream antioxidant systems, such as *MnSOD* (49).

To the best of our knowledge, the condition of mitochondrial dysfunction caused by *PINK1* mutation and oxidative stress remains to be determined. Although the Wnt signaling pathway is linked to the FOXO signaling pathway via β-catenin (50), the present results suggested that *Wnt2* did not change the β-catenin protein expression level, but did affect the mRNA expression levels of *PGC-1α* and *FOXO*, and the expression levels of their target protein MnSOD. Based on these results, it can be speculated that *Wnt2* may be directly involved in the PGC-1α/FOXO/MnSOD signaling pathway; however, the specific mechanism remains to be investigated. Moreover, the regulation of signaling pathways is complex and there will be cross-effects between the pathways (47-48), with both upregulation and inhibition of the expression of related factors leading to cascade reactions. Therefore, it will be beneficial to examine how the Wnt2 pathway interacts with or influences the PGC-1α/FOXO/MnSOD pathway in future experiments.

The present study demonstrated that *Wnt2* overexpression protected $PINK1^{B9}$ transgenic flies by improving flight muscle and mitochondrial morphology, and enhancing mitochondrial complex I and II function. Furthermore, it was found that *Wnt2* overexpression exhibited a protective effect on early mitochondria and oxidative stress-related PD by improving mitochondrial function and reducing oxidative stress damage. However, the present study does have some limitations. First, the model is monotonous and limited to fruit flies, and thus requires further examination in higher animal models such as mice. Secondly, further research into the underlying pathways and mechanisms is required, such as whether it is the experimental effects that are related to the non-classical Wnt signaling pathway. Furthermore, how *Wnt2* cross-links with the PGC-1α/FOXO/MnSOD signaling pathway is not fully understood. In the *PINK1* mutant PD *Drosophila* model of mutation constructed by Park *et al* (24), mitochondrial dysfunction and oxidative stress injury are primarily exhibited in the early stage, while DA neuronal loss predominantly occurs in the middle and late stages of the *Drosophila*, which is after 25 days (24,50). This is consistent with the progressive DA neuronal loss observed in clinical patients with PD.

Thus, this may indicate that damage to DA neurons in PD is the result of further neurological damage caused by these pathogenic factors.

To the best of our knowledge, there are currently no treatments that mitigate disease progression or prevent neurodegeneration in all neurodegenerative disease. Therefore, it is necessary to develop interventions that are effective prior to severe damage of the neuronal in the early stages of PD. The present results indicated that *Wnt2* overexpression had a protective effect on early mitochondrial damage and oxidative stress in PD, improving mitochondrial function and reducing oxidative stress damage. Due to the limitations of the present study, the specific mechanism of action of *Wnt2* is not fully understood. However, the improvement of mitochondrial function and the reduction of oxidative stress damage can support the experimental results of the *Drosophila* model, providing a strong basis for future experiments.

In conclusion, the present results suggested that the *Wnt2* gene may have a protective effect on $PINK1^{B9}$ transgenic *Drosophila*. Therefore, it can be hypothesized that the reduction of oxidative stress and the restoration of mitochondrial function via *Wnt2* gene overexpression in the *PINK1* mutant transgenic *Drosophila* may be related to the PGC-1α/FOXO/MnSOD signaling pathway.

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Availability of data and materials

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

Authors' contributions

SRX was responsible for construction of the *Drosophila* models, western blotting, MDA and ROS level determination, and data analysis. XYW was responsible for construction of the *Drosophila* models, morphological observation of *Drosophila*, O2K detection and data statistics. SRX and XYW wrote the first draft of this article. XLF was responsible for electron microscopy analysis. XRC performed the ATP determination experiment. ZWW was responsible for mRNA expression level detection. QHL and LS undertook project funding, project design, manuscript revision and quality control. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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