STAT3-mediated ferroptosis is involved in α-synuclein pathology

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Research Article

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Abstract

Background

Oligomeric α-synuclein (α-syn) can activate microglia to drive the early stages of Parkinson's disease (PD) pathogenesis. Our previous studies have found a significant expression difference between the IncRNA IL6ST-AS and its antisense RNA IL6ST in the cerebrospinal fluid of PD patients. Furthermore, in α-syn-induced HMC3 cells, a decline in IL6ST and its downstream target JAK2/STAT3 were also observed. Accumulating investigations have illustrated that STAT3 regulates the expression of ferroptosis-related genes and further influences the proliferation of cells.

Methods

The role and mechanisms of IL6ST/JAK2/STAT3 axis in α-syn induced HMC3 cells and PD mouse models were explored by Western blot or immunohistochemistry. Transcriptome sequencing of HMC3 cells exposed to α-syn oligomers and PD mouse models were performed. The STAT3 activator and the STAT3 inhibitor were used to regulate the expression of STAT3. qPCR was used to detect the expression of ferroptosis regulation genes (FRG) in HMC3 cells induced by α-syn or STAT3 inhibitor. ROS, lipid peroxidation and iron levels were measured by flow cytometry.

Results

We found that α-syn could impair cell activity and stably inhibit the IL6ST/STAT3/HIF-1α pathway in α-syn-induced HMC3 cells. Besides, we performed transcriptomic analysis for α-syn-induced HMC3 cells and in α-syn-induced PD mouse models and GSEA indicated an association with ferroptosis. The reduction in P-STAT3 resulted in the lower expression of HIF-1α and the transcriptional activation of ferroptosis positive regulation (FPR) genes. P-STAT3 mediated ferroptotic cell death in α-syn-induced HMC3 cells by modulating lipid peroxidation and iron metabolism levels. An in vivo study revealed that the IL6ST/JAK2/STAT3/HIF-1α pathway was upregulated in PD mouse models.

Conclusions

STAT3 was an important factor that regulates ferroptosis in α-syn pathology via the JAK2/STAT3/HIF-1α axis. Our research illustrated the relationship of the JAK2/STAT3/HIF-1α axis and ferroptosis in the pathological process of α-syn both in vitro and in vivo, providing new topics of interest regarding the inflammation damage hypothesis and pathogenesis in PD.

Background

Parkinson's disease (PD) is the second most common neurodegenerative disorder and a major public health concern. Neuropathologically, abnormal deposition of α-synuclein (α-syn) aggregation in neuronal cell bodies is a major driver of PD. α-Syn, a 14 kDa soluble disordered protein, is primarily produced in neurons and exists in monomeric, oligomeric, and fibril forms in the disease. α-Syn can activate the inflammatory
responses of microglia through neuron-to-microglia transmission. The results from recent studies suggested that various pathways react to cellular dysfunction caused by α-syn toxicity, including mitochondrial dysfunction, autophagy–lysosomal impairment, endoplasmic reticulum stress and more\textsuperscript{4,5}. Our previous study demonstrated that α-syn triggered inflammatory reactions in microglial cells and revealed that Microglial cells rely on the autophagy–lysosome pathway (ALP) to affect their ability to phagocytose and eliminate toxic substances\textsuperscript{6}.

Microglial cells are the resident innate immune cells of the central nervous system (CNS) and react to misfolded proteins by activating inflammatory pathways and pattern recognition receptor ligation\textsuperscript{7}. The activation of microglial cells plays a critical role in ferroptosis and subsequent neurodegeneration\textsuperscript{8}. Following activation, microglia may elicit either anti-inflammatory or proinflammatory responses, or both, depending on the target. Similarly, in vivo evidence has indicated that distinct accumulation of the neuronal protein α-syn in microglia is a prominent feature in PD mouse models\textsuperscript{9,10}.

We previously reported that the overexpression of IL6ST-AS induced by exogenous α-syn could inhibit the expression of IL6ST and the activation of JAK2/STAT3/HIF-1α pathway in HMC3 cells\textsuperscript{11}. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is the major signaling mechanism for a broad array of cytokines and growth factors, widely participating in biological processes such as immunomodulatory cell proliferation, differentiation, and apoptosis\textsuperscript{12}. Previous research has demonstrated that STAT3 is involved in ferroptosis in multiple diseases, such as ulcerative colitis\textsuperscript{13}, hypertension\textsuperscript{14} and a variety of cancers\textsuperscript{15}. Ferroptosis, a unique iron-dependent lipid peroxidation cell death pathway, plays a crucial role in the development and progression of PD. Despite these promising results, whether STAT3 directly contributes to ferroptosis in PD regulation has not been clarified. In this study, we aimed to explore the effects and detailed mechanisms of the STAT3 gene in modulating ferroptosis after stimulation with α-synuclein (Fig. 1).

**MATERIALS AND METHODS**

**Cell Culture**

HMC3 cells were purchased from ATCC(CRL-3304). HMC3 cells were cultured under 5% CO\textsubscript{2} and 37 °C. HMC3 cells were grown in minimum essential medium (MEM) with NEAA containing 10% FBS, 100 µg/mL streptomycin and 100 U/mL penicillin. The cells were periodically passaged every 2–3 days. HMC3 cells were seeded in six-well plates before they were treated with α-syn oligomers. In Stattic + α-syn group, HMC3 cells were treated with α-syn oligomers (25 µmol/L) for 24h followed by treatment with 20 µmol/L Stattic for 4h. In TFA + α-syn group, HMC3 cells were treated with α-syn oligomers (25 µmol/L) for 24h followed by treatment with 10 µmol/L TFA for 24h.

**Preparation and characterization of the α-syn oligomer**
The α-syn monomer solution was purchased from Genemei Biotech Co., Ltd. (Guangzhou, China). Then, the solution was incubated in a rotary shaker at 280 rpm and 37°C for 72 hours. Transmission electron microscopy (TEM) was used to characterize the morphology of α-syn oligomers.

**Cell viability**

CCK-8 assays were performed to evaluate cell viability. HMC3 cells were seeded in a 96-well plate at a density of approximately 4000 cells per well with five replicates. Overnight, HMC3 cells were observed to a suitable density, and different concentrations of α-syn oligomers were then added to the experimental group. Ten microliters of CCK-8 were added to each well for 1 h. Finally, absorbance was obtained at 570 nm using a microplate reader (Thermo MK3). The cell survival rate was subsequently calculated.

**RNA Extraction and qPCR**

First, the cell medium was aspirated from the culture dish, and the cells were subsequently rinsed twice with PBS at room temperature. Then, the Fast Tissue RNA Purification Kit (EZBioscience, USA) was used to extract total RNA according to the protocol. RNA concentration and quality were determined by IMPLEN N60 Touch. cDNA was synthesized using the PrimeScript™ RT Reagent Kit (Takara, Japan) according to the product instructions. It was diluted 1:5 in double-distilled water before qPCR plate preparation. Then, all qPCRs were performed on 96-well plates and were carried out on a Roche LightCycler 96 using SYBR(R) Premix Ex Taq™. β-Actin was used as an internal control in qRT–PCR for HMC3 cells. In this study, relative gene expression was calculated using the comparative 2^(-ΔΔCt) method. Primer sequences were detailed as follows (Table 1).
### Table 1
The primer sequences of FRG.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’to3’)</th>
</tr>
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<tbody>
<tr>
<td>CHAC1-F</td>
<td>GAACCCTGGTTACCTGGGC</td>
</tr>
<tr>
<td>CHAC1-R</td>
<td>GCGAGCAAGTATTTCAAGTTG</td>
</tr>
<tr>
<td>FTH1-F</td>
<td>CGAGGTGGCCGAATCTTCC</td>
</tr>
<tr>
<td>FTH1-R</td>
<td>GTTTGTGCAGTTCCAGTGTG</td>
</tr>
<tr>
<td>HSPB1-F</td>
<td>ACGGTCAGACCAAGGATGG</td>
</tr>
<tr>
<td>HSPB1-R</td>
<td>AGCGGTATTTCCGCCTGA</td>
</tr>
<tr>
<td>GPX4-F</td>
<td>GAGGCAAGACCGAAGTAAACTAC</td>
</tr>
<tr>
<td>GPX4-R</td>
<td>CCGAACTGTTACACGGGAA</td>
</tr>
<tr>
<td>NFE2L2-F</td>
<td>TCAGCGACGGAAGAATGATGA</td>
</tr>
<tr>
<td>NFE2L2-R</td>
<td>CACCTTGTTTCTGACTGGATGT</td>
</tr>
<tr>
<td>TFRC-F</td>
<td>GGCTACTTGCGCTATTGAAAGG</td>
</tr>
<tr>
<td>TFRC-R</td>
<td>CAGTTTCTCCGACAACTTCTCT</td>
</tr>
<tr>
<td>β-actin-F</td>
<td>CTACCTCATGAAGATCCTCACCAGA</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>TTCTCCTTAATGTCACGCACGATT</td>
</tr>
</tbody>
</table>

### Protein extraction and western blot analysis

HMC3 cells were seeded at different densities depending on treatment. The medium was carefully aspirated, and the cells were washed three times with prechilled PBS on ice. Cells were lysed in RIPA lysis buffer (Cwbiotech, Beijing, China) supplemented with phosphatase inhibitor cocktail (Cwbiotech, Beijing, China) (100*) and protease inhibitor cocktail (Cwbiotech, Beijing, China) (100*). In brief, we used 20–30 µg of protein per lane, and the proteins in each sample were loaded on 4–12% gradient gels. Then, the proteins were transferred from the gel to 0.2 µm polyvinylidene fluoride (PVDF) membranes (Merck Millipore, USA). Membranes were blocked with 5% bovine serum albumin (BSA, Saibao Biotechnology, Yancheng, China) for 1 h. Subsequently, primary antibodies (1:1000) were incubated at 4°C overnight. The next day, the blots were washed in TBST and incubated with secondary antibodies (1:5000) for at least 1 h at room temperature. Finally, each blot was visualized using an Omni-ECL™ Enhanced Pico Light Chemiluminescence Kit (Epizyme Biomedical Technology Co., Ltd, Shanghai, China). GAPDH and TUBULIN were used as normalization controls. The quantitative expression of each protein was determined by ImageJ software. The information on the antibodies used in this study were listed in the Supplementary Table S1.
Detection of Intracellular ROS

Intracellular ROS were measured using an oxidation-sensitive fluorescent probe (DCFH-DA) with a Reactive Oxygen Species Assay Kit (Beyotime, Shanghai, China). Probes were diluted to achieve a final concentration of 10 µM with MEM. Then, the cells were incubated with a suitable concentration of DCFH-DA for 20 min at 37°C in the dark and washed with fresh serum-free medium three times. Finally, the fluorescence of DCFH was immediately detected by flow cytometry. The excitation and emission wavelengths were measured at 488 nm and 525 nm, respectively.

Detection of lipid peroxidation and intracellular Ferrous ion

The level of intracellular Ferrous ion in HMC3 cells was examined using FerroOrange dye following the manufacturer’s instructions (Dojindo Laboratories, Shanghai, China). The level of lipid peroxides in HMC3 cells was quantified using the lipid peroxide fluorescent probe Liperfluo dye following the manufacturer’s instructions (Dojindo Laboratories, Shanghai, China).

Experimental animals

C57BL/6 male mice (30±5 g) were provided by the Laboratory Animal Centre of Sun Yat-Sen University (Guangzhou, China). The animals were settled in cages at a standard and constant animal facility and had free access to water and rodent chow. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-Sen University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Sun Yat-Sen University.

Stereotaxic injection of α-syn oligomers

For α-syn oligomer injection, stereotaxic surgery was performed in the left striatum. Two-month-old mice were anesthetized with 10% chloral hydrate (Leagene, Beijing, China) (3.5 ml/kg, intraperitoneal injection). Then, the mice were placed on a stereotaxic frame (RWD, Guangdong, coordinates from bregma: anterior posterior (AP): + 0.2 mm; mediolateral (ML): −2.0 mm; dorsal ventral (DV): −2.6 mm, based on the Franklin and Paxinos mouse brain atlas. A total of 8 µg of α-syn oligomers were injected at constant flow using a 10 µl Hamilton syringe (Hamilton, Graubünden, Switzerland) with a 26 s-gauge custom needle. After each injection, to prevent reflux of the solution, the needle was allowed to remain in place for 5 minutes and withdrawn slowly. Finally, the incision was closed using sutures. The anesthetized animals were placed on heating blankets until they recovered from anesthesia.

Tissue preparation

Mice were anesthetized with 10% chloral hydrate and then transcardially perfused with 30 ml of saline, followed by cold 4% paraformaldehyde. Intact brain tissues were removed and then fixed in 4%
paraformaldehyde overnight. After gradient ethanol dehydration and clearing using xylene, the brain tissues were paraffin-embedded for coronal sectioning.

Transcriptome sequencing

Total RNA isolation, RNA-Seq library construction and transcriptome sequencing were performed by Huayin Health Medical Group Co., Ltd. (Guangzhou, China). GO enrichment analyses were performed using the GO consortium web tool (www.geneontology.org). KEGG pathway analysis was performed using the KEGG web server (http://www.genome.jp/kegg/mapper.html). GSEA was performed using GSEA software (v4.3.2, www.broadinstitute.org/gsea).

Immunohistochemistry

Immunohistochemical staining of the brain sections was conducted using an SP Rabbit & Mouse HRP Kit (DAB) (CWBIO, Beijing, China) according to the manufacturer’s protocol. First, the tissue sections were baked at 60°C for an hour. After deparaffinization with xylene and hydration with gradient ethanol, the sections were subjected to heat-mediated antigen retrieval using citrate buffer (Solarbio, Beijing, China) for 30 min. Then, the sections were blocked using 2.5% normal goat serum for 10 min and incubated with anti-TH antibodies (1:300) diluted in antibody diluent solution overnight at 4°C. The next day, the sections were rinsed and incubated with biotinylated secondary antibody for 10 min at room temperature. The immunohistochemical signal was visualized using 3,3’-diaminobenzidine (DAB) (CWBIO, Beijing, China). Immunohistochemical staining for α-syn, IL6ST, P-STAT3 and HIF-1α was performed in the same way. Images were observed under PANNORAMIC DESK (3DHISTECH, Hungary) and quantified using ImageJ software (NIH, Bethesda, MD, USA). The information on the antibodies used in this study were listed in the Supplementary Table S2.

Statistical Analysis

SPSS software v24.0 (IBM, NY, USA) was used for statistical analyses. All experiments were repeated three times. Triplicate measurements were used for statistical analyses. Data are expressed as the mean ± standard deviation (SD). Student’s t test was performed to compare two groups to each other. P values < 0.05 were considered statistically significant.

Results

1. The toxicity of exogenous α-syn oligomers to HMC3 cells

To examine whether and how the HMC3 cells population responds to the clearance and phagocytosis of pathogenic α-syn oligomers, HMC3 cells were exposed to well-characterized recombinant human α-syn oligomers. All subsequent experiments were performed using α-syn oligomers. First, we used transmission electron microscopy images to characterize the size and shape of α-syn oligomers (Fig. 2A-B). We found that the oligomeric α-syn species appeared to be approximately spherical or cylindrical in the TEM images,
with very high uniformity. The protein molecular mass of α-syn oligomers was measured by western blot analyses (Fig. 2C). In this study, the final composition of oligomeric α-syn was assessed by SDS–PAGE, with an apparent molecular weight greater than 100 kDa. Nevertheless, some monomers remain, which is likely due to the dissociation of some unstable oligomers.

Cell activity was detected by a Cell Counting Kit-8 (CCK-8) assay kit (Fig. 2D). The results showed that cell activity was significantly decreased when the concentration of α-syn reached 25 µmol/L. As the concentration increased, the cytotoxicity of α-syn to HMC3 cells also increased.

In addition, we measured the content of ROS in different concentrations of α-syn-induced HMC3 cells to assess the level of oxidative stress. When HMC3 cells were exposed to α-syn at a lower concentration (less than 5 µmol/L) for 24 h, an increased level of intracellular ROS was observed (Fig. 2E). This illustrated that brief stimulation could exacerbate oxidative stress. However, the level of intracellular ROS decreased when HMC3 cells were exposed to α-syn at a higher concentration (10–25 µmol/L) for 24 h.

2. IL6ST/JAK2/STAT3/HIF-1α activation was inhibited during α-syn oligomer treatment

In this part, we proceeded to explore the effect of α-syn on IL6ST/JAK2/STAT3/ HIF-1α in HMC3 cells. The relative amounts of both phosphorylated (P-JAK2 and P-STAT3) and nonphosphorylated (JAK2 and STAT3) proteins were quantified.

To explore the effect of the different concentrations of α-syn oligomers in HMC3 cells, we conducted a dose–response assay. Five gradients of stimulation times were set in this experiment in a range of 0–25 µmol/L (Fig. 3A). The suppression of IL6ST in HMC3 cells increased with increasing concentrations of α-syn. By regulating the expression of IL6ST, α-syn has an effect on IL6/IL6R/IL6ST complex downstream pathways such as the JAK2/STAT3 pathway. In HMC3 cells induced by α-syn, activation of the JAK2/STAT3 pathway was inhibited. With the higher α-syn treatment concentration, the inhibitory effect was obviously enhanced.

Additionally, considering the effect of processing time, we also conducted a time-response study. We designed four time points at 3 h, 9 h, 12 h and 24 h (Fig. 3B). Similarly, the protein expression of the IL6ST/JAK2/STAT3 pathway was decreased with the same trend. IL6ST/JAK2/STAT3 protein expression in HMC3 cells was decreased after α-syn treatment in a time-dependent manner.

Based on our findings above, we focused on the downstream signaling pathways modulated by JAK2/STAT3 in HMC3 cells. The restraint of the JAK2/STAT3 pathway led to a significant reduction in HIF-1α. According to the study performed by Hannah Scheiblich et al., we examined the level of IL6ST/JAK2/STAT3/HIF-1α during α-syn phagocytosis. As displayed in Fig. 3C, IL6ST/JAK2/STAT3/HIF-1α protein expression was strongly inhibited by α-syn treatment during phagocytosis.
3. Differential expression analysis and verification of α-syn-induced transcriptomic changes in HMC3 cells.

To identify specific α-syn-induced functional changes and the downstream signaling pathways modulated by JAK2/STAT3/HIF-1α in HMC3 cells, transcriptome sequencing of controls and cells exposed to α-syn oligomers were performed.

Based on the microarray analysis results, a heatmap of differentially expressed genes between control and α-syn-treated HMC3 cells was generated (Fig. 4A). Volcano plots indicated that there were 75 upregulated mRNAs and 100 downregulated mRNAs in HMC3 cells treated with α-syn (Fig. 4B). Gene Ontology (GO) enrichment analysis showed that the differentially expressed genes were mainly enriched in cell death within molecular functions (Fig. 4C). In this study, we used datasets to explore the role of ferroptosis and FRG alterations in α-syn-induced HMC3 cells. The GSEA results showed that the ferroptosis pathway exhibited a higher enrichment score (FDR < 25%, p < 0.05, |NES| > 1). The GSEA results of the ferroptosis-marker gene set database regulatory network showed enrichment of 6 mRNAs, namely, TFRC, CHAC1, NFE2L2, FTH1, GPX4 and HSPB1 (Fig. 4E). These mRNAs were verified in later qPCR experiments. The ferroptosis geneset in this study were listed in the Supplementary Table S3.

4. STAT3-mediated ferroptosis is involved in α-syn-induced HMC3 cells

We next examined whether STATS induced the expression of the classically identified α-syn-inducible genes described above. First, the STAT3 activator Colivelin TFA\textsuperscript{17,18} and the STAT3 inhibitor Stattic\textsuperscript{19,20} were used to regulate the expression of STAT3. Confirmation of the results of the regulator was performed through western blotting, as shown in Fig. 5A. P-STAT3 could positively regulate the expression of HIF-1α (Fig. 5B). The results, shown in Fig. 5C, revealed that restraining STAT3 seemed to lead to an increase in TFRC, CHAC1 and FTH1 in α-syn-induced group, Stattic group and TFA + α-syn-induced group. The expression of the GPX4 gene decreased in the Stattic group but increased in the α-synuclein-induced group and TFA + α-syn-induced group. The expression of the HSPB1 gene increase in Stattic group and TFA + α-syn-induced group. The level of lipid peroxides was increased in α-syn-induced group, Stattic group and Stattic + α-syn-induced group (Fig. 5D). Furthermore, the level of lipid peroxides was higher in Stattic + α-syn-induced group compared with α-syn-induced group and Stattic group. The level of lipid peroxides was decreased in the TFA + α-syn-induced group compared with α-syn-induced group (Fig. 5D). The level of intracellular Ferrous ion also revealed the same trend in Fig. 5E.

5. Confirmation of the α-syn-induced models and IL6ST/STAT3 staining of the substantia nigra in α-syn-induced PD model mice

To clarify whether α-syn-induced mouse models of PD were successfully established, TH and α-syn staining data were used in our study. In α-syn-induced models, Immunohistochemical staining of TH in the substantia nigra (SN) was lower in left side than right side. α-syn-induced PD model mice both of left and right SN presented with lower TH levels than controls (Fig. 6A). Following α-syn treatment, the density of DA
neurons was significantly lower than that in control mice. In vivo, we found by α-syn staining that α-syn did increase significantly (Fig. 6B). To summarize, the immunohistochemical results suggested that injection of α-syn caused PD-like pathological changes in mice. As shown in Fig. 6C-E, immunostaining analysis indicated a significant increase in IL6ST, P-STAT3 and HIF-1α levels in the α-syn group.

6. Differential expression analysis and verification of α-syn-induced transcriptomic changes in PD model mice

We further elaborated on the biological functions that were altered by α-syn in PD mouse models. A heatmap shows differentially expressed genes between control and α-syn-treated PD models (Fig. 7A). Volcano plots indicated that 92 upregulated mRNAs and 103 downregulated mRNAs were induced by treatment with α-syn (Fig. 7B). GO enrichment analysis also showed that the differentially expressed genes were mainly enriched in cell death within molecular functions (Fig. 7C). In this study, we used datasets to explore the role of ferroptosis and FRG alterations in PD mouse models. The GSEA results also showed that the ferroptosis pathway exhibited a higher enrichment score (FDR < 25%, p < 0.05, │NES│ >1) (Fig. 7D).

Discussion

In the present study, we explored the interactions among α-syn, STAT3 and related ferroptosis. First, we found that cell activities were significantly decreased in α-syn oligomer-induced HMC3 cells. We observed that the higher concentration and the longer exposure period of α-syn oligomers resulted in the stronger suppression of IL6ST/JAK2/STAT3/HIF-1α in HMC3 cells. The STAT3 was core transcriptional factor regulating IL6ST/JAK2/STAT3/HIF-1α axis. The inhibition of STAT3 led to an increase in TFRC and CHAC1, which suggested aggravated ferroptosis by iron and GSH homeostasis. In parallel, we found that α-syn mediated ferroptotic cell death by inhibiting the expression of STAT3 thereby promoting lipid peroxidation and iron metabolism levels.

In addition, we verified that the construction of α-syn-induced animal models was successful through TH+ and α-syn staining. We found that α-syn injection in these animal models significantly upregulated the expression of IL6ST/STAT3/HIF-1α. The results from IL6ST/STAT3/HIF-1α staining further corroborated the involvement of STAT3 in α-syn-related mechanisms. In verifying the differentially expressed genes identified through bioinformatic analysis above, we confirmed that both α-syn induction and inhibition of STAT3 significantly upregulated the expression of ferroptosis-related genes. Herein, these data indicated that ferroptosis was modulated by STAT3 in α-syn-related pathology.

In PD and other synucleinopathies, the monomeric protein α-syn becomes destabilized, misfolded and aggregated into insoluble, highly structured fibrils that form part of Lewy bodies (LBs)21. These forms resulted in distinct toxicity or seeding and spreading properties. Aggregated α-syn was predominantly found in neurons, but it also appeared frequently in glial cells during disease progression. Moreover, α-synuclein oligomers induce mitochondrial dysfunction, ultimately leading to neuronal death by activating the mitochondrial PTP 5. Oligomeric α-syn spreads more efficiently than higher molecular weight structures22.
Previous studies pointed strongly toward α-synuclein oligomers being the main toxic participants in PD\textsuperscript{23}. It is currently accepted that α-syn oligomers are the most toxic among all α-syn morphological forms\textsuperscript{24}. Based on the above analysis, α-syn oligomers were used in our study. We described the detailed characterization of toxic oligomeric species formed by α-syn, in accordance with a previous study\textsuperscript{24}. We successfully constructed a traditional α-syn-injection animal model verified by the depletion of DA neurons and increase in α-syn staining, in accordance with a previous study\textsuperscript{25,26}.

Our previous research showed that activation of the IL6ST/STAT3/ HIF-1α pathway was inhibited in α-syn-treated HMC3 cells at a specific time point and concentration (25 µmol/L). According to our dose–response analysis, the activities of the IL6ST/JAK2/STAT3 pathway in HMC3 cells were steadily downregulated in a time-dependent manner. It was observed that the higher the concentration and longer the exposure period of α-syn, the stronger the suppression of IL6ST/JAK2/STAT3. According to the study performed by Hannah Scheiblich et al.\textsuperscript{7}, α-syn treatment within 30 min was regarded as phagocytic processes. Similarly, the pathway was also inhibited during phagocytosis in our research.

Plamena et al. confirmed that α-syn aggregation induced ferroptosis by an interplay of iron, calcium and lipid peroxidation\textsuperscript{27}. Ferroptosis is a newly discovered form of cell death driven by iron-dependent lipid peroxidation\textsuperscript{28}. The key molecular mechanisms of ferroptosis include oxidative damage, iron toxicity and lipid peroxidation. Research has revealed that STAT3 is an important negative regulator of the ferroptosis pathway. In this research, we found that the inhibition of STAT3 could exacerbate the level of intracellular Ferrous ion and lipid peroxides induced by α-syn. Our results also demonstrated the activation of STAT3 could attenuate α-syn induced the accumulation of intracellular Ferrous ion and lipid peroxides.

In other disorders, STAT3 is capable of inducing ferroptosis through multiple pathways. However, the detailed mechanism of ferroptosis in α-syn pathology has not been clearly identified. Thus, we suspected that the mechanism of the JAK2/STAT3/HIF-1α signaling pathway induced the pathological processes in ferroptosis regulation in α-syn. To further investigate the molecular mechanisms between α-syn, STAT3 and ferroptosis, we used transcriptome sequencing of control and HMC3 cells exposed to α-syn oligomers. Gene set enrichment analysis (GSEA) is widely used to implement gene set enrichment testing, which is based on mainly enriched molecular signatures rather than differential gene expression\textsuperscript{29,30}. GSEA showed a significant enrichment in the ferroptosis-related gene set. Previous studies proposed several characteristic genes related to ferroptosis (such as GPX4\textsuperscript{31,32}, FTH1\textsuperscript{33}, HSPB1\textsuperscript{34}, NFE2L2\textsuperscript{35,36}, CHAC1\textsuperscript{37} and TFRC\textsuperscript{38}), the differential expression of which was also observed in our research. We speculated that STAT3 may upregulate the expression of the FPR genes CHAC1 and TFRC by binding to their promoters. α-Syn inhibited JAK2/STAT3/HIF-1α signaling pathway and induced the overexpression of CHAC1 and TFRC, which promoted the progression of ferroptosis in HMC3 cells. Besides, α-syn could also suppress the expression of NFE2L2 and subsequently accelerate ferroptosis but not through JAK2/STAT3/HIF-1α axis. Notably, in our study, ferroptosis negative regulation (FPR) genes such as GPX4, FTH1 and HSPB1 were upregulated in α-syn-induced HMC3 cells, which may indicate the mitigation in ferroptosis, but not participate in the process of α-syn-induced ferroptosis. We found that STAT3 acted as a core negative regulator of ferroptosis in α-syn pathology through a several-fold mechanism and that inhibition of STAT3 facilitated ferroptosis.
through a multipronged mechanism associated with lipid peroxidation and iron metabolism. Our studies identified CHAC1 and TFRC as novel and potential targets for later investigations in ferroptosis involved in α-synuclein pathology.

Some research has investigated whether STAT3 mediates the accumulation of ROS. It was demonstrated that several compounds improved intracellular inflammation through the inhibition of ROS production and the STAT3 signaling pathway\textsuperscript{39,40}. Serene et al. confirmed that exposing neuronal cells to increasing concentrations of both oligomeric and fibrillar α-syn (10–40 nmol/L) resulted in stepwise increases in cellular ROS production\textsuperscript{24}. The pathways and mechanisms by which ROS transition remain uncertain. In the rat 6-hydroxydopamine (6-OHDA) model of PD, Ye Tian et al. demonstrated that FTH1 was involved in iron accumulation and the ROS pathway\textsuperscript{41}. They also found that the knockdown of FTH1 in PC-12 cells significantly inhibited cell viability and caused mitochondrial dysfunction. In our research, we found that ROS was increased in α-syn-induced HMC3 cells in low concentrations (less than 5 µmol/L) of α-syn oligomer treatment. However, we found that ROS was decreased in α-syn-induced HMC3 cells in higher concentrations (10–25 µmol/L) of α-syn oligomer treatment. Our results suggested high concentrations of α-syn may activated the potential clearance mechanism of ROS in HMC3 cells, which still deserved further explorations. Although ROS can be cleared, the cellular damage induced by ROS accumulating still existed, which also aggravated ferroptosis.

Injection of α-syn significantly upregulated the expression of IL6ST/STAT3/HIF-1α in the substantia nigra, in agreement with a previous study using α-syn-induced rat PD models\textsuperscript{42}. These results are in agreement with the in vitro studies above. We speculated that if the concentration of α-syn was excessively high, the intracellular antioxidant defense mechanisms were activated to maintain cellular homeostasis. We performed transcriptome sequencing to compare gene expression. In this study, we used datasets to explore the role of ferroptosis and FRG alterations in PD mouse models. The GSEA results also showed that the ferroptosis pathway exhibited a higher enrichment score.

The present study has the following limitation. the number of animals in each group (n = 3–5) was too small in the animal study. These methodological flaws might cause biased results and should be ameliorated in our future studies.

**Conclusion**

Altogether, in the present study, we found that ferroptosis was the downstream target regulated by STAT3 in α-syn-exposed subjects, where STAT3 played a critical regulatory role. These results imply the main probable mechanisms of the α-syn/STAT3/FERROPTOSIS pathway in PD-related α-syn pathology. In this potential pathway, we found that intervention with α-syn and STAT3 contributed to improving PD symptoms. However, the effects of STAT3 intervention remain unclear in vivo. Since the members of the α-syn/STAT3/FERROPTOSIS pathway interact and might affect PD symptoms, our future investigation will focus on the effects of ferroptosis-related gene intervention. We believe the findings of this study are helpful for exploring novel diagnostic and therapeutic targets against PD. Overall, our results demonstrate a
novel feedback mechanism of STAT3-mediated maintenance of redox homeostasis in ferroptosis during α-synuclein pathology.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α-syn</td>
<td>α-synuclein</td>
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<tr>
<td>PD</td>
<td>Parkinson's disease</td>
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<td>FPR</td>
<td>ferroptosis positive regulation</td>
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<td>ALP</td>
<td>autophagy–lysosome pathway</td>
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<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>JAK/STAT</td>
<td>The Janus kinase/signal transducers and activators of transcription</td>
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<tr>
<td>MEM</td>
<td>minimum essential medium</td>
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<tr>
<td>FRG</td>
<td>ferroptosis-relative genes</td>
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<tr>
<td>SN</td>
<td>substantia nigra</td>
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<tr>
<td>LBs</td>
<td>Lewy bodies</td>
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<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
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<tr>
<td>FPR</td>
<td>ferroptosis negative regulation</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
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**Declarations**

**Acknowledgements**

Not applicable.

**Author contributions**

Conceptualization: HZ, XJ, JZ, DL and ET; Data curation: HZ, XJ, JZ, KH, YC, QS, DL and ET; Funding acquisition: DL and ET; Investigation: HZ, XJ, JZ, KH, YC, QS, DL and ET; Methodology: HZ, XJ, JZ, KH; Supervision: ET and DL; Writing - original draft: HZ, XJ, JZ, DL and ET; Writing - review & editing: All authors.

**Founding**

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Availability of data and materials

The authors declared that the datasets used and analyzed in this study were available from the corresponding authors upon reasonable request.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-Sen University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Sun Yat-Sen University (Ethics protocol number: 2020-001058).

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no conflicts of interest.

References


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**Figures**
Figure 1

A schematic diagram showed that STAT3-mediated ferroptosis was involved in α-synuclein pathology.
The toxicity of exogenous α-syn oligomers to HMC3 cells.

A (Scale bars: 200 nm) and B (Scale bars: 100 nm) Transmission electron microscopy images were used to characterize the size and shape of α-syn oligomers. C The protein molecular mass of α-syn oligomers was measured by western blot analyses. D Cell activity was detected by CCK8 assay. E Quantitative data of ROS fluorescence intensities in different experimental groups. Data were obtained from three biological
replicates. Data = mean ± SD, * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$, **** indicated $p < 0.0001$.

Figure 3

IL6ST/JAK2/STAT3/HIF-1α axis activation was inhibited in HMC3 cells during α-syn oligomer treatment
A The suppression of IL6ST/JAK2/STAT3/HIF-1α axis in HMC3 cells increased with the increasing concentrations of α-syn in a range of 0–25 μmol/L. B IL6ST/JAK2/STAT3/HIF-1α axis protein expression in HMC3 cells was decreased after α-syn treatment in a time-dependent manner in a range of 0-24h. C The level of IL6ST/JAK2/STAT3/HIF-1α axis was strongly inhibited by α-syn treatment during phagocytosis (5min and 15min). The protein expression above was determined by western blot and quantification of immunoblots was carried out. Data were obtained from three biological replicates. Data = mean ± SD, * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$, **** indicated $p < 0.0001$. 

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**Figure A:** Heatmap showing the expression levels of various genes in different conditions.

**Figure B:** Volcano plot depicting the log2 fold change vs. -log10(p-value) for gene expression.

**Figure C:** Bar chart illustrating the number of genes affected by treatment.

**Figure D:** Enrichment score graph showing the enrichment of specific gene sets.

**Figure E:**Heatmap of gene expression levels in different conditions.
**Figure 4**

**Differential expression analysis and verification of α-syn-induced transcriptomic changes in HMC3 cells.**

A The heat map of the enriched genes between control (n=3) and α-syn-induced HMC3 cells (n=3). Red dots represented upregulated genes, and blue dots represented down-regulated genes. B. Volcano plot of 175 differentially expressed genes between control and α-syn-treated HMC3 cells. Red dots represent upregulated genes, and blue dots represent down-regulated genes. C. GO enrichment analysis showed the differentially expressed genes were mainly enriched in cell death within biological process. D The GSEA result showed that the ferroptosis pathway exhibited a higher enrichment score (FDR<25%, p < 0.05, \(|\text{NES}|>1\)). E Ferroptosis regulation genes between control group and α-syn-induced HMC3 cells. Data were obtained from three biological replicates.
STAT3-mediated ferroptosis was involved in α-syn-induced HMC3 cells

A The protein expression P-STAT3 in different groups were measured by western blot and quantification of immunoblots was carried out. B The protein expression HIF-1α in different groups were measured by western blot and quantification of immunoblots was carried out. C TFRC, CHAC1, NFE2L2, FTH1, GPX4 and HSPB1 mRNA expression were assessed by qPCR. D Quantitative data of lipid peroxides probe fluorescence.
Intensities in different experimental groups. Quantitative data of ferrous ion probe fluorescence intensities in different experimental groups. Data were obtained from three biological replicates. Data = mean ± SD. * represented compared to control, * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$, **** indicated $p < 0.0001$. @ represented compared to α-syn group, @ indicated $p < 0.05$, @@@ indicated $p < 0.001$.

Figure 6
Confirmation of the α-syn-induced models and IL6ST/STAT3/ HIF-1α staining of the substantia nigra in α-syn induced PD mouse models.

A Positive Area of TH+ cells in the substantia nigra (SN) and the quantitative data of the TH+ staining in control groups (n=3) and α-syn induced PD mouse models (n=3). B Positive Area of α-syn in the SN and the quantitative data of the α-syn staining in control groups (n=3) and α-syn induced PD mouse models (n=3). C Positive Area of IL6ST in the SN and the quantitative data of the IL6ST staining in control groups (n=3) and α-syn induced PD mouse models (n=3). D Positive Area of P-STAT3 in the SN and the quantitative data of the P-STAT3 staining in control groups (n=3) and α-syn induced PD mouse models (n=3). E Positive Area of HIF-1α in the SN and the quantitative data of the HIF-1α staining in the substantia nigra (SN) in control groups (n=3) and α-syn induced PD mouse models (n=3). Data = mean ± SD, * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p< 0.001$, **** indicated $p < 0.0001$. 
Figure 7

Differential expression analysis and verification of α-syn-induced transcriptomic changes in PD mouse models.

A The heat map of the enriched genes between control (n=3) and PD mouse models (n=3). Red dots represented upregulated genes, and blue dots represented down-regulated genes. B. Volcano plot of 195 differentially expressed genes between control (n=3) and PD model mice (n=3). Red dots represented
upregulated genes, and blue dots represented down-regulated genes. C GO enrichment analysis showed the differentially expressed genes were mainly enriched in cell death within molecular functions. D The GSEA result showed that the ferroptosis pathway exhibited a higher enrichment score (FDR<25%, $p < 0.05$, $|\text{NES}| > 1$).

**Supplementary Files**

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- SupplementaryFigureS1.tif
- SupplementaryFigureS2.tif
- SupplementaryFigureS3.tif
- SupplementaryTableS1.The information on the antibodies used in this study for Western Blot..docx
- SupplementaryTableS2.The information on the antibodies used in this study for immunohistochemical..docx
- SupplementaryTableS3.The ferroptosis geneset in this study.xls