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Influence of RNA interference on the mitochondrial subcellular localization of alpha-synuclein and on the formation of Lewy body-like inclusions in the cytoplasm of human embryonic kidney 293 cells induced by the overexpression of alpha-synuclein☆

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Abstract

The specific and effective *α-synuclein* RNA interference (RNAi) plasmids, and the α-synuclein-pEGFP recombinant plasmids were co-transfected into human embryonic kidney 293 (HEK293) cells using the lipofectamine method. Using an inverted fluorescence microscope, α-synuclein proteins were observed to aggregate in the cytoplasm and nucleus. Wild-type α-synuclein proteins co-localized with mitochondria. Hematoxylin-eosin staining revealed round eosinophilic bodies (Lewy body-like inclusions) in the cytoplasm of some cells transfected with α-synuclein-pEGFP plasmid. However, the formation of Lewy body-like inclusions was not observed following transfection with the RNAi pSYN-1 plasmid. RNAi blocked Lewy body-like inclusions in the cytoplasm of HEK293 cells induced by wild-type *α-synuclein* overexpression, but RNAi did not affect the subcellular localization of wild-type *α-synuclein* in mitochondria.

Key Words: RNA interference; *α-synuclein* gene; subcellular localization; inclusion; Parkinson's disease; neural regeneration

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INTRODUCTION

Parkinson's disease (PD) is usually observed in the middle-aged and elderly, and is a chronic, progressive, neurological, degenerative disease. The primary pathological characteristics include degeneration of dopaminergic neurons in the substantia nigra and the formation of Lewy body inclusions in the cytoplasm of remaining neurons[1-5]. The etiology and pathogenesis of PD remains poorly understood, with no clinically proven effective therapeutic method to cure the disease[6-10]. *α-synuclein* is the pathogenic gene for familial PD, and its protein is the major component of Lewy body inclusions[1-5]. Many studies have shown that pathological aggregation of α-synuclein protein is responsible for the occurrence, development, and involvement of clinical symptoms in familial PD[7-8, 11-14]. Previous studies from our group have confirmed that α-synuclein overexpression induces pathological aggregation of α-synuclein protein in human embryonic kidney 293 (HEK293) cells and the formation of a substance that is similar to Lewy body-like inclusions[15]. Our previous studies have also shown that RNA interference (RNAi) can block the aberrant aggregation of *α-synuclein* by overexpression of wild-type *α-synuclein*[16].

RNAi is a fairly new and simple method to downregulate specific genes, with the hope of treating diseases caused by gene overexpression or gene mutations[15-20]. In this study, we designed a specific and effective RNAi plasmid to block *α-synuclein* gene expression to investigate the influence of RNAi on mitochondrial subcellular localization of α-synuclein and on the formation of Lewy body-like inclusions in the cytoplasm of HEK293 cells induced by *α-synuclein* overexpression.

RESULTS

Reverse transcription-PCR (RT-PCR) of *α-synuclein* mRNA expression

RT-PCR and fluorescent semi-quantitative detection results showed that, compared with non-transfection and transfected negative plasmid control groups, *α-synuclein* mRNA levels were significantly reduced in the

transfected pSYNi-1 group (69.6%, $P < 0.01$). Expression decreased by 9.6% in the pSYNi-2 group, with no significant changes in *α-synuclein* mRNA expression in the pSYNi-3 and pSYNi-4 groups ($P > 0.05$; Figure 1).

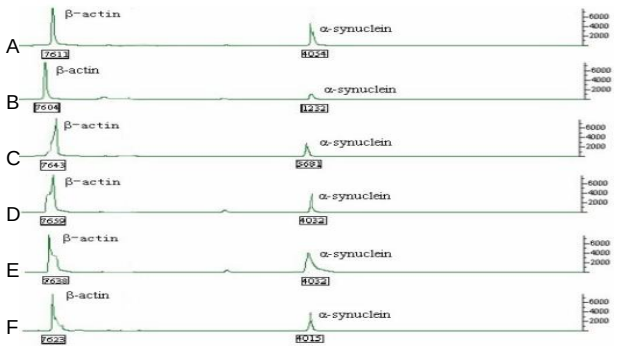

Figure 1 *α-synuclein* mRNA expression in human embryonic kidney 293 cells (semi-quantitative polymerase chain reaction).

(A) non-transfected group; (B) pSYNi-1 group; (C) pSYNi-2 group; (D) pSYNi-3 group; (E) pSYNi-4 group; (F) negative control group. Numerical values on the right data are scales; numerical values below peaks are gene peak values that exhibit amplified outputs.

Western blot analysis of α-synuclein protein expression

At 48 hours after transfection of HEK293 cells, western blot results revealed significantly decreased α-synuclein protein expression in the pSYNi-1 group compared with the negative control group ($P < 0.01$). The pSYNi-2, pSYNi-3, and pSYNi-4 groups exhibited no changes in expression (Figure 2).

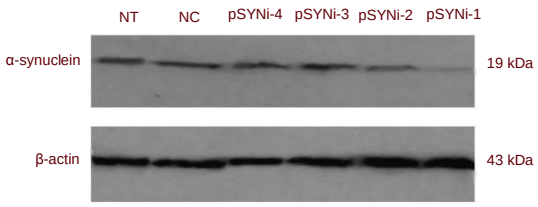

Figure 2 Western blot analysis of α-synuclein protein expression in human embryonic kidney 293 cells following RNAi plasmid transfection. α-synuclein protein expression significantly decreased in the pSYNi-1 group. NT: Non-transfected group; NC: negative control group.

Effects on the mitochondrial subcellular localization of α-synuclein following transfection of RNAi pSYNi-1 and α-synuclein-pEGFP

At 48 hours after transfection, mitochondrial staining as well as immunofluorescence results revealed that α-synuclein proteins could aggregate in the cytoplasm. Wild-type α-synuclein proteins co-localized with mitochondria. Transfection with RNAi plasmid did not change the subcellular localization of α-synuclein in mitochondria (Figure 3).

Effects on eosinophilic bodies formation following transfection of RNAi pSYNi-1 and α-synuclein-pEGFP

Hematoxylin-eosin staining revealed round, eosinophilic bodies (Lewy body-like inclusions) in the cytoplasm of some cells of the control group at 48 hours after transfection. However, following transfection with the RNAi pSYN-1 plasmid, the formation of Lewy body-like inclusions was not observed (Figure 4).

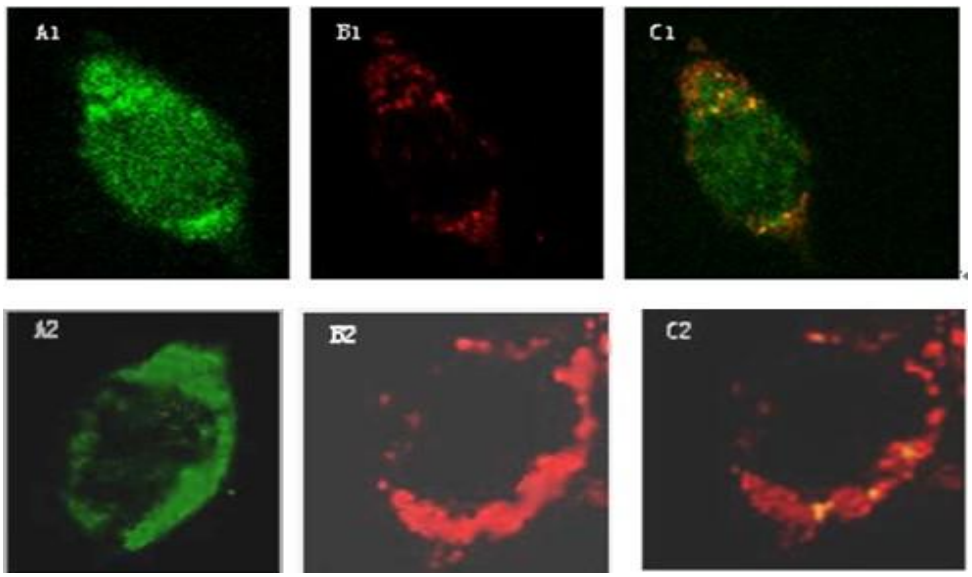

Figure 3 Subcellular localization of α-synuclein protein expression in the mitochondrion after transfection with the RNA interference vector pSYNi-1 and α-synuclein-pEGFP (inverted fluorescence microscope, × 1 000). Green shows EGFP fluorescence (A), *i.e.* α-synuclein localization; red: Mito Tracker staining (B), *i.e.* subcellular localization in the mitochondrion; yellow: co-localization (C). A1, B1, C1 are the irrelative interference group (α-synuclein-pEGFP eukaryocyte expression vector and RNA interference negative vector); A2, B2, C2 are the co-transfection group (RNA interference vector pSYNi-1 and α-synuclein-pEGFP). EGFP: Enhanced green fluorescent protein.

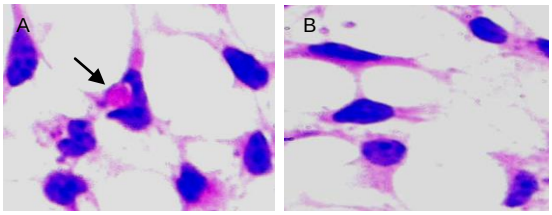

Figure 4 Eosinophilic inclusions in the cytoplasm of cultured human embryonic kidney 293 cells (hematoxylin-eosin staining, × 400).

(A) Irrelative interference group (α-synuclein-pEGFP eukaryocyte expression vector and RNA interference negative vector), exhibiting eosinophilic body formation (arrow).

(B) Co-transfection group (RNA interference vector pSYNi-1 and α-synuclein-pEGFP), without eosinophilic bodies. EGFP: Enhanced green fluorescent protein.

DISCUSSION

RNAi, first described in 1998[17], refers to the specific degradation of a homologous sequence of mRNA mediated by exogenous or endogenous double-stranded RNA, leading to gene silencing. Because this occurs at the post-transcriptional level, it is also referred to as "post-transcriptional gene silencing", which is a ubiquitous control mechanism in eukaryotes to resist viral invasion, prevent activation of transposons, and regulate gene expression[21-24]. RNAi is characterized by the following: (1) RNAi is a post-transcriptional gene silencing mechanism[25-26]; (2) has high specificity–it only degrades homologous mRNA with corresponding sequences, has no effects on mRNA expression of other genes, and single nucleotide changes in siRNA can block RNA interference[27-28]; (3) has high efficiency–several magnitudes below the antisense oligodeoxynucleotide concentration, siRNA increases the catalytic function and significantly inhibits or blocks gene expression[29]; (4) has strong penetration–RNAi inhibits gene expression across cell boundaries and can be transferred over long distances between different cells, thereby maintaining signaling throughout the entire organism, and can even be transmitted to offspring[30]. In recent years, the mechanisms of RNAi have been revealed[31-33]. RNAi technology has gradually improved, and applications have expanded from genomics to medical fields and the development of gene therapies.

The application of RNAi technology in the nervous system was initially tested on invertebrates, such as C. elegans and Drosophila. RNAi technology was successfully used to block the expression of embryonic tyrosine phosphatase genes in wild-type drosophila[34]. Cationic transfection reagents[27] have been used to introduce microtubule-associated protein 2 siRNA into the cerebral cortex of cultured rat hippocampal cells to block expression of microtubule-associated protein 2. The number of successful RNAi technology applications in the nervous system has demonstrated that this method can be applied to neuroscience research and has exhibited broad prospects[35-36].

In this study, *α-synuclein* expression was silenced in HEK293 cells by vector-mediated RNAi using the pBSHH1 plasmid. This vector was selected for several reasons; first, the cloning site of the vector contains a 4-nucleotide overhang on the 5' end of each DNA strand, allowing for directional cloning of the shRNA of interest; and second, the H1 promoter of the vector is recognized by RNA polymerase III resulting in high-level, constitutive expression of shRNA in most mammalian cell types. We identified an effective targeting sequence for RNAi, which was localized to the C-terminal coding sequence of the human *α-synuclein* gene, and used this to generate HEK293 cells in which *α-synuclein* expression was stably silenced. Due to the endogenous expression of *α-synuclein* and the dopaminergic characteristics of HEK293 cells, HEK293/*α-synuclein* is a good cellular model for studying the normal function of *α-synuclein* and examining its role in PD pathogenesis.

Studies have shown that RNAi exhibits positional effects[25-30]; it affects different regions of mRNA in targeted genes, such as the coding region, 3' untranslated region (3'UTR) and 5' untranslated region (5'UTR), and blocking efficiency varies. RNAi is ineffective in certain areas of the coding region[26]. The present study focused on *α-synuclein* and four target sites were chosen. Because compounded or transfected siRNA easily degrades *in vitro* due to widely spread RNA enzymes, it is unstable and difficult to handle. Therefore, the development of DNA vectors containing siRNA, which can be highly expressed in cells, is very important. In the present study, PBSHH1 plasmids, using the H1 promoter, were used. Four DNA vectors, which targeted the *α-synuclein* gene and effectively express hairpin siRNA, were designed and constructed. Western blot and RT-PCR semi-quantitative fluorescence revealed that the PSYNi-1 plasmid effectively blocked *α-synuclein* expression with 69.6% efficiency.

Evidence suggests that mitochondrial dysfunction can induce *α-synuclein* misfolding and lead to increased oxidative stress. Oxidative damage to lipids, proteins and DNA[37] as well as a decrease in the levels of the important antioxidant glutathione[38], has been detected in autopsy tissue from the brains of individuals with PD. These findings provide a plausible link between oxidative damage and the formation of Lewy body protein aggregates that are characteristic of PD, as oxidative damage induces α-synuclein aggregation and impairs proteasomal ubiquitination and degradation of proteins. Recent advances in metabolomic, proteomic and transcriptomic approaches are anticipated to permit further identification of alterations at the molecular level that are relevant to mitochondrial metabolism in PD[39].

In our study, the mitochondrial subcellular localization of α-synuclein was measured by mitochondrial staining as

well as immunofluorescence. At 48 hours after transfection, mitochondrial staining and immunofluorescence results revealed that α-synuclein proteins aggregated in the cytoplasm. Wild-type α-synuclein proteins co-localized with mitochondria. Transfection with RNAi plasmid did not change the subcellular localization of α-synuclein in mitochondria. Fibrillar α-synuclein is thought to be the building block of Lewy bodies. Indeed, α-synuclein is also the most sensitive marker for Lewy bodies, implying that it is necessary for Lewy body formation[40]. A number of studies have shown that *α-synuclein* expression induces pathological aggregation of α-synuclein protein[7-8]. Our previous studies have also shown that *α-synuclein* gene transfer to HEK293 cells, results in overexpression of *α-synuclein*, induces pathological aggregation of α-synuclein protein and the formation of Lewy body-like inclusions[16]. However, whether RNAi would have an influence on the formation of acidophilic bodies in the cytoplasm remains unclear.

At 48 hours after transfection, hematoxylin-eosin staining revealed no formation of acidophilic bodies in the cytoplasm of the RNAi pSYN-1 plasmid group, which suggests that RNAi blocked the formation of acidophilic bodies in the cytoplasm due to α-synuclein overexpression.

The present study suggested that RNAi successfully blocked the formation of acidophilic bodies in the cytoplasm due to wild-type *α-synuclein* overexpression. RNAi did not change the subcellular localization of α-synuclein in mitochondria. These results provide a basis for the pathogenesis of PD.

MATERIALS AND METHODS

Design

Engineered cytological controlled experiment.

Time and setting

Experiments were performed at the National Key Laboratory of Medical Genetics in China in 2005, and some experiments were supplemented in 2010.

Materials

HEK293 cells were supplied by the National Key Laboratory of Medical Genetics in China.

Methods

HEK293 cell culture

HEK293 cells were cultured as previously described[16]. Cells were incubated in RPMI1640 culture medium (Gibco, Carlsbad, CA, USA), containing 15% (v/v) fetal bovine serum (Gibco) in 5% CO_2 at 37°C. At 24 hours prior to transfection, cells were seeded in six-well culture plates at a density of 2×10^5 cells/well. When cells were approximately 80% confluent, they were transfected.

Construction and transfection of α-synuclein-pBSHH1 RNAi carriers

Short hairpin RNA was designed to target specific regions of human *α-synuclein* mRNA. Four pairs of Oligo DNA were prepared (Table 1), which were produced by Shanghai Sagon Biological Engineering. The same amount of paired Oligo DNA (2.5 μg/μL) was added to a PCR tube.

Table 1 OligoDNA of the *α-synuclein* gene

Code	OligoDNA (5'–3')
pSYNi-1	gATCCgCAgTggCTgAgAAgACCAATTCAAgA-gATTggTCTTCTCAgCCACTgTTTTTTggAAAA-gCTTTTCCAAAAAACAgTggCTgAgAAgAC-CAATCTCTTgAATTggTCTTCTCAgCCACTgCg
pSYNi-2	gATCCgACCAAAgAgCAAgTgACATTCAAgA-gATgTCACTTgCTCTTTggTCTTTTTTggAAA AgCTTTTCCAAAAAAgACCAAAgAg-CAAgTgACATCTCTTgAATgTCACTTgCTCTTTggTCg
pSYNi-3	gATCCgTgACAAATgTTggAggAgTTCAAgA-gACTCCTCCAACATTTgTCACTTTTTTggAAA AgCTTTTCCAAAAAAgTgACAAATgTTggAggAg-TCTCTTgAACTCCTCCAACATTTgTCACg
pSYNi-4	gATCCATgTTggAggAgCAgTggTTTCAAgAgAACCAC-TgCTCCTCCAACATTTTTTTggAAA AgCTTTTCCAAAAAAATgTTggAggAgCAg-TggTTCTCTTgAAACCACTgCTCCTCCAACATg

Following annealing, double-stranded hairpin siRNA DNA was formed and cloned into the pBSHH1 plasmid (provided by the National Lab of Medical Genetics, Changsha, Hunan Province, China[16]). This mixture was then used to transform JM109 competent bacteria. Following selection, the plasmids were small-scale extracted. The plasmids were extracted and purified according to the QIAprep Spin Miniprep Kit Protocol (Invitrogen, Carlsbad, CA, USA). Restriction enzymes were used to obtain pSYNi-1, pSYNi-2, pSYNi-3, and pSYNi-4 in combination with the QIAGEN Tip500 Protocol (Invitrogen)[16]. The recombined plasmids were transfected according to the Lipofectamine 2000 Protocol (Invitrogen)[26]. Non-transfected and negative plasmid transfect groups were also established.

RT-PCR and western blot detection of α-synuclein expression

Total RNA was extracted using Trizol (Gibco). The RNA was then reverse-transcribed into cDNA using the reverse transcription kit (Promega, Madison, WI, USA) and the following conditions: 70°C for 10 minutes, 42°C for 60 minutes; 95°C for 5 minutes, and 4°C for 5 minutes. The sequences were then amplified using the following parameters: 30 cycles of 94°C for 30 seconds, 64°C for 30 seconds, and 72°C for 45 seconds. The PCR products were electrophoresed on a 1.2% (w/v) agarose gel and fluorescently scanned using an ABI PRISM 3100 automated sequencer (ABI, Foster City, CA, USA). GeneScan Analysis 3.0 software and Genotyper 2.5 software were used (Gibco) to semi-quantify fluorescence. *α-synuclein* gene primers were as follows: upstream - 5'-HEX-ATG GAT GGA TGT ATT CAT GAA AGG ACT T-3'; downstream - 5'-GGC CTT AAG GGC TTC AGG TTC GTA GTC TTG-3' (Shanghai Sagon Biological Engineering, Shanghai, China). Conventional methods were used for western

blot analysis. A total of 40 μg total protein was electrically transferred onto polyvinylidene fluoride membranes. α-synuclein and β-actin primary antibodies (Sigma, St. Louis, MO, USA), were provided by the American Promega Company, and goat anti-mouse horse radish peroxidase was used as the secondary antibody (Thermo, Barrington, IL, USA)[16, 36].

Transfection of RNAi vector and α-synuclein-pEGFP

HEK293 cells were incubated in a 96-well culture plate, five wells/group. The cells were transfected according to the Lipofectamine 2000 Protocol (Invitrogen)[16]. HEK293 cells were transfected with α-synuclein-pEGFP containing 0.5 μL Lipofectamine 2000 and pSYNi-1 mixed with 0.1 μg DNA plasmid (1: 1) as the transfection group. Expression vectors of α-synuclein-pEGFP and RNAi negative carriers were transfected as the negative control group. HEK293 cells were transfected with blank plasmid as the blank control group.

Detection of mitochondrial subcellular localization of α-synuclein following transfection of RNAi pSYNi-1 and α-synuclein-pEGFP

Cells were seeded on a 12-well plate with glass coverslips and maintained in pre-heated (37°C) culture medium containing 1.8 μmol/L Mito Tracker (Sigma). Cells were incubated in 5% CO_2 at 37°C for 40 minutes. Cells were fixed in 3.7% (w/v) paraformaldehyde, and washed three times with phosphate-buffered saline (PBS) for five minutes each. The cells were incubated in 0.2% (v/v) PBS-Tween-20 for 5 minutes at room temperature and washed three times with PBS. The cells were coverslipped with 50% (v/v) glycerin and images were collected using an Axiovert200 type inverted fluorescence microscope with a laser excitation wavelength of 488 nm and 637 nm. Green fluorescence= enhanced green fluorescent protein, red fluorescence= Mito Tracker, and yellow fluorescence = α-synuclein, which indicated co-localization with mitochondria.

Hematoxylin-eosin staining

Cells were washed three times in pre-cooled PBS, fixed for 15 minutes with 95% (v/v) pre-cooled alcohol at −20°C, washed twice with PBS for 1 minute each, incubated in 250 μL hematoxylin for 10 minutes at room temperature, and rinsed in tap water for 30 minutes. Diluted hydrochloric acid alcohol was added to separate the colors for several seconds, cells were rinsed in tap water until the cell nuclei became blue, incubated in 300 μL of 0.5% (w/v) eosin for 10 minutes at room temperature, rinsed in tap water, and sequentially dehydrated in 70%, 80%, 90%, and 95% (v/v) alcohol for 1 minute. The cells were then incubated in 100% (v/v) alcohol for 3 minutes, xylene for 1 minute, mounted in 100% (v/v) glycerin, and images were collected using an inverted microscope (Leica, Solms, Germany).

Statistical analysis

Data are expressed as the mean ± SD and comparisons of the mean of two samples were analyzed using the Student's *t*-test. All data were included in the statistical analysis and SPSS 10.0 (SPSS, Chicago, IL, USA) was used to conduct statistical analysis. $P < 0.05$ was considered statistically significant.

Author contributions: Tao Chen had full access to all data and participated in data integrity and data analysis accuracy. Guoqiang Wen, Yidong Deng participated in data collection. Min Guo, Zhigang Long, and Feng Ouyang participated in data analysis and interpretation. Xiaoping Liao participated in study design, study supervision, and manuscript development.

Conflicts of interest: None declared.

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Ethical approval: The project received full ethical approval from the People's Hospital of Hainan Province.

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