RNA interference mediated silencing of α -synuclein in MN9D cells and its effects on cell viability

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Abstract: Objective To silence the expression of α -synuclein in MN9D dopaminergic cells using vector mediated RNA interference (RNAi) and examined its effects on cell proliferation and viability. **Methods** We identified two 19-nucleotide stretches within the coding region of the α -synuclein gene and designed three sets of oligonucleotides to generate double-stranded (ds) oligos. The ds oligos were inserted into the pENTRTM/H1/TO vector and transfected into MN9D dopaminergic cells. α -Synuclein expression was detected by RT-PCR, real-time PCR, immunocytochemistry staining and Western blot. In addition, we measured cell proliferation using growth curves and cell viability by 3-(4, 5)-dimethylthiazolium (-z-y1)-3, 5-diphenyltetrazoliumromide (MTT). **Results** The mRNA and protein levels of α -synuclein gene were significantly down-regulated in pSH2/ α -SYN-transfected cells compared with control MN9D and pSH/CON-transfected MN9D cells, while pSH1/ α -SYN-transfected cells showed no significant difference. Silencing α -synuclein expression does not affect cell proliferation but may decrease cell viability. **Conclusion** Our results demonstrated pSH2/ α -SYN is an effective small interfering RNA (siRNA) sequence and potent silencing of mouse α -synuclein expression in MN9D cells by vector-based RNAi, which provides the tools for studying the normal function of α -synuclein and examining its role in Parkinson's disease (PD) pathogenesis. α -Synuclein may be important for the viability of MN9D cells, and loss of α -synuclein may induce cell injury directly or indirectly.

Keywords: α -Synuclein; RNA interference; Parkinson's disease

1 Introduction

α -Synuclein is a 140-amino acid protein, which has been strongly implicated in the pathogenesis of Parkinson's disease (PD)^[1,2]. Previous studies have shown that α -synuclein mutations (A53T, A30P, E46K) or triplications are associated with some autosomal-dominant PD^[3-6]. Although there is no α -synuclein gene mutation identified in idiopathic PD^[7,8], aggregated α -synuclein has been found to be the major com-

ponent of Lewy bodies and Lewy neurites, the pathological hallmarks of PD^[9-14]. Although the normal function of α -synuclein and its role in the pathogenesis of PD remain unclear, several hypotheses have been proposed based on its physical properties or interacting partners^[15-21]. One theory suggested that α -synuclein might be linked to PD via the regulation of dopamine (DA) homeostasis^[19-23]. Several studies have proposed that α -synuclein may be involved in regulating the biosynthesis, vesicles storage and release, as well as reuptake of DA^[24-31]. In addition, α -synuclein, as a chaperone, has been reported to interact with many other proteins, including 14-3-3, ERK1/2, parkin, etc.^[32-37]. However, some of these findings remain controversial. Further studies are required to examine the normal function of α -synuclein.

RNA interference (RNAi) is a new strategy for silencing gene expression. The mechanism of RNAi is that the short

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double-stranded (ds) RNA can mediate the degradation of sequence specific mRNA^[38-40]. It has been reported that introducing small interfering RNAs (siRNAs) or short hairpin RNA (shRNA) into mammalian cells could specifically silence the expression of target genes^[41-43]. It has become a powerful tool to study gene function, with high specificity, efficiency and convenience. In the present study, we silenced the expression of α -synuclein using vector mediated RNAi in dopaminergic MN9D cells to study the normal function of α -synuclein.

2 Materials and Methods

2.1 Construction of shRNA expression plasmids Short hairpin RNAs were designed to target specific regions of mouse α -synuclein mRNA (Gene Bank Accession No: AF179273). We identified two 19-nucleotide stretches within the coding region of the α -synuclein gene that were located within one exon, 40%-50% GC rich, and unique within the mouse genome. A nine-nucleotide loop structure was designed to form the hairpin. We designed three sets of oligonucleotides as instructed by the BLOCK-iTTM Inducible H1 RNAi Entry Vector Kit (Invitrogen, Carlsbad, CA, USA): SH1/ α -SYN: 5'-CACCGAGCAAGTGACAAATTTGTTCAAGAGAC AACATTTGTCACTTGCTC-3' (forward), 5'-AAAAGAG CAAGTGACAAATGTTGTCTCTTGAACAACATTTGTCACTTG CTC-3' (reverse); SH2/ α -SYN: 5'-CACCGAGC TACCAAGACTATGAGTTCAAGAGACTCATAGTC TTGGTAGCC-3' (forward), 5'-AAAAGGCTACCAAG ACTATGAGTCTCTTGAACCTCATAGTCTTGGTAGCCT-3' (reverse); and SH/CON: 5'-CACCGGATCGCCAGAA CAAGTATTTCAAGAGAATACTTGTCTGGCGATCC-3' (forward), 5'-AAAAGGATCGCCAGAACAAGTAT TCTCTTGAAATACTTGTCTGGCGATCC-3' (reverse). Single-stranded (ss) DNA oligos (BioSia Co. Ltd., Shanghai, China) were synthesized. Equal amounts of each pair of ss oligos were annealed to generate ds oligos. The ds oligos were inserted into the pENTRTM/H1/TO vector (Invitrogen) using T4 DNA Ligase and transformed into One Shot[®] TOP10 Chemically Competent *E. coli* (Invitrogen). The transformants were verified by PCR and sequencing. The positive plasmids were named pSH1/ α -SYN, pSH2/ α -SYN and pSH/CON, respectively.

2.2 Cell Culture The MN9D dopaminergic cell line was generated by fusion of rostral mesencephalic neurons from

embryonic C57BL/6J (embryonic d 14) mice with N18TG2 neuroblastoma cells^[31] (gift from Dr Bastian Hengerer, Novartis AG). Cells were cultured in DMEM/F12 media supplemented with 10% neonatal calf serum (GIBCO BRL, Grand Island, NY, USA) (pH 7.2) and incubated at 37 °C in an atmosphere of 5% CO₂. MN9D cells endogenously express α -synuclein and TH, and produce measurable levels of DA.

2.3 Transfection and selection MN9D cells were stably transfected with pSH1/ α -SYN, pSH2/ α -SYN or pSH/CON. For transfection, cells were plated in 6-well tissue culture plates pre-coated with poly-l-lysine (Sigma, St. Louis, MO, USA) at 80%-85% confluency. Transfections were performed using LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instructions. Four to six hours after transfection, the media was replaced with fresh growth media. The following day, cells were trypsinized and replated into a larger-sized tissue culture format. Polyclonal pools of stably transfected cells and monoclonal cells were selected using 400 μ g/mL ZeocinTM (Invitrogen) and maintained with media containing 50 μ g/mL ZeocinTM.

2.4 RNA isolation, RT-PCR and real-time RT-PCR Total RNA was extracted from pSH1/ α -SYN, pSH2/ α -SYN and pSH/CON stably transfected MN9D cells using Trizol reagent. First-strand cDNA was synthesized from 5 μ g of total RNA, using the SuperScriptTM First-strand synthesis System for RT-PCR (Invitrogen). The reaction mixture for PCR consisted of 1 μ L of cDNA template, 17 μ L of sterile ddH₂O and 10 pmol (1 μ L) of each specific primer. The following primers were used: α -synuclein: 5'-ATAAGAATGCGGCCGCATGGATGTATTCATGAAAG-3' (forward) and 5'-CCGCTC-GAGGCTTCAGGTTCTAGTCTTGA-3' (reverse). As an internal control, actin cDNA was co-amplified using the following primer sequences: 5'-CCCATCTACGAGGGCTACGC-3' (forward) and 5'-AGGAAGGAGGGCTGGAACA-3' (reverse). Each PCR was started by predenaturation at 96 °C for 3 min. Each PCR cycle consisted of 94 °C for 30 s, 50 °C for 55 s, and 72 °C for 55 s, followed by a final 10 min extension at 72 °C. PCR amplification was carried out for 35 cycles for α -synuclein and 22 cycles for actin using a Perkin-Elmer DNA thermal cycler 480 (Applied Biosystems, Foster City, CA, USA).

The reaction mixture (20 μ L) for real-time PCR consisted of 10 μ L SYBR GREEN PCR Master Mix (Applied Biosystems), 0.5 μ L of cDNA template, 0.4 μ L of each spe-

cific primer (10 $\mu\text{mol/L}$) and 9.7 μL of sterile ddH₂O. The following primers were used: α -synuclein: 5'-TGACG-GGTGTGACAGCAGTAG-3' (forward) and 5'-CA-GTGGCTGCTGCAATGC-3' (reverse); β -actin: 5'-AC-CACCATGTACCCAGGCATT-3' (forward) and 5'-CCA-CACAGAGTACTTGCGCTCA-3' (reverse). Quantitative PCR was performed in duplicate or triplicate using a 7300 real time PCR thermal cycler (Applied Biosystems). Each reaction was started by 50 °C for 2 min, 95 °C for 10 min and 96 °C for 3 min, followed by 40 cycles of 94 °C for 15 s, 59 °C for 20 s and 72 °C for 30 s.

2.5 Immunocytochemistry staining Cells were washed with 0.01 mol/L PBS, fixed in 4% paraformaldehyde (30 min), permeabilized with 0.5% Triton-X (20 min), and blocked in 5% normal goat serum (30 min). Samples were incubated with a specific monoclonal antibody against α -synuclein (2E3; 1:1000) (gift from Dr Shun YU in Xuanwu Hospital, Capital Medical University, Beijing, China) overnight at 4 °C. Cells were washed with 0.01 mol/L PBS and incubated with biotinylated goat anti-mouse IgG (1:400, Zhongshan, Beijing, China) at 37 °C for 2 h and 1 h with avidin-biotin-peroxidase complex (1:400, Zhongshan). Cells were finally visualized in a solution containing 3,3-diaminobenzidine (Sigma) and H₂O₂ in 50 mmol/L of Tris-HCl buffer. After immunostaining, cells were examined by a fluorescence microscopy.

2.6 Cell proliferation and viability assay For the cell proliferation assay, the different MN9D cells were plated at 5.0×10^3 cells per well in 24-well plates and cultured at 37 °C. The cell number per milliliter media was counted using a hemacytometer for 8 d. To measure cell viability, the different MN9D cells were plated into 96-well plates (1.0×10^4 cells per well) and cultured for 24 h before the cells were assayed using a MTT Kit (Promega, Wisconsin, USA). Sixteen wells were measured for each MN9D cell line.

2.7 Western blot analysis Lysates of MN9D cells were prepared as previously described^[44]. Protein concentrations were determined using BCA relative to BSA protein standards according to the manufacturer (Pierce, Rockford, IL, USA). Whole cell protein extracts were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Prestained protein standards were used to determine the relative molecular mass of proteins. The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline, then incubated with the α -synuclein specific monoclonal antibody (2E3;

1:1 000) separately at room temperature for 3 h, followed by 1 h incubation with a peroxidase-conjugated secondary antibody (1:3 000, ZhongShan) at room temperature. The blots were washed and immunodetection was carried out by chemiluminescence using SuperSignal (Pierce). Blots were then stripped and re-probed with an antibody against β -actin (1:500, Zhongshan) and detected as described above. Blots were scanned using Kodak Image Station (440 CF; Kodak, Rochester, USA) and the optical densities of the bands relative to β -actin within each lane were obtained to account for any variations in proteins.

2.8 Statistical analysis ANOVA followed by Bonferroni post hoc multiple comparisons were used to examine the significance of differences among the experimental groups. A value of $P < 0.05$ was considered to be statistically significant. All data are expressed as mean $\pm$ SEM.

3 Results

3.1 Construction of the shRNA expression plasmids After synthesis, each pair of the ss oligos was annealed to generate ds oligos. The annealing efficiency was analyzed by 4% agarose gel electrophoresis (Fig. 1). Each ds oligo annealing reactant showed a clearly detectable molecular weight band around 50 bp (only data for SH1/ α -SYN were shown), as expected for the length of the designed ds oligos. The 50 bp bands were brighter than the corresponding lower molecular weight band, which represented the unannealed ss oligos in the annealing reactant. These data indicate that each pair of ss oligos annealed effectively to form ds oligos. The ds oligos were ligated with linearized pENTR™/H1/TO vectors and the positive clones were expanded and verified by PCR (Fig. 2). Bands near 350 bp were found in all PCR reactants, indicat-

Fig. 1 Analysis of the annealing reaction by 4% agarose gel electrophoresis. Lane 1, forward ss oligo of pSH1/ α -SYN; Lane 2, reverse ss oligo of pSH1/ α -SYN; Lane 3, ds oligo annealing reaction mixture; Lane 4, 50 bp DNA ladder.

ing that the ds oligos were ligated into the pENTRTM/H1/TO vectors. Further analysis by sequencing confirmed that the orientation and sequence of the inserted ds oligos were correct.

3.2 Analysis α -synuclein expression in MN9D transfected cells pSH1/ α -SYN, pSH2/ α -SYN and pSH/CON were transfected into MN9D cells and were selected by 400 μ g/mL ZeocinTM for 12 d. The ZeocinTM resistant cell clones were then picked and expanded. The gene expression of α -synuclein in these clones was assayed by RT-PCR, real-time RT-PCR, immunocytochemistry and Western blot. RT-PCR analysis revealed that the α -synuclein mRNA levels were decreased in pSH2/ α -SYN-transfected MN9D cells compared with MN9D and pSH/CON-transfected MN9D cells (Fig. 3). However, α -synuclein mRNA levels in pSH1/ α -SYN-transfected cells were not significant different to levels observed in control MN9D and pSH/CON-transfected MN9D cells (Fig. 3). These data suggest that α -synuclein expression may be inhibited by transfection with pSH2/ α -SYN. In addition, real-time RT-PCR revealed that α -synuclein mRNA levels in

pSH2/ α -SYN-transfected cells ($9.8 \pm 2.4\%$) were significantly lower than in MN9D and MN9D/CON-transfected ($81.6 \pm 4.9\%$) cells (Fig. 4). The immunocytochemistry data also indicated that α -synuclein gene expression was down-regulated in pSH2/ α -SYN-transfected cells (Fig. 5). Weak immunocytochemistry staining was observed in pSH2/ α -SYN-transfected cells compared to strong α -synuclein staining in control MN9D, pSH/CON-transfected and pSH1/ α -SYN-transfected MN9D cells. Furthermore, Western blot analysis revealed that α -synuclein protein levels were significantly lower in pSH2/ α -SYN-transfected cells (0.150 ± 0.004) than in MN9D cells (0.401 ± 0.038) (Fig. 6). Thus, α -synuclein expression was inhibited after transfection of MN9D cells with pSH2/ α -SYN. These data suggest that SH2/ α -SYN was an effective siRNA sequence and that α -synuclein expression was stably silenced in pSH2/ α -SYN-transfected MN9D cells. The pSH2/ α -SYN and pSH/CON stably transfected cells were then named MN9D/ α -SYN⁻ and MN9D/CON, respectively.

3.3 Effects of silencing α -synuclein expression on cell proliferation and viability No significant differences in cell morphology were observed among the three groups of MN9D cells (data not shown). As shown in the growth curve in Fig. 7A, cell proliferation of MN9D/ α -SYN⁻ and MN9D/CON cells was decreased compared with control MN9D cells. However, there were no significant differences in cell proliferation between MN9D/ α -SYN⁻ and MN9D/CON cells (Fig. 7A). Thus,

Fig. 2 Analysis of the PCR reaction mixture of pSH1/ α -SYN, pSH2/ α -SYN and pSH/CON transformant by 4% agarose gel electrophoresis. Lanes 1, 2 and 3 are pSH1/ α -SYN, pSH2/ α -SYN and pSH/CON PCR reaction mixture, respectively; Lane 4, 50 bp DNA ladder.

Fig. 3 Analysis of α -Synuclein mRNA levels by RT-PCR. Lane 1, 2 and 3 are pSH1/ α -SYN, pSH2/ α -SYN and pSH/CON transfected MN9D cells; Lane 4, MN9D cells; Lane 5, 100 bp DNA ladder.

Fig. 4 Statistical analysis of α -synuclein gene expression by real-time RT-PCR. The α -synuclein mRNA level was decreased by approximately 90% in pSH2/ α -SYN transfected cells (MN9D/ α -SYN⁻) compared with normal MN9D cells. It showed significant difference in α -synuclein gene expression in pSH2/ α -SYN transfected cells compared with MN9D and MN9D/CON cells ($n = 5$, $**P < 0.01$), while no significant difference was shown in pSH1/ α -SYN transfected cells.

Fig.5 Immunocytochemistry staining of MN9D cells, pSH1/ α -SYN, pSH2/ α -SYN, and pSH/CON transfected MN9D cells with anti- α -synuclein antibody. Strong intensity of α -synuclein immunocytochemistry was found in normal MN9D cells and pSH1/ α -SYN transfected, pSH/CON transfected MN9D cells except pSH2/ α -SYN transfected MN9D cells. A: normal MN9D cells; B, C, D are pSH1/ α -SYN, pSH/CON, pSH2/ α -SYN transfected MN9D cells, respectively. Scale bar, 10 μ m.

Fig.6 α -Synuclein protein expression was inhibited in pSH2/ α -SYN transfected cells. A: Western blot analysis of α -synuclein protein levels; Lane 1, MN9D cells; Lane 2, MN9D/CON cells; Lane 3, MN9D/ α -SYN⁻ cells. B: Statistical analysis of α -synuclein protein levels in MN9D, MN9D/CON, MN9D/ α -SYN⁻ cells by linear density ratio of α -synuclein/ β -actin. α -Synuclein protein level decreased significantly in MN9D/ α -SYN⁻ cells (0.150 ± 0.004) compared with MN9D (0.401 ± 0.038) and MN9D/CON (0.321 ± 0.011) cells ($n = 5$, $**P < 0.01$).

Fig.7 Effects of silencing α -synuclein on cell proliferation and viability. A: Growth curves of normal MN9D and MN9D/CON, MN9D/ α -SYN⁻ cells. —Normal MN9D cells, ----MN9D/CON cells, ····MN9D/ α -SYN⁻ cells; B: Cell viabilities assayed by MTT. The cell viability decreased in MN9D/ α -SYN⁻ and MN9D/CON cells. It decreased significantly in MN9D/ α -SYN⁻ cells compared with normal MN9D and MN9D/CON cells (** $P < 0.01$).

the differences in cell proliferation are probably not due to α -synuclein silencing. Although no differences in proliferation were observed between MN9D/ α -SYN⁻ and MN9D/CON cells, cell viability was affected. As shown in Fig. 7B, the cell viability of MN9D/ α -SYN⁻ cells was significantly decreased compared with control MN9D and MN9D/CON cells ($n = 5$, $P < 0.01$). Cell viability was decreased by 58.3% and 50% in MN9D/ α -SYN⁻ cells (0.091 ± 0.006) compared with MN9D (0.217 ± 0.012) and MN9D/CON (0.189 ± 0.005) cells, respectively (Fig. 7B). These data suggest that α -synuclein may be important for the viability of MN9D cells.

4 Discussion

In the present study, we silenced the expression of α -synuclein in MN9D dopaminergic cells by vector mediated RNAi using the BLOCK-iTTM inducible H1 RNAi entry vector. We identified an effective targeting sequence for RNAi, which localized in the coding region of the mouse α -synuclein gene, and used this to generate MN9D/ α -SYN⁻ cells in which the expression of α -synuclein was stably silenced. Due to the endogenous expression of α -synuclein and the dopaminergic characteristics of MN9D cells, MN9D/ α -SYN⁻ is a good cellular model for studying the normal function of α -synuclein and examining its role in PD pathogenesis.

We chose two 19-nucleotide stretches within the coding region of the α -synuclein gene as the target sequences, because longer sequences may induce non-specific responses in mammalian cells^[45]. The sequences locate within

one same exon, and do not contain homology to other genes within the mouse genome. Furthermore, we selected the target sequences that lie in NAC domain and acid tail of α -synuclein structure separately for avoiding a site of RNA-protein interaction.

The BLOCK-iTTM inducible H1 RNAi entry vector was selected for several reasons. Firstly, the vector contains a kanamycin resistance gene for selection in *E. coli* and a ZeocinTM resistance marker to allow generation of stable cell lines that express the shRNA of interest. Secondly, the cloning site of the vector contains a 4-nucleotide overhang on the 5' end of each DNA strand, allowing directional cloning of the shRNA of interest. Thirdly, 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. Finally, the vector is Gateway[®]-adapted to allow easy transfer of the H1/TO RNAi cassette into any appropriate expression system for other RNAi applications (e.g. lentiviral system for stable delivery of regulated shRNA in hard-to-transfect or non-dividing mammalian cells).

The ds oligos annealing reaction showed a clearly detectable, higher molecular weight band that differed in size from each component ss oligos. Remaining unannealed ss oligo was also detectable. In this study, we estimated that the efficiency of the annealing reaction was greater than 50%.

We assessed the cell proliferation and viability of MN9D/ α -SYN⁻ cells and found that the cell viability of MN9D/ α -SYN⁻ cells was significantly decreased compared with MN9D and

MN9D/CON cells while cell proliferation was unaffected. These findings suggested that loss of α -synuclein might induce cell injury directly or indirectly. Based on previous reports, we propose that in PD pathology, a loss of functional α -synuclein, whether it has reduced expression or aggregation, will result in increased DA in neurons^[46]. A marked increase in cytosolic DA levels may account for decreased cell viability. DA can readily oxidize to generate hydrogen peroxide, superoxide and reactive DA quinones^[47,48], which are cytotoxic due to their inhibitory effects on the mitochondrial respiratory system^[49,50]. In addition, superfluous DA itself may be cytotoxic via inhibition of mitochondrial complex I activity^[51]. Thus, it is also possible that increased cytosolic DA levels induce mitochondrial dysfunction, which results in decreased viability in MN9D/ α -SYN⁻ cells.

In conclusion, we identified an effective targeting sequence for RNAi, which localized in the C-terminal coding sequence of the mouse α -synuclein gene. This work demonstrates effective gene silencing of α -synuclein in MN9D cells by a plasmid vector mediated RNA interference mechanism and provides the tool for studying the normal function of α -synuclein. Our data also demonstrate that silencing α -synuclein expression results in decreased cell viability. We propose that in cells DA itself and its oxidized products including reactive oxygen species and reactive DA quinones may lead to cell injury or even death, because silencing α -synuclein expression cannot remove excess DA from the cytosol. Further understanding the normal function of α -synuclein in maintaining DA homeostasis may help to identify novel preventative or therapeutic strategies for PD.

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RNA 干扰介导的 MN9D 细胞中 α -synuclein 表达抑制及其对细胞活力的影响

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摘要: 目的 使用载体介导的 RNA 干扰(RNA interference, RNAi)方法抑制 MN9D 细胞中的 α -synuclein 基因表达, 并检测其对细胞增殖和活力的影响。方法 在 α -synuclein 的开放读码区选择两段 19 个核苷酸的片段, 并选择一段与小鼠基因无同源性的阴性对照序列, 分别设计合成单链寡核苷酸, 退火融合成双链寡核苷酸。将双链寡核苷酸克隆入 pENTR/H1/TO 质粒载体, 鉴定正确后, 转染 MN9D 细胞, 通过筛选获得稳定的细胞克隆。使用 RT-PCR、Real-time PCR、免疫细胞化学染色和 Western blot 方法检测细胞内的 α -synuclein 的表达水平, 鉴定 RNAi 的效率。利用生长曲线和 MTT 法分别检测比较各组细胞的增殖和活力。结果 转染 pSH2/ α -SYN 的细胞与正常和转染 pSH/CON 的 MN9D 细胞相比, α -synuclein mRNA 及蛋白水平均明显减少; 而转染 pSH1/ α -SYN 的细胞 α -synuclein mRNA 及蛋白水平与两组对照细胞没有明显差别。抑制 α -synuclein 表达不影响细胞增殖, 但可以降低细胞活力。结论 结果表明, SH2/ α -SYN 是有效的小干扰 RNA (small interfering RNA, siRNA) 序列, 通过载体介导的 RNAi 方法可以有效抑制 MN9D 细胞中的 α -synuclein 表达, 这为进一步研究 α -synuclein 蛋白的生理功能及其在帕金森病(Parkinson's disease, PD)发病中的作用提供了良好的细胞模型; α -synuclein 在维持 MN9D 细胞活力中起到重要作用, α -synuclein 功能缺失会导致细胞直接或间接的损伤。

关键词: α -Synuclein; RNA 干扰; 帕金森病