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Knockdown of α -Synuclein Enhances Susceptibility to Staurosporine-Induced Apoptosis in Human Melanoma SK-MEL28 Cells

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Abstract: α -Synuclein (α -Syn) is a central player in the pathophysiology of dopaminergic neurodegeneration that occurs in Parkinson's disease. Recently, it has been demonstrated that α -Syn is highly expressed in human melanoma cell line, SK-MEL28 but is undetectable in non-melanocytic cutaneous carcinoma and normal skin. Its wild-type form was also found to inhibit apoptosis in response to various pro-apoptotic stimuli, making it another attractive candidate in oncogenesis besides γ -Syn. Therefore, the objective of this study was to investigate the cyto-toxic/protective roles of α -Syn in melanoma SK-MEL28 cells, by knockdown and subjecting cells to apoptotic stimulus, staurosporine. For knockdown, short hairpin RNAs (shRNAs) targeting α -Syn were constructed in the expression vector, pLKO.1 and then transfected into SK-MEL28 cells with pLKO.1-TRC control and pLKO.1-scramble shRNA as controls. Stably transfected cells were established by selection with puromycin and α -Syn protein expression was then confirmed by Western blotting. The effects of α -Syn knockdown on cell viability, morphology, expression of Bcl-2, Bcl-XL, Bax and cleaved caspase 9 and proliferative index after staurosporine treatment were studied. Cell viability MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay showed that the survival of SK-MEL28 with α -Syn knockdown was significantly reduced as compared to controls. Depletion of endogenous α -Syn was found to enhance staurosporine-induced cytotoxicity in SK-MEL28 with greater Bax/Bcl-2 and Bax/Bcl-xL ratios and cleaved caspase 9 level. α -Syn knockdown also reduced the proliferative index of SK-MEL28. Overall, these results suggest that the endogenously high α -Syn confers resistance against apoptosis in SK-MEL28 cells.

Key words: α -Synuclein, shRNA-knockdown, melanoma, staurosporine, apoptosis

INTRODUCTION

α -Synuclein (α -Syn) is an abundant presynaptic protein belonging to the family of proteins that include α -, β - and γ -Syn. Structurally, it is a 140 amino acid protein consisting of a N-terminal repeat region (1-95) which contains seven conserved repeat motifs believed to be involved in α -Syn interactions with lipids; a central hydrophobic NAC region (61-95) which is essential for the aggregation of α -Syn and an acidic C-terminus (96-140) which exhibits chaperone activity (El-Agnaf and Irvine, 2000; George *et al.*, 1995; Perrin *et al.*, 2000; Souza *et al.*, 2000). α -Syn has attracted considerable attention due to its involvement in neurodegenerative diseases. Extensive studies show that α -Syn is neurotoxic and it is implicated in the pathophysiology of Parkinson's Disease (PD) (Maries *et al.*, 2003). On the contrary, many evidences from previous literatures have reported a neuroprotective effect for α -Syn since it was found to inhibit apoptosis induced by a broad range of apoptotic stimuli or to

modulate the expression levels or activity of proteins implicated in the regulation of apoptosis onset (Da Costa *et al.*, 2000, 2002). Therefore, the exact cyto-toxic/protective roles conferred by α -Syn still remain elusive.

Apart from its central role in the pathophysiology of PD, α -Syn has recently been identified as another attractive candidate in carcinogenesis besides γ -Syn which has been detected in elevated levels in various types of cancer including breast, ovarian, gastric, liver, colorectal, uterine and lung cancers, particularly in advanced stages of the diseases (Ahmad *et al.*, 2007). Matsuo and Kamitani (2010) demonstrated that α -Syn is positively detected in primary and metastatic melanoma sections but is undetectable in non-melanocytic cutaneous carcinoma and normal skin. SK-MEL28 is one of the melanoma cell lines found to be highly overexpressing α -Syn. Thus far, not many studies have explored the role of α -Syn in cancer cells.

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In this present study, we assessed the ability of α -Syn to resist staurosporine-mediated apoptosis in human melanoma cell line, SK-MEL28 by stable short hairpin RNA (shRNA) knockdown. Hallmarks of apoptosis studied included cell viability, expression of apoptotic markers and cell cycle and proliferative index analysis, revealing that the endogenously high α -Syn confers resistance against apoptosis in SK-MEL28 cells.

MATERIALS AND METHODS

Cell culture, plasmids and transfection: Human melanoma cell line, SK-MEL28 was kindly donated by Dr. Yang-Mooi Lim, Faculty of Medicine and Health Science, UTAR. Cells were cultured in Minimum Essential Medium (MEM) (Cellgro® Media tech, Inc., VA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (i-DNA Biotechnology, Singapore) and 1% penicillin/streptomycin (Millipore, Billerica, MA) at 37°C in a 5% CO₂ humidified incubator. For efficient gene silencing, three short interfering RNA (siRNA) oligonucleotides specifically targeting α -Syn mRNA were determined using the siRNA Selection Program hosted by Whitehead Institute for Biomedical Research (Yuan *et al.*, 2004). Table 1 shows the target regions of each designated shRNA oligonucleotides which are indicated on the basis of the base sequences and nucleotide numbering as shown in the human α -Syn mRNA (BC108275). The forward and reverse oligonucleotides were then annealed and linked into pLKO.1-TRC cloning vector (Moffat *et al.*, 2006) sourced from Addgene, MA. Linearized pLKO.1-TRC control, pLKO.1-scramble shRNA and three pLKO.1 vectors, each cloned with different shRNA sequence targeting the human α -Syn mRNA were transfected into SK-MEL28 by electroporation (ECM 830 ElectroSquarePorator; BTX Harvard Apparatus, MA). Stably expressing transfectants were selected using 3 μ g mL⁻¹ puromycin (BioWORLD, OH) for 10-14 d and polyclonal pools of stably transfected cells were maintained.

Western blot analysis: Cells were harvested in lysis buffer (10 mM Tris/HCl, pH 7.8, 100 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.05% (v/v) nonidet-P40, 10 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride) containing protease inhibitor cocktail (Sigma, MO). Concentration of supernatant protein lysates were quantified using bicinchoninic acid (BCA) based on colorimetric detection at 562 nm using the BCA™ Protein Assay Kit (Thermo Scientific, MA). Equal amounts of total protein (40 μ g well⁻¹) were resolved on 15% polyacrylamide gel and transferred to polyvinylidene

Table 1: shRNA oligonucleotides against human α -Syn mRNA sequence (BC108275)

Name	Target region in human α -Syn mRNA	
	Base sequences	Nucleotide numbering
HuAsynRNAi1	5'-AAAGTGGCGGTTAGGATATAT-3'	2575-2595
HuAsynRNAi2	5'-AATGAGTGACTATAAGGATGG-3'	2385-2405
HuAsynRNAi3	5'-AAGGACCAGTTGGCAAGAAT-3'	552-572

difluoride membranes (Millipore, MA). The membranes were first blocked in Phosphate-Buffered Saline-Tween 20 (PBS-T) containing 5% non-fat milk and then probed with primary antibodies, mouse anti- α -Syn, clone 7B12.2 (1:1000) (Chemicon, CA), rabbit anti-Bax, anti-Bcl-2, anti-Bcl-xL or anti-cleaved caspase 9 (Asp 315) (1:1000) (Cell Signaling Technology, MA), followed by secondary HRP conjugated rabbit anti-mouse IgG (Calbiochem, CA) or goat anti-rabbit IgG (1:10,000) (Nacalai Tesque, Kyoto, Japan). Bands were visualized via enhanced chemiluminescence (ECL) detection system (Thermo Scientific, MA) according to the manufacturer's instructions and image acquisition was performed using FluorChem FC2 system (Alpha Innotech, CA). For repeated hybridization with anti-actin clone C4 as loading control, the membrane was stripped in stripping buffer (0.4 M glycine, 2% v/v Tween-20, 0.2% SDS, pH 2.2), washed and further incubated in anti-actin (Millipore, MA) at 1:5000 dilution. AlphaView® (TX) software was used for densitometry analysis.

Immunofluorescence microscopy: Cells were seeded onto coverslips and grown to 50% confluency for a period of 48-72 h. Cells were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) in PBS and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Cells were then blocked with 2% goat serum (Sigma-Aldrich) in PBS and then incubated with primary antibody, anti- α -Syn clone 7B12.2 at dilution of 1:200. The coverslips were then washed with PBS and incubated with secondary antibody, goat anti-mouse IgG Dylight 488 conjugated (Thermo Scientific) at dilution of 1:100. After extensive washing with PBS, coverslips were then mounted on slides using buffered glycerol (90% glycerol in PBS). Cells were visualized using inverted-type fluorescence microscope (Nikon Eclipse TS100) and photomicrographs were captured at 100 x magnification.

Staurosporine treatment and MTT assay: Staurosporine (Millipore) of 10mM master stock was prepared in Dimethyl Sulfoxide (DMSO). Untransfected and stably transfected SK-MEL28 cells were plated on 96-well plates (BD Falcon, MD) at density of 1×10^4 cells per well. After overnight incubation at 37°C in a humidified 5% CO₂

atmosphere, cells were treated with staurosporine at concentrations of 0.5 and 1.0 μM . Untreated untransfected cells were used as control samples. Cell morphology was examined under the inverted microscope. Following 24h incubation, 20 μL of aqueous MTT solution (5 mg mL^{-1}) (Biobasic Inc, Ontario, Canada) was added into each well for 4 h. The MTT solution was carefully decanted off and 100 μL DMSO was added into each well to solubilize the purple formazan crystals. The plate was kept in the dark and gently shaken for 15 min. The plate was then transferred to a microplate plate reader (Bio-Rad, Model 680 with Microplate Manager Software) and the absorbance was measured at 550 nm. The intensity of color produced is directly proportional to the number of viable cells. Percentage cell viability could be calculated from the absorbance values. Graph of percentage cell viability against the corresponding concentrations of drug was plotted. LD_{50} , the concentration of a drug substance required to kill half the members of a tested population after a specified test duration was determined directly from the graph.

Propidium iodide staining for flow cytometric analysis:

Stably-transfected SK-MEL 28 cells were seeded into 6-well plate with optimal seeding densities of 2×10^5 cells/well. After overnight incubation, cells were treated with staurosporine at the concentration of LD_{50} for 24 h. Untransfected cells served as control. The cells were then harvested, washed with PBS and fixed with 700 μL of ice-cold ethanol for at least 2 h at 4°C . Ethanol was added dropwise while vortexing the cells to ensure fixation of cells and to minimize clumping. Fixed cells were pelleted, washed with PBS, stained with 10 $\mu\text{g mL}^{-1}$ of propidium iodide (Sigma Aldrich, MO) in PBS, treated with 100 $\mu\text{g mL}^{-1}$ of RNaseA (Fermentas, Burlington, Canada) and were incubated for 30 min at 37°C . A laser-based flow cytometer (FACSCalibur, Becton Dickinson, NJ) was used for this cellular DNA content analysis. Propidium iodide fluorescence could be detected in the FL2 channel. For each acquisition of flow cytometric analysis, 10,000 of cells were collected and the flow rate was kept under 400 events sec^{-1} . Results were displayed as dot plot and histograms. Percentages of cells in the sub G_0 , G_0/G_1 , S, G_2/M phases of the cycle were calculated using Scripps Research Institute's WINMDI™ software. The Proliferative Index (PI) was calculated using the formula:

$$\text{PI} = \frac{(S+G_2)}{(G_1+S+G_2)}$$

Statistical analysis: Data were expressed as Mean \pm Standard Error of the mean (SEM) of three

independent experiments which were performed in triplicates, unless otherwise stated. Statistical analysis was performed by one-way Analysis of Variance (ANOVA) followed by LSD's post hoc test for multiple comparisons using the SPSS 15.0 software (SPSS Inc., IL), A p-value of less than 0.05 was considered as statistical significant.

RESULTS

α -Syn-shRNA down-regulates α -Syn expression:

Western blot revealed a single band of 19 kDa that is consistent with the monomeric α -Syn for untransfected SK-MEL 28 cells which has been demonstrated by Matsuo and Kamitani (2010) to be highly expressing α -Syn (Fig. 1a). Compared with controls, α -Syn-shRNAs HuAsynRNAi1 and HuAsynRNAi2 were found to inhibit α -Syn protein expression by 12.0 and 2.50% only while α -Syn-shRNA HuAsynRNAi3 inhibited α -Syn protein expression significantly with approximately 49% reduction (Fig. 1b). HuAsynRNAi3 appeared to be more effective than HuAsynRNAi1 and HuAsynRNAi2 in α -Syn gene silencing. This sequence has also been confirmed to be valid for α -Syn gene silencing by two previous reports (Sapru *et al.*, 2006; Wu *et al.*, 2009). pLKO.1-TRC control and pLKO.1-scramble shRNA had no effect on α -Syn protein level. These results demonstrate that α -Syn-shRNA transfection is an effective and long-term suppression method of endogenous α -Syn. Human U6 promoter could effectively drive shRNA transcription in SK-MEL28 cells and shRNA HuAsynRNAi3 could successfully bring about knockdown of *SNCA* mRNA expression. Immunofluorescence microscopy further confirmed expression of human α -Syn in SK-MEL28 cells. Untransfected SK-MEL28, pLKO.1-TRC control-transfected SK-MEL28, pLKO.1-scramble shRNA-transfected SK-MEL28, pLKO.1-HuAsynRNAi1-transfected SK-MEL28 and pLKO.1-HuAsynRNAi2-transfected SK-MEL28 cells exhibited a strong homogenous expression of α -Syn, whereas pLKO.1-HuAsynRNAi3-transfected SK-MEL28 showed lower fluorescence intensity, indicative of lower α -Syn immunoreactivity (Fig. 1c). pLKO.1-HuAsynRNAi3-transfected SK-MEL28 with significant down-regulation of α -Syn was then chosen to be used in subsequent experiments.

α -Syn knockdown enhances staurosporine-induced cytotoxicity:

To determine the effect of α -Syn expression against staurosporine-induced cell death, the relative cytotoxic response to staurosporine treatment in untransfected SK-MEL28 (as control), pLKO.1-TRC

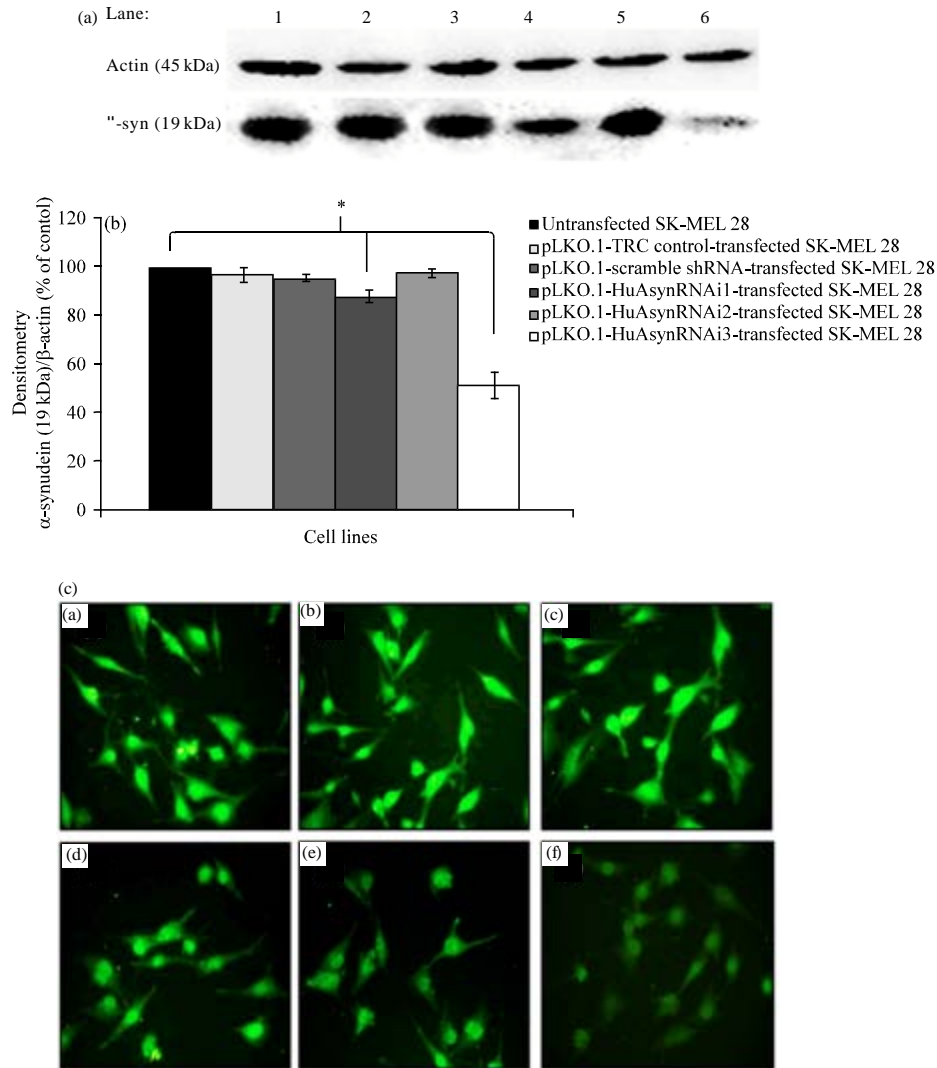


Fig. 1: Expression of α -Syn in SK-MEL28 cells. (a) Western blot analysis with anti- α -Syn clone 7B12.2. Lane 1: Untransfected SK-MEL28; Lane 2: pLKO.1-TRC control-transfected SK-MEL28; Lane 3: pLKO.1-scramble shRNA-transfected SK-MEL28; Lane 4: pLKO.1-HuAsynRNAi1-transfected SK-MEL28; Lane 5: pLKO.1-HuAsynRNAi2-transfected SK-MEL28; Lane 6: pLKO.1-HuAsynRNAi3-transfected SK-MEL28. (b) The relative densitometry. Each bar represents the Mean \pm SEM of two different experiments. (c) Immunofluorescence photomicrographs of SK-MEL28. (a) Untransfected SK-MEL28 (b) pLKO.1-TRC control-transfected SK-MEL28 (c) pLKO.1-scramble shRNA-transfected SK-MEL28 (d) pLKO.1-HuAsynRNAi1-transfected SK-MEL28 (e) pLKO.1-HuAsynRNAi2-transfected SK-MEL28 (f) pLKO.1-HuAsynRNAi3-transfected SK-MEL28. Cells were visualized using inverted-type fluorescence microscope (Nikon Eclipse TS100) and photomicrographs were captured at 100 \times magnification. $p < 0.05$ (*) indicates comparison between pLKO.1-HuAsynRNAi3-transfected and untransfected SK-MEL28 cells by one-way-ANOVA

control-transfected SK-MEL28, pLKO.1-scramble shRNA-transfected SK-MEL28 and pLKO.1-HuAsynRNAi3-transfected SK-MEL28 were analyzed. All four cell lines were exposed to 0.50 and 1.00 μ M staurosporine for 24 h

before MTT assay was conducted. Based on the MTT assay, a concentration-dependent decrease in cell viability was observed (Fig. 2). Following exposure to 0.50 and 1.00 μ M staurosporine, pLKO.1-HuAsynRNAi

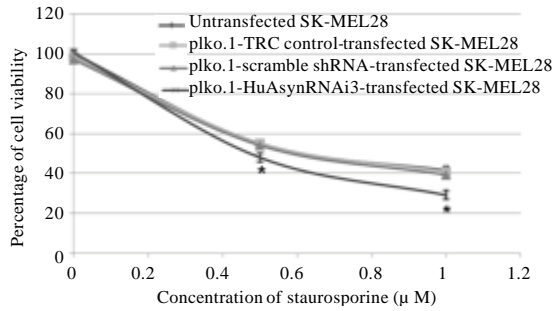


Fig. 2: α -Syn knockdown enhances staurosporine-induced cytotoxicity. Cells were exposed to staurosporine at two serial diluted concentrations (0.50 and 1.00 μ M) for 24 h before MTT assay was performed and absorbance was read at 550 nm. Results represent Mean \pm SEM of three separate experiments. $p < 0.05$ (*) indicates comparison between pLKO.1-HuAsynRNAi3-transfected and untransfected SK-MEL28 cells by one-way-ANOVA

3-transfected SK-MEL28 with α -Syn knocked-down significantly exhibited a maximal decrease in cell viability. LD₅₀ of staurosporine for untransfected SK-MEL28 cells was determined to be 0.50 μ M and this concentration was used for subsequent experiments. The results demonstrate that endogenously high α -Syn expression can protect SK-MEL28 cells from staurosporine-induced cell death.

Expression of apoptotic markers in SK-MEL28: To determine whether the knockdown of α -Syn expression in SK-MEL28 could further elicit apoptotic stress induced by staurosporine, the expression of downstream pro- and anti-apoptotic molecules of the intrinsic (mitochondrial) apoptotic pathway was investigated. Elevated Bax level has been shown to promote apoptosis in response to numerous cell death-inducing stimuli (Oltvai and Korsmeyer, 1994). pLKO.1-HuAsynRNAi3-transfected SK-MEL28 had significantly greater Bax protein expression, both endogenously and after staurosporine treatment, as compared to untransfected SK-MEL28 (control), pLKO.1-TRC control-transfected SK-MEL28 and pLKO.1-scramble shRNA-transfected SK-MEL28 (Fig. 3a, b).

Bcl-2 and Bcl-xL have been shown to prevent apoptotic cell death induced by a variety of stimuli in several different systems (Zhang *et al.*, 2003; Li *et al.*, 2001). SK-MEL28 cells transfected with pLKO.1-HuAsynRNAi3 had the lowest basal Bcl-2 and

Bcl-xL levels (Fig. 3a, c, d). After exposure to 0.50 μ M staurosporine for 24 h, the Bcl-xL level further significantly decreased in the same trend, whereas Bcl-2 expression in all the cells was significantly increased. This increased Bcl-2 expression could be a compensatory response of the cells against the staurosporine insult. However, pLKO.1-HuAsynRNAi3-transfected SK-MEL28 was shown to have significantly lower Bcl-2 expression, compared with control.

Caspase 9 is an important member of the cysteine aspartic acid protease (caspase) family (Duan *et al.*, 1996). Upon apoptotic stimulation, cytochrome c released from mitochondria associates with procaspase 9 (47 kDa)/Apaf-1. This complex processes procaspase 9 into a large active subunit (35 or 17 kDa) and a small subunit (10 kDa) by self cleavage at Asp315 (Li *et al.*, 1997). Cleaved caspase 9 could only be detected in staurosporine-treated cells and pLKO.1-HuAsynRNAi3-transfected SK-MEL28 had the greatest cleaved caspase 9 level as compared to untransfected SK-MEL28 (control), pLKO.1-TRC control-transfected SK-MEL28 and pLKO.1-scramble shRNA-transfected SK-MEL28 (Fig. 3a, e).

Bax/Bcl-2 and Bax/Bcl-xL ratios determine the apoptotic potential of the cells (Chang *et al.*, 2005; Raisova *et al.*, 2001). pLKO.1-HuAsynRNAi3-transfected SK-MEL28 was shown to have elevated Bax/Bcl-2 and Bax/Bcl-xL ratios significantly as compared to controls, both before and after staurosporine treatment (Fig. 3f, g). Taken together, these results suggest that the endogenously high α -Syn expression in SK-MEL28 can increase Bcl-2 and Bcl-xL expression, attenuate Bax upregulation and cleavage and activation of caspase 9, thereby confers protective effect against staurosporine-induced cell death.

α -Syn knockdown reduces the proliferative index of SK-MEL28: To explore the possible roles of α -Syn in controlling cell proliferation, the cell cycles of untransfected and stably transfected cells were examined by flow cytometric analysis with propidium iodide staining. As depicted in Fig. 4a and b, in general, treatment with staurosporine led to an increase of cells in the G₂/M phase of cell cycle, with a corresponding decrease of cells in the G₁ and S phase of cell cycle when compared to untreated cells. Cells subjected to staurosporine treatment also displayed an increase in the sub G₀ (hypodiploid) portion, as a consequence of partial DNA content loss due to fragmentation. No significant difference could be observed between these four cell types for all the phases after staurosporine treatment. However, the basal mean proliferative index of pLKO.1-HuAsynRNAi3-transfected SK-MEL28 with

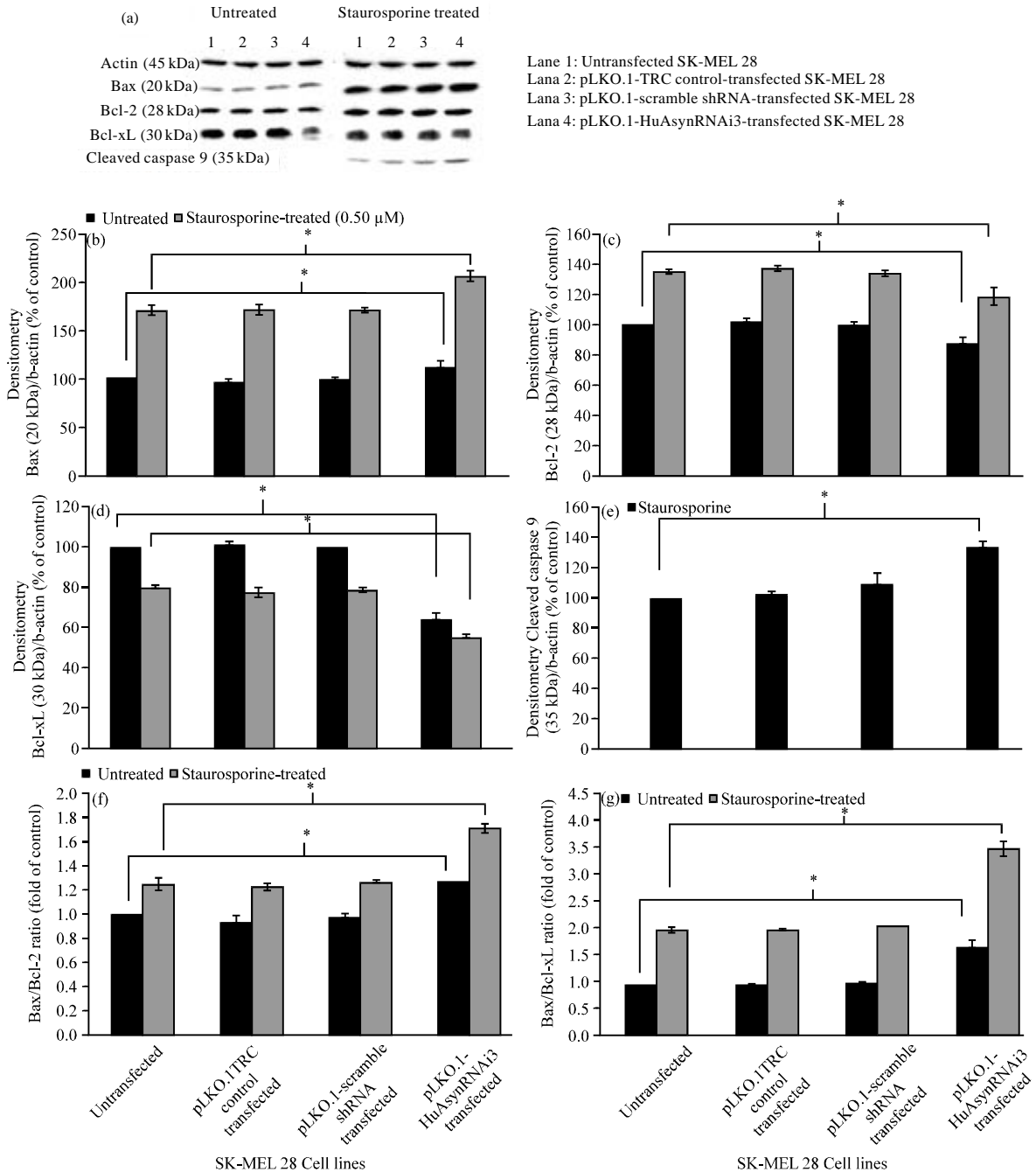


Fig. 3: Depletion of endogenous α -Syn in SK-MEL28 cells increases Bax/Bcl-2 and Bax/Bcl-xL ratios and cleaved caspase 9 level. (a) Expression of Bax, Bcl-2, Bcl-xL and cleaved caspase 9 in SK-MEL28 cell lines by Western blot analysis and (b-e) show their relative densitometries. Ratios of (f) Bax/Bcl-2 and (g) Bax/Bcl-xL in SK-MEL28 were quantified. Cells were incubated in the absence or presence of 0.50 μ M staurosporine for 24 h and cell lysate were prepared and subjected to Western blot analysis. The Bcl-2 /Bax and Bcl-xL/Bax ratios in control (untreated and untransfected) cells were set as 1.00. Results represent Mean \pm SEM of two separate experiments. $p < 0.05$ (*) indicates comparison between pLKO.1-HuAsynRNAi3-transfected and untransfected SK-MEL28 cells under the same treatment by one-way-ANOVA

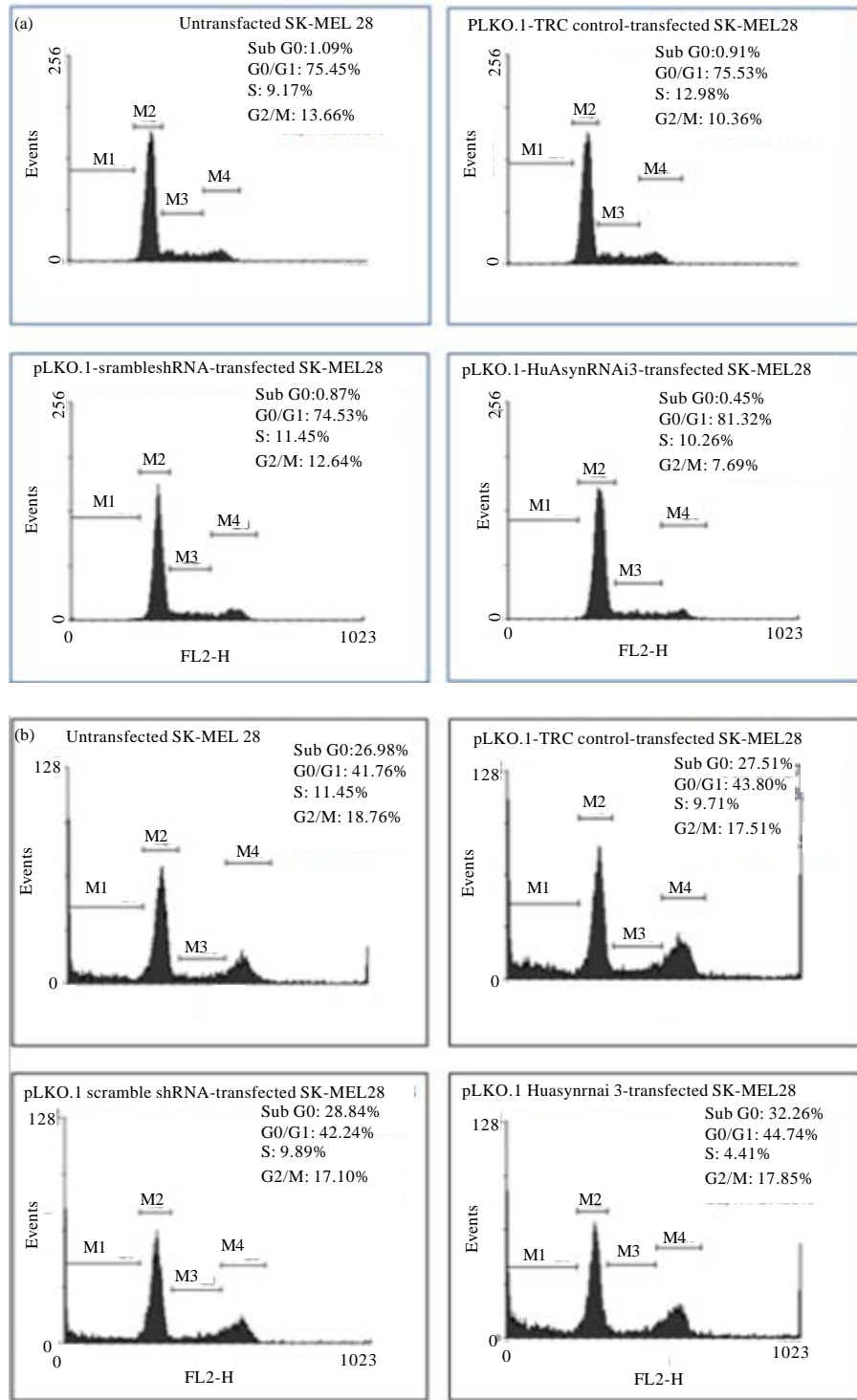


Fig. 4: Continued

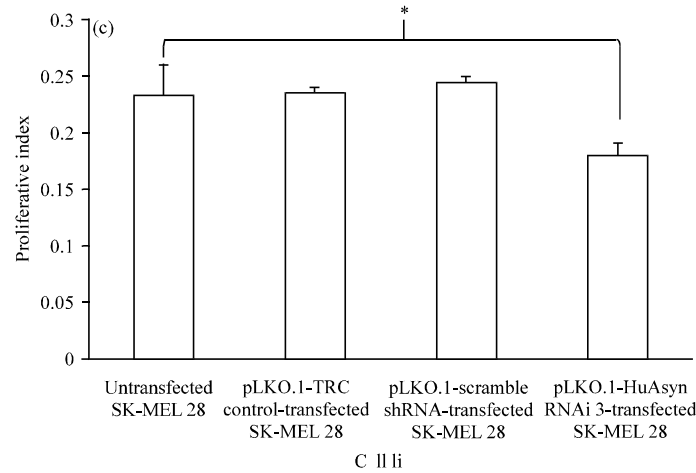


Fig. 4: α -Syn knockdown reduces the proliferative index of SK-MEL28. Cell cycle distribution of (a) untreated and (b) staurosporine-treated SK-MEL28 cell lines. SK-MEL28 cell lines were incubated in the absence or presence of staurosporine for 24 h before being harvested, fixed with 70% ethanol for up to 2hr followed by propidium iodide staining and flow cytometric analysis. The percentage of cells in each phase of the cell cycle was evaluated using the WinMDI software. Representative histograms from one experiment are shown. M1 = Sub G₀/G₁ phase, M2 = G₀/G₁ phase, M3 = S phase, M4 = G₂/M phase. (c) Basal mean proliferative index was calculated using the equation $(S + G_2)/(G_1 + S + G_2)$. These results represent Mean \pm SEM of three independent experiments. $p < 0.05$ (*) indicates comparison between pLKO.1-HuAsynRNAi3-transfected and untransfected SK-MEL28 cells by one-way-ANOVA

α -Syn expression being downregulated was significantly lower than those of untransfected SK-MEL28, pLKO.1-TRC control-transfected SK-MEL28 and pLKO.1-scramble shRNA-transfected SK-MEL28 (Fig. 4c). The endogenously high α -Syn expression could promote G₀/G₁ to S transition and G₂/M in SK-MEL28 and therefore, might have contributed to the enhanced proliferation rate.

DISCUSSION

This study assessed the cytoprotective role of α -Syn in melanoma SK-MEL28 cells. The main novel finding is that the endogenously high expression of α -Syn is essential for survival of SK-MEL28 cells as its gene silencing by shRNA interference resulted in enhanced staurosporine-induced cytotoxicity with greater levels of Bax/Bcl-2 and Bax/Bcl-xL ratios and cleaved caspase 9. α -Syn is only recently discovered to be highly expressed in cancer cells. Therefore not many studies have elucidated its anti-apoptotic property in cancer cells. The cytoprotective effect shown by endogenously strong α -Syn expression in SK-MEL28 cells against staurosporine toxicity could only be generally related to the well-known studies of another Synuclein family member, γ -Syn which plays vital role in carcinogenesis

(Ahmad *et al.*, 2007) and also could be related to the anti-apoptotic property of α -Syn in neuronal cell lines (Da Costa *et al.*, 2000).

Both α - and γ -Syn share substantial sequence homology as each has a series of loosely repeated motifs throughout the first 93 amino acids and an acid region toward the C-terminus (Lavedan, 1998). γ -Syn has been reported to be 50% identical and 74% homologous to α -Syn (Czekierdowski and Czekierdowska, 2007). γ -Syn, initially termed as Breast Cancer-specific Gene 1, has been found at abundance levels in majority of advance-stage breast, ovarian, gastric, liver, colorectal, uterine and lung cancers, compared to its almost undetectable levels in normal or benign counterparts (Bruening *et al.*, 2000; Liu *et al.*, 2007; Morgan *et al.*, 2009; Yanagawa *et al.*, 2004; Ye *et al.*, 2008; Zhao *et al.*, 2006). Oncogenic activation of γ -Syn was found to contribute to the development of breast and ovarian cancer by promoting tumour cell survival under adverse conditions and by providing resistance to certain chemotherapeutic drugs via modulation of MAPK pathway. γ -Syn is associated with two major MAPKs, i.e., ERK1/2 and JNK1 and it has been shown that overexpression of γ -Syn leads to constitutive activation of ERK1/2 and down-regulation of JNK1 in response to a host of environmental stress signals (Pan *et al.*, 2002). Knockdown of γ -Syn sensitized

human breast cancer cells to endoplasmic reticulum stress-induced apoptosis. Induction of apoptosis by endoplasmic reticulum stress when γ -Syn was down-regulated was reported to be dependent on modulation of JNK or involvement of caspase 3 and 7 activation (Hua *et al.*, 2009). These data suggest that the up-regulation of γ -Syn (and α -Syn) expression in these human malignancies promotes increase in cancer cell motility and disease progression. The mechanism underlying the anti-apoptotic effect of cellular α -Syn in SK-MEL28 cells against staurosporine could be therefore, speculated to be linked to the activation of ERK1/2 and down-regulation of JNK1, similar to that of γ -Syn in various malignancies.

The precise cellular mechanism underlying the anti-apoptotic function of α -Syn remains to be elucidated. However, one could postulate on the involvement of the chaperoning property of α -Syn. Studies have shown that α -Syn shares a physical and functional homology with the chaperone protein 14-3-3 and its interactions with various cellular proteins like dephosphorylated Bad, protein kinase C (Ostrerova *et al.*, 1999), ERK, PI3/Akt kinase (Seo *et al.*, 2002) and cytochrome c (Ostrerova *et al.*, 1999) have been thus far well established. These further support the concept that α -Syn might counteract the activation of cell death signaling molecules during apoptotic insult.

To date, studies have suggested that the anti-apoptotic effect of α -Syn in neuronal cells seems to be mediated through a variety of factor including decreased expression of p53 (Da Costa *et al.*, 2000), stimulation of the PI3/Akt signaling pathway (Gomez-Santos *et al.*, 2002), inactivation of JNK (Hashimoto *et al.*, 2002), increase in the expression levels of the anti-apoptotic protein Bcl-2 and inhibition of the activity of pro-apoptotic proteins (Sidhu *et al.*, 2004). Thus, one could speculate that these few mechanisms might at least in some part be accounted for the anti-apoptotic properties exhibited by α -Syn against staurosporine treatment in our current study.

In this study, we could observe that knockdown of α -Syn in SK-MEL28 reduced the cell proliferative index. In support of these findings, overexpression of α -Syn in PC12 cells resulted in enhanced proliferation rate and enrichment of cells in the S phase of the cell cycle (Lee *et al.*, 2003). This was associated with increased accumulation of the mitotic factor cyclin B, down-regulation of the tumour suppressor retinoblastoma 2 and increased phosphorylation of ERK1/2, the key molecules in proliferation signaling (Lee *et al.*, 2003). Therefore, it is suggested that α -Syn expression could promote cell cycle progression and thus contribute to enhanced proliferation rate.

As α -Syn overexpression has been implicated to be involved in the melanoma cells' resistance to apoptosis which leads to cancer progression, the possibility of using α -Syn as a potential target for melanoma drug therapy could be examined. Moreover, one could consider using α -Syn as a target to overcome staurosporine-related drug resistance, for example in the case of metastatic melanoma Mel-RM cell line which is partially resistant to staurosporine (Zhang *et al.*, 2004).

CONCLUSION

In conclusion, α -Syn knockdown was found to aggravate staurosporine-induced cytotoxicity, indicating that endogenous α -Syn confers protective effect in SK-MEL28 cells. This study, thus, provides insights concerning the molecular mechanisms of α -Syn in melanoma cancer biology. The underlying influences of α -Syn against staurosporine-induced apoptosis highlighted could facilitate prospective translation into a therapeutic target for melanoma.

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