ELUCIDATION OF THE ROLES OF γ-SYNUCLEIN IN THE INVASIVENESS AND SURVIVAL OF COLORECTAL CANCER CELL LINE, LS 174T

By

GOH KAI WEY

A dissertation submitted to the Department of Biomedical Science,
Faculty of Science,
Universiti Tunku Abdul Rahman,
in partial fulfilment of the requirements for the degree of
Master of Science
June 2015

ABSTRACT

ELUCIDATION OF THE ROLES OF γ-SYNUCLEIN IN THE INVASIVENESS AND SURVIVAL OF COLORECTAL CANCER CELL LINE, LS 174T

Goh Kai Wey

γ-Synuclein, a neuronal protein of the synuclein family, is involved in carcinogenesis. To investigate its role in colorectal cancer carcinogenesis, γ-synuclein was over-expressed in LS 174T colon adenocarcinoma cell line (termed LS 174T-γsyn). When compared with untransfected or mock transfectants, LS 174T-γsyn had higher mobility in scratch wound assay, tend to scatter more in cell-scattering assay, and had enhanced lamellipodia and filopodia formation in cell-spreading assay. Enhanced adhesion of LS 174T-γsyn to fibronectin and collagen and significantly higher proliferation rate showed that γ-synuclein was able to increase extracellular matrix interaction and promoted proliferation of LS 174T. Higher invasiveness of LS 174T-γsyn was evidenced by enhanced invasion to the bottom of the basement membrane in Boyden chamber assay. However, LS 174T-γsyn was significantly more vulnerable to doxorubicin, vincristine and hydrogen peroxide insults, via apoptotic cell death. LS 174T-γsyn also had reduced anchorage-independent growth as shown by reduced colony formation and reduced anoikis resistance. The over-expression of γ-synuclein was found to confer both pro-invasive and doxorubicin-mediated pro-apoptotic properties to LS 174T, where the former was mediated through enhanced cyclic adenosine monophosphate response

element binding protein (CREB) phosphorylation, while the latter involved hepatocyte growth factor (HGF) down-regulation and subsequent downstream signalling pathways possibly involving extracellular signal-regulated kinases (ERK)1/2, p38α, c-JunNterminal kinase (JNK) pan and Signal Transducers and Activators of Transcription (STATs). This unexpected contrasting finding as compared to other similar studies on colon cancer cell lines might be correlated with the degree of tumour advancement from which the cell lines were derived from.

ACKNOWLEDGEMENT

Upon completion of this dissertation, I would like express my gratitude to my supervisor, Dr. Say Yee How and co-supervisor Dr. Michelle Ng Yeen Tan for their priceless advices and guidance throughout the progression of this research project. Special thanks to UTAR Research Fund IPSR/RMC/UTARRF/C210/S1 and IPSR/RMC/UTARRF/2012-C2/G05 for funding this project.

Furthermore, I would like to utter my distinctive thanks to all the helpful lab mates for their valuable supports and accompaniment in making my research such a wonderful and enjoyable experience. I would like to thank them for all their helps, interests and generosity in sharing their knowledge with me.

Last but not least, I would like to thank my family for the never ending loves and supports they had bestowed upon me in the completion of this research project.

APPROVAL SHEET

This dissertation entitled "**ELUCIDATION OF THE ROLES OF γ-SYNUCLEIN IN THE INVASIVENESS AND SURVIVAL OF COLORECTAL CANCER CELL LINE, LS 174T**" was prepared by GOH KAI WEY and submitted as partial fulfilment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

Approved by:

_____
(Dr. Say Yee How)
Date:……..
Associate Professor/Supervisor
Department of Biomedical Science
Faculty of Science
Universiti Tunku Abdul Rahman

_____
(Dr. Michelle Ng Yeen Tan)
Date:……..
Assistant Professor/Co-supervisor
Department of Biomedical Science
Faculty of Science
Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

SUBMISSION OF DISSERTATION

It is hereby certified that **GOH KAI WEY** (ID No: **11ADM03677**) has completed this dissertation entitled "ELUCIDATION OF THE ROLES OF γ-SYNUCLEIN IN THE INVASIVENESS AND SURVIVAL OF COLORECTAL CANCER CELL LINE, LS 174T" under the supervision of Dr. Say Yee How (Supervisor) from the Department of Biomedical Science, Faculty of Science, and Dr. Michelle Ng Yeen Tan (Co-Supervisor) from the Department of Biomedical Science, Faculty of Science.

I understand that University will upload softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____
(GOH KAI WEY)

DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

Name _____

Date _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	One-way analysis of variance
BCSG-1	Breast Cancer-specific Gene 1
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CBP	CREB-binding protein
CMV	Cytomegalovirus
CREB	cAMP response element-binding protein
DCF	2',7'-dichlorofluorescein
DCFH	2',7' dichlorofluorescin
DCFH-DA	2', 7'-dichlorofluorescein diacetate
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin hydrochloride
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyante
HER	Human epidermal growth factor receptor
HGF	Hepatocyte Growth Factor
hr	Hour
HRP	Horseradish peroxidase
HSP60	Heat Shock Protein 60
JAK	Janus kinases
JNK	c-Jun N-terminal kinase
H_2O_2	Hydrogen peroxide

LC_{75}	Lethal concentration 75
LB	Luria-Bertani
LSD	Least Significant Difference
mAb	Monoclonal antibody
MAP2	Microtubule associated protein 2
MAPK	Mitogen-activated protein kinase
min	Minute
MMP	Matrix metalloproteinases
MMP-9	Matrix metalloproteinase 9
MSK	Mitogen and stress activated protein kinase
MT1-MMP	Membrane type 1 metalloproteinase
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NAC	Non-Aβ-component
OD	Optical density
PCR	Polymerase chain reaction
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PLD2	Phospholipase D2
Poly-HEMA	Poly(2-hydroxyethyl methacrylate)
PS	Phosphotidylserine
PVDF	Polyvinylidene difluoride
RFU	Relative fluorescence unit
s	Second

ROS	Reactive oxygen species
shRNA	Short hairpin RNA
siRNA	Small Interfering RNA
SEM	Standard error of mean
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
STAT	Signal transducers and activators of transcription
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
VIN	Vincristine sulphate

CHAPTER 1

INTRODUCTION

Synuclein belongs to a highly soluble neuronal protein family consists of three isoforms which includes α-synuclein, β-synuclein, γ-synuclein (Ahmad et al., 2007; Surguchov, 2013). The biological and biochemical functions of all the three members are not entirely understood. All three synuclein genes are composed of five similar sizes of coding exons, and the overall gene organizations are very well conserved (Lavedan, 1998). Synuclein proteins are small, constituting only 127–140 amino acids (Surguchov, 2013). They are a natively unfolded group of proteins that are characterized by five to six KTKEGV amino acid sequence repeats, constituting the amino end of the proteins (Lavedan, 1998; Lavedan et al., 1998). These repeats probably result in the formation of conserved amphipathic class A2-helices when it reversibly binds to phospholipid membranes since it resembles the characteristics of apolipoproteins (Lavedan, 1998; George, 2002; Ahmad et al., 2007). This property supports the role of synucleins in vesicular release at presynaptic nerve terminals (Clayton and George, 1998). However, the acidic carboxyl-terminal domain is less conserved, which contributes to the functional differences of synuclein proteins (George, 2002; Surguchov, 2013).

α-Synuclein is well-known to be involved in Alzheimer's and Parkinson's diseases. α-Synuclein is well-known as the major component of Lewy bodies in Parkinson's disease (Spillantini et al., 1997). α-Synuclein constitutes the non-amyloid component of amyloid deposition in Alzheimer's disease (Ueda et al., 1993). Meanwhile, β-synuclein is the least-explored member of synucleins. It is assumed to have a neuro-protective role by inhibiting α-synuclein aggregation (Park and Lansbury, 2003). Synuclein proteins have been shown to be involved in neuronal functions due to their brain tissue-specific expression characteristics (Lavedan, 1998). γ-Synuclein was reported to involve in human oncogenesis when γ-synuclein was documented as the Breast Cancer-specific Gene 1 (BCSG-1). This is due to its over-expression in advanced infiltrating breast cancer as compared with normal tissues or benign breast lesions (Ji et al., 1997). γ-Synuclein was also being detected in breast ductal carcinomas and up-regulated in ovarian carcinomas as compared with normal breast tissue and normal ovarian epithelium (Bruening et al., 2000). Therefore, it is being proposed as a potential breast and ovarian cancer marker (Ji et al., 1997; Gupta et al., 2003a). More recently, γ-synuclein was found to possess chaperone functional activity in breast cancer cells (Jiang et al., 2004) and it was reported to be able to modulate intracellular signalling pathway in breast and ovarian cancers (Pan et al., 2002; Hua et al., 2009). Therefore, γ-synuclein is able to be developed as a therapeutic target. Additional studies suggested that the stage-specific expression of γ-synuclein may link to progression of tumourigenesis (Ji et al., 1997; Bruening et al., 2000; Zhao et al., 2006). Furthermore, the high expression of γ-synuclein is not confined to breast and ovarian cancers but

also in cancer of the prostate, lung, liver, cervical, colon, gastric, and oesophagus (Liu et al., 2005). However, γ-synuclein was surprisingly reported to be down-regulated in human oesophageal squamous cell carcinoma (Zhou et al., 2003). Therefore, it is believed that the expression of γ-synuclein maybe cancer specific and γ-synuclein may exhibit different functions in variety of cancer types.

The deviation from normal to malignancy can be discussed from the six hallmarks of malignant cells, include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, programmed cell death evasion, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). To add to the current six hallmarks of cancer, deregulation of cellular energetic, avoidance of immune destruction, genomic instability and mutation, and tumour-promoting inflammation have been pointed out recently (Hanahan and Weinberg, 2011). Metastasis is a complex process which involves the detachment of cancer cells from a primary tumour to distant locations resulting in dissemination of cancer cells. Although many steps in the metastatic process are thought to contribute to metastatic efficiency, but the key factor that contributes to metastasis is still unknown.

There are evidences that showed γ-synuclein may play roles in promoting invasion and metastasis by supporting cell motility and cell transformation, and at the same time, provide protective characteristics against apoptosis in non-neuronal tumour tissues. γ-Synuclein was shown to be able to

mediate invasion and metastasis of breast cancer by up-regulating the expression of matrix metalloproteinase 9 (MMP-9) (Chen et al., 2013a). Apart from that, the pro-invasive properties of γ-synuclein were evidenced in breast cancer cells. Cell motility and invasiveness through Matrigel invasion assay of γ-synuclein over-expressing breast cancer MDA-MBA-435 cells was shown to be enhanced as shown by Jia et al., (1999). On the other hand, silencing of γ-synuclein in LNCaP prostate cancer cells contributed to the induction of G1 cell-cycle arrest and inhibition of cellular proliferation (Chen et al., 2012). Down-regulation of γ-synuclein was shown to be able to suppress the colony formation of the HCT 116 colorectal cancer cells (Ye et al., 2009). γ-Synuclein also contributed to the development of breast and ovarian cancers by providing resistance to chemotherapeutic drugs and enhancing cells survival under stress conditions (Pan et al., 2002). Meanwhile, knockdown of γ-synuclein was able to sensitise human breast cancer cells to apoptosis as a result of endoplasmic reticulum stress (Hua et al., 2009). Not many studies have explored the role of γ-synuclein in colorectal cancer cells. Therefore, whether γ-synuclein has any pro-invasion and pro-apoptotic effects in colorectal cancer cell lines that would eventually lead to disease progression needs to be elucidated.

To date, despite the long focus on γ-synuclein, the physiological roles of γ-synuclein in human Dukes' type B colorectal adenocarcinoma remain unknown. This study focuses on the biological aspects of invasion and metastasis and programmed cell death evasion of cells. The study was strategised to over-express γ-synuclein in colorectal adenocarcinoma cells, LS

174T and non-cancerous human embryo kidney cells, HEK 293 in revealing the roles of γ-synuclein in colorectal carcinogenesis. Therefore, the objectives of this study were:

(a) To investigate the levels of endogenous γ-synuclein expression in LS 174T and HEK 293 cell lines, and thereby to stably over-express γ-synuclein.

(b) To evaluate the effects of over-expressing γ-synuclein on pro-invasive/anti-invasive properties of LS 174T and HEK 293.

(c) To evaluate the pro-apoptotic/anti-apoptotic and pro-oxidative/anti-oxidative properties of γ-synuclein in LS 174T and HEK 293 over-expressing γ-synuclein.

(d) To investigate whether γ-synuclein monoclonal antibody (mAb) could reverse the pro-apoptotic/anti-apoptotic property of γ-synuclein.

CHAPTER 2

LITERATURE REVIEW

2.1 Colorectal Cancer Facts

Cancer is a growing problem and becomes increasingly important as a public health concern. Cancer is the leading cause of mortality worldwide, with approximately 8.2 million of deaths around the world in 2012 (GLOBOCAN, 2012a; World Health Organization, 2014). An amount of 14.1 million new cancer cases were reported and 32.6 million people are living with cancer (within 5 years of diagnosis) in 2012 worldwide (GLOBOCAN, 2012a). International Agency for Research on Cancer (2014) reported that the burden of cancer will continue to increase to 23.6 million new cases per year by 2030, representing an increase of 68% compared with 2012. The incidence of cancer was slightly higher, about 25%, among males as compared with females, with rates of 205 and 165 per 100,000, respectively (GLOBOCAN, 2012a). Majority of the patients were diagnosed at the late stage of the disease (Lim, 2002) while metastasis was the major cause of cancer death (World Health Organization, 2014). In Malaysia, the incidence of cancer was reported increased from 32,000 new cases in 2008 to approximately 37,000 in 2012 (Cancer Research Initiative Foundation, 2013).

Colorectal cancer was the second most common cancer after breast cancer and the first among males and also second among females in Peninsular Malaysia. A total of 2,866 cases were registered with National Cancer Registry, Malaysia in 2006 and represented 13.2% of all cases registered. The incidence was slightly higher among males as compared with females. From the projection of ethnics, the incidence was the highest among Chinese as compared with Malay and Indian populations (National Cancer Registry, 2006). Globally, colorectal cancer was a major cause of mortality throughout the world. In men, colorectal cancer ranked the third most common cancer with 746,000 cases or 10.0% of the total reported (GLOBOCAN, 2012b). It was also the second most common cancer in women with 614,000 cases or 9.2% of the total reported worldwide in 2012 (GLOBOCAN, 2012b).

Therefore, there is a need for research on molecular cell biology of colorectal cancer so that colorectal cancer development could be better understood. Wide research in molecular cancer biology had identified many key molecules involved in the tumourigenesis of cancer. One possible target which plays a role in colorectal cancer is a small cytosolic protein called γ-synuclein that belongs to the synuclein family.

2.2 *In Vitro* Model to Study Colorectal Cancer

2.2.1 Human Colon Adenocarcinoma Cell, LS 174T

LS 174T cells, deposited by Northwestern University, were derived from a 58 years old female Caucasian with Dukes type B adenocarcinoma of

the colon. The colon-derived cells which are epithelial-like, tend to grow in islands form and pile on top of each other as shown in Figure 2.1. Mice injected with LS 174T cells occasionally developed solitary hepatic and pulmonary tumour colonies in nude mice proved the tumourigenicity of LS 174T cells. Moreover, LS 174T expressed oncogenic c-*myc*, n-*myc*, *H-ras, N-ras, myb*, *fos,* and p53 mRNA showed the oncogenic property. The cells are also able to grow in anchorage-independent condition (Trainer et al., 1988). Electron microscopy studies revealed abundant microvilli and intracytoplasmic mucin vacuoles present in LS 174T (Tom et al., 1976).

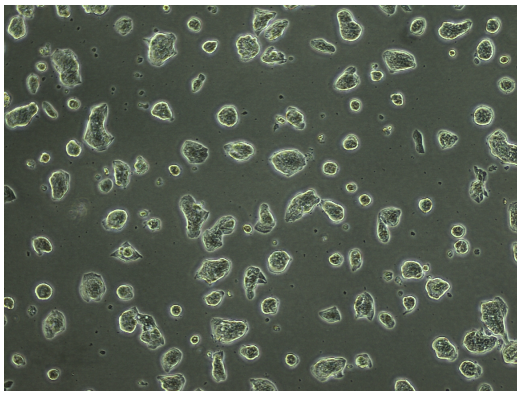


Figure 2.1 Cell morphology of LS 174T. Photograph of cells was taken using Nikon Eclipse TS100 inverted microscope at 100× magnification

2.2.2 Human Embryo Kidney Cell, HEK 293

Human embryonic kidney (HEK) 293 cell line, a general non-cancer control cell line used in previous studies (Taherian et al., 2011; Parmar et al., 2014), is a adherent epithelial-like cell line as shown in Figure 2.2. The cell line was originally harvested from an unknown fetus, but the current commercially available source of HEK 293 cell is from the original transformation done by Graham et al. (1977). The transformation of human

embryonic kidney (HEK) cells with sheared human adenovirus type 5 DNA generated the widely used expression tool known today as the HEK 293 cell line. As highlighted by Thomas and Smart (2005), its biochemical machinery is capable of carrying out most of the post-translational folding and processing required to generate functional, mature protein from a wide range of nucleic acids. The cell type has also been widely generated in stably transfected forms to study various cell-biological questions. Since HEK 293 cells are embryo-kidney origin and assumed to be non-cancerous, therefore it is proposed to be used as control cell line in this study to elucidate the role of neuronal protein γ-synuclein in colorectal cancer and non-cancerous cells.

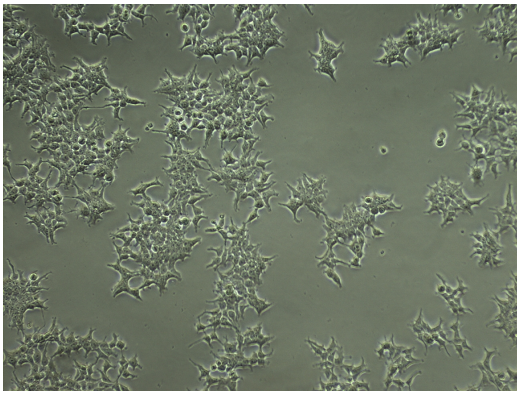


Figure 2.2 Cell morphology of HEK 293. Photograph of cells was taken using Nikon Eclipse TS100 inverted microscope at 100× magnification

2.3 The Synuclein Family

2.3.1 Synuclein Genes

The synuclein family includes three highly homologous members that have been extensively described in vertebrates which include α-synuclein, β-synuclein and γ-synuclein (George, 2002).

α-Synuclein was isolated from the pacific electric ray fish *Torpedo californica* using anti-sera against purified cholinergic synaptic vesicles. This protein was found to be located at the inner nuclear envelope and presynaptic nerve terminals and hence named synuclein, based on the combination of the words syn (synapse) and nuclein (nucleus) (Maroteaux et al., 1988). β-Synuclein was initially isolated from bovine brain as a 134 amino acid residues phosphoneuroprotein (Nakajo et al., 1990; Nakajo et al., 1993). In human homologue, it is 61% identical in sequence to the α-synuclein, and therefore renamed as β-synuclein (Jakes et al., 1994). γ-Synuclein was the last member to be discovered. γ-Synuclein was first identified as Breast Cancer-specific Gene 1 (BCSG1) due to its abundant levels in advanced infiltrating breast carcinoma as compared with normal or benign breast lesions (Ji et al., 1997). Due to its high sequence homology with α-synuclein and β-synuclein, it was inducted to the synuclein protein family (Lavedan et al., 1998).

The human α-synuclein, β-synuclein, and γ-synuclein genes are mapped to chromosome 4q21.3-q22, 5q35, and 10q23, respectively (Spillantini et al., 1995; Lavedan et al., 1998). All synuclein genes compose of five coding exons of similar sizes. The overall organisation of these genes is very well conserved. Figure 2.3 illustrates the α-synuclein gene has 7 exons while the β-synuclein gene has 6 exons, but only 5 exons among all are protein-coding. However, the γ-synuclein gene has 5 exons which are all protein-coding (Lavedan, 1998). γ-Synuclein intron 1 contains two closely located AP1 promoter sites. Mutation of the sequence markedly reduces the

promoter activity, suggesting that AP1 is an important activator for γ-synuclein transcription (Lu et al., 2002).

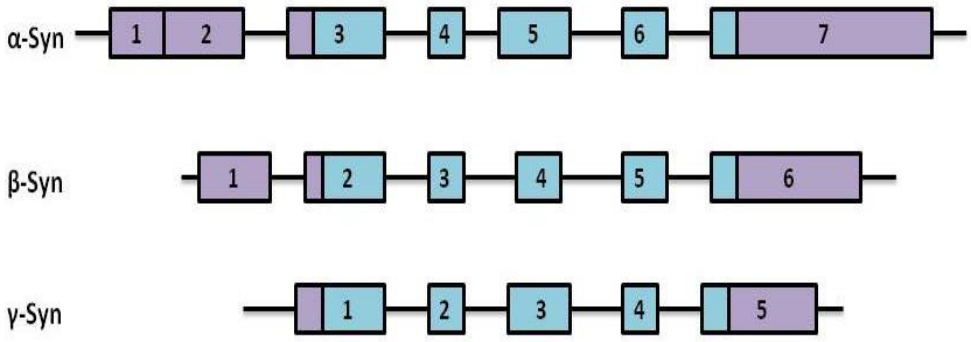


Figure 2.3 Organisation of the human synuclein genes. Exons are represented by boxes, blue or purple, for the coding or untranslated regions respectively. Introns are shown as interrupted horizontal lines (adapted from Lavedan, 1998).

2.3.2 Synuclein Proteins

Synuclein proteins are small and soluble, constituting only 127–140 amino acids (Surguchov, 2013). All the proteins do not contain cysteins or tryptophans throughout their sequence (Surguchov, 2013). They are natively unfolded with the characteristics of high conservation of amino-terminal domain with an 11-residue repeated motifs containing five to six repeats of the amino acid sequence, KTKEGV (Lavedan, 1998; Lavedan et al., 1998). These repeats result in the formation of conserved amphipathic class A2-helices suggesting its lipid binding properties (Lavedan, 1998; George, 2002; Ahmad et al., 2007). However, the acidic carboxyl-terminal domain is less conserved which contributes to the functional differences of synuclein proteins (George, 2002; Surguchov, 2013). Figure 2.4 illustrates the common structure of all the

three highly homologous members and Figure 2.5 shows the protein sequence alignment of human synuclein members.

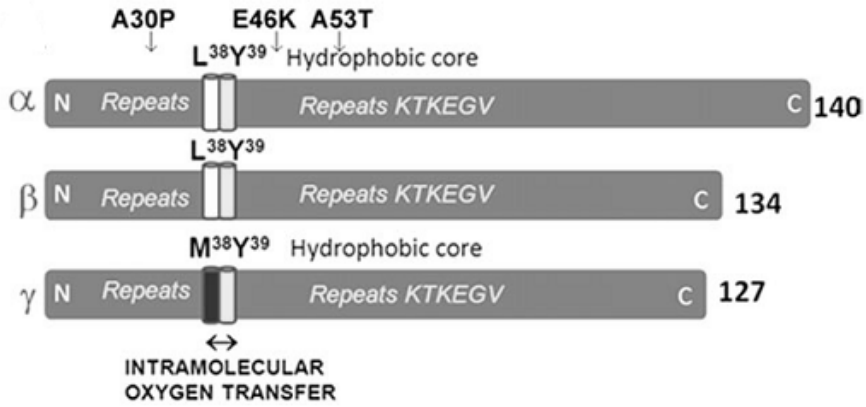


Figure 2.4 Schematic drawing of α-synuclein, β-synuclein, and γ-synuclein protein. N-termini of three members of the synuclein family have similar amino acid sequences and contain several repeats (KTKEGV). Hydrophobic core (NAC domain) is present in α-synuclein and γ-synuclein but not in β-synuclein (adapted from Surguchov, 2013).

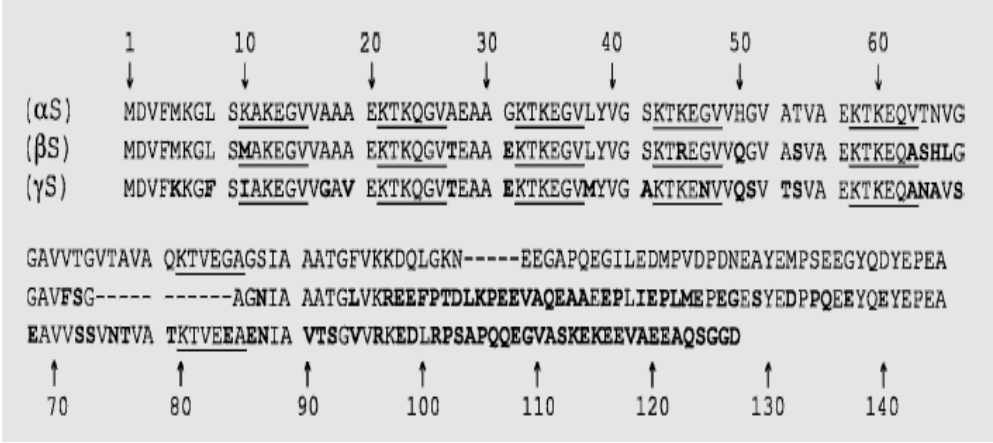


Figure 2.5 Protein sequence alignment of human α-synuclein (αS), β-synuclein (βS), and γ-synuclein (γS). The imperfect 11-residue repeats are delineated by spaces. The KTKEGV consensus sequence within each repeat is underlined. Boldface characters indicate a difference from the α-synuclein sequence (adapted from Sung and Eliezer, 2006).

α-Synuclein with molecular size of 19 kDa comprises 140 amino acids (George, 2002). To add to the above characteristics, as shown in Figure 2.4, α-synuclein has hydrophobic central region that contains the 35-amino acid-non-Aβ-component (NAC) peptide, which is thought to render the protein

susceptibility to toxicity and aggregation (Lavedan, 1998; Kim et al., 2009). β-Synuclein shares the common characteristics of α-synuclein and γ-synuclein, with a deletion of 11 amino acids overlapping the last repeat motif that positioned within the hydrophobic NAC region, as shown in Figure 2.5. This renders it to be less prone to aggregation as compared with α-synuclein and γ-synuclein (Sung and Eliezer, 2007). γ-Synuclein is the least conserved synuclein members with large divergent at its acidic C-terminal domain which lacks of tyrosine-rich sequence. α-Synuclein and γ-synuclein tend to aggregate *in vitro* since γ-synuclein resembles α-synuclein in its free state residual secondary structure (Sung and Eliezer, 2007). All the three synuclein members adopt helical structures in their N-terminal domains upon binding to lipid components (Davidson et al., 1998; Sung and Eliezer, 2006).

2.3.3 Functions of α-Synuclein and β-Synuclein

The physiological functions of α-synuclein as well as β-synuclein and γ-synuclein, are not clear. α-Synuclein has attracted considerable attention due to its involvement in Parkinson's disease and Alzheimer's disease (Ueda et al., 1993; Spillantini et al., 1997). The neuro-pathological hallmark of Parkinson's disease and dementia is the presence of intra-neuronal α-synuclein inclusions, known as Lewy bodies (Trojanowski and Lee, 1998; Lippa et al., 2001).

α-Synuclein is proposed to play a role in regulation of synaptic functions, neurotransmitter release and homeostasis, and modulating proteasomal activity (Lavedan, 1998; Abeliovich et al., 2000; Snyder et al.,

2005; Oaks and Sidhu, 2011,) while β-synuclein is thought to have the anti-aggregation functions by interacting with α-synuclein (Hashimoto et al., 2001; Park and Lansbury, 2003).

α-Synuclein and β-synuclein may be important regulatory components of the vesicular transport processes by selectively inhibit phospholipase D2 (PLD2), which is a key factor in the synthesis and fusion of synaptic vesicles (Jenco et al., 1998). Other evidences to prove the involvements of α-synuclein in neurotransmitter release and homeostasis include co-immunoprecipitation with tyrosine hydroxylase, which is an enzyme in the biosynthesis of dopamine (Perez et al., 2002), regulating the release of neurotransmitter in presynaptic vesicles by sustaining the N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complex assembly in the presynaptic terminal (Burre et al., 2010), and accelerating cellular dopamine uptake by forming complexes with the human presynaptic dopamine transporter (Lee et al., 2001). The proteasomal activity functions of α-synuclein was evidenced when α-synuclein protofibrils was reported to markedly inhibit the proteasomal degradation activities (Zhang et al., 2008).

Since selectively inhibit PLD2 release, β-synuclein may be involved in the synaptic vesicles release (Jenco et al., 1998). Interestingly, β-synuclein was assumed to have neuro-protective role when it was shown to be able to inhibit α-synuclein aggregation and toxicity (Hashimoto et al., 2001; Windisch et al., 2002; Park and Lansbury, 2003). Transgenic mouse models had demonstrated that β-synuclein could ameliorate α-synuclein neurotoxicity by

reducing the α-synuclein protein aggregation with improvements in motor performance and survival extension (Fan et al., 2006).

2.3.4 Functions of γ-Synuclein

The physiological function of γ-synuclein is still arguable but it is believed to be involved in some of the cellular processes including tumour progression (Jia et al., 1999; Liu et al., 2000; Hibi et al., 2009; Ye et al., 2009), chaperone-like activity (Jiang et al., 2004), cytoskeleton regulation (Zhang et al., 2011), and lipid metabolism (Oort et al., 2008).

2.3.5 γ-Synuclein Tissue Distribution and Subcellular Localization

Reported as neuronal cytoplasmic protein, γ-synuclein is expressed predominantly in the cell bodies and axons of primary sensory neurons, sympathetic neurons, and motor neurons (Buchman et al., 1998b). In the neuronal tissues, it is highly expressed in thalamus, subthalamic nucleus, substantia nigra, hippocampus and amygdala. Corpus callosum and caudate nucleus express γ-synuclein but to lesser extent relative to the mentioned tissues (Lavedan, 1998). Besides the nervous system, it is also expressed in the heart, skeletal muscle, pancreas, kidney, and liver tissues (Lavedan, 1998). Furthermore, it was shown to be expressed in stratum granulosum of the epidermis of neonatal and adult mice (Ninkina et al., 1999). However, the significance of the presence of γ-synuclein in those tissues is still unknown. γ-

Synuclein protein was also observed in human adipose tissues, suggesting it plays an important role in adipocyte physiology (Oort et al., 2008).

Intracellularly, the cytoplamic protein identity was confirmed when γ-synuclein was reported to favour cytoplasmic localisation identical to α-synuclein due to the similarity of their N-terminal and core regions (Specht et al., 2005). However, the dynamic localisation of γ-synuclein was reported when it was shown that γ-synuclein was present in the perinuclear area and localised to centrosomes in several types of human interphase cells. (Surguchov et al., 2001). In late mitosis, γ-synuclein is not associated with centrosomes but redistributes to the midbody in telophase. Under higher temperature, γ-synuclein tends to translocate from the perinuclear area to the nucleus (Surgucheva et al., 2006). The literature suggested that γ-synuclein may change its intracellular localisation.

2.4 Roles of γ-Synuclein in Cancer Biology

2.4.1 Hallmarks of Cancer

The alterations in cell physiology leading to malignant growth manifestations can be referred to the six hallmarks of cancer: growth signals self-sufficiency, anti-growth signals insensitivity, programmed cell death evasion, endless replicative potential, sustained angiogenesis, and tissue metastasis and invasion (Hanahan and Weinberg, 2000).

Normal cells require mitogenic growth signals before they can actively proliferate. Such behaviour is strongly different from that of a tumour which is greatly independent on the exogenous growth stimulation. Cancer cells possess the ability to generate many of their own growth signals (Hanahan and Weinberg, 2000). Furthermore, cancer cells have the ability to evade the anti-proliferative signals that govern the transition of cells through G1 phase of their cell cycle to maintain their proliferative ability (Hanahan and Weinberg, 2000).

Unlike normal cells, cancer cells also have the ability to evade apoptosis and thus grow and divide uncontrollably. Apoptotic events are typically manifested through apoptotic mitochondrial death signalling via cytochrome c release, which are governed by the members of the Bcl-2 family of proteins. The protein family composes both the pro-apoptotic and anti-apoptotic functions (Green and Reed, 1998). Ultimately, cytochrome c will stimulate the release of caspases that execute the death program through destruction of subcellular structures including organelles and genome (Thornberry and Lazebnik, 1998). Resistance to apoptosis can be acquired by cancer cells through loss of a pro-apoptotic regulators (Hanahan and Weinberg, 2000).

The acquired abilities mentioned earlier lead to deregulation of proliferation program that enable the generation of the vast cell populations. However, limitless replication can only be achieved by telomeres lengthening, mainly through telomerase enzyme. This prevents the cells from entering

senescent state and irreversibly into a G_0-like state (Bryan and Cech, 1999; Hanahan and Weinberg, 2000). Besides, cancer cells have intrinsic ability to induce angiogenesis to sustain supply of essential nutrients and oxygen through the counterbalancing between positive and negative signals that encourage or block angiogenesis (Hanahan and Weinberg, 2000). The ability for invasion and metastasis enables cancer cells to detach from the primary tumour and enter into lymphatic or blood vessel to form a secondary tumour in distant location mediated through up-regulation of extracellular proteases (Chambers and Matrisian, 1997; Hanahan and Weinberg, 2000). To further enable the metastasis process, cancer cells develop alterations in their shape as well as in their attachment to other cells through down-regulation of E-cadherin (Christofori and Semb, 1999). Additionally, adhesion molecules that favour the cell migrations, for example, N-cadherin is up-regulated in some highly aggressive carcinomas (Hazan et al., 2000; Yang et al., 2007).

To add to the current six hallmarks of cancer, deregulation of cellular energetic, avoidance of immune destruction, genomic instability and mutation, and tumour-promoting inflammation have been pointed out recently (Hanahan and Weinberg, 2011). In their explanation, cancer cells exhibit alteration in cellular energetic, such as increased aerobic glycolysis which enables a reprogrammed metabolism for the support of neoplastic proliferation. Avoiding immune destruction further allows cancer cells to evade immune surveillance that can recognize and eliminate majority of cancer cells before they can establish themselves and form masses. The malignancy is dependent on the multiple alterations in the genomes of cancer cells, including increase

the rate of mutation. The event is accelerated by inactivating genes responsible for DNA maintenance which contribute to the increased rate of tumourigenesis. Tumour-promoting inflammation describes that tumour-associated inflammation may enhance tumourigenesis and cancer progression by supplying the tumour microenvironment with bioactive molecules that favours the invasion, metastasis and tumourigenesis, include growth factors, survival factors, and pro-angiogenic factors (Hanahan and Weinberg, 2011). The complexity of cancer cell biology nevertheless can be referred to any of the hallmarks as summarised in Figure 2.6.

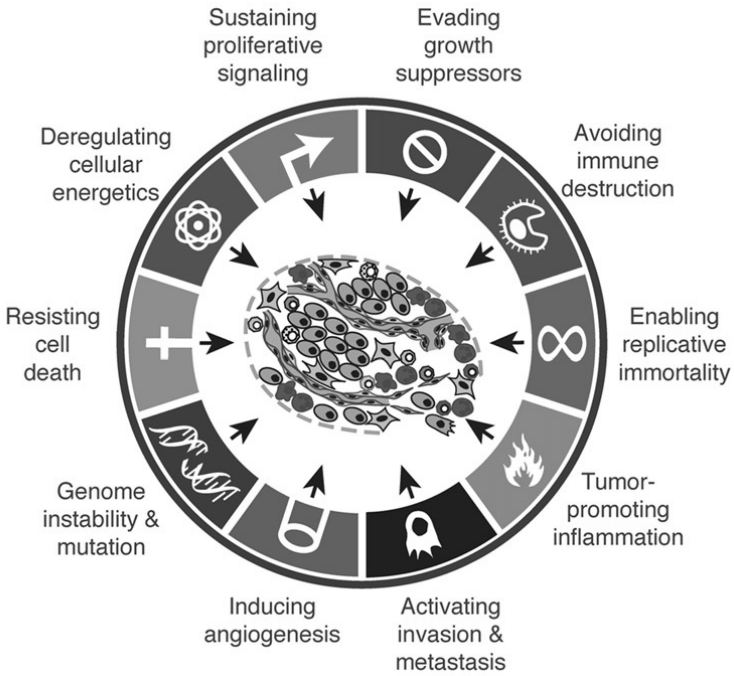


Figure 2.6 Ten hallmarks of cancer (adapted from Hanahan and Weinberg, 2011).

2.4.2 Stage-specific Expression of γ-Synuclein Leads to Biomarker Proposal

During the discovery of BCSG-1, γ-synuclein was undetectable in normal or benign breast lesions, but was up-regulated in advanced infiltrating breast cancer (Ji et al., 1997). The expression pattern that increases with the clinical cancer stage suggested that γ-synuclein maybe a potential breast cancer marker (Ji et al., 1997). Highly γ-synuclein expression was also observed in many ovary pre-neoplastic lesions as compared with normal ovarian epithelium thus suggesting that γ-synuclein was up-regulated in concern with the development of some ovarian tumours (Bruening et al., 2000). Since its neoplastic stage-specific expressing property, γ-synuclein has emerged as a promising biomarker, which is not only restricted to hormone-dependent carcinomas, but also in majority of glioblastomas (Fung et al., 2003). Elevated levels of γ-synuclein were also detected in diverse cancer types, including liver, oesophagus, colon, gastric, lung, prostate, cervical, and breast cancer, but rarely in their adjacent non-neoplastic tissues. In addition, the abundant expression was found in the late II-IV stages but little expression was detected in the early stage I of those cancer types (Liu et al., 2005).

γ-Synuclein expression also correlated with the cancer advanced stages and lymph node invasion suggesting its contribution to metastasis regardless of the cancer types (Wu et al., 2003; Li et al., 2004; Zhao et al., 2006; Wu et al., 2007; Zou et al., 2012). In its relation to the adverse clinical outcome, expression of the gene has a negative impact on the overall patient survival.

Patients with a γ-synuclein-positive tumour had a significant lower overall survival rate and a high probability of death as compared with its undetectable conditions (Guo et al., 2007; Wu et al., 2007; Morgan et al., 2009; Wu et al., 2013). The proofs supported γ-synuclein as a potential marker for cancer progression and possibly as a target for cancer therapy. Diverse reports supported γ-synuclein as the pro-cancerous protein. However, only one report proposed γ-synuclein as a negative regulator in carcinoma. γ-Synuclein down-regulation was observed in majority of the human oesophageal squamous cell carcinoma (ESCC) and over-expression of γ-synuclein could inhibit the growth and transformation abilities of the ESCC 9706 cells (Zhou et al., 2003).

γ-Synuclein is an emerging biomarker in the evaluation of progression of cancers (Amsterdam et al., 2012). In colorectal cancer, it is reported to be more sensitive to predict the lymph node invasion and advanced stage by more than one synuclein proteins detection (Ye et al., 2010b). It is indeed contradicted with the findings reported by the similar group that γ-synuclein up-regulation in colorectal cancer was sufficient to correlate with lymph node involvement, and distant metastasis (Ye et al., 2008; Ye et al., 2009; Ye et al., 2010a; Amsterdam et al., 2012).

There were also other researchers who were interested in identifying γ-synuclein as a cancer marker through the non-surgical means. Quantitative analysis by Western blotting in urine found that 40.2% from 112 bladder cancer patients and only 3.5% from 230 control patients were found positive

for γ-synuclein (Iwaki et al., 2004). In pancreatic adenocarcinoma, the protein level of γ-synuclein was undetectable in normal serum samples, but γ-synuclein was positive in 38% of 56 pancreatic carcinoma samples by using Western blot analysis (Li et al., 2004). Using a specific sandwich ELISA for γ-synuclein, it was shown that γ-synuclein levels between the sera of patients with colorectal cancer, gastric adenocarcinomas, oesophageal cancer and healthy individuals were significantly different. γ-Synuclein was more sensitive in the early detection of sera of the patients with colorectal cancer, gastric adenocarcinomas and oesophageal cancer thus suggesting it as a promising biomarker for early detection of these cancers (Liu et al., 2012).

2.4.3 γ-Synuclein and Regulation of the Cytoskeleton

γ-Synuclein was reported to involve in the cytoskeleton regulation when it was concluded to be able to increase the susceptibility of neurofilament-H of the intermediate filament to calcium-dependent proteases (Buchman et al., 1998a). The direct association of γ-synuclein with cytoskeleton was reported in another study by Zhang et al. (2011) that suggested the over-expression of γ-synuclein may reduce cell chemo-sensitivity of breast and ovarian cancer cells by decreasing the microtubule rigidity. The group demonstrated that γ-synuclein can bind to microtubule and promote tubulin polymerisation in the presence of microtubule associated protein 2 (MAP2). The co-localisation of γ-synuclein with microtubules was also confirmed by Western blot analysis and *in vivo* by fluorescence microscopy after transfection with a γ-synuclein construct in HeLa cells.

(Zhang et al., 2011). Over-expression of γ-synuclein had also led to microtubule-dependent mitochondria clustering at perinuclear area in A2780 ovarian cancer cells thus suggesting γ-synuclein's involvement in the microtubule-dependent cellular processes including mitochondria trafficking (Zhang et al., 2011). Involvement of γ-synuclein in microtubule-dependent mitosis and cytokinesis was suggested to be associated with centrosomes (Surguchov et al., 2001) and in telophase, γ-synuclein was found to bound to the midbody (Surgucheva et al., 2006).

As a microtubule-associated protein, its over-expression was reported to decrease the effectiveness of anti-microtubule drugs like paclitaxel and vinblastine. On the other hand, its interruption was found to be able to increase the efficacy of those drugs (Pan et al., 2002; Zhou et al., 2006; Singh et al., 2007b).

2.4.4 Role of γ-Synuclein in Tumour Progression

2.4.4.1 γ-Synuclein and Cell Growth and Proliferation

The role of γ-synuclein in cell growth and proliferation is well-documented under both anchorage-dependent and -independent conditions. In infiltrating ductal carcinoma H3922 cells, cytokine Oncostatin M-induced growth inhibition occurred as a result of the down-regulation of γ-synuclein expression in both transcriptional and translational levels (Liu et al., 2000). Over-expression of γ-synuclein significantly increased the growth of human breast cancer MCF-7 cells, both in monolayer culture and in the form of soft

agar colony (Liu et al., 2000). In the similar approach, inhibition of γ-synuclein expression through γ-synuclein-specific small interfering RNA (siRNA) and mRNA anti-sense technologies significantly inhibited the anchorage independent growth through the clonogenicity measurement in MCF-7 and T47D breast cancer cell lines (Lu et al., 2002; Liang et al., 2013). The involvement of γ-synuclein in tumour growth was confirmed when short hairpin RNA (shRNA)-knockdown γ-synuclein in MCF-7 formed smaller tumour masses and in nude mice xenograft model (Shen et al., 2011). Similar effects were also observed in colorectal cancer cell line HCT116 (Ye et al., 2009). The silencing of γ-synuclein through siRNA significantly attenuated SW1116 cell growth and colony formation *in vitro*, but the opposite effects could not be obtained when γ-synuclein was over-expressed in the cells *in vitro*. Meanwhile in *in vivo* model, γ-synuclein knock-down suppressed the tumourigenicity of SW1116 colon cancer cells in mice with significant smaller tumour masses in nude mice model and the over-expression of γ-synuclein has led to prominent metastasis phenotype (Ye et al., 2013).

The intracellular program regulated by γ-synuclein determined the cell proliferative ability. Over-expression of γ-synuclein stimulated the ligand-dependent cell anchorage-dependent and -independent proliferation through the binding of oestrogen while suppression of endogenous γ-synuclein expression significantly inhibited cell growth in response to oestrogen in breast and ovarian cancer cells. The result suggested that γ-synuclein was required for oestrogen receptor-α signalling (Jiang et al., 2003). To further elaborate the action of γ-synuclein, the same group showed that γ-synuclein is

a new chaperone protein in the heat-shock protein-based multi-protein chaperone complex for stimulation of oestrogen receptor-α signalling by enhancing oestrogen binding (Jiang et al., 2004). Intracellularly, γ-synuclein was shown to reduce the protein level of the mitotic check point protein called BubR1 which led to mitotic checkpoint compromise, subsequently allowing the cells to continue with the cell cycle progress (Gupta et al., 2003b). These findings supported the role of γ-synuclein in cell growth and proliferation.

2.4.4.2 γ-Synuclein and Cell Invasion and Metastasis

Apart from stimulating growth and proliferation, the pro-invasive properties of γ-synuclein were also evidenced in breast cancer cells when over-expression of γ-synuclein in MDA-MBA-435 breast cancer increased their cell motility and invasiveness as observed through Matrigel invasion assay (Jia et al., 1999). Immunohistochemical results also showed nude mice which had been given the implants of γ-synuclein positive cells into mammary fat pads displayed an increase in tumour growth, and metastasis into axillary lymph nodes and lungs, compared with mice which had been given the control γ-synuclein-negative cells implants (Jia et al., 1999). Consistent with this finding, γ-synuclein suppression mediated by RNA interference (RNAi) inhibited the clonogenicity and invasiveness of transfected breast cancer MCF-7 cells in Boyden chamber cell invasion assay (Liang et al., 2013). In pancreatic cancer, Hibi et al., (2009) reported that γ-synuclein over-expression correlated with perineural invasion and lymph node metastasis clinically. In the same reported, γ-synuclein suppression by shRNA was shown to reduce

the incidence of perineural invasion and liver as well as lymph node metastasis in mouse.

The invasive property of γ-synuclein was reported to be modulated by matrix metalloproteinases (MMP), a family of endopeptidases capable of degrading and remodelling components of the extracellular matrix (ECM) and contributes to cellular migration and metastasis (Vu and Werb, 2000). γ-Synuclein and MMP-9 are also significantly correlated with each other in breast cancer, whereby γ-synuclein may promote the invasion and metastasis of breast cancer mediated by up-regulating the expression of MMP-9 (Chen et al., 2013a). Therefore, aberrant expression of MMPs as a result of γ-synuclein over-expression may promote the invasiveness of colorectal cancer by enhancing cell motility and metastatic properties. The finding was further confirmed when γ-synuclein over-expression up-regulated MMP-9 gene expression through the activation of the AP-1 cis element in retinoblastoma Y79 cells (Surgucheva et al., 2003).

The invasion and metastasis processes must be assisted by cancer cell motility. The effect of γ-synuclein on cell motility was further demonstrated in breast and ovarian cancer cell lines. γ-Synuclein was proposed to enhance metastasis in breast and ovarian cancer by enhancing cell motility through activation of the Rho family GTPases and extracellular signal-regulated kinases (ERK) (Pan et al., 2006). The enhanced motility and invasion effects of γ-synuclein were verified in colorectal cancer HCT 116 cells when

inhibition of cell migration and invasion occurred in Boyden chamber invasion assays due to γ-synuclein knock-down (Ye et al., 2009).

2.4.4.3 γ-Synuclein and Cell Survival/Apoptosis

Cell survival and its ability to evade apoptosis are important for the metastasis process. Studies have shown that γ-synuclein was able to bind to transcriptional factors and modulate different gene expressions (Surgucheva and Surguchov, 2008), therefore influencing the cell survival ability. γ-Synuclein was shown to increase the resistance to mitotic arrest and apoptosis in breast cancer cells induced by paclitaxel (Zhou et al., 2006). Many different protective mechanisms related to γ-synuclein in cancers have been reported. γ-Synuclein contributed to the development of breast and ovarian cancer by providing resistance to chemotherapeutic drugs and enhancing cells survival under stress conditions through ERK 1/2 and c-Jun N-terminal kinase (JNK) 1 (Pan et al., 2002). γ-Synuclein down-regulation was found to be able to enhance endoplasmic reticulum stress-induced apoptosis in human breast cancer cells (Hua et al., 2009). In the same study, the apoptosis induction was dependent on JNK and caspases activation (Hua et al., 2009). These findings suggested that γ-synuclein was able to regulate cancer malignancies possibly through mitogen-activated protein kinase (MAPK) pathways. Apart from that, the binding of γ-synuclein targeting peptide inhibitor to γ-synuclein was shown to be able to enhance the sensitivity of breast cancer cells to anti-microtubule drugs (Singh et al., 2007b).

2.4.5 Synuclein and Oxidative Stress

Reactive oxygen species (ROS) is a collection oxygen-derived free radicals like superoxide anion ($O2•^-$), hydroxyl (HO•), peroxyl (RO_2•), and alkoxyl (RO•) radicals, as well as oxygen-derived non-radical species such as hydrogen peroxide (H_2O_2) (Halliwell and Cross, 1994). Several studies on ROS suggested that ROS was involved in the pathogenesis of tumours (Gao et al., 2013; Chae et al., 2014; Chen et al., 2014). ROS like H_2O_2 is able to cross cell membranes and brings cellular changes to macromolecules like proteins, lipids, and nucleic acids (Bienert et al., 2006; Bienert et al., 2007). It is described as a signalling molecule which is responsible in the growth of tumour cells by regulating related gene expression in human tumours. Increase in ROS coupled with decrease in anti-oxidant and disruption of intracellular redox homeostasis might result in oxidative stress and ultimately lead to the initiation of apoptosis (Circu and Aw, 2010).

H_2O_2 can regulate MAPK signalling pathways that result in activation of apoptotic pathways. This in turn leads to release of cytochrome *c* which activates the caspase cascade (Green and Reed, 1998; Bienert et al., 2006). H_2O_2-treated HeLa cells resulted in a time- and dose-dependent apoptosis induction which was accompanied by activation of extracellular signal-regulated protein kinase, c-Jun N-terminal kinase/stress-activated protein kinase and p38 MAPKs (Wang et al., 1998). The activation pathways in turn activate pro-apoptotic p73, Bax and inhibit anti-apoptotic protein Bcl-XL. The events subsequently stimulate the release of cytochrome *c* and the caspase-3

and caspase-9 activations (Singh et al., 2007a). Similar pathways had also been seen in hydrogen peroxide treated HPV-containing SiHa and CaSki cells (Singh and Singh, 2008).

Currently, there is no evidence on the action of γ-synuclein on oxidative stress modulated by H_2O_2. Furthermore, there is still no known data to describe the cellular pathways exerted by γ-synuclein in relation to H_2O_2 oxidative challenge. The involvement of synuclein family in the regulation of oxidative stress can only be referred to α-synuclein. A30P and A53T mutants of α-synuclein failed to protect H_2O_2-induced cell death, while its wild type could block H_2O_2-induced cytotoxicity in PC12 cells and SK-N-MC neuroblastoma cells (Wersinger and Sidhu, 2003; Jiang et al., 2007). Over-expression of wild type α-synuclein but not the mutants was found to be able to rescue SH-SY5Y neuroblastoma cells from H_2O_2 challenge after rotenone and maneb treatments (Choong and Say, 2011). It was proven that the oxidative protective effect of α-synuclein was mediated through the inactivation of JNK pathway (Hashimoto et al., 2002). Furthermore, α-synuclein was reported to be able to protect neurons against oxidative stress through the phosphoinositide 3-kinase (PI3K) / Akt signalling pathway, and subsequently increased the expression of Bcl-2 expression (Seo et al., 2002). However, the high expression of synuclein may bring toxicity to the cells and the protective effects is reduced (Seo et al., 2002, Wersinger and Sidhu, 2003). Therefore, it is believed that oxidative cyto-protective role of α-synuclein varies according to its expression level.

CHAPTER 3

MATERIALS AND METHODS

3.1 List of Solutions and Buffers Formulations

Formulations of solutions and buffers used in this study are shown in Table 3.1.

Table 3.1: List of solutions and buffers formulations

Solutions/Buffers	Formulations
TAE buffer	20 mM Tris, 2 mM Glacial acetic acid, 1 mM EDTA, pH 8.0
Lysis buffer	50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, pH 7.8,
Laemmli sample buffer	125 mM Tris, 5% Sodium dodecyl sulfate, 0.02% Bromophenol blue, 20% Glycerol, pH 6.8
Tris-glycine electrophoresis buffer	25 mM Tris, 190 mM Glycine, 1% SDS, pH 8.6
Transfer buffer	150 mM Glycine, 20 mM Tris, 20% Methanol
Phosphate buffered saline Tween-20 (PBS-T)	0.1% Tween-20 in PBS
Stripping buffer	0.2 M Glycine, 0.1% SDS, 1.0% Tween-20, pH 2.2,
Binding buffer	10 mM Tris, 150 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, and 1.8 mM $CaCl_2$, pH 8.4

3.2 Cloning of γ-Synuclein cDNA into pcDNA™ 3.1 Vector

3.2.1 pcDNA™ 3.1 Vector

The pcDNA™3.1D/V5-His TOPO® (Invitrogen, USA), a 5.5 kilobase plasmid as shown in Figure 3.1, was used as a shuttle to deliver γ-synuclein cDNA into LS 174T colorectal cancer cells and HEK 293 human embryo kidney cells. The plasmid is designed to facilitate rapid directional cloning of blunt-end Polymerase Chain Reaction (PCR) products for expression in mammalian cells. The presence of Cytomegalovirus (CMV) promoter allows the high-level expression of protein in mammalian cells and neomycin selectable marker allows the selection of stable cell clones upon transfection.

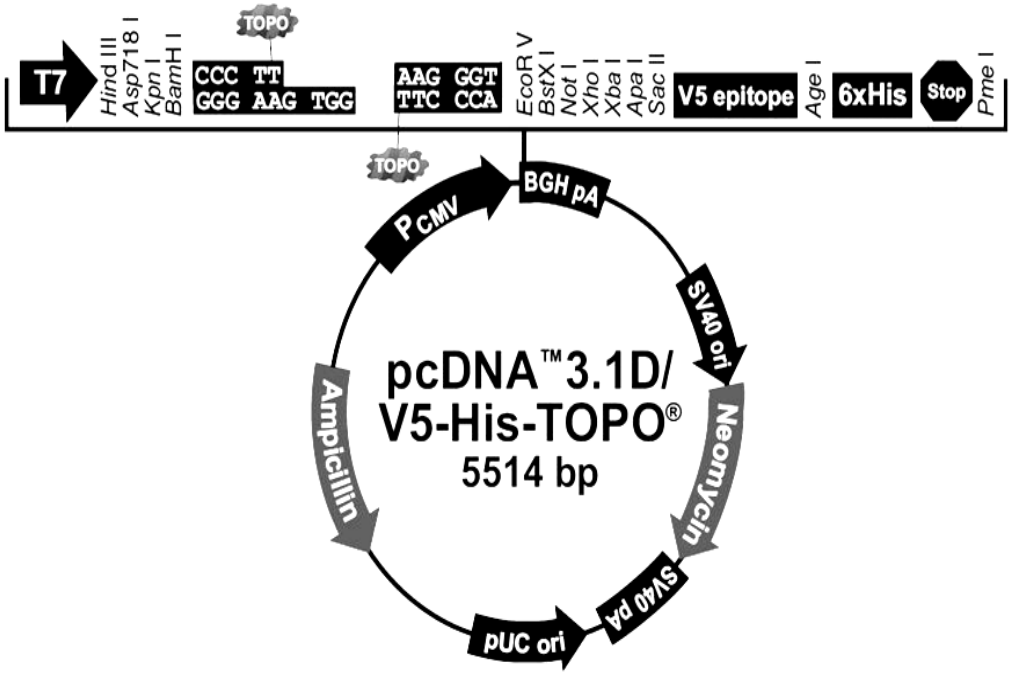


Figure 3.1 Map of pcDNA™ 3.1 vector (adapted from Invitrogen™, 2010).

3.2.2 Sourcing of γ-Synuclein cDNA Clone

Plasmid pOTB7 shown in Figure 3.2 with full length human γ-synuclein (accession number: BC014098) cDNA (Clone ID: 4546444) cloned within the flanking *EcoR*I and *Xho*I restriction sites was purchased from Addgene, USA.

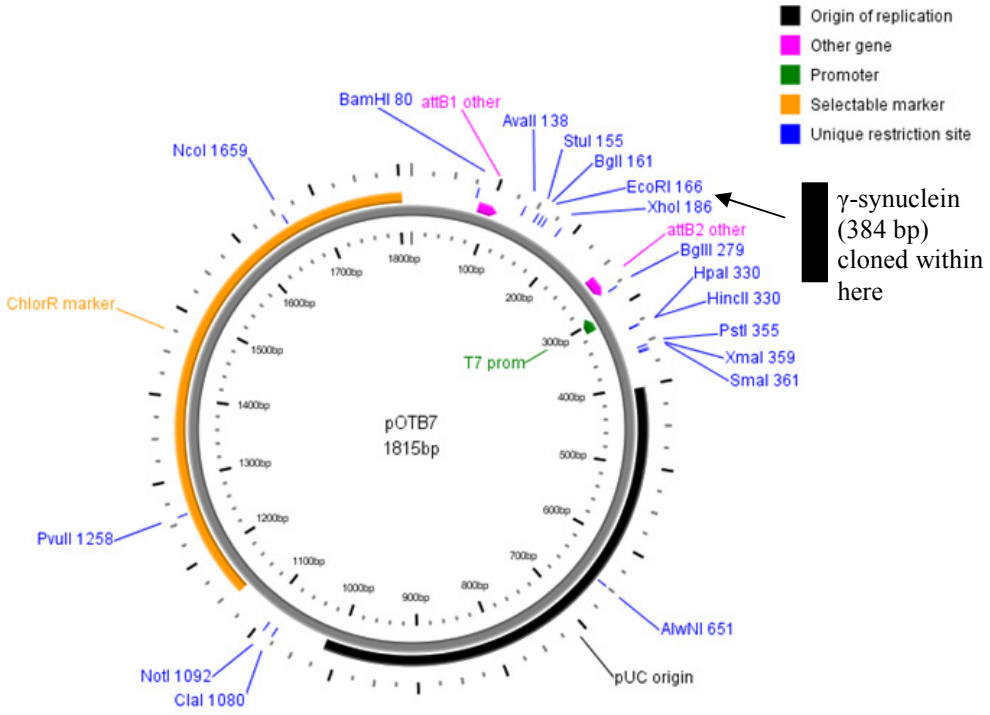


Figure 3.2 Map of pOTB7 (adapted from BDGP Resources, 2014). Arrow shows the position where the full length human γ-synuclein is inserted.

3.2.3 Plasmid Extraction

3.2.3.1 Mini-preparation

The glycerol *E. coli* stock containing pOTB7 was streaked on Luria-Bertani (LB) agar plate supplemented with 25 µg/ml chloramphenicol. The plate was incubated at 37^{o}C overnight.

A single colony was picked from the plate and inoculated into 5 ml LB medium containing 25 μg/ml of chloramphenicol. The bacterial culture was incubated for 16 hr at 37°C with shaking at approximately 220 rpm. Bacterial cells were centrifuged at 6,000 rpm for 5 min at 4°C and the supernatant was discarded. Plasmid DNA was extracted using GF-1 Plasmid DNA Extraction Kit (Vivantis, USA) according to the manufacturer's protocols. The extracted DNA was eluted with 50 μl distilled water.

3.2.3.2 Midi-preparation

Upon cloning, the correct clones were transformed into competent *E. coli* TOP10 strain and plasmid purification was carried out using QIAGEN® Plasmid Midi Kits (QIAGEN, Netherlands). A colony was picked from LB-ampicillin agar and inoculated into 5 ml LB-ampicillin medium (ampicillin was added to agar and medium to the final concentration of 50 μg/ml). The medium was vigorously shaken at 300 rpm at 37°C for 8 hr. The culture was diluted at the ratio of 1:500 into 25 ml LB medium and shaking was continued for subsequent 16 hr. The bacteria cells were harvested with centrifugation at 6,000 rpm for 15 min. Plasmid DNA was then extracted according to the manufacturer's protocols.

The resultant DNA pellet was air-dried before the DNA was diluted in appropriate volume of distilled water. DNA concentration was determined by SmartSpec™ Plus Spectrophotometer (Bio-Rad, USA) with absorbance at 260

nm and 280 nm. The ratio of A_{260}:A_{280} (DNA: protein) of the extracted DNA should be greater than 1.8 for better purity.

3.2.4 Amplification of γ-Synuclein cDNA Sequence

Full length human γ-synuclein cDNA was amplified using PCR forward primer 5'-CACCATGGATGTCTTCAAGAAGG-3' and reverse primer 5'- CTAGTCTCCCCCACTCTGG-3' in MJ Mini^{TM} Personal Thermal Cycler (Biorad, USA). The preparation of PCR mixtures and thermal cycling programme are presented in Table 3.2 and Table 3.3, respectively.

Table 3.2: PCR reaction mixture

Components	**Stock Concentration**	**Final Concentration/ Amount**	**Final Volume**
Pol Buffer B (EURx, Poland)	10×	1×	5 µl
dNTP mix	10 mM	0.2 mM	1µl
Forward primer (First Base, Malaysia)	100 µM	0.5 µM	0.25 µl
Reverse primer (First Base, Malaysia)	100 µM	0.5 µM	0.25 µl
pOBT7 Plasmid Template	1 ng/µl	10 ng	10 µl
Sterile distilled water	-	-	33 µl
Taq DNA Polymerase (EURx, Poland)	5 U/µl	2.5 U	0.5 µl
Total volume			**50 µl**

Table 3.3: Thermal cycling programme applied to generate blunt end γ-synuclein cDNA clone

Step	Temperature	Time	Number of Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 s	25
Annealing	55°C	30 s	
Extension	72°C	30 s	
Final Extension	72°C	7 min	1
Cooling	4°C	indefinite	

3.2.5 Agarose Gel Electrophoresis PCR Product Purification

The PCR products were resolved in a 1.0% agarose gel in TAE buffer, electrophoresed at 90 V for 30 to 40 min until the loading dye approached three quarters of the gel, stained with ethidium bromide. To prepare a 1.0% agarose gel for electrophoresis, 1.0 g of agarose powder (Vivantis, USA) was added into 100 ml of TAE buffer and dissolved in a microwave oven. The gel solution was then cooled and poured into a pre-set gel rack to allow polymerisation. PCR products were added into the wells along with a 1 kb DNA ladder (Vivantis, USA) for determination of product size.

Purification of DNA was performed using GF-1 Gel DNA Recovery Kit (Vivantis, USA). Briefly, agarose gel band containing blunt end PCR product was excised using a scalpel and solubilised in an equal volume of Buffer GB. The gel was completely melted at 50^{o}C in a water bath. The sample was then transferred into a column assembled in a clean collection tube for DNA binding. Column was spun at 10,000 rpm for 1 min to remove the

remaining sample followed by washing step with 750 µl of Wash Buffer at 10,000 rpm centrifugation for another 1 min. The column was then dried by 1 min centrifugation at 10,000 rpm. The purified DNA was eluted with 30 µl of distilled water, at 10,000 rpm of 1 min centrifugation.

3.2.6 TOPO® Cloning Reaction and Transformation of Plasmid into One Shot® TOP10 Competent Cells

The purified blunt end PCR product was subcloned into pcDNA™ 3.1 Vector using pcDNA™3.1/V5-His TOPO® TA Expression Kit (Invitrogen, USA) according to manufacturer's protocol. The components in reaction mixture presented in Table 3.4 was prepared and incubated for 10 min at room temperature. The reaction mixture was placed on ice and proceeded to transformation into One Shot® TOP10 Competent Cells. Two microlitres of the mixture in Table 3.4 was added into a vial containing 50 µl of One Shot® TOP10 Chemically Competent *E. coli*, and gently mixed and incubated for 15 min. The cells were heat-shocked for 30 s at 42°C without shaking and immediately transferred to ice. Two hundred fifty microlitres of room temperature LB medium was then added and incubated with 200 rpm shaking at 37°C for 1 hr. Two hundred microlitres of the cells was spread on a prewarmed LB agar plate supplemented with 50 µg/ml ampicillin and the plate was incubated overnight at 37^{o}C.

Table 3.4: TOPO® cloning reaction

Reagents	volume
Purified PCR products	1 µl
Salt solution	1 µl
Sterile water	3 µl
TOPO® vector	1 µl
Final volume	**6 µl**

3.2.7 Restriction Digest Analysis

For positive clones screening, plasmids DNA were isolated from the transformants using GF-1 Plasmid DNA Extraction Kit (Vivantis, USA), according to the manufacturer's protocol.

A 50 µl restriction digest mixture consisting of cloned DNA, 0.5 µl of bovine serum albumin (BSA) (10 mg/ml), 5 µl 10× Buffer and 1 µl of *Xho*I (Vivantis, USA) was used to screen for cloned inserts and those with presence of the insert were sent for an out-sourced sequencing service provided by First BASE Laboratories, Malaysia using T7 forward primer. The presence and exact orientation of γ-synuclein in the clone and its sequence was then confirmed by aligning with human γ-synuclein cDNA sequence (Accession no. BC014098) using Multiple Sequence Alignment (MultAlin).

3.2.8 Preparation of Glycerol Stock for Long-Term Storage

The glycerol stock of the correct clone was made for long-term storage at -80°C while a stock of plasmid DNA was stored at -20°C. To prepare the

glycerol stock, a colony was inoculated into 2 ml of LB broth containing 50 µg/ml of ampicillin. The bacterial culture was incubated overnight at 37°C with shaking at 220 rpm before 0.85 ml of bacteria culture was mixed with 0.15 ml of sterile glycerol. The mixture was then transferred to a cryovial and kept at -80°C.

3.3 Cell Culture

3.3.1 Cell lines Maintenance

LS 174T and HEK 293 cell lines were grown in complete culture Eagle's Minimum Essential Medium (EMEM). To prepare complete culture EMEM, 9.53 g of complete culture Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich, USA) was dissolved in one litre of water supplemented with 2.2 g of sodium bicarbonate (SYSTERM, Malaysia), 10% v/v heat-inactivated fetal bovine serum (FBS) (PAA lab, Austria) and Penicillin-Streptomycin antibiotics (Millipore, USA). All cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Complete media were replenished every two days and subculturing of cells was performed depending on the cells' confluency.

3.3.2 Passaging of Cell Lines

Subculture was carried out when cells reached 80% confluency. Depleted medium was removed from culture flask and the cells were then washed twice with 5 ml of Phosphate Buffered Saline (PBS) (OXOID Ltd,

UK). One millilitre of 0.05% trypsin-EDTA (Gibco Inc, USA) was added to allow the cells to detach. The flask was incubated at 37°C for 3 min and 3 ml of fresh complete medium were added to inhibit trypsin. Cells were then centrifuged at 2,000 rpm for 3 min. The cell pellets were resuspended in fresh medium and seeded at preferred density.

3.3.3 Freezing and Thawing of Cells

To freeze the cells, cells that reach 60 to 80% confluency were trypsinised and pelleted at 2,000 rpm for 3 min. Freezing media (50 µl of dimethyl sulfoxide (DMSO, Merck, USA) in 950 µl of fresh culture medium) were added and the cells were resuspended and transferred to cryovials. The cells were cooled to -80°C overnight before they were transferred to liquid nitrogen for long-term storage.

To thaw the frozen cells, the cryovial was removed from liquid nitrogen and immersed in 37°C for immediate thawing. Three millilitres of complete culture medium was added to the thawed cells and pelleted at 2,000 rpm for 3 min. The medium was replaced with fresh medium to get rid of DMSO and seeded at preferred density.

3.4 Generation of Stable Cell Lines Over-expressing Human γ-Synuclein

Cells transfection was done using TransPass D1 Transfection Reagent (TPD1TR) (New England Biolabs, USA) in the absence of serum. Cells were seeded onto a 100 mm cell culture dish (BD Biosciences, USA) to reach 60-70% confluency. OPTI®-MEM (Gibco Inc, USA) was prepared by dissolving 13.6 g of OPTI®-MEM powder and 2.4 g of $NaHCO_3$ into 1 L of deionised water. Five microgrammes of DNA was added into 4 ml of sterile serum-free OPTI®-MEM. TPD1TR was vortexed to allow even mixing before 12.5 µl of TPD1TR was added into each reaction. The mixture was mixed gently. The mixture was allowed to form transfection complexes by standing at room temperature for half an hour. The cells were washed once with OPTI®-MEM at the time of transfection. The medium was then replaced with the prepared transfection mixture and incubated for 4 hr at 37°C to allow the cells to take up the transfection complexes. After 4 hr, transfection medium was removed and replaced with fresh complete EMEM medium. To obtain stable clones, transfected cells were selected in medium containing G418 sulphate antibiotics (A.G. Scientific Inc, USA) after 48 hr post-transfection at a concentration of 1 mg/ml for 21 days. Selection process was carried out for a series of passaging by introducing fresh medium and antibiotic to the cells. Protein expression was then confirmed by Western blotting and immunofluorescence microscopy.

3.5 Protein Expression Analysis

3.5.1 Immunoblotting

3.5.1.1 Cell Lysates Preparation

Cells were grown to 60-70% confluency and washed with PBS for 3 times. Cells were then detached from the cell culture flasks using a cell scraper. Cell suspension was centrifuged at 2000 rpm for 3 min at 4°C to pellet the cells. Subsequently, the supernatant was discarded. Approximately 100 µl of ice-cold lysis buffer was added to lyse the cells completely. Protease inhibitor cocktail (Sigma-Aldrich, USA) was added into the lysate at a dilution factor of 1:100 and incubated on ice for 60 min. Then, cell debris was pelleted by centrifugation at 13,000 rpm for 10 min at 4°C and the supernatant was collected. Protein concentration of lysate was quantified using Pierce® Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, USA) as described in section 3.5.1.3.

3.5.1.2 Medium Preparation

Cells were incubated with serum-free EMEM medium. The culture supernatants were then collected after 48 hr and concentrated using Pierce® Concentrator, 9K MWCO (Thermo Scientific, USA). Briefly, pre-rinsing was done by adding 4 ml of distilled water to the upper chamber and centrifuged until more than 3 ml of filtrate was produced. The supernatant was added into the upper chamber of the concentrator and centrifuged at 3,000 *g* until approximate dead-stop volume was achieved. Approximately 100 µl of

concentrated supernatants were collected from upper chamber. Protein concentration was determined by standard BCA assay as described in section 3.5.1.3.

3.5.1.3 BCA Protein Assay

Bovine serum albumin (BSA) standards of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml were prepared. BCA working reagent was then prepared by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B. Each unknown sample was diluted to 1:20 and 20 µl was added into the wells along with BSA standards. Subsequently, 200 µl of BCA working reagent was added to each well. The mixtures were mixed thoroughly on a plate shaker for 30 s followed by incubation at 37°C for 30 min. The absorbance at 562 nm was measured on a microplate reader, Infinite 200 PRO multimode reader (Tecan, Switzerland). The assay was carried out in duplicates.

3.5.1.4 Denaturing Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Formulations of stacking gel and resolving gel are shown in Table 3.5. Resolving gel solution was prepared and poured into the gel cassette. The gel was then overlaid with butanol to ensure a flat interface on the resolving. The resolving gel was then allowed to polymerise for 30 min. Butanol was removed by inverting the gel and rinsing the top of the gel with water. Stacking gel solution was then prepared and the top of the gel cassette was

filled with the gel solution. The comb was inserted. The stacking gel was left to polymerise for 30-60 min and subsequently transferred to Mini-PROTEAN® III electrophoresis cell (Bio-Rad, USA) which was filled with Tris-glycine electrophoresis buffer.

Fourty microgrammes of proteins were mixed with Laemmli sample buffer in 1:1 ratio supplemented with 5% v/v β-mercaptoethanol and pre-heated at 95°C for 5 min. Heated samples were then being loaded onto the polyacrylamide gel and separated by electrophoresis at 170 V for approximately 90 min.

Table 3.5: Formulations for SDS-PAGE resolving gel and stacking gel

Component	**15% resolving gel**	**12% resolving gel**	**5% stacking gel**
30% acrylamide/bis solution	2.5 ml	2.0 ml	330 µl
1.5 M Tris (pH 8.8)	1.3 ml	1.3 ml	-
1 M Tris (pH 6.8)	-	-	0.25 ml
H_2O	1.1 ml	1.6 ml	1.4 ml
10% SDS	50 µl	50 µl	20 µl
10% APS	50 µl	50 µl	20 µl
TEMED	2 µl	2 µl	2 µl
Total Volume	**5 ml**	**5 ml**	**2 ml**

3.5.1.5 Western Blot Analysis

Transfer buffer was used to pre-wet the SDS-PAGE gel and thick blot paper (0.84 mm thick) (Bio-Rad, USA). The membrane sandwich was

prepared in a way that the protein from SDS-PAGE gel in the cathode could be transferred to polyvinylidene difluoride (PVDF) membrane placed in the anode in Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, USA). All air bubbles were rolled out before the safety cover was positioned on the cell unit. The protein in the gel was blotted to the PVDF membrane for 1 hr at 12 V.

The membrane was then fixed with 0.4% paraformaldehyde (PFA) (Sigma-Aldrich, USA) in PBS for 30 min at room temperature to enhance detection efficiency. The additional step was done for γ-synuclein expression study as synuclein monomers tend to easily detach from blotted membranes that would result in no or very poor detection during subsequent treatment and washing steps (Lee and Kamitani, 2011). The membrane was blocked with 5% w/v skim milk powder (OXOID, UK) in PBS-T for 1 hr at room temperature. The desired protein was probed with primary antibody diluted in PBS-T containing 3% Bovine Serum Albumin (BSA) Fraction V (Bio Basic Inc., Canada) for overnight incubation at 4°C. The membrane was extensively washed with PBS-T before the membrane was incubated in secondary antibody conjugated with horseradish peroxidase (HRP) in PBS-T and 3% BSA at room temperature for 1 hr. Again, the membrane was washed extensively with PBS-T. The desired protein bands were detected using Pierce® enhanced chemiluminescence (ECL) system (Thermo Scientific, USA) and visualised using FluorChem FC2 imager (Alpha Innotech Corp, USA). Image processing and analysis software, ImageJ 1.44p by National Institutes of Health (http://rsb.info.nih.gov/ij/index.html) was used for densitometric analysis. Antibody dilutions are shown in Table 3.6.

Table 3.6: Antibodies for Western blotting analysis and immunofluorescence microscopy

Antibody	Source	Supplier	Dilution
Anti-γ-synuclein, clone EP1539Y	Rabbit	Millipore, USA	1:1000
Anti-actin, clone C4	Mouse	Millipore, USA	1:5000
Anti-HGF	Mouse	R&D Systems, USA	1 μg/ml
Anti-rabbit IgG-HRP conjugate	Goat	Nacalai Tesque, Inc., Japan	1:10000
Anti-mouse IgG- HRP conjugate	Rabbit	Thermo Scientific, USA	1: 10000
Anti-rabbit IgG (H+L), CF™ 488A	Chicken	Sigma-Aldrich, USA	1:100

3.5.1.6 Stripping of PVDF Membrane for Reprobing

After bands visualisation, the membrane was immersed in 10 ml of stripping buffer for 10 min at room temperature to remove the bound antibodies. The membrane was washed extensively with PBS-T before reprobing with desired antibody.

3.5.2 Immunofluorescence Microscopy

Cells were seeded onto coverslips and grown for a period of 48 hr. Cells were then fixed with 4% w/v PFA in PBS for 15 min at room temperature and further permeabilised with 0.5% Triton X-100 (Fisher Scientific Ltd, UK) in PBS for 5 min. The cells on coverslips were then blocked with 5% goat serum (Sigma Aldrich, USA) in PBS for 1 hr at room

temperature prior to incubation with primary antibody, anti-γ-synuclein, clone EP1539Y at dilution of 1:100 overnight at 4°C. The coverslips were then entensively washed with PBS before incubating with chicken anti-rabbit IgG (H+L), CF™ 488A antibody (Sigma-Aldrich, USA), at a dilution of 1:200 for 1 hr in the dark. After extensive washing with PBS, the coverslips were then mounted on slides with the monolayer facing downward using mounting agent, DPX (Merck, Germany). Cells were then visualised using Nikon Eclipse TS100 inverted fluorescence microscope equipped with Nikon B-2A filter.

3.6 Cell Mobility Activity Measurement

3.6.1 Scratch Wound Assay

Cells were grown to 100 % confluency in series of passaging in 35 mm cell culture dishes. Cell culture medium was then removed and the cells were washed with PBS. A wound was scratched in the middle of well using P100 pipette tips. Cells were washed again to eliminate any cell debris and detached cell. The wound was micrographed to indicate the initial size. The wound was then micrographed every 24 hr for 2 days using Nikon Eclipse TS100 inverted microscope at 40× magnification. Prior to photographing, cells were fed with fresh complete medium. The wound size was then analysed using TScratch programme, which was developed to automatically analyse wound healing assays by the Koumoutsakos group (CSE Lab) at ETH Zürich (Geback et al., 2009). This software determines percent wound closure (the space remaining

in the scratch wound). The percentage of wound areas of day 2 and 3 were then normalised to the wound area at day 1.

3.6.2 Cell Scattering Assay

Cell scattering could be induced under serum starvation condition (Jun et al., 2007; Chen et al., 2010). Briefly, 3.0×10^4 of cells were seeded in 6-well plate and cultured for 3 days. Prior to experiment, the morphology of cell was observed and micrographed at 200× magnification using Nikon Eclipse TS100 inverted microscope. Subsequently, the complete culture medium was replaced with serum deprived-EMEM. The cell colony 'spider-like' scattering effect as a result of motility was observed after 24 hr and micrographed.

3.6.3 Cell Spreading Assay

Cell spreading could be induced by serum as reported by previous studies (Wiechen and Dietel, 1995; Shen et al., 2007). Therefore, cells were plated in 6-well plate in serum deprived-EMEM and cultured overnight at 37°C. The starved cells were stimulated by 10% FBS in EMEM and reincubated at 37°C for 1 hr. Filopodia and/or lamellipodia formation as a result of protruded membrane and cell spreading were observed and micrographed using Nikon Eclipse TS100 inverted microscope at 200× magnification.

3.7 Extracellular Matrix Interaction Measurement Using Cell Adhesion Assay

The adhesive property on fibronectin and collagen I, which are components of ECM, was revealed through cell adhesion assay in 96-well flat bottom plate (BD Falcon, USA). Fibronectin (R&D Systems, USA) was serially diluted to different concentrations (5, 2.5, 1.25, 0.625 and 0 μg/ml) with PBS while rat tail collagen I (Millipore, USA) was serially diluted to different concentrations (40, 20, 10, 5, 0 μg/ml) with PBS. Fifty microlitres of diluted fibronectin or collagen I was added to each well in triplicates and tapped to disperse. The plate was covered and incubated at room temperature for 1 hr to allow coating. The solutions containing fibronectin or collagen I were removed and 0.15 ml of 1.0% of BSA in PBS was added to each well to block any remaining protein binding site for 1 hr. The wells were washed 3 times with 0.15 ml of PBS before the cells (1×10^5 cells per well) were added into the wells. The plate was then incubated at 37°C for 30 min for fibronectin and 15 min for collagen I to allow the cells to adhere to the coated wells. Non-adhered cells were washed away gently 3 times with 100 μl of PBS. Adhered cells were fixed with 50 μl of 4% PFA (Sigma-Aldrich, USA) and stained using 50 μl of crystal violet for 15 min at room temperature. The wells were then washed extensively with PBS to remove excess stain. The dye was solubilised using 50 μl of 0.1% Triton-X (Fisher Scientific Ltd, UK) in PBS and the optical density (OD) was measured at 570 nm using Infinite 200 PRO multimode reader (Tecan, Switzerland).

3.8 Cell Invasion Measurement

Invasion through the ECM is one of the important steps in tumour metastasis. Cell invasion ability was measured using Millipore® QCM™ 24-well Invasion Assay kit according to manufacturer's protocol. Cells with 80% confluency were used in the assay. Cells were starved by incubating them with serum-free medium for 24 hr. All the plates and reagents were brought to room temperature prior to assay. Pre-warmed 300 µl serum-free medium was added to the interior of the inserts and ECM layer of the insert was allowed to be rehydrated for 30 min. Two hundred fifty microlitres of medium was carefully removed from the insert without disturbing the membrane. The cells were then trypsinised to detach and brought to a volume of 1.0×10^6 cells per ml of serum-free medium. Five hundred microlitres of prepared cell suspension was added into the insert and subsequently 500 µl of serum containing medium was added into the lower chamber, in which the serum acted as the chemo-attractant. The plate was covered and incubated for 24 hr at 37°C in a CO_2 incubator.

Cells suspension in the insert was carefully removed from the top of insert by pipetting and the invasion chamber insert was allocated into a clean well containing 225 µl of prewarmed Cell Detachment Solution to allow the invaded cells to be detached from the membrane for 30 min at 37°C. Seventy five microlitres of Lysis buffer/Dye Solution (prepared by dilution of CyQuant GR® Dye 1:75 with 4× Lysis Buffer) was added into each well and incubated for 15 min at room temperature to allow cell lysis and detected by CyQuant

GR® Dye. The fluorescent dye would exhibit strong fluorescence enhancement when bound to cellular nucleic acids. Two hundred microlitres of Cell Detachment Solution/Lysis Buffer/ Dye Solution was then transferred to a 96-well plate and Relative Fluorescence Unit (RFU) was read using 480/520 nm filter in a microplate reader, Infinite 200 PRO multimode reader (Tecan, Switzerland).

3.9 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay as Cell proliferation Measurement

In order to determine the proliferative ability of cell, 2.5×10^3 / 100 µl of cells were seeded per well into 96 well plates in triplicates. The cells were cultured for 24 hr (day 0) prior to MTT assay to determine the initial absorbance. Following 24 hr incubation, 20 µl of MTT solution (Nacalai Tesque, Japan) (5 mg/ml in PBS) was added into each well. The plate was then incubated for 4 hr in the dark at 37°C in CO_2 incubator to allow purple formazan crystal development. Seventy microlitres of the medium-containing MTT was removed without disturbing the formazan crystal and 150 µl of DMSO was added to dissolve the formazan crystals. Absorbance was then determined by microplate reader, Infinite 200 PRO multimode reader (Tecan, Switzerland) at 550 nm. The intensity of colour produced is directly proportional to the number of viable cells.

The procedures were repeated for day 1, 3, and 5. The absorbance values were then normalised to day 0.

3.10 Cell Anchorage-Independent Growth Measurement

3.10.1 Soft Agar Colony Formation Assay

Soft agar colony formation assay is an anchorage-independent growth assay to measure the transformation ability of the cells. An amount of 3.0×10^4 of cells was plated in 0.35% agarose (Choice-care, Malaysia) top agar (0.70% of sterile agarose diluted 1:1 with 2× complete culture medium) on the top of 0.50% base agar (1.00% of sterile agarose diluted 1:1 with 2× complete culture medium) in a 35 mm culture dish. The culture dishes were then maintained at 37°C in a CO_2 incubator for 1 month to allow anchorage independent growth of the cells. The cultures were supplied with complete culture medium every three days to ensure humidity and continuous supply of nutrients. The colonies formed were fixed with 10% PFA prior to staining with cystal voilet. Each plate was photographed and visible colonies were counted using image processing and analysis software, ImageJ.

3.10.2 Anoikis Assay

Poly(2-hydroxyethyl methacrylate) (Poly-HEMA) (Sigma-Aldrich, USA)-coated 96-well plates were used due to the inability of the non-transformed cells to adhere to Poly-HEMA-coated plates, and therefore suspended in the medium. As a result, anoikis was initiated due to loss of cell adhesion to the ECM (Paoli et al., 2013).

To coat the plates, Poly-HEMA was dissolved in 95% of ethanol at a final concentration of 20 mg/ml. Each well was coated with 50 µl of Poly-HEMA solution. The plates were then periodically rocked to ensure even coating until ethanol was totally evaporated and Poly-HEMA had completely solidified.

Equal number of cells (1.0×10^5 cells/well) was seeded in triplets in Poly-HEMA-coated wells for 3 days. Viability of the cells was then determined by MTT assay as described in Section 3.9.

3.11 Cell Treatments

3.11.1 Treatment with Anti-cancer Drugs and Hydrogen Peroxide

A total of 2.0 x 10^4 of cells per well was seeded in 96-well plate overnight to allow the cells to adhere to the bottom of the well. To induce cell death, cells were incubated with different concentrations of filtered sterile-doxorubicin hydrochloride (DOX) (Calbiochem, Germany) and vincristine sulphate (VIN) (Calbiochem, USA). DOX was serially diluted to different concentrations (20, 10, 5 and 0 µM) while VIN was serially diluted to 0.1, 0.05, 0.025 and 0 µM. Oxidative stress was induced by exposing the cells to 0.8, 0.4, 0.2 and 0 mM of H_2O_2 (SYSTERM, Malaysia). The drugs were dissolved in sterile distilled water before added into cell culture medium for treatment purposes. The cells were incubated for 48 hr during the treatments before the cell viability was determined by MTT assay as described in section 3.9.

3.11.2 γ-Synuclein Monoclonal Antibody (mAb) Treatment

To investigate the effect of γ-synuclein monoclonal antibody (mAb) on the viability of untreated and DOX-treated LS 174T cells, a total of 2.0 x 10^4 of cells per well was seeded in 96-well plate to allow for adherence overnight. Different concentrations of anti-γ-synuclein, clone EP1539Y (1, 5 and 10 μg/ml) and 6 μM of DOX were co-administered into the wells. Wells with anti-γ-synuclein treatments alone were also set up to investigate the effect of anti-γ-synuclein on cells. The cells were incubated for 48 hr during the treatments before the cell viability was determined by MTT assay as described in Section 3.9.

3.12 Detection of Reactive Oxygen Species (ROS) using 2', 7'-Dichlorofluorescein Diacetate (DCFH-DA)

Intracellular Reactive Oxidative Species (ROS) generated were detected using fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, USA). Non-fluorescent DCFH-DA was used to determine intracellular H_2O_2 in LS 174T and HEK 293 cells. In brief, non-polar DCFH-DA diffused into the cell and is deacetylated by esterases inside the cell to non-fluorescent 2',7' dichlorofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of intracellular ROS. The DCF-associated fluorescence in the cells could be monitored by fluorescence microscope and indicate the resultant oxidative stress is due to over-production of ROS.

Briefly, cells were seeded for 24 hr for attachment. Cells were incubated with 10 μM of DCFH-DA diluted in DMSO for 30 min in dark and washed with PBS. The cells were then stressed with 3 mM of H_2O_2 for 30 min at 37°C and washed again with PBS before immediately visualised using inverted fluorescence microscope (Nikon Eclipse TS100, USA). The negative control was loaded with DCFH-DA alone, without H_2O_2 treatment.

3.13 Apoptosis Detection Using FITC-Annexin V/ PI Dual Staining

To further elucidate the effect of DOX treatments towards cell viability and assurance of apoptotic cell death, cells were subjected to fluorescein isothiocyante (FITC)-Annexin V / propidium iodide (PI) dual staining. The staining method is based on the observation that cells can translocate the membrane phosphotidylserine (PS) from the inner surface of the plasma membrane to the cell surface after initiating the apoptosis. Once on the surface, PS can be easily detected by staining with fluorescent conjugate of Annexin V, a protein that has high affinity to PS. PI staining further provides evidence of loss of plasma membrane integrity. Cells that have bound to AnnexinV-FITC would show green staining in the plasma membrane. Cells that have lost membrane integrity would show red staining, but the healthy cells with intact membrane structure would repeal the stains when observed under fluorescence microscopy.

Apoptosis was induced by treating the cells with 6 μM of DOX for 48 hr after allowing the cells to attach onto cover slips. Cells were washed with

PBS and added with Annexin V - FITC / PI staining solution in dark for 15 min. To prepare the Annexin V - FITC / PI staining solution, a total of 25 µl of Annexin V – FITC (Life Technology, USA) was added to 100 µl of binding buffer and PI (Calbiochem, Germany) was then added into the solution to yield the final concentration of 10 µg/ml. Negative controls were prepared by incubating the cells in the absence of DOX. The cells were then washed with binding buffer and fixed with 4% PFA for 10 min. The coverslips were then mounted on slides using mounting agent, DPX (Merck, Germany). Cells were then visualised using Olympus BX 41 inverted fluorescence microscope installed with NB and NG filters. Two random fields were captured from each slide. Unstained cells, cells stained with FITC-Annexin V only and cells showing apoptotic characteristics were counted.

3.14 Phospho-Kinase Antibody Array

Protein substrate phosphorylation was analysed using Human Phospho-Kinase array kit (ARY003B, R&D Systems, USA), according to the manufacturer's protocol.

Briefly, treated and untreated cells were solubilised in lysis buffer at 4 °C for 30 min. Protein concentrations were determined by BCA assay. Membranes with spotted catcher antibodies were then incubated at 4°C overnight with 200 µg per membrane of protein diluted in 2 ml of Array Buffer 1. Then, the array was washed to remove unbound protein, incubated with a cocktail of biotinylated detection antibody at room temperature for 2 hr.

Upon extensive washing, phosphorylated proteins were revealed using streptavidin-horseradish peroxidase and chemiluminescent detection reagents. The resultant dot spots were captured using FluorChem FC2 imager (Alpha Innotech Corp, USA) and the intensity of the dot spots were analysed through National Institutes of Health, ImageJ software.

3.15 Statistical Analysis

Statistical Package for the Social Sciences (SPSS) software version 16.0 (SPSS Inc., USA) was used to analyse the data obtained. All results were analysed with one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD)'s *post hoc* comparison test. A p value less than 0.05 is considered as statistically significant. The results presented were expressed as mean ± standard error of mean (SEM) based on at least two independent experiments performed in triplicates, unless otherwise stated.

CHAPTER 4

RESULTS

4.1 Over-expression of γ-Synuclein in LS 174T and HEK 293

4.1.1 Cloning of γ-Synuclein cDNA into pcDNA™ 3.1 Vector

Blunt-end PCR product of γ-synuclein with the size of 384 bp was first produced [Figure 4.1 (a)]. The PCR product was then purified before directionally cloned into the TOPO cloning site of pcDNA™3.1 expression vector, downstream to cytomegalovirus promoter. Figure 4.1 (b) shows the *Xho*I-linearised pcDNA™3.1 empty vector with the size of 5514 bp and pcDNA™3.1-γ-synuclein with 5898 bp. Sequence result as shown in Figure 4.2 was confirmed to be human γ-synuclein cDNA (Accession no. BC014098).

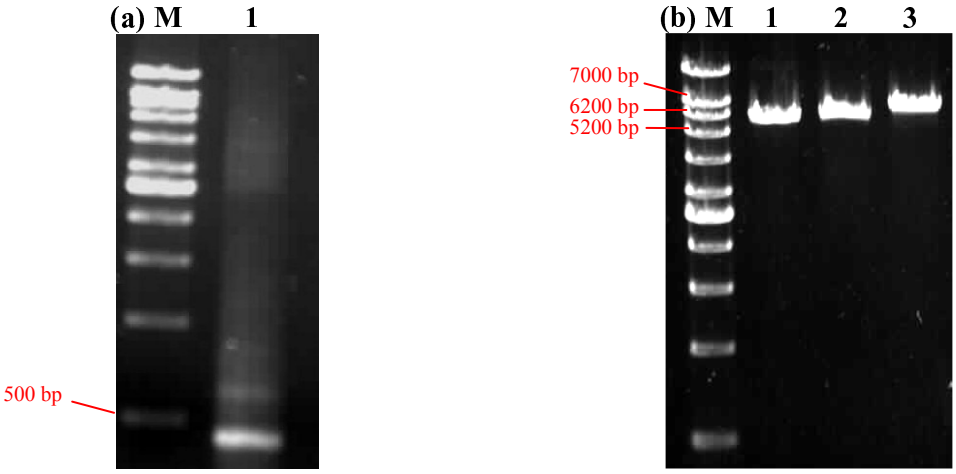


Figure 4.1 Human γ-synuclein cDNA cloning into pcDNA™ 3.1 vector. Agarose gel analysis. (a) Lane 1, blunt end γ-synuclein PCR product (384 bp), M, 1 kb DNA ladder. (b) Lane 1, pcDNA™ 3.1 vector (5,514 bp), lane 2, pcDNA™ 3.1 vector (5,514 bp), lane 3, pcDNA™ 3.1with insert γ-synuclein (5898 bp).

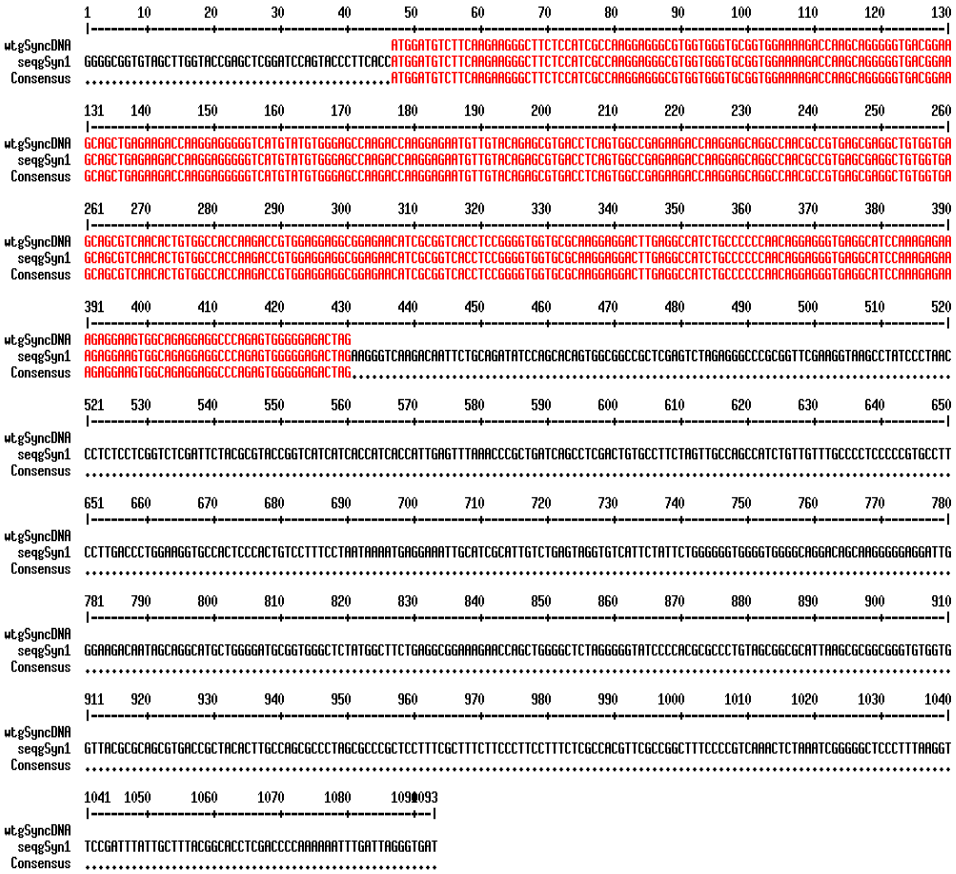


Figure 4.2 MultAlin alignment of a positive clone with human γ-synuclein cDNA sequence (Accession no. BC014098). Human γ-synuclein cDNA sequence was designated as 'wtgSyncDNA' and 'seqgSyn1' was the positive clone. The red abbreviations represent fit match.

4.1.2 Generation of Stable Human Cell Lines Over-expressing γ-Synuclein

Upon transfection, the stable expression of γ-synuclein was verified using Western blotting analysis using anti-γ-synuclein and the membrane was reprobed with actin antibody to confirm equal protein loading in each lane. The stably over-expressing γ-synuclein pcDNA™3.1-γ-synuclein transfected LS 174T cells was designated as LS 174T-γsyn, pcDNA™3.1 vector-transfected LS 174T cells as LS 174T-3.1, stably over-expressing γ-synuclein

pcDNA™3.1-γ-synuclein transfected HEK 293 cells as HEK 293-γsyn and pcDNA™3.1 vector-transfected HEK 293 cells as HEK 293-3.1.

Figure 4.3 (a) shows strong expression of a 16 kDa human γ-synuclein protein bands in HEK 293 and LS 174T cells stably transfected with pcDNA™3.1-γ-synuclein construct but not in the untransfected and pcDNA™3.1 vector-transfected cells. Densitometric analysis revealed that γ-synuclein protein expression was 10.5 fold higher in HEK 293-γsyn compared with untransfected cells, whereby γ-synuclein protein expression was 7.6 fold higher in LS 174T-γsyn compared with untransfected cells [Figure 4.3 (b)].

Stable expression of human γ-synuclein was further confirmed by immunocytochemical analysis (Figure 4.4). A bright immunofluorescence staining pattern in the LS 174T-γsyn and HEK 293-γsyn revealed that the γ-synuclein protein was distributed throughout the cytoplasm and γ-synuclein is predominantly a cytosolic protein. The result was consistent with the finding by Buchman et al. (1998b). No immunoreactivity was detected in the HEK 293 untransfected and HEK 293-3.1 cells whereas low endogenous expression was detected in untransfected LS 174T and LS 174T-3.1 cells.

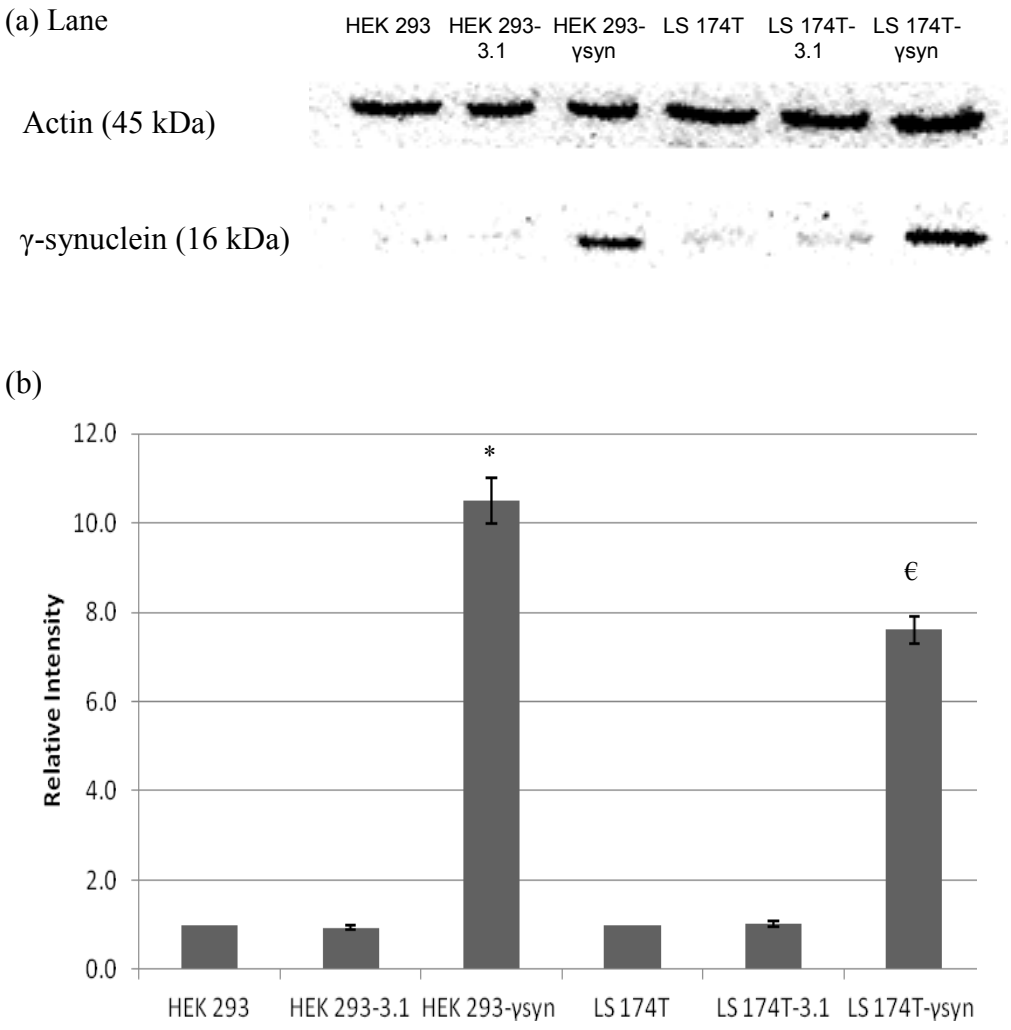


Figure 4.3: Examination of stable over-expression of human γ-synuclein in HEK 293 and LS 174T by Western blotting. (a) Lysates were electrophoresed in 15% resolving SDS polyacrylamide gel. Stable expression of γ-synuclein was then determined by Western blot analysis using anti-γ-synuclein, clone EP1539Y; a 16 kDa band corresponding to the molecular mass of human γ-synuclein protein was detected in HEK 293-γsyn and LS 174T-γsyn. (b) Densitometric analysis of protein expression in Western blot shown in (a) was performed using ImageJ 1.44p. The relative intensity (RI) of the γ-synuclein band was normalised to the respective untransfected cell which was set at 1.0. * $p < 0.05$ compared with untransfected HEK 293 and HEK 293-3.1. € $p < 0.05$ compared with untransfected LS 174T and LS 174T-3.1.

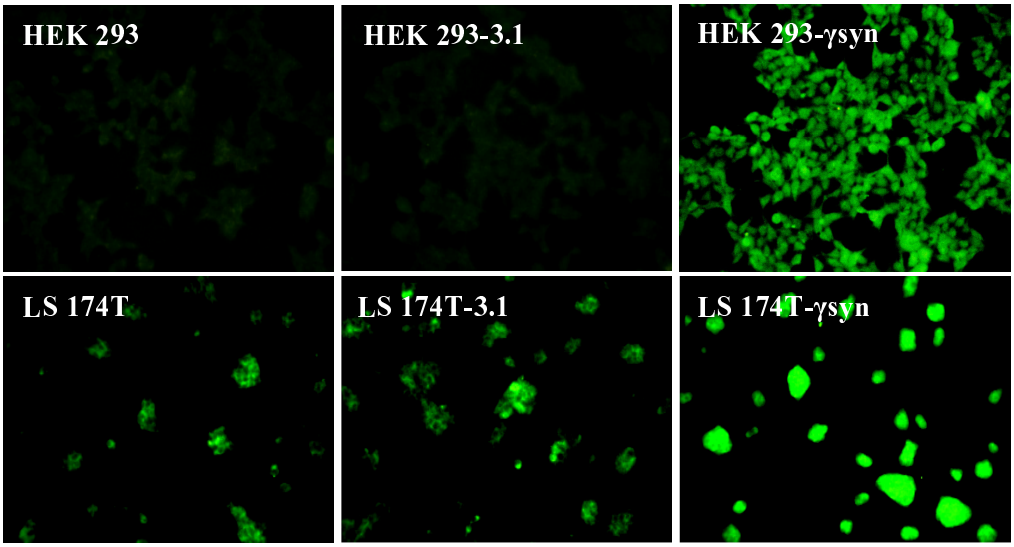
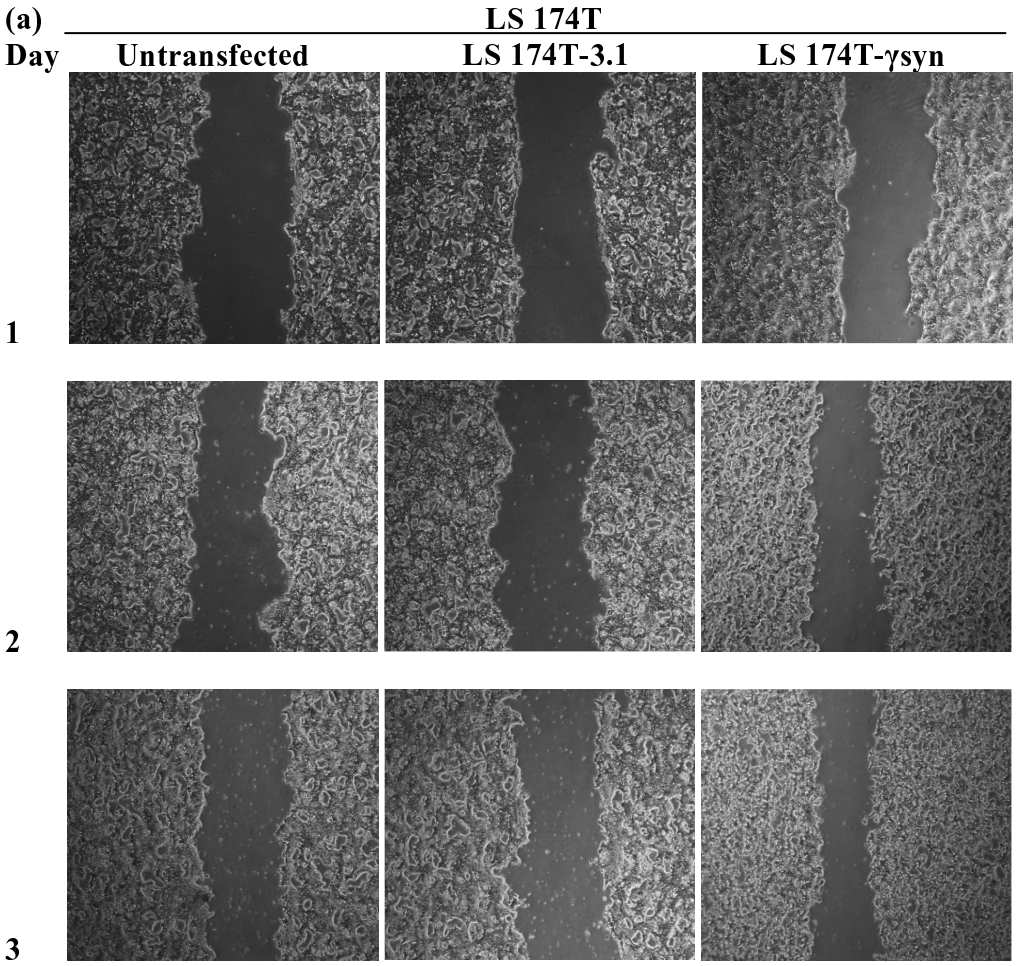


Figure 4.4: Examination of stable over-expression of human γ-synuclein in HEK 293 and LS 174T by immunoflourescence microscopy. Immunofluorescence microscopy bright staining revealed that γ-synuclein was evenly distributed in stably transfected HEK 293-γsyn and LS 174T-γsyn. (Magnification = 200×).

4.2 γ-Synuclein Enhanced Cancer Cell Motility

To address the role of γ-synuclein on cell migration, cells were induced to migrate into a wound created by scratching confluent cultures with a pipette tip to examine the migration of LS 174T-γsyn and HEK 293-γsyn in comparison with their respective untransfected and empty vector transfected cells [Figure 4.5 (a) and Figure 4.6(a)]. Closure of wounded area was monitored for 48 hr. As shown in Figures 4.5, γ-synuclein significantly increased the migratory ability of LS 174T cells. The open area was more rapidly covered by LS 174T-γsyn. However, γ-synuclein had no noticeable effect on cell migration of HEK 293 cells. The open area of HEK 293-γsyn was similar with that of untransfected HEK 293 and HEK 293-3.1 cells. ANOVA revealed that there was no statistical significant difference (Figure 4.6). Quantification of wound closure was represented in Figure 4.5 (b) for LS

174T and Figure 4.6 (b) for HEK 293. The quantified open area in untransfected and vector control of LS 174T cells were reduced, 48 hr after onset of experiment, but for LS 174T-γsyn cells, quantified open area was dramatically reduced [Figure 4.5 (b)]. ANOVA and *post hoc* LSD comparison test showed that there was significant reduction of quantified open area of LS 174T-γsyn compared with untransfected LS 174T and LS 174T-3.1.



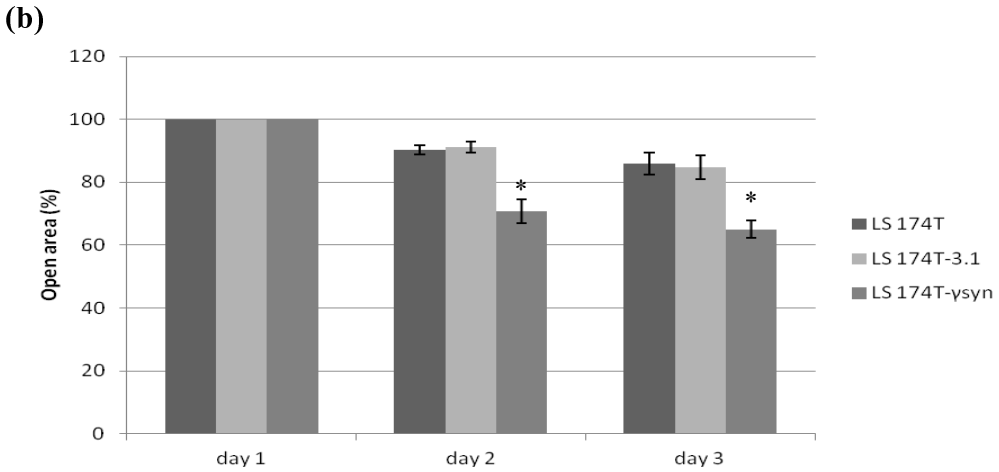


Figure 4.5 γ-Synuclein enhanced motility of LS 174T cells. Cell migration was analysed by using the scratch wound assay. (a) Wound scratched for LS 174T (b) Open area of LS 174T analysed from TScratch software. Data of wound open area was expressed in % and represented the mean ± SEM of 3 independent experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test. * $p < 0.05$ compared with LS 174T and LS 174T-3.1.

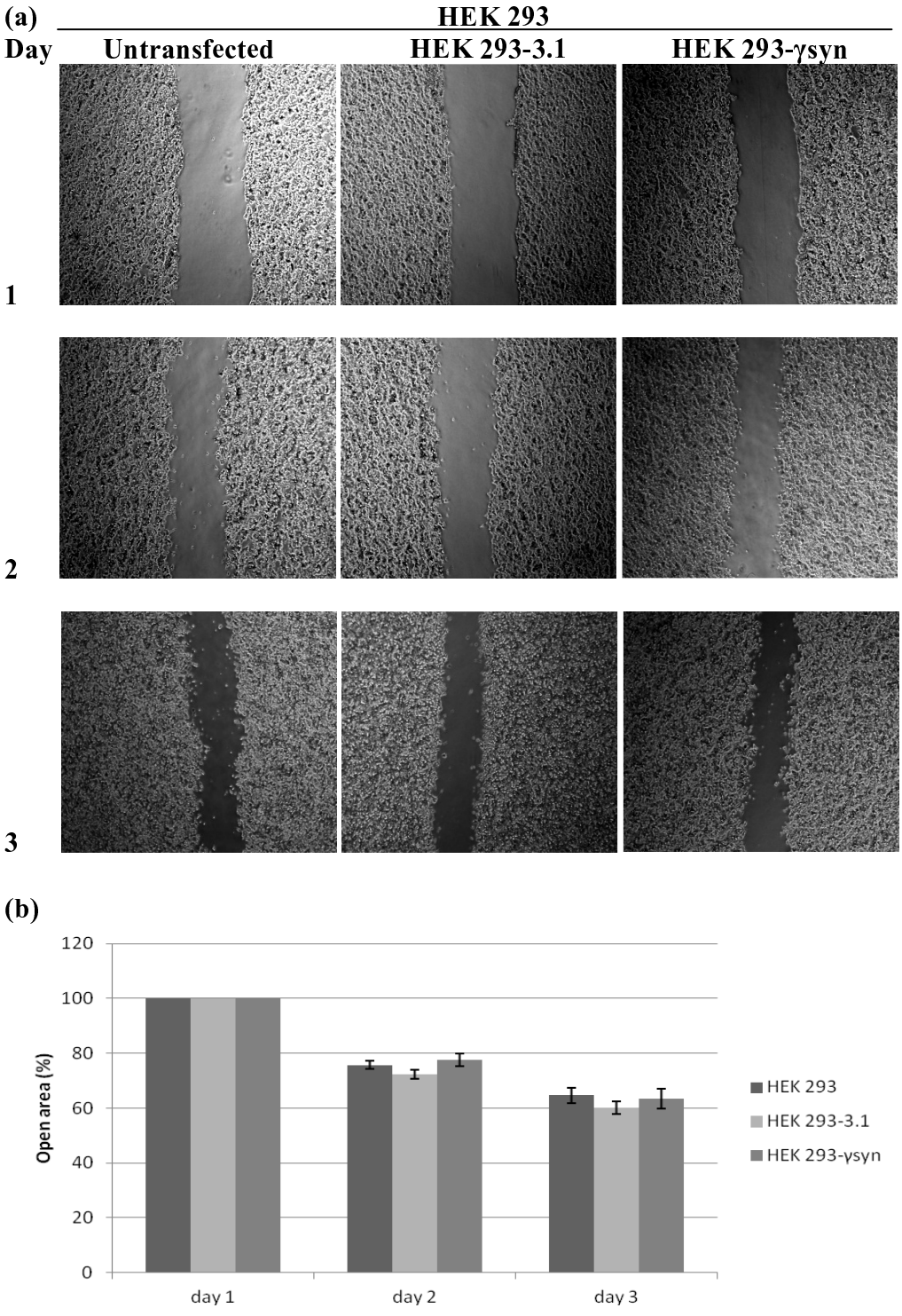


Figure 4.6 γ-Synuclein did not affect motility of HEK 293 cells. Cell migration was analysed by using the scratch wound assay. (a) Wound scratched for HEK 293 (b) Open area of HEK 293 analysed from TScratch software. Data of wound open area was expressed in % and represented the mean ± SEM of 3 independent experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test.

Involvement of γ-synuclein in LS 174T cells motility was further examined by cell colony morphological changes in cell scattering assay and cell spreading assay. Cells are considered scattered when they lose contact with their neighbours and exhibit a fibroblast-like phenotype. LS 174T-γsyn tended to scatter in a spider-like manner under serum-deprived condition. Intercellular adhesion among LS 174T-γsyn cells was relatively weak as compared with untransfected LS 174T and LS 174T-3.1 [(Figure 4.7 (II)]. HEK 293 did not grow into islands and therefore scattering morphology could not be seen either in serum containing medium or serum-deprived conditions [(Figure 4.8 (II)].

In cell spreading assay, formation of lamellipodia and filopodia could be detected with replenishment of serum containing medium after serum starvation. Filopodia are rod-like plasma membrane protrusions in a form of 'finger-like' structure (Arjonen et al., 2011). The sheet-like protrusions at the cell front are termed as lamellipodia (Abercrombie et al., 1970). It is widely accepted that lamellipodia and filopodia are important for cell motility and therefore assist cancer metastasis and cell invasion (Small et al., 2002; Machesky, 2008). Filopodia are also essential for many cellular processes like cell adhesion, migration, angiogenesis and the formation of cell-cell contacts (Arjonen et al., 2011). The ability to form lamellipodia and filopodia was greatly enhanced in LS 174T-γsyn compared with untransfected LS 174T and LS 174T-3.1 [(Figure 4.7 (III)] whereby there was no significant morphology difference between HEK 293-γsyn and untransfected HEK 293 and HEK 293-3.1 cells [(Figure 4.8 (III)]. These data suggested that γ-synuclein was able to

enhance the motility of LS 174T colorectal cancer cells but not HEK 293 non-cancerous cells.

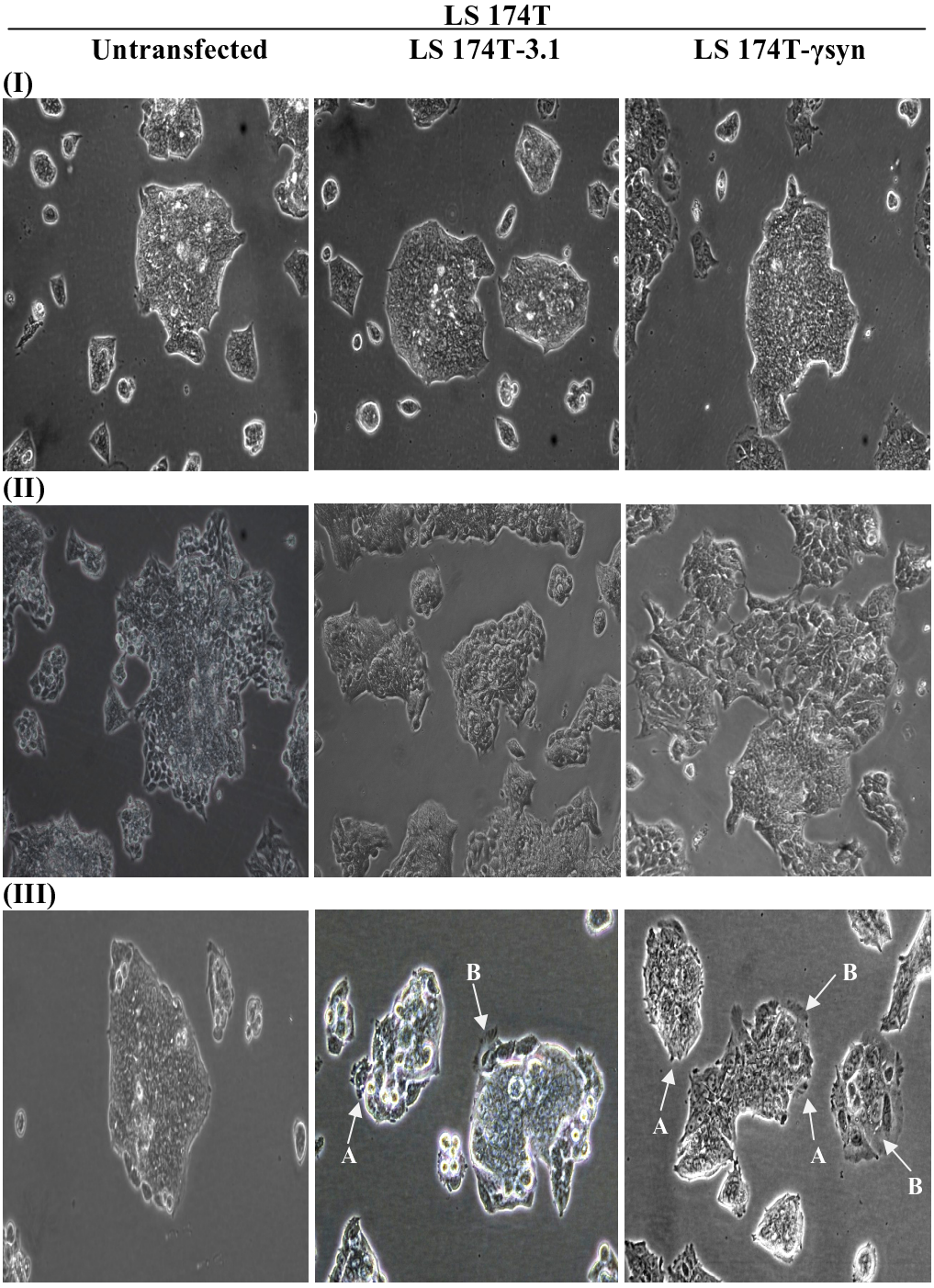


Figure 4.7 Cell scattering and cell spreading morphology of LS 174T. (Magnification power = 200×). (I) Control (II) Cell scattering (III) cell spreading. Cells were showing scattering pattern and spider-like pattern after serum starvation for 24 hr in cell scattering assay. A – Filopodia and B – lamellipodia formation after serum induction.

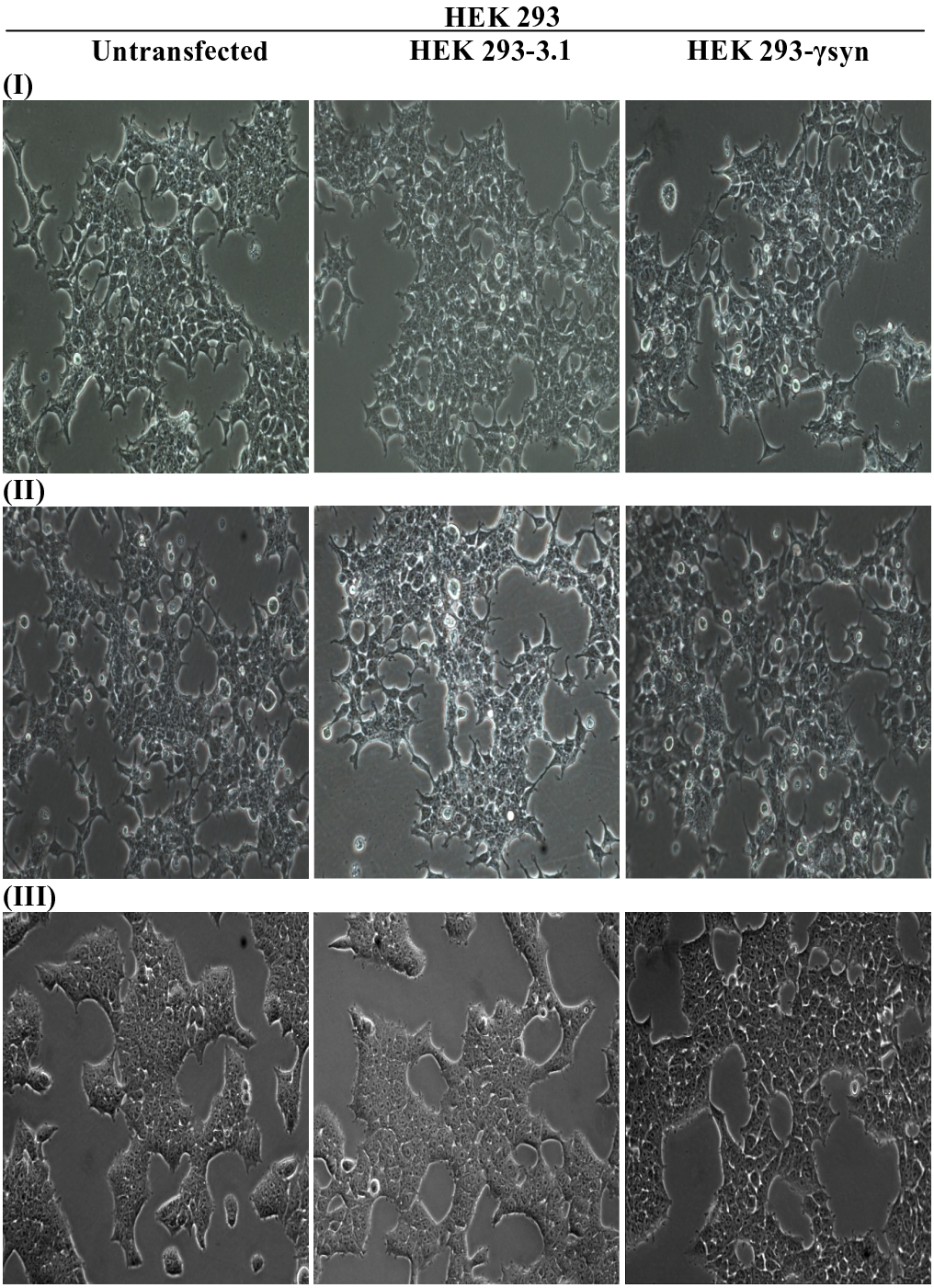


Figure 4.8 No observable cell scattering and cell spreading morphology in HEK 293. (Magnification power = 200×). (I) Control (II) Cell scattering (III) cell spreading. Cells showed no scattering pattern after serum starvation for 24 hr in cell scattering assay. Cell spreading morphology was not observable after serum induction.

4.3 γ-Synuclein Enhanced Cancer Cell Interaction with the ECM

The ECM is composed of two main classes of macromolecules: proteoglycans and fibrous proteins (Jarvelainen et al., 2009; Schaefer and Schaefer, 2010). The main fibrous proteins are collagens, elastins, fibronectins and laminins (Bosman and Stamenkovic, 2003; Oskarsson, 2013). The cell-ECM interactions are important in cell migration and invasion, which are a part of cancer metastasis components whereby cells can pass through the basal membrane and are able to invade the surrounding tissue and vessel walls, involving the ECM (Brunner and Tzankov, 2007; Lu et al., 2012). Therefore, two major ECM components, collagen and fibronectin were chosen to evaluate the ability of cell interaction with ECM in cell adhesion assay.

γ-Synuclein was able to enhance the adhesion of LS 174T to fibronectin at concentration 0.625 μg/ml but could not achieve statistical significance (p=0.064). At concentrations of 1.25, 2.5 and 5 μg/ml, the adhesion ability was markedly increased [Figure 4.9 (a)]. γ-Synuclein only showed significant increase in LS 174T adhesion to collagen I at concentrations of 20 and 40 μg/ml [(Figure 4.9 (c)]. Figure 4.9 (a) shows the increase in cells-fibronectin interactions from 0 to 2.5 μg/ml in LS 174T cells and plateaued beyond the concentration of 2.5 μg/ml. Figure 4.9 (c) shows similar trend whereby increase in cells-collagen I interactions from 0 to 20 μg/ml in LS 174T cells and plateaued beyond the concentration of 20 μg/ml. There was no significant difference for OD values between HEK 293, HEK 293-3.1 and HEK 293-γsyn cells for all concentrations of fibronectin and

collagen I [Figure 4.9 (b) and (d)]. Generally, LS 174T-γsyn had high adhesion ability to collagen and fibronectin while γ-synuclein cells did not alter the adhesion property of HEK 293 cells. The data from this study was the only one that reported on the interaction of γ-synuclein with collagen and fibronectin.

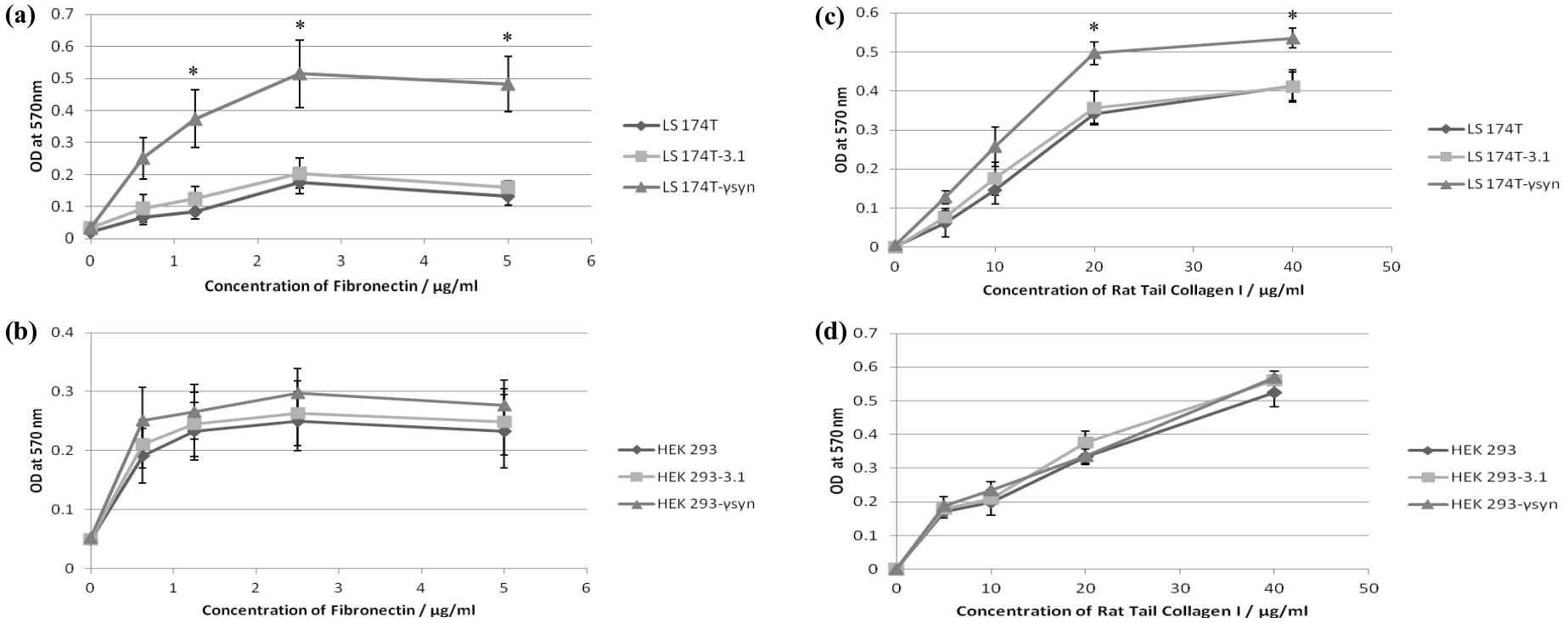


Figure 4.9 γ-Synuclein enhanced adhesion of LS 174T to fibronectins and collagen I but not HEK 293 cells. (a) and (c) LS 174T and (b) and (d) HEK 293 cells were incubated with various concentrations of fibronectins for 30 min and rat tail collagen I for 15 min. Adhered cells were stained, lysed and quantified at OD_{570}. Qualitative variations in adhesion were measured as changes in OD values. Data of OD expressed in mean ± SEM were obtained from 3 independent experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test. * $p < 0.05$ compared with LS 174T and LS 174T-3.1.

4.4 γ-Synuclein Enhanced Cancer Invasion

The Millipore® QCM™ Invasion Assay kit was used to evaluate the invasiveness of LS 174T cells performed in an invasion chamber, based on the Boyden chamber principle. Invasion through the ECM is one of the important steps in tumour metastasis. In the assay, invasive cells migrated through the ECM layer and invaded the bottom of the ECMatrix™, which is a reconstituted basement membrane matrix of protein, using FBS as the chemo-attractant. Invaded cells at the bottom of the insert membrane were dissociated from the membrane and quantified as described in section 3.8. The experiment on HEK 293 cells was not performed due to non-cancerous property of HEK 293 and HEK 293 does not possess any invasive ability. The RFU unit of LS 174T-γsyn was significantly increased as compared with untransfected LS 174T and LS 174T-3.1 cells, thus showing that LS 174T-γsyn exhibited high invasive capability (Figure 4.10).

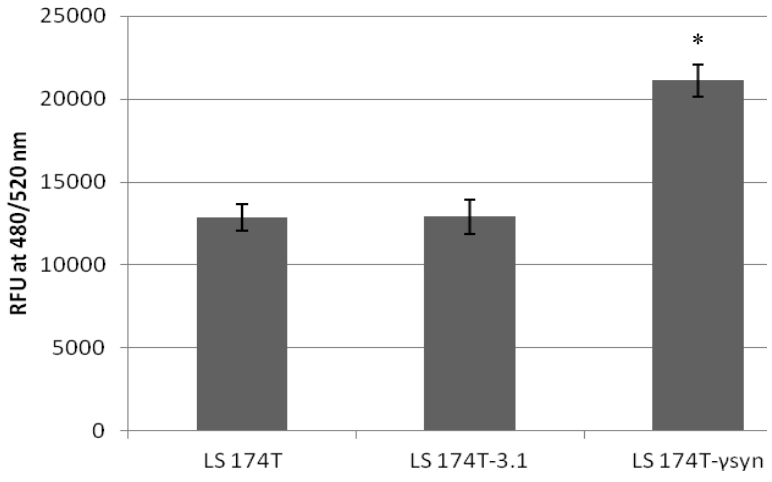
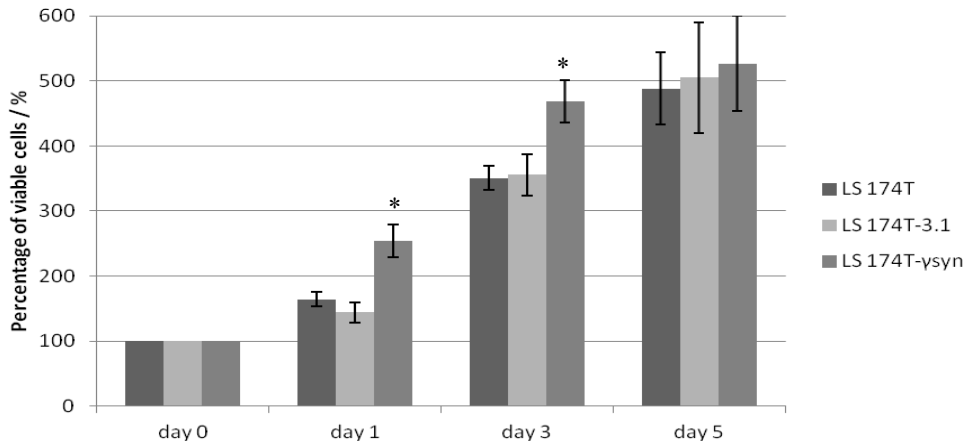


Figure 4.10 γ-Synuclein enhanced cell invasion of LS 174T. Cells were allowed to invade towards 10% FBS for 24 hr. Fluorescence measurements were taken according to manufacturer's protocol. Data of RFU expressed in mean ± SEM were obtained from 2 independent experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test. * $p < 0.05$ compared with LS 174T and LS 174T-3.1.

4.5 γ-Synuclein Enhanced Cancer Cell Proliferation

To measure the proliferation ability of cells, cells were allowed to proliferate for day 1, 3 and 5 before the number of cells was determined by MTT assay. As shown in Figure 4.11, significant increases of cell proliferation were found on day 1 and 3 of LS 174T-γsyn, whereby the percentage of cells of LS 174T-γsyn was significantly higher than that of untransfected LS 174T and LS 174T-3.1. However, percentage of HEK 293, HEK 293-3.1 and HEK 293-γsyn cells did not differ significantly throughout the experiment. The data suggested that γ-synuclein was able to promote proliferation of LS 174T but not HEK 293 under anchorage-dependent condition.

(a)



(b)

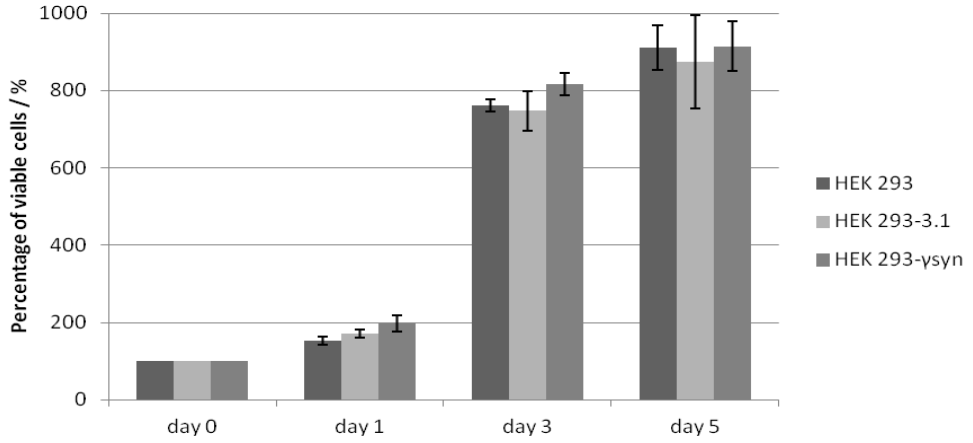


Figure 4.11 γ-Synuclein enhanced cell proliferation of LS 174T but not HEK 293. (a) LS 174T and (b) HEK 293 were seeded into 96-well plates. The cells were cultured for 24 hr (day 0) prior to MTT assay. The procedures were repeated for day 1, 3, and 5. The absorbance values at 550 nm in MTT assay were then normalised to day 0. Data of percentage of cells expressed in mean ± SEM obtained from 3 independent experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test. * $p < 0.05$ compared with LS 174T and LS 174T-3.1.

4.6 Anchorage-Independent Growth of Cells

4.6.1 γ-Synuclein Reduced Transformation Ability of Cancer Cells

Colony formation assay is designed to assay a cell's ability to grow to an unattached surface also known as anchorage-independent growth. Cells fail to grow when suspended in semi-solid medium like agar or gel. The cells

which have retained the capacity to divide will proliferate indefinitely and produce a large colony of cells. The ability of a single cell to grow into a large colony that can be visualised with the naked eye is proof that the cells have transformed. Figure 4.12 shows that colony formation of LS 174T-γsyn was significantly reduced. Colonies formed were smaller and lesser under unaided eye compared with untransfected LS 174T and LS 174T-3.1 cells Over-expression of γ-synuclein did not alter the characteristics of HEK 293 and HEK 293-γsyn did not form any visible colony under anchorage-independent condition (results not shown).

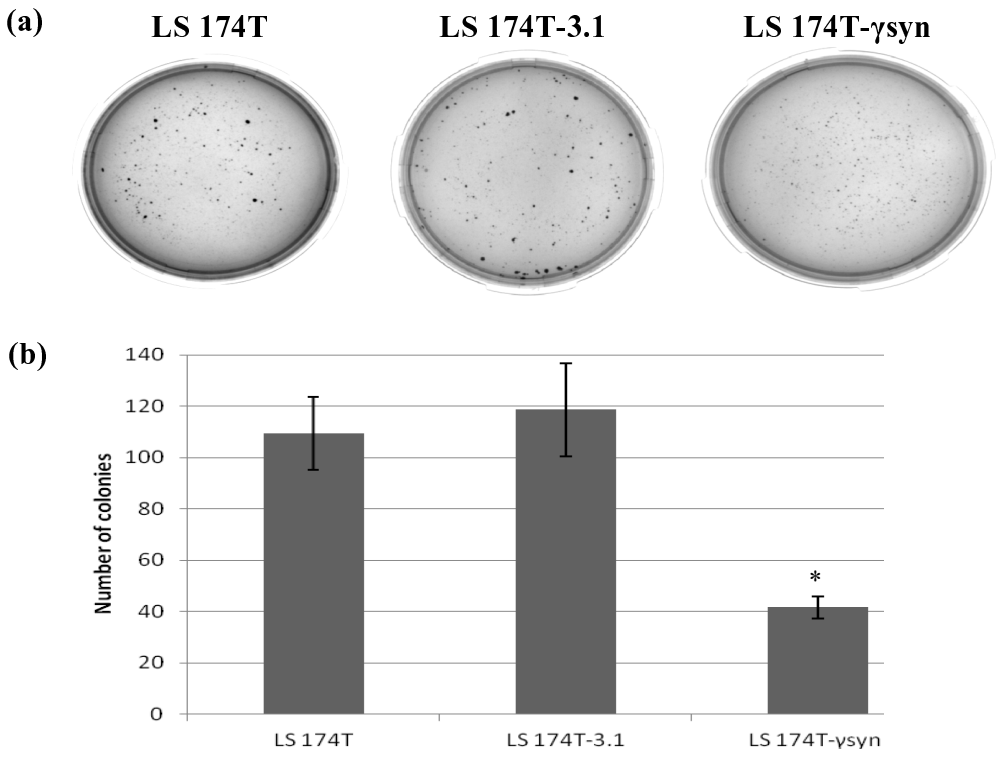
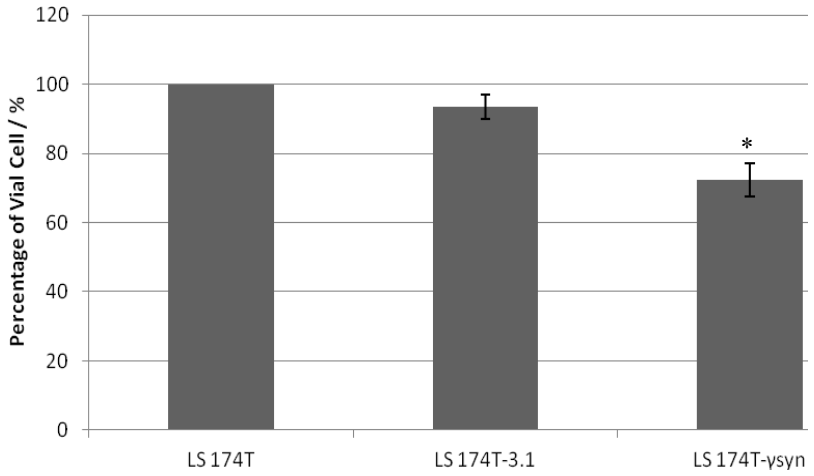


Figure 4.12 γ-Synuclein reduced transformation ability of LS 174T. Cells were plated in 0.35% agarose top agar on the top of 0.5% base agar for 1 month. Colonies formed were fixed with PFA and stained with crystal violet prior to photographing. (a) Photographed visible colonies. (b) Number of colonies counted. Data of number of colonies expressed in mean ± SEM were obtained from 3 independent experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test. * $p < 0.05$ compared with LS 174T and LS 174T-3.1.

4.6.2 γ-Synuclein Promoted Anoikis of Cancer Cells

Anoikis assay was performed to investigate whether γ-synuclein over-expressing cells could evade anoikis to give insight on the contribution of γ-synuclein to the anchorage-independent property of the cells. Anoikis is a programmed cell death induced upon cell detachment from extracellular matrix, as strategy used by organisms to eliminate unattached cells (Paoli et al., 2013). Anoikis avoidance and adapting to new microenvironment allow tumour cells to become anchorage-independent and metastasize, as one of the hallmarks of cancer cells (Hanahan and Weinberg, 2000). LS 174T-γsyn had significant lower viable cells in anoikis assay compared with untransfected LS 174T and LS 174T-3.1, indicating over-expression of γ-synuclein was able to promote anoikis in LS 174T cells [Figure 4.13 (a)]. There was no significant difference in terms of viability between untransfected HEK 293, HEK 293-3.1 and HEK 293-γsyn cells [Figure 4.13 (b)].

(a)



(b)

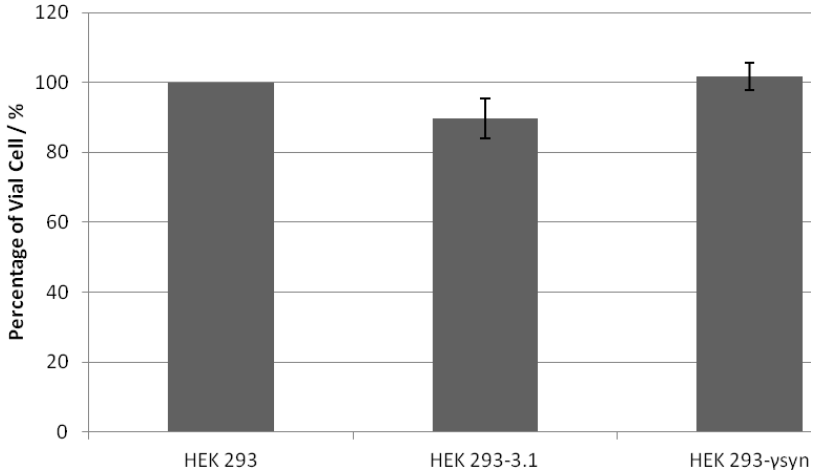


Figure 4.13 γ-Synuclein promoted anoikis of LS 174T but not HEK 293. (a) LS 174T and (b) HEK 293 were seeded in Poly-HEMA-coated wells for 3 days. Viability of the cells was then determined by MTT assay. The absorbance values at 550 nm in MTT assay were then normalised to untransfected cells. Data of percentage of vial cell expressed in mean ± SEM were obtained from 3 independent experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test. * $p < 0.05$ compared with LS 174T and LS 174T-3.1.

4.7 Effects of γ-Synuclein against Cancer Drug Treatments

4.7.1 γ-Synuclein Sensitised LS 174T towards Cancer Drug-induced Cell Death

To determine the pro-cytotoxic effects of γ-synuclein towards cancer cells in cancer drug-induced cell death, LS 174T and HEK 293 cells were challenged with two cancer drugs DOX and VIN for 48 hr under different concentrations. Cell viabilities were then assessed by MTT assay to indicate the degree of cell death. A concentration-dependent decrease in cell viability was observed.

MTT assay revealed that LS 174T-γsyn showed significant lower cell viability upon treatment with DOX at concentrations of 10 and 20 μM for 48 hr as compared with untransfected LS 174T and LS 174T-3.1 cells. Although the cell viability of LS 174T-γsyn was lower at 5 μM, it could not achieve statistical significance (p=0.097) [Figure 4.14 (a)]. There was no significant difference in terms of viability for all doses of DOX between untransfected HEK 293, HEK 293-3.1 and HEK 293-γsyn cells [Figure 4.14 (b)]. Lethal concentration 75 (LC_{75}) of DOX determined for LS 174T-γsyn cells was 6 μM and this concentration was used for subsequent apoptosis induction experiments.

Cells were further tested with VIN to ensure that the pro-cytotoxic property was not drug-dependent. Parallel with the result obtained with DOX, the MTT assay revealed that LS 174T-γsyn showed significant lower cell

viability upon treatment with VIN at concentrations of 0.025 and 0.05 μM for 48 hr as compared with untransfected LS 174T and LS 174T-3.1 cells. Although the cell viability of LS 174T-γsyn was lower at 0.1 μM, it could not achieve statistical significance (p=0.098) [Figure 4.14 (c)]. There was no significant difference in the viability for all doses of VIN between untransfected HEK 293, HEK 293-3.1 and HEK 293-γsyn cells [Figure 4.14 (d)].

The results demonstrated that high γ-synuclein expression could promote LS 174T cells towards DOX-induced cell death by decreasing cell viability.

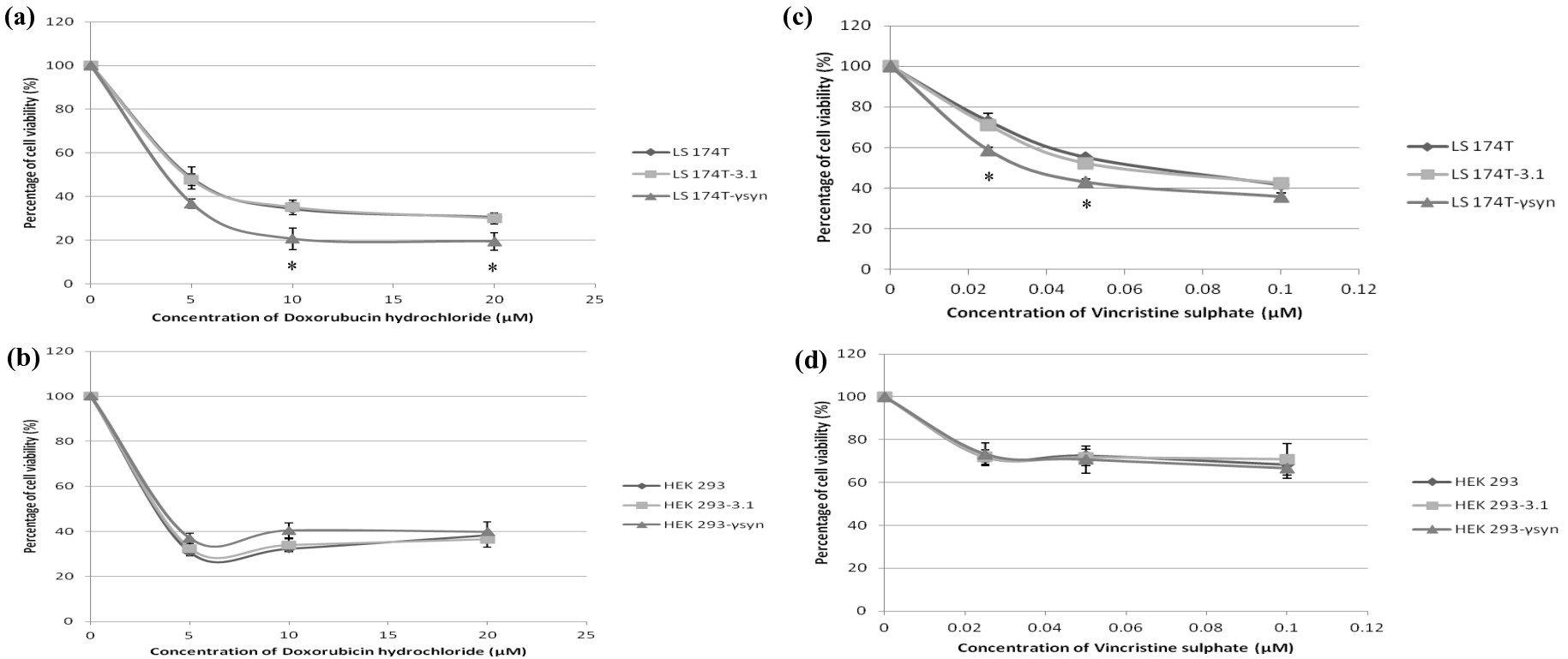


Figure 4.14 Effect of DOX and VIN on percentage of cell viability of LS 174T and HEK 293. Cells were exposed to DOX at concentrations of 20, 10 and 5 µM [(a) and (b)] and VIN at concentrations of 0.1, 0.05, and 0.025 µM [(c) and (d)] for 48 hr before MTT assay was performed and absorbance was read at 550 nm. These results represented mean ± S.E.M of three separate experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test. * $p < 0.05$ compared with LS 174T and LS 174T-3.1.

4.7.2 Observation of Morphological Changes in LS 174T and HEK 293 following DOX and VIN Treatments

Cytotoxic effects of DOX and VIN administered elicited extensive morphological changes on LS 174T and HEK 293 cells. These could be easily observed under an inverted microscope. Generally, the morphological changes of the cells correlated to the cell viability MTT results. In Figure 4.15, LS 174T cells were recognised as healthy when displaying standard island-like morphology with well-defined membrane and exhibited usual anchorage properties. Upon treatment with DOX and VIN, the growth of cells was greatly reduced. Apoptotic morphological changes were significant with the presence of chromatin condensation, nuclear fragmentations, cytoplasm blebbing followed by fragmentation of the cell into membrane-bound apoptotic bodies. Dead cells were shrunken and detached from the surface. As the concentration of DOX and VIN increased, the degree of confluence and number of healthy cells gradually decreased and apoptotic bodies gradually increased. Pro-cytotoxic properties of γ-synuclein in LS 174T could be appreciated when the island-like structure was less prominent in LS174T-γsyn. Cellular toxicity of HEK 293 was observed when the cells displayed nuclear condensation, became rounded and clumped on top of each other and a lack of uniform monolayer. No morphological changes were observed between the untransfected HEK 293, HEK 293-3.1 and HEK 293-γsyn cells (Figure 4.16).

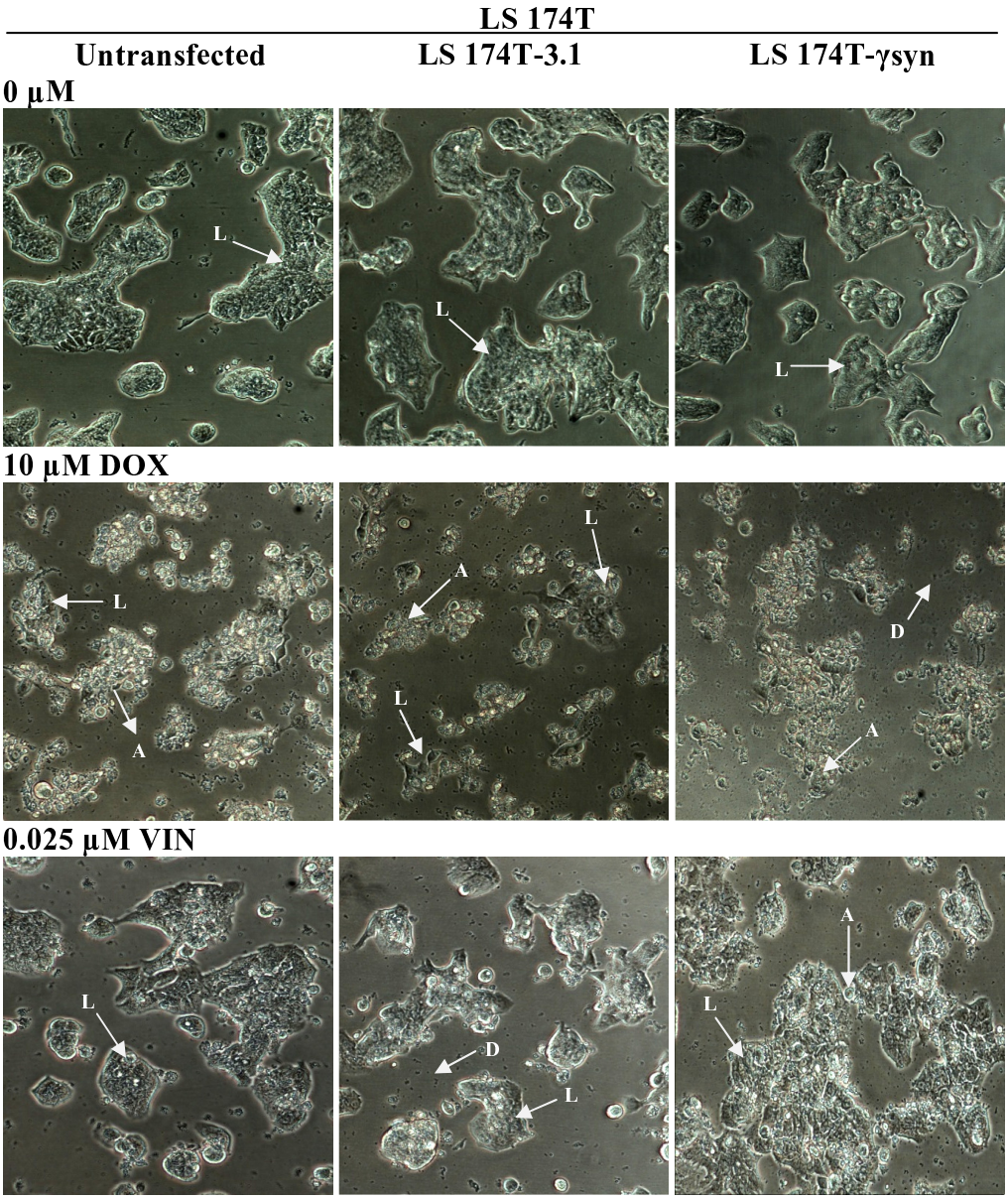


Figure 4.15 Morphological changes of LS 174T cells after treatment with DOX and VIN for 48 hr. Apoptotic bodies were visibly distinguished in the DOX and VIN-treated LS 174T cells. Photographs of cells were taken using Nikon Eclipse TS100 inverted microscope at 200× magnification. L-live cells, D-cell debris, A-apoptotic bodies.

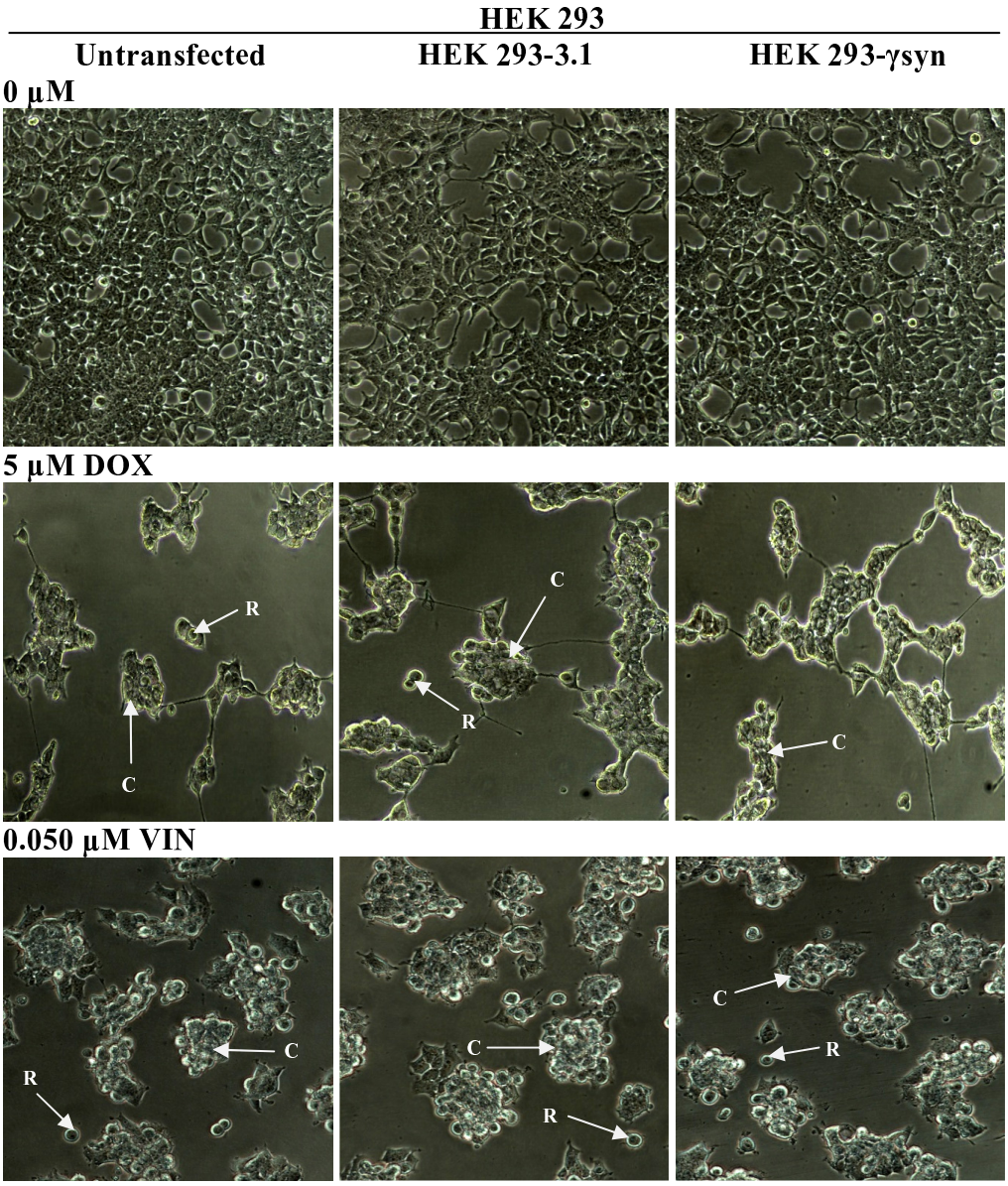


Figure 4.16 Morphological changes of HEK 293 cells after treatment with DOX and VIN for 48 hr. Rounded cells and cell clumping were observed in DOX and VIN-treated HEK 293 cells. Photographs of cells were taken using Nikon Eclipse TS100 inverted microscope at 200× magnification. R-rounded cells, C-cell clumping.

4.8 Effects of γ-Synuclein against Oxidative Stress

4.8.1 γ-Synuclein was Pro-cytotoxic during Oxidative Stress-induced LS 174T Cancer Cells Death

Section 4.7 has shown the pro-cytotoxic effects of γ-synuclein towards cancer drugs-induced cell death. This experiment was intended to ascertain the pro-cytotoxic property of γ-synuclein during oxidative stress-induced cell death as the role of synuclein as an antioxidant has been reported in previous study (Zhu et al., 2006). H_2O_2 is one of the versatile oxidants. Due to its high cell permeability, it can regulate signalling pathways and therefore promote cell death (Nogueira-Pedro et al., 2013; Sinha et al., 2013). Exposure of cells to increasing concentrations of H_2O_2 resulted in dose-dependent cell death, which was detectable 48 hr after the treatments. There was an overall significant decrement on cell viability in LS 174T-γsyn. In parallel with DOX and VIN treatments, MTT assay revealed that LS 174T-γsyn showed significant lower cell viability upon treatment with H_2O_2 at concentrations of 0.4 and 0.8 mM for 48 hr as compared with untransfected LS 174T and LS 174T-3.1 cells. Although the cell viability of LS 174T-γsyn was lower at concentration of 0.2 mM, it could not achieve statistical significance (p=0.114) [Figure 4.17 (a)]. There was no significant difference in terms of viability for all doses of H_2O_2 tested in untransfected HEK 293, HEK 293-3.1 and HEK 293-γsyn cells [Figure 4.17 (b)]. These results implicated γ-synuclein was able to play role in promoting H_2O_2-induced cell death in LS 174T colorectal cancer cells but not HEK 293 non-cancerous cells and further proved that γ-synuclein could enhance cell death in LS 174T cells.

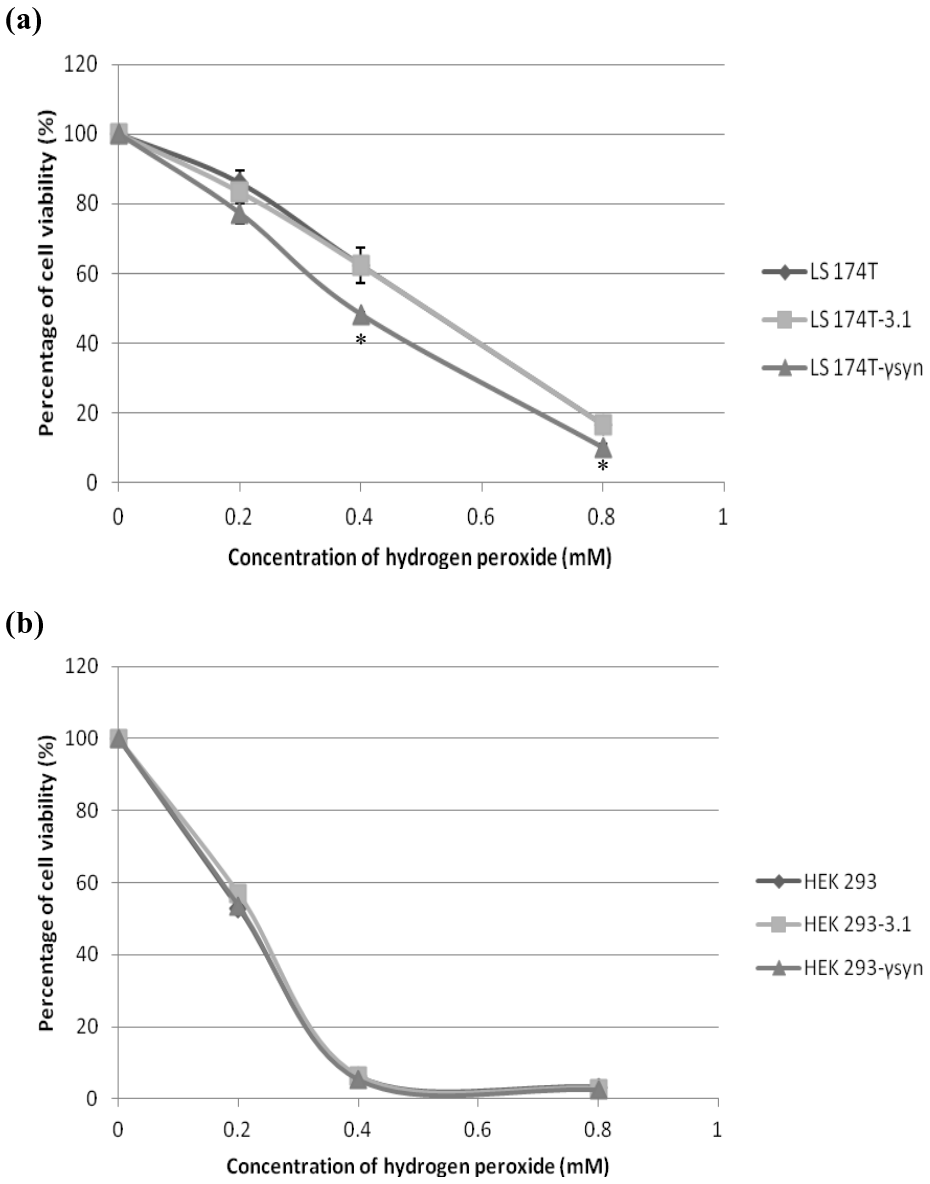


Figure 4.17 Effect of H_2O_2 on percentage of cell viability of LS 174T and HEK 293 cells. Cells were exposed to H_2O_2 at concentration of 0.8, 0.4 and 0.2 mM for 48 hr before MTT assay was performed and absorbance was read at 550 nm. These results represented mean ± S.E.M of three separate experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test. * $p < 0.05$ compared with LS 174T and LS 174T-3.1.

The mechanism of cell death mediated by H_2O_2 was examined via inverted microscope to detect morphology changes. Resembling the characteristics shown by DOX and VIN-treated cells, classical hallmarks of apoptosis like shrinkage of cell, membrane blebbing, cell rounding and loss of anchorage properties were observed in the H_2O_2-treated groups (Figure 4.18 and Figure 4.19).

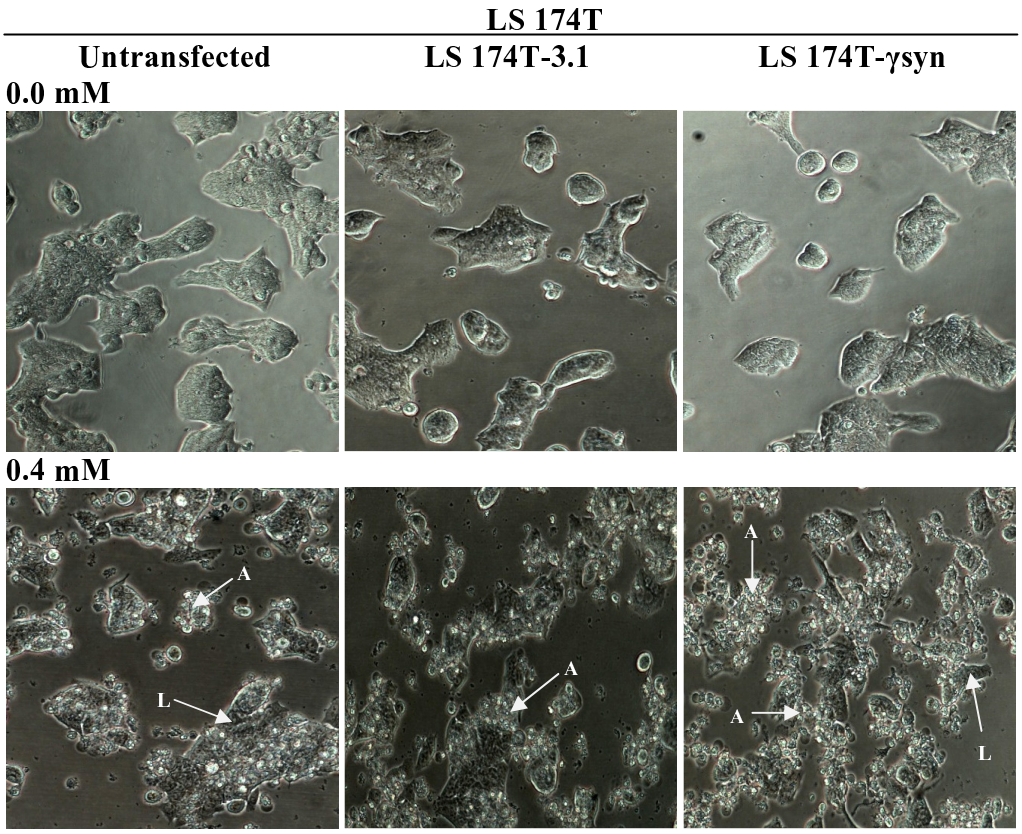


Figure 4.18 Morphological changes of LS 174T cells after treatment with H_2O_2. Apoptotic bodies were visibly distinguished in the H_2O_2-treated LS 174T. Photographs of cells were taken using Nikon Eclipse TS100 inverted microscope at 200× magnification. L-live cells, A-apoptotic bodies.

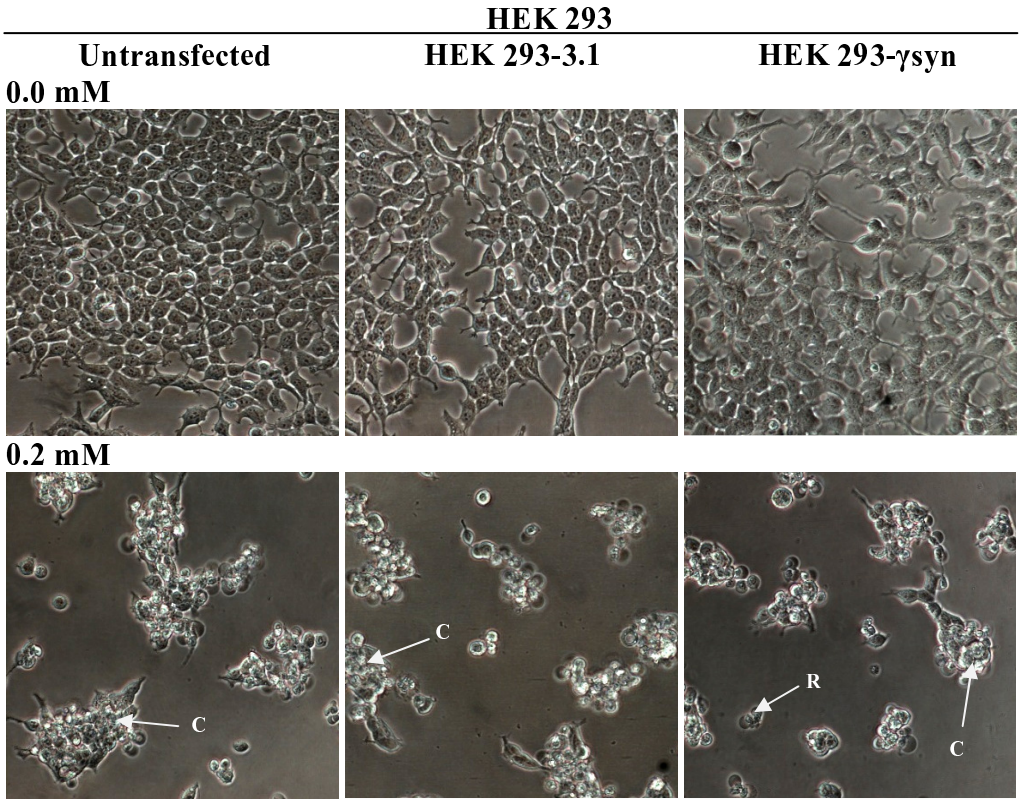


Figure 4.19 Morphological changes HEK 293 cells after treatment with H_2O_2. Photographs of cells were taken using Nikon Eclipse TS100 inverted microscope at 200× magnification. R-rounded cells, C-cell clumping

4.8.2 γ-Synuclein Enhanced H_2O_2-induced Reactive Oxidative Species Generation in LS 174T but not HEK 293

To further determine whether γ-synuclein over-expression modulates oxidative stress, the intracellular ROS generation was monitored using the redox-sensitive fluorophore, DCFH-DA following H_2O_2 treatment.

The amount of DCF formed increases proportionally to the cellular oxidant production, particularly H_2O_2. As shown in Figure 4.20, LS 174T-γsyn had significant elevated ROS levels upon H_2O_2 treatment compared with untransfected LS 174T and LS 174T-3.1 cells. On the other hand, HEK 293-γsyn showed similar ROS level when compared with untransfected cells and

HEK 293-3.1 cells upon exposure to 3 mM of H_2O_2 for 30 min. Interestingly, the basal ROS level did not change in negative control of LS 174T and HEK 293 cells. This indicated that ROS level only increased upon cell stress in LS 174T-γsyn.

Therefore, these results demonstrated that γ-synuclein has the novel ability to enhance ROS production by acting as a pro-oxidant in LS 174T. The ability of γ-synuclein in modulating ROS generation might be an explanation for its pro-cytotoxic effects during oxidative stress-induced apoptosis and further contributed to the low cell viability during H_2O_2-induced cell death in only LS 174T.

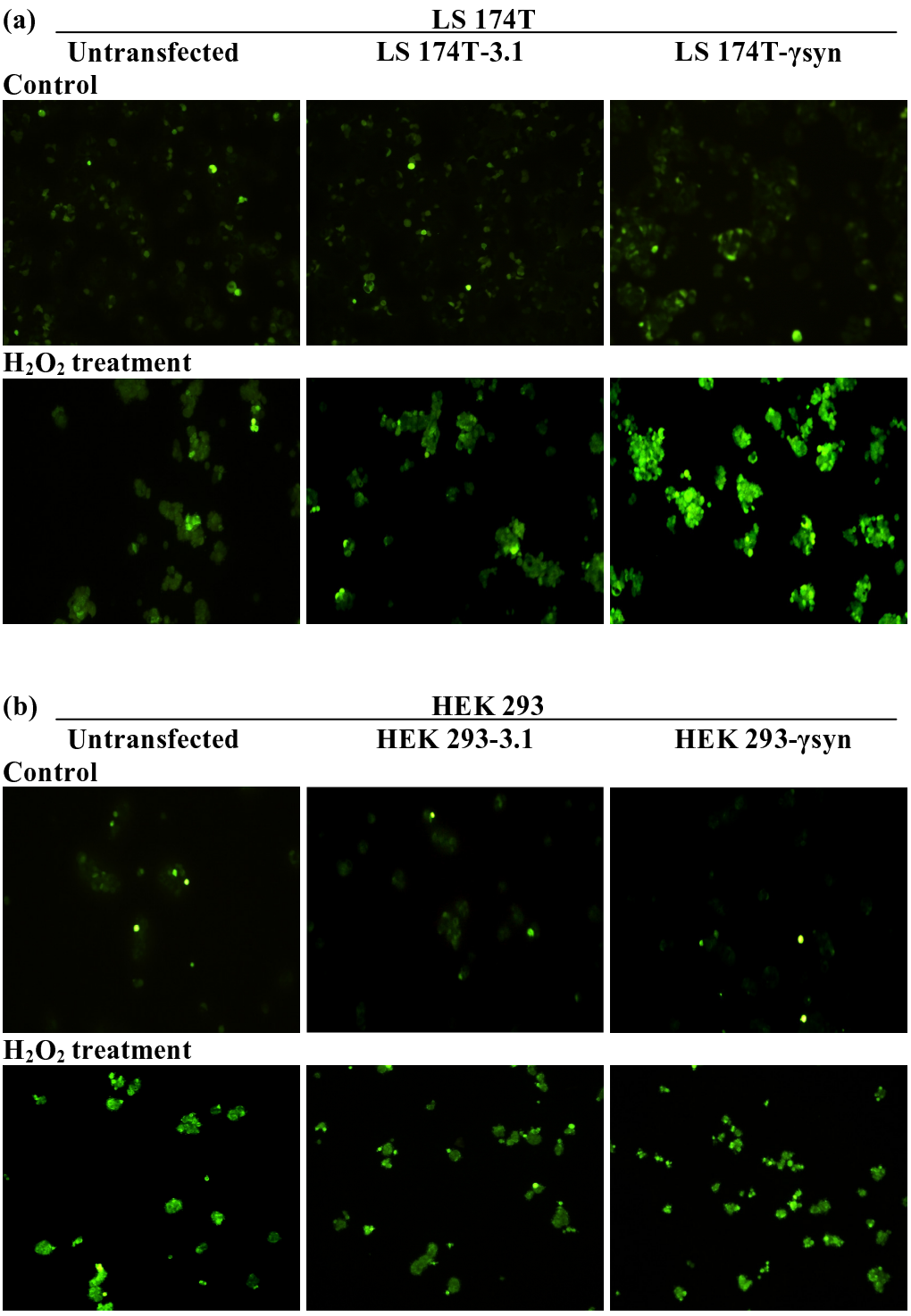


Figure 4.20 γ-Synuclein modulated H_2O_2-induced burst of ROS in LS 174T cells (a) but not HEK 293 (b). ROS generated within cells was detected using fluorescent probe DCFH-DA upon treatment with 3 mM of H_2O_2 for 30 min. Nikon Eclipse TS100 fluorescence microscope image with enhanced fluorescence revealed that ROS was elevated in LS 174T-γsyn. (Magnification = 200×).

4.9 Apoptosis Detection using FITC-Annexin V/ PI Dual Staining in LS 174T

The induction of apoptosis upon treatment with DOX at 6 μM for 48 hr in LS 174T cells is shown in Figure 4.21 (a). The binding of Annexin V-FITC to cells indicates apoptosis induction. Hence, the mechanism of cell death induced by DOX was confirmed via apoptosis as proven by Annexin V-FITC binding. As shown in Figure 4.21 (b), the healthy cells in LS 174T-γsyn was significantly lower compared with the untransfected LS 174T and LS 174T-3.1 cells. However, in comparison to the untransfected LS 174T and LS 174T-3.1, LS 174T-γsyn showed a remarkable increment in the percentage of cells with Annexin V tagging. On the other hand, stainings were being repealed by all the intact and healthy cells. These data suggested that in the presence of γ-synuclein, apoptosis process could be sped up with faster transition of PS exposure to membrane integrity loss and this result further supported the MTT cell viability assay upon DOX treatment.

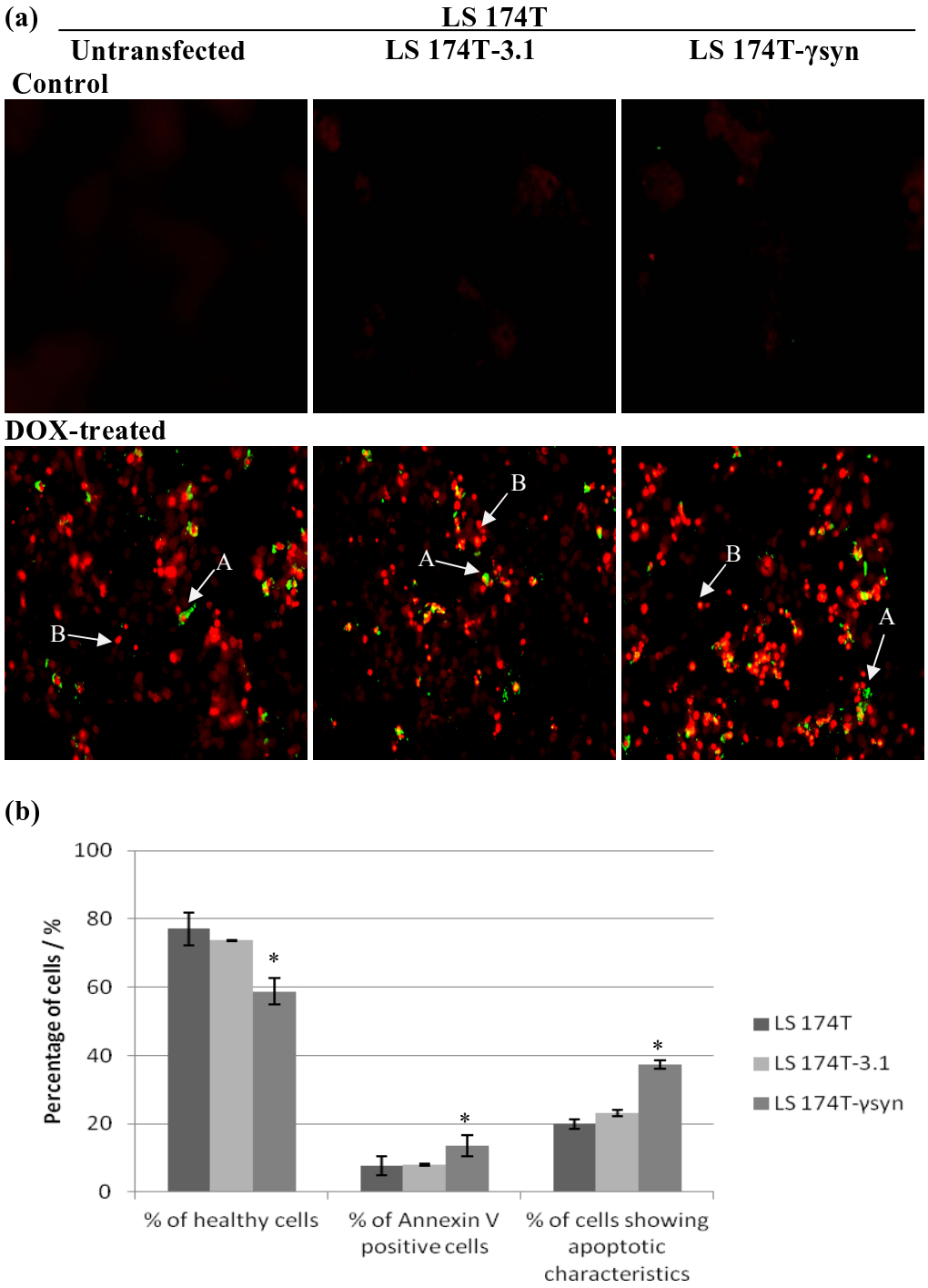


Figure 4.21 Pro-apoptotic effects of γ-synuclein in DOX-treated LS 174T cells. (a) Fluorescence microscopy of Annexin V-FITC / PI dual staining. Cells were seeded on cover slips and allowed to adhere overnight and treated with DOX for 48 hr prior to Annexin V-FITC/PI dual staining. A shows green stained cells representing cells with PS translocation. B shows red stained cells with apoptotic characteristics. (b) Statistical graph of Annexin V-FITC / PI dual staining. Data were expressed in percentage of cells represented the mean ± SEM of 2 independent experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test. * $p < 0.05$ compared with LS 174T and LS 174T-3.1.

4.10 mAb Against γ-Synuclein Inhibits DOX-mediated Pro-apoptotic Effect of γ-Synuclein in LS 174T Cells

Different mAbs have been developed as therapeutic tool for tumour treatments. Various effects of mAb on tumour cells had been reported, which include growth inhibition, apoptosis promotion (Chen et al., 2013b) and their ability to regulate proliferation signalling pathway in cancer cells *in vitro* and *in vivo* (Damiano et al., 2013). Therefore, the aim of this study was to investigate the effects of γ-synuclein mAb on the viability of untreated and DOX-treated LS 174T cells. The finding may serve as an early indicator whether γ-synuclein mAb could be used for colorectal cancer treatment. Cells were treated separately with different concentrations of γ-synuclein mAb (1, 5 and 10 μg/ml), 6 μM of DOX and combination of γ-synuclein mAb and DOX.

γ-Synuclein mAb significantly increased percentage of cell viability of DOX-treated LS 174T-γsyn at all experimental concentrations compared with the cells treated only with DOX, in which γ-synuclein exhibited cancer cell-specific pro-apoptotic characteristic towards DOX treatments. The pro-apoptotic property of γ-synuclein was not evident in HEK 293 cells. Therefore, it is believed that mAb against γ-synuclein could inhibit pro-apoptotic effect of γ-synuclein specific to LS 174T only. Surprisingly, when the cells were treated with γ-synuclein mAb alone, the growths of the cells were decreased. Cell viability of LS 174T-γsyn was significantly decreased when incubated with 1, 5 and 10 μg/ml of γ-synuclein mAb as compared with its non-treated cells. However, γ-synuclein mAb could retard the growth of LS

174T only at concentrations of 5 and 10 μg/ml. Therefore, γ-synuclein mAb could give a better growth reduction at concentrations of 1, 5 and 10 μg/ml in LS 174T-γsyn when compared with untransfected LS 174T cells [Figure 4.22 (a)]. The result indicated that the reduction in cell viability was not a direct effect exerted by γ-synuclein mAb in LS 174T. On the other hand, γ-synuclein mAb did not affect the basal growth of HEK 293, in which the cell viability did not vary when compared with non-treated cells [Figure 4.22 (b)].

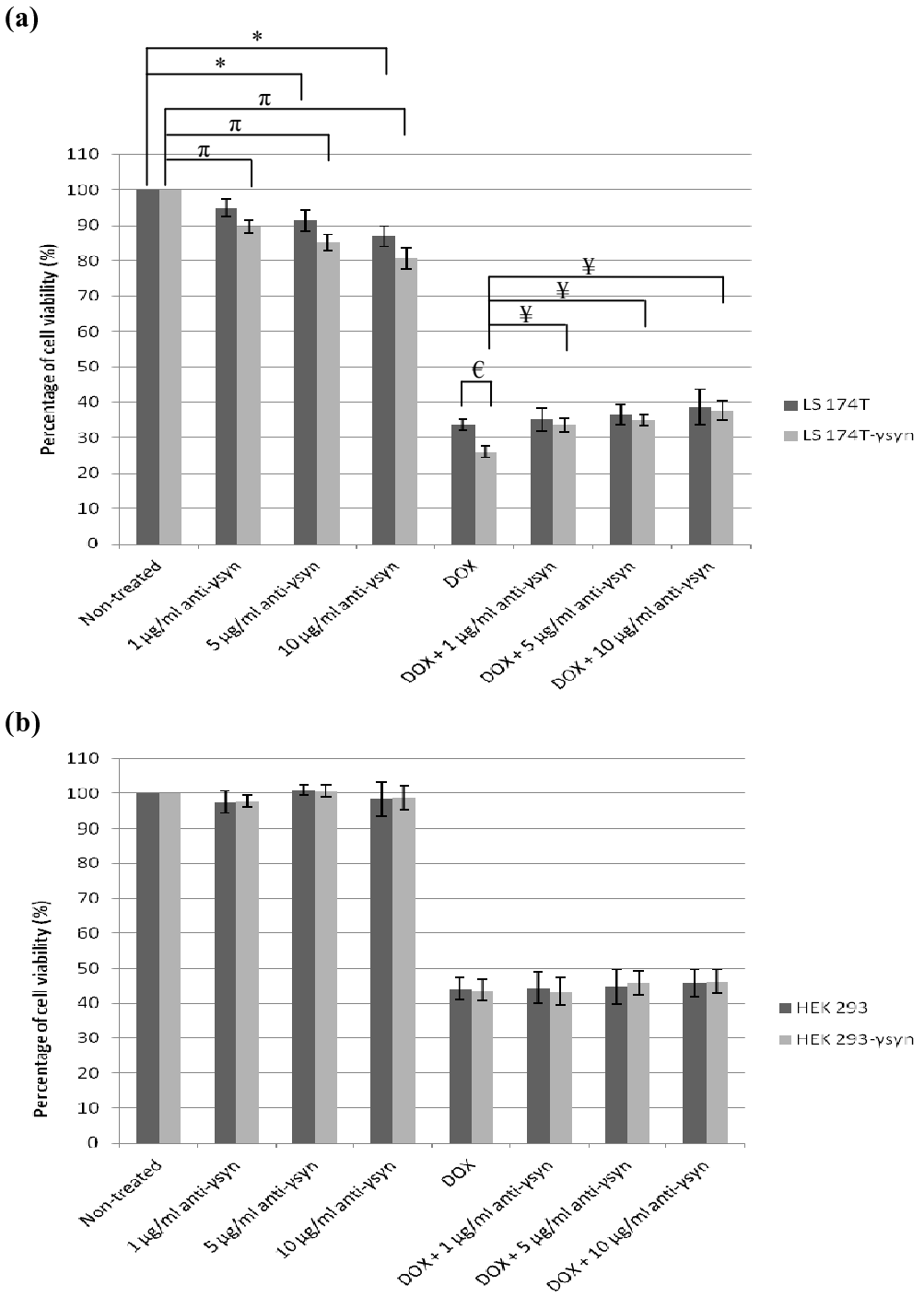


Figure 4.22 Cell viability of monoclonal anti-γ-synuclein treated and DOX-treated (a) LS 174T and (b) HEK 293. Cells were incubated with different monoclonal anti-γ-synuclein concentrations and additionally treated with 6 µM of DOX for 48 hr before cell viability were determined by MTT assay and absorbance was read at 550 nm. Data were expressed in percentage of cell viability represented the mean ± SEM of 3 independent experiments. Mean values were compared using ANOVA followed by LSD's *post hoc* test. * represents $p < 0.05$ when compared with non-treated LS 174T. π represents $p < 0.05$ when compared with non-treated LS 174T-γsyn. € represents $p < 0.05$ when compared with DOX-treated untransfected LS 174T. ¥ represents $p < 0.05$ when compared with DOX-treated LS 174T-γsyn.

4.11 Hepatocyte Growth Factor (HGF) Down-regulation in DOX-treated LS 174T-γsyn

γ-Synuclein was having pro-invasive property and could promote metastasis in only colorectal cancer LS 174T cells but it was shown to be pro-apoptotic to stress stimuli like anchorage-independent condition, oxidative challenge and chemotherapeutics drugs. A protein would not have pro-invasive and pro-apoptotic properties at the same time. Therefore, subsequent experiments were done to study the possible molecular pathways regulated by γ-synuclein in LS 174T cells which turned γ-synuclein to be pro-apoptotic under cancer chemotherapeutic drug DOX treatment.

Hepatocyte Growth Factor (HGF) expression was studied to examine whether the over-expression of γ-synuclein would modulate the expression and secretion of HGF and convert the LS 174T pro-apoptotic to DOX and VIN. HGF has been reported to be able to enhance apoptosis in human ovarian cancer cells treated with chemotherapeutics drugs, such as cisplatin and paclitaxel. The pro-apoptotic effect was shown to be dependent on the p38 MAPK signalling pathway (Coltella et al., 2006).

In this study, LS 174T cells were incubated in the absence or presence of DOX at 6 μM for 48 hr before cell culture medium conditioning for detection of HGF was prepared. As shown in Figure 4.23, all LS 174T cells expressing HGF as secretory protein was detected from culture supernatant but not as intracellular protein, evidenced from band detection corresponded to the

30 kDa of β-subunit of HGF. Expression of HGF in cell lysates could not be detected by using immune-blotting (result not shown). Over-expressing γ-synuclein in LS 174T did not alter the expression of HGF in the untreated cells. However, HGF secretion was decreased in LS 174T-γsyn after treatment with DOX, while lower HGF level was not observed in DOX-treated untransfected LS 174T and LS 174T-3.1 after the treatment. The HGF immuno-blot suggested that γ-synuclein could suppress the HGF expression after DOX treatment in LS 174T-γsyn cells.

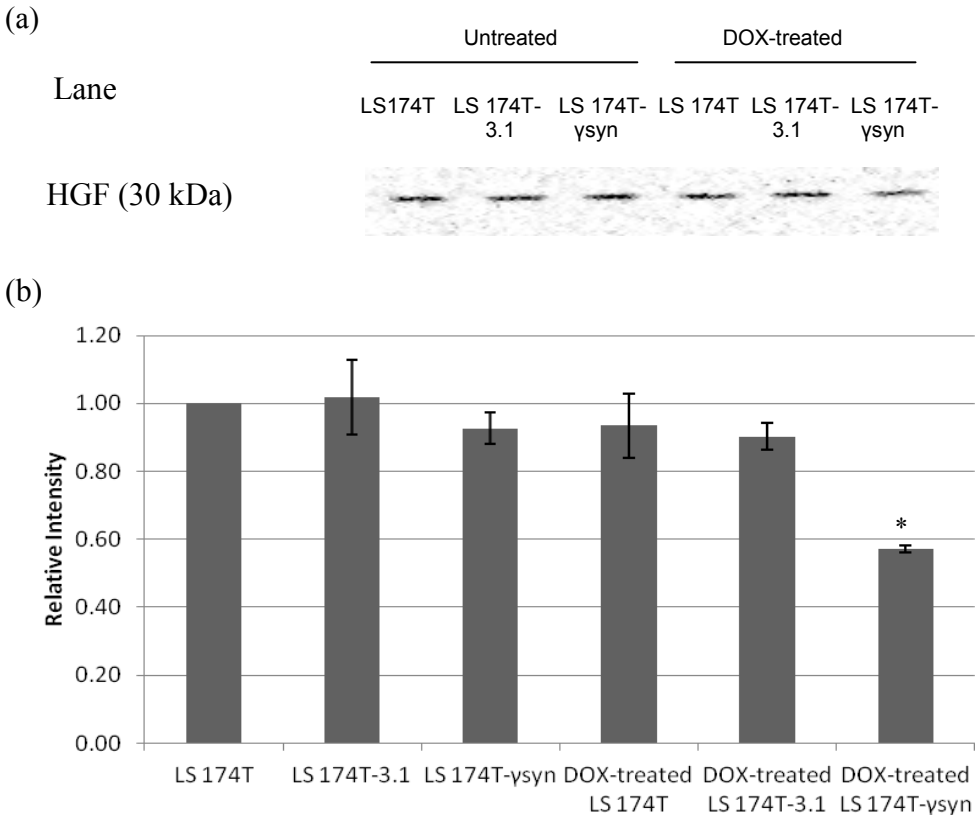


Figure 4.23 Expression of HGF in LS 174T cells by (a) Western blot analysis and (b) its relative densitometric analysis. Lysates were electrophoresed in 12% resolving SDS polyacrylamide gel. Cells were incubated in the absence or presence of 6 μM of DOX for 48 hr and cell culture supernatant concentrates were prepared and subjected to Western blot analysis. Densitometric analysis of HGF expression in Western blot shown in (b) was performed using ImageJ 1.44p software. The relative intensity of the HGF bands (30 kDa) was normalised to the untransfected LS 174T which was set at 1. * $p < 0.05$ compared with LS 174T, LS 174T-3.1, LS 174T-γsyn, DOX-treated LS 174T and DOX-treated LS 174T-3.1.

4.12 γ-Synuclein Resulted in Distinct Alterations of Phospho-kinase Activity in Untreated and Treated LS 174T

The effect of γ-synuclein on the protein phosphorylation of the cells and the determination of the possibly pathways involved were investigated in untreated cells and DOX treated cells. Forty three kinase phosphorylation sites were assayed, and among these, only one phospho-protein being detected in the untreated cells while ten types of phospho-protein were differently expressed in DOX-treated cells. As shown in Figures 4.24 (a), (b) and Figure 4.25 (a), γ-synuclein over-expression was able to enhance cAMP response element-binding protein (CREB) phosphorylation at S133 site. Figures 4.24 (c), (d) and Figure 4.25 (b) show expression of phospho-proteins such as CREB, p38α, ERK1/2, JNK pan, mitogen and stress activated protein kinase (MSK) 1/2, signal transducers and activators of transcription (STAT)2, STAT6, STAT5a, STAT5b, and c-Jun, which were involved in the regulation of the cell survival and proliferation pathways were down-regulated in DOX-treated LS 174T-γsyn (less than 20% as compared with DOX-treated untransfected LS 174T). At the same time, equal loading of the sample can be referred to the equal density of Heat Shock Protein 60 (HSP60) spots. Therefore, the assay showed that γ-synuclein exerted different molecular pathways in both untreated and DOX-treated LS 174T cells.

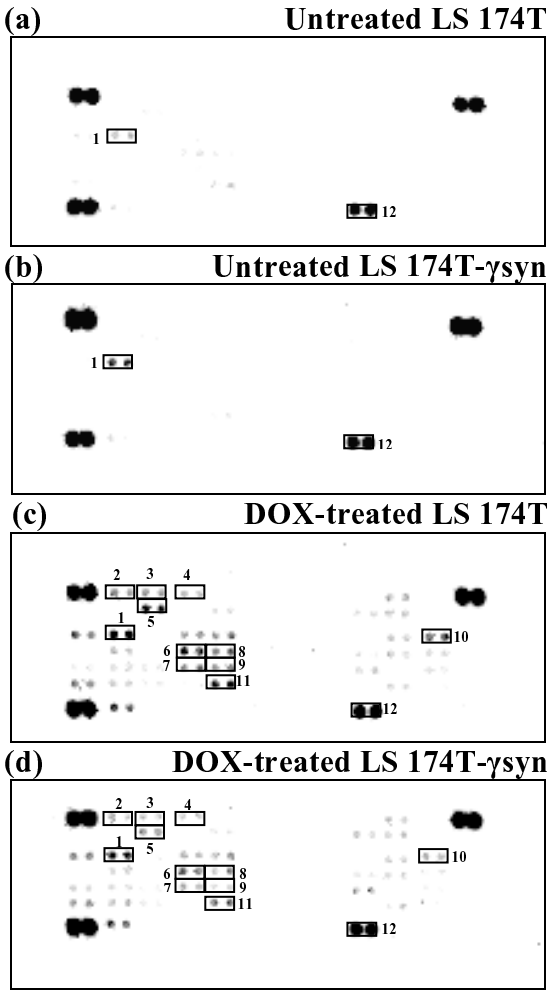
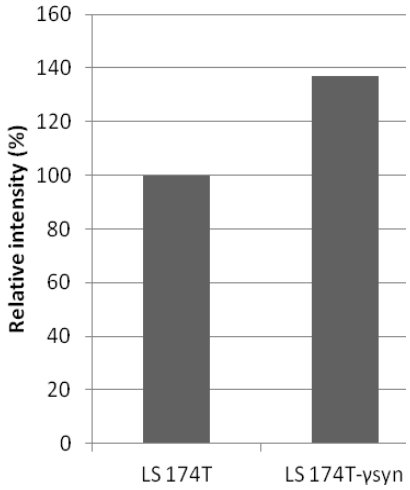


Figure 4.24 Phospho-kinase antibody array analysis. Cells were incubated in the absence or presence of 6 μM of DOX for 48 hr. Cell lysates were prepared and subjected to human phospho-kinases level determination using Human Phospho-Kinase kit (ARY003B, R&D Systems, USA), according to manucfacturer's protocol. Boxes 1–12 indicate phospho-CREB, phospho-p38α, phospho-ERK1/2, phospho-JNK pan, phospho-MSK1/2, phospho-STAT2, phospho-STAT6, phospho-STAT5a, phospho-STAT5b, phospho-c-Jun, phospho-STAT5a/b and HSP60 respectively.

(a)



(b)

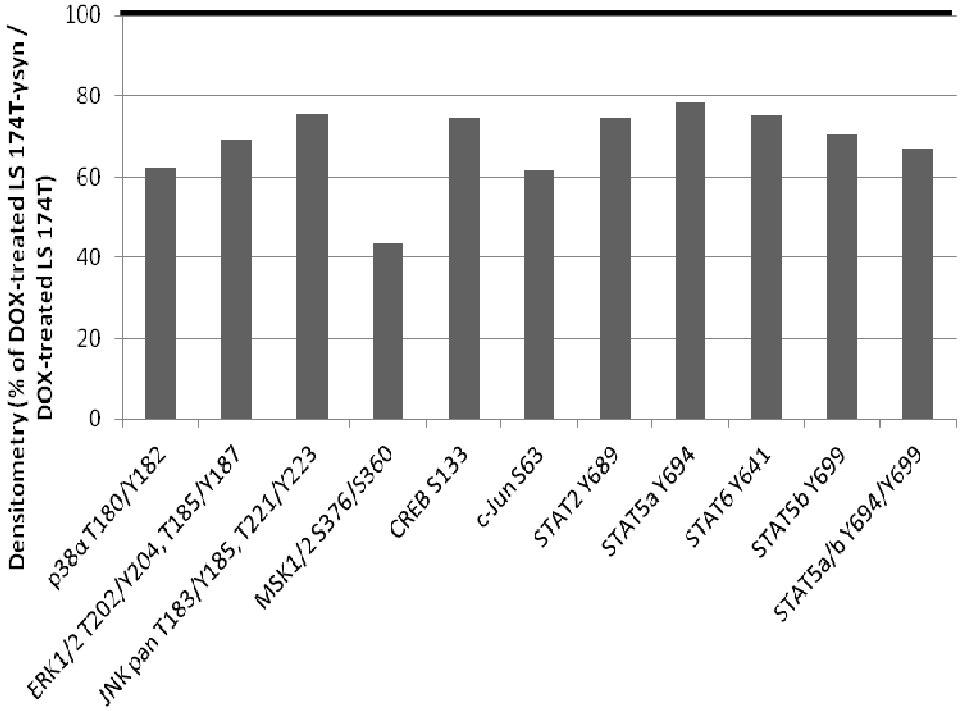


Figure 4.25 Densitometric analysis of phospho-kinase antibody array. (a) Densitometry of CREBS133 expression in untreated cells. The relative intensity was normalised to the untreated, untransfected LS 174T which was set at 100%. (b) Selective results were expressed as percentage of the DOX-treated LS 174T-γsyn as compared with DOX-treated LS 174T.

.

CHAPTER 5

DISCUSSION

5.1 Introduction

This study first analysed the possible pro-invasive roles of γ-synuclein in human colorectal cancer, LS 174T cells as compared with non-cancerous, HEK 293 cells, by γ-synuclein over-expression. Biological features of the cells like cell motility, cell attachment to extracellular matrix, cell proliferation, and cell invasion were assessed *in vitro* as the indicators for cell invasiveness. Although similar studies have been conducted previously, a concrete conclusion on the effects of γ-synuclein expression in colorectal cell lines still could not be drawn, due to the different cell lines used in previous studies (Ye et al., 2009; Ye et al., 2013; Ye et al., 2014). Besides, different colorectal cancer cell lines had been proven to give widely different patterns of growth and metastatic spread in *in vivo* orthotopic mice model (Flatmark et al., 2004). Therefore, it is believed that elucidation of the role and involvement of γ-synuclein in colorectal cancer cell lines should be directly referred to specific type of cell line, in which the human Dukes' type B colorectal adenocarcinoma LS 174T cell was emphasised in this study.

5.2 The Role of γ-Synuclein in Cell Motility

Metastasis is a multistep process by which cancer cells disseminate from primary tumours and establish secondary masses in distant locations. The metastatic process can be divided into a series of different stages. These stages include primary tumour detachment, lymphatic or vascular systems intravasation, circulation survival, host defence mechanisms avoidance, new site arrest, tissue extravasation and growth at the new site (Fidler, 1990; Chambers et al., 2002). Motility is one of the key steps in the penetration of the basement membrane and connective tissue in which cancer cells appear to move in several different ways either collectively as a group of mass or as single cell. The present study provided further evidence to support the role of γ-synuclein in colorectal cancer cell motility. γ-Synuclein was found to be able to enhance motility of LS 174T in scratch wound assay. Consistent with the finding by Ye et al. (2009), the amount of migrated colorectal cancer cells HCT116 with knock-down γ-synuclein in the lower chamber of Boyden chamber was much lesser than that of untransfected HCT116 cells, and its control HCT116/vector cells. In another study reported by Chen et al. (2012), serum chemotaxis of siRNA γ-synuclein knock-down androgen-dependent human advanced prostate cancer cell line, LNCaP, through the Matrigel in Transwell chambers was significantly reduced.

Considering the nature of cell motility, morphological changes of cells as a result of dynamic rearrangements of the cytoskeleton are common to enable cell to move from primary location to others. As such, membrane

ruffling, characterised by fluctuating movement of membrane protrusions consisting of lamellipodia and filopodia which is induced during cell movement to give the observation of cell spreading (Ridley, 1994). γ-Synuclein over-expression in LS 174T was found to be able to induce cell scattering in the cell scattering assay. The ability to form lamellipodia and filopodia was enhanced in LS 174T cell over-expressing γ-synuclein in spreading assay. The morphological changes observed further supported the motility of LS 174T cells modulated by γ-synuclein. The structure of lamellipodia and filopodia are collectively termed as invadopodia when the structures extend vertically from ventral cell membrane into the ECM by matrix degradation (Chen, 1989). During invasion, invadopodia are believed to be important for tumour cells to penetrate the basement membrane of blood vessels, which contribute to the metastatic process (Condeelis and Segall, 2003). From the structure point of view, lamellipodia are a network of branched actin filaments assembly (Chhabra and Higgs, 2007) whereas filopodia are originated from the lamellipodial actin networks but in a more parallel and tightly bundled manner (Small, 2010). Actin at the area of invadopodia is thought to depolymerise and repolymerise continuously during cell movement. Polymerisation occurs continuously at a steady state and must be balanced by depolymerisation (Mitchison and Cramer, 1996). Interactions of actin filaments with microtubules and intermediate filaments are also evidenced during invadopodia extensions (Schoumacher et al., 2010).

γ-Synuclein was reported to function as microtubule associated protein and was able to affect microtubule properties. It was reported to reduce cell

chemo-sensitivity of tumour cells by decreasing microtubule rigidity (Zhang et al., 2011). A recent study by Panneerselvam et al. (2013) found that there was strong interaction between γ-synuclein and the tail regions of microtubule subunits. This interaction altered the binding site of microtubule targeting drug like Taxol which in turn contribute to development of resistance against the drug in cancer cells.

Members of small GTPases of the Rho family were reported to be able to signal the formation of lamellipodia and filopodia (Nobes and Hall, 1995). Pan et al. (2006) had identified the activation of the Rho family small-GTPases and ERK to be the factor for γ-synuclein-enhanced cell motility in late stage breast and ovarian cancer metastasis. Therefore, this explained how γ-synuclein was able to increase the motility of cells by enhancing formation of invadopodia, which in turn contributed to the scattering morphology as observed in cell scattering assay.

5.3 Enhanced ECM Interactions Contributed to Increased Invasion

Invasion and metastasis occur as a result of the interactions between cancer cells and extracellular environment that modify the local ECM, stimulate migration, and promote proliferation and survival of cancer cells (Hanahan and Weinberg, 2000). A tumour cell must be able to degrade and remodel the ECM to assist in its migration upon adhesion. Adhesion to the ECM therefore can direct cytoskeletal remodelling and contribute to cell

motility possibly through invadopodia formation (Schmidt and Friedl, 2010), and which in turn, contribute to invasion property.

ECM is a complex network of different combinations of collagens, proteoglycans, hyaluronic acid, laminin, fibronectin, and many other glycoproteins (Frantz et al., 2010). Two major ECM components, such as collagen and fibronectin were chosen as the substrates for the cell adhesion assays as collagen is the most abundant fibrous protein within the ECM microenvironment. Collagen is also involved in the regulations of cell adhesion, chemotaxis and migration (Frantz et al., 2010). Meanwhile, fibronectin is involved in directing the organisation of ECM and is important in mediating cell migration during tumour metastasis (Frantz et al., 2010). Abnormal changes in the amount and composition of the ECM can greatly potentiate the oncogenic effects during malignant transformation. Abnormal ECM dynamics are also well-documented in clinical studies. Collagens depositions were shown to be increased during tumour formation (Zhu et al., 1995; Kauppila et al., 1998; Huijbers et al., 2010). Apart from that, fibronectin expression was related to breast cancer tumour size, histological grade, and MMP-9 expression. In the same study, tumours cells with high fibronectin were reported to be significantly associated with a higher probability of metastasis, poorer overall survival, and expression of various MMP and its inhibitors, called tissue inhibitor of metalloproteinases (Fernandez-Garcia et al., 2014).

The current results indicate that the pro-invasive potential of γ-synuclein was further convinced by the enhanced cell adhesion to ECM components. The interaction between γ-synuclein with ECM was further supported when Surgucheva et al. (2003) reported that γ-synuclein over-expression up-regulated MMP-9 gene expression. The up-regulation of MMP-9 was possibly through the activation of the AP-1 cis element in retinoblastoma Y79 cells. Therefore, it is speculated that the expression of MMP-9 could be the underlying cause to γ-synuclein-enhanced interaction of cell with ECM. Subsequently, this interaction can contribute to invasive phenotype of the cells. γ-Synuclein may promote the invasion and migration of LS 174T cells towards the basement membrane matrix of protein as highlighted in the cell invasion assay. This enhanced invasive property could be mediated through the enhanced ability to degrade and remodel components of the ECM through MMP over-expression. Comparable with the results of the enhanced invasion and adhesion property of γ-synuclein in LS 174T cells, γ-synuclein was reported to facilitate colon cancer SW1116 cells to pass through Matrigel and filter membrane and elevated the adherence of SW1116 cells to human umbilical vein endothelial cells *in vitro* (Ye et al., 2014).

5.4 γ-Synuclein in Cell Proliferation

γ-Synuclein was able to promote proliferative ability of LS 174T under anchorage-dependent condition in this study. In line with the previous studies, the expression of γ-synuclein increased breast cancer cell growth through interaction with a mitotic checkpoint kinase, BuBR1. The expression of γ-

synuclein was shown to be able to reduce the BubR1 protein expression and therefore compromised the mitotic checkpoint control (Gupta et al., 2003b). Inaba et al. (2005) further explained that γ-synuclein expression in breast cancer cells might override the mitotic checkpoint control through inhibition of the normal function of BubR1 and by promoting genetic instability. Meanwhile, silencing of γ-synuclein by siRNA in prostate cancer LNCaP cells was reported to contribute to the cellular proliferation inhibition by induction of G1 phase cell-cycle arrest (Chen et al., 2012). Furthermore, γ-synuclein had also been demonstrated to induce highly proliferative pregnancy-like phenotype of mammary epithelial cells (Liu et al., 2007). Thus, the aberrant expression of γ-synuclein was able to stimulate breast and prostate cancer progression and mammary cells development through different signalling pathways modulation and cell cycle transition.

Surprisingly, under anchorage-independent condition, the growth and colony formation ability of the γ-synuclein over-expressing LS 174T cells were remarkably low. This gave rise to the idea that γ-synuclein could be pro-apoptotic apart from being pro-invasive in LS 174T cells. The pro-apoptotic characteristics of γ-synuclein will be explained in the later section. Being proposed as the pro-invasive protein, majority of the findings reported γ-synuclein was able to enhance the growth of cancer cells by over-expression. Meanwhile, its down-regulation could suppress the growth of the cells under anchorage-dependent and -independent manners *in vitro* (Lu et al., 2002; Jiang et al., 2003; Ye et al., 2009; Liang et al., 2013). However, Zhou et al. (2003) reported that over-expression of γ-synuclein in human oesophageal squamous

carcinoma ESCC9706 cells could inhibit the growth and transformation abilities of the cells. This is the only currently known finding reporting γ-synuclein served as the negative regulator for the both anchorage-dependent and -independent growth of cells.

5.5 γ-Synuclein was Pro-apoptotic Protein in LS 174T

The anoikis promotion property of γ-synuclein under anchorage-independent condition directed the study towards apoptosis aspects of γ-synuclein in LS 174T cells. In the assessment of apoptotic characteristic of γ-synuclein, the cell viability was examined by MTT assay upon apoptosis induction by cancer drugs treatments and oxidative challenge. The results highlighted that γ-synuclein sensitised LS 174T to cancer-drugs treatments and oxidative stress induction. The findings concluded that γ-synuclein was pro-apoptotic in LS 174T whereas γ-synuclein over-expressing showed no functional difference in non-cancerous HEK 293. Thus, these supported the fact that the action of γ-synuclein was only in cancerous cells, particularly colorectal adenocarcinoma as shown in this study. The non-functional property of γ-synuclein in non-cancerous cells is still unknown at the moment.

Cells must acquire proliferative properties in response to an inappropriate environment. When cells lose their normal cell–ECM interactions, the cell cycle is arrested and apoptosis process also known as anoikis is initiated (Frisch and Ruoslahti, 1997). This may lead to the activation of caspases and downstream molecular pathways, culminating in the

activation of endonucleases, DNA fragmentation and cell death (Chiarugi and Giannoni, 2008). The balance between inducers and repressors stimuli will determine whether the cell enters proliferative or apoptotic pathways and may contribute to tumourigenesis and tumour progression (Merlino, 1994; Steller, 1995). γ-Synuclein over-expressing LS 174T cells were prone to cell death due to anoikis promotion under anchorage-independent condition. The anoikis promotion effect contributed to high apoptosis rate and subsequently low transformation ability of cells and further contributed to low tumourigenesis of cells. The anoikis assay results were in-line with soft agar colony assay result Anchorage-independent condition might be a stressful stimulus in γ-synuclein over-expressing LS 174T cells. The current novel finding directly associates γ-synuclein expression with anoikis and the result has not been reported elsewhere particularly in colorectal adenocarcinoma. MAPK pathway is one of the signalling cascades triggered during anoikis (Paoli et al., 2013). Therefore, γ-synuclein may promote the anoikis effect through MAPK pathway in LS 174T cells since γ-synuclein had been shown to be involved in apoptosis through MAPK pathway regulation in breast and ovarian cancers (Pan et al., 2002; Hua et al., 2009).

Unlike the protective roles in human breast and ovarian cancer (Pan et al., 2002; Hua et al., 2009), the multiple-drug resistance property of γ-synuclein contrasts with the novel finding of this study where the over-expression of γ-synuclein was essential for DOX- and VIN-induced cell death. The effect was drug independent of two different categories of drugs. DOX is an anti-tumour antibiotic (anthracyclines) known to bind to DNA-associated

enzymes and able to intercalate with DNA base pairs (Tacar et al., 2013), while VIN is a vinca alkaloid found to prevent the formation of spindle microtubules, therefore disables the movements of chromosomes (Himes et al., 1976). The possible γ-synuclein cytotoxicity effect towards cancer drug-induced cell death was further confirmed with Annexin V- FITC/PI dual staining where it showed that the cytotoxicity effect of γ-synuclein against cancer drug-induced cell death was via an apoptotic mechanism, instead of necrotic. Apoptotic mechanism was confirmed when the apoptotic cells were tagged with Annexin V which had high affinity to PS and PI was able to enter apoptotic bodies. This was indeed corresponded to morphological changes under light microscopy when fragmentation of the cell into membrane-bound bodies was observable.

5.6 γ-Synuclein was Pro-oxidant in LS 174T Cells

Sensitisation of γ-synuclein to H_2O_2-induced cell death further convinced that γ-synuclein is pro-apoptotic in LS 174T. The ability of γ-synuclein to modulate ROS level under oxidative stress was also being studied and the DCFH-DA based assay highlighted that γ-synuclein failed to suppress but instead enhanced intracellular ROS level upon oxidative stress induction. The results concluded that γ-synuclein is pro-oxidant and can be described as a free radical enhancer during oxidative stress in LS 174T.

Oxidative stress is an important contributing factor in carcinogenesis. Oxidative stress can bring oxidative damage to crucial biomolecules including

DNA. Accumulation of oxidative species will eventually result in impairment of cellular functions as a result of the alteration in signal transduction and gene expression. The change in these cellular events contributes to alterations of mitogenesis, transformation, mutagenesis and cell death (Hunt et al., 1998; Mills et al., 1998). Under oxidative stress condition, post-translational modifications of the other synuclein protein family, α-synuclein, have been implicated to promote oligomerisation of α-synuclein, therefore exerts toxicity to the cells (Xiang et al., 2013). It is widely accepted that accumulation of excess ROS can oxidize cellular components, including proteins leading to impaired function and eventually cell death. γ-Synuclein was shown to be the most easily oxidized member of the synuclein family and it formed oligomer aggregates that accumulate in cells after oxidation (Surgucheva et al., 2012). The γ-synuclein aggregates formation was regulated by oxidation of two adjacent amino acids, Met^{38} and Tyr^{39} (Surgucheva et al., 2012). Thus, oxidative stress may initiate the process of protein aggregation and lead to the accumulation of toxic protein inclusions. The toxic inclusion may be the reason of cellular toxicity behaviour of γ-synuclein under oxidative stress. Therefore, oxidative stress and post-translational modifications could significantly modify γ-synuclein's functional properties in LS 174T cells.

In the monitoring of intracellular ROS generation using the redox-sensitive fluorophore, DCFH-DA following H_2O_2 treatment, it is important to note that under normal physiological condition, γ-synuclein did not have influence on the production of ROS, but ROS production was only being enhanced after H_2O_2 treatment in LS 174T cells. Although there is no evidence

in the current study, it could be possibly explained that under oxidative stress, the accumulation of protein aggregation may in turn enhance the regeneration of ROS (Tabner et al., 2005; Stefani and Rigacci, 2013). The accumulation of ROS possibly due to the cell impaired ROS scavenger capability. Also, misfolded proteins may permeabilise membranes and increase intracellular free calcium ions, which in turn activate mitochondrial metabolic processes and produce ROS (Gregersen and Bross, 2010). Another possible mechanism is the accumulation of the misfolded proteins may inhibit the proteasome (Gregersen and Bross, 2010). α-Synuclein protofibrils was reported to markedly inhibit the proteasomal degradation activities and further caused α-synuclein aggregation and accumulation (Zhang et al., 2008). The protein aggregation as a result of H_2O_2 treatment may in turn cause cellular toxicity by regeneration of ROS in LS 174T. However, there is no oxidative toxicity effect in HEK 293-γsyn cells. This may be due to the cell ROS scavenger capability to counter balance the oxidative stress. Therefore, it is speculated that the oxidative stress induction in γ-synuclein over-expressing LS 174T cells is able to promote cellular toxicity which in turn further enhance the degree of ROS production.

It is important to note that anchorage-independence and loss of cell adhesion can initiate metabolic and oxidative stresses which have opposing effects on cell survival (Martindale and Holbrook, 2002). Meanwhile, production of ROS correlates with anoikis (Li et al., 1999). Therefore, the low tumourigenicity of γ-synuclein over-expressing LS 174T might be due to inability of γ-synuclein to reduce ROS level under oxidative stress

environment and inability to suppress anoikis promotion. It is in contrast to breast cancer when Jiang et al. (2004) reported that γ-synuclein expressing cells enhanced mammary cells tumourigenesis. The cell-specific action of γ-synuclein can be concluded as having both pro-invasive and pro-apoptotic properties in colorectal adenocarcinoma LS 174T cells. However, γ-synuclein has no functional effect in non-cancerous HEK 293 cells.

5.7 γ-Synuclein mAb as a Treatment for Colorectal Adenocarcinoma

Although chemotherapy has been considered the standard therapeutic means for cancer, agents with superior anti-tumour activity are needed to prolong lives in cancer patients. Monoclonal antibody therapy emerges as a new approach due to its various advantages. This therapy can alter antigens or receptors function, modulate the immune system and deliver a specific drug which is conjugated to an antibody targeting a specific antigen (Scott et al., 2012). Trastuzumab, a recombinant humanised anti-human epidermal growth factor receptor (HER)-2 monoclonal antibody is able to bind to its antigen on cell surface and triggers various anti-tumour activities such as in increasing HER-2 receptor destruction by endocytosis, inhibiting intracellular pathway signalling, inducing G1 phase arrest and triggering of antibody-dependent cell-mediated cytotoxicity in gastric cancer (Meza-Junco et al., 2011). In colorectal cancer, usage of A7-Neocarzinostatin mAb-drug conjugate in clinical trial showed the survival rate of the patients with postoperative liver metastasis treated with the conjugate was higher than the patients treated with conventional chemotherapy (Takahashi et al., 1992).

To look into the possible colorectal adenocarcinoma therapeutic mechanism in relation to γ-synuclein, LS 174T and HEK 293 cells were treated with increasing concentrations of either γ-synuclein mAb alone or a combination of γ-synuclein mAb and DOX. This preliminary result demonstrated a suppressive effect on cell viability when only LS 174T cells were treated with γ-synuclein mAb but not HEK 293. A dose response effect of γ-synuclein mAb concentrations could be observed. In addition, this study also demonstrated the ability of γ-synuclein mAb to suppress the efficacy of DOX in LS 174T-γsyn. Considering the choice of therapeutic means, a decrease in cell viability was observed in γ-synuclein mAb treated cells, but the decrease was not comparable to DOX-treated cells. Without considering the toxicity incurred by DOX, treating γ-synuclein expressing LS 174T cells with DOX alone is definitely a better option. This is because the cell viability of DOX-treated LS 174T-γsyn was the lowest among all experimental treatments due to the fact that γ-synuclein is pro-apoptotic in LS 174T. However, treating γ-synuclein mAb in γ-synuclein expressing LS 174T cells along with DOX is not recommended. This is because γ-synuclein mAb might reduce the efficacy of DOX and increase the cell viability. This might favour the cell survival and cell replication and therefore promoting the malignancy potential of the cells.

The mechanism on how γ-synuclein antibody participates in the cellular activities has been studied recently. It was being reported that γ-synuclein antibody was able to change the expression of proteins involved in apoptotic pathways. The events was triggered by the γ-synuclein antibody

internalisation in neuroretinal cells observed by presence of γ-synuclein antibody containing vesicles in living cells (Wilding et al., 2014). The γ-synuclein mAb uptake possibly uses the process of endocytosis which can happen in a more rapid way (Fewou et al., 2012). Upon entering the cells, γ-synuclein mAb binds to its intracellular antigen and alters the cellular function by binding to transcriptional factors and modulating different gene expressions. Apart from this, there are other possible ways where γ-synuclein mAb can exert its anti-tumour effects on LS 174T. This includes signalling mediated by surface antigen cross-linking that leads to cell death, blocking an activation signal that is necessary for continuous cell growth, antibody-dependent cellular cytotoxicity, complement mediated cytotoxicity and the ability of mAb to alter the cytokine production (Weiner, 2007). The mechanism under the regulation of γ-synuclein mAb in this study is still unknown. Therefore, more studies are needed to look into the possibility of using γ-synuclein mAb to suppress the invasive property of γ-synuclein expressing and non-expressing colorectal adenocarcinoma cells.

5.8 Influence of γ-Synuclein on Intracellular Signalling Pathways in LS 174T

To identify the possible intracellular pathway(s) associated with γ-synuclein, phospho-kinase antibody array screening revealed that γ-synuclein was able to enhance CREB phosphorylation at S133 site intracellularly. Up-regulation of CREB might in turn promote the invasive potential of LS 174T including enhanced motility, adhesion to ECM components, and anchorage-

dependent proliferation. The expression of HGF was not affected by γ-synuclein over-expression. Therefore, up-regulation of CREB is independent of the expression of HGF.

Transcription of the down-stream target genes can be regulated by CREB. The transcriptional function of CREB is activated when CREB is being phosphorylated at S133, which triggers the recruitment of transcriptional co-activators, CREB-binding protein (CBP) which in turn, mediates transcriptional activation through its association with RNA polymerase II complexes (Mayr and Montminy, 2001). The phosphorylation of CREB can be catalysed by a variety of kinases, including calcium/calmodulin-dependent kinases, Akt or p90Rsk, and protein kinase A, which is activated by cAMP (Shaywitz and Greenberg, 1999; Sakamoto and Frank, 2009,). Therefore, it is believed that γ-synuclein is involved in either pathway in promoting the S133 phosphorylation of CREB and affecting gene transcription. CREB S133 correlated positively with enhanced expression of ECM protein fibronectin and cell cycle progression factor cyclin D1 (Devi et al., 2011). Besides, CREB S133 which activates both membrane type 1 metalloproteinase (MT1-MMP) and MMP2 expression in various melanoma cells further convinced that CREB S133 can regulate cell invasion (Melnikova et al., 2006). CREB also interacts with Rho GTPase (Sordella et al., 2002) which is a possible γ-synuclein interacting protein candidate. The γ-synuclein-enhanced cell invasion property might be modulated by CREB S133 since CREB S133 was reported to be involved in the regulation of cell proliferation, differentiation and survival (Shaywitz and Greenberg, 1999; Ishida et al., 2007).

In the investigation on how γ-synuclein provides pro-apoptotic characteristic under DOX treatments, γ-synuclein was found to be able to modulate multiple molecular pathways in untreated and DOX-treated LS 174T cells. Unlike the protective effects of HGF reported elsewhere, HGF was shown to sensitise ovarian cancer cells to low dose of paclitaxel and cisplatin (Rasola et al., 2004). HGF was also shown to enhance apoptosis in human ovarian cancer cells treated with paclitaxel and cisplatin through the p38 MAPK signalling pathway (Coltella et al., 2006). Therefore, HGF was described to possess dual ability either as a pro-invasive signal or cellular toxicity signal. In conjunction with that, HGF expression was determined in LS 174T cells.

The current novel finding indicates the paracrine effect of HGF in LS 174T is down-regulated by γ-synuclein over-expressing in LS 174T, which in turn down-regulates phospho-proteins involved in the transduction of cell survival and proliferation signals possibly through CREB, STAT protein family, JNK pan; ERK1/2, p38α, c-Jun, MSK1/2. The schematic diagram in Figure 5.1 explains the possible signalling pathways that contribute to the pro-apoptotic condition in γ-synuclein over-expression DOX-treated LS 174T.

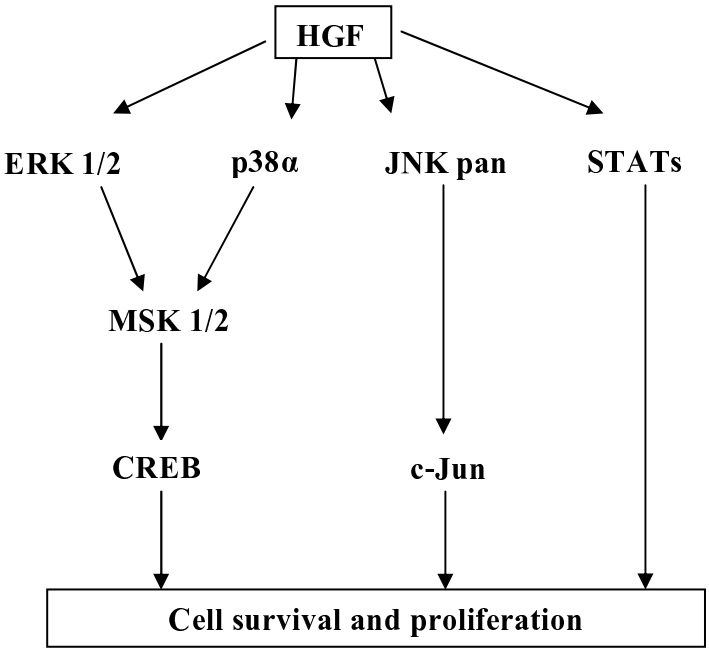


Figure 5.1 Possible signalling pathway induced by the over-expression of γ-synuclein in DOX-treated LS 174T cells.

HGF is a multifunctional protein, originally identified as a mitogen for mature hepatocytes. It is also reported to elicit different functions in different cell types. HGF is not only able to support organogenesis and morphogenesis of various tissues and organs but it also elicits potent organotrophic function in supporting regeneration of organs (Matsumoto and Nakamura, 1996). HGF is also involved in tumour invasion and metastasis (Scarpino et al., 1999), and blocks the induction of apoptosis (Fan et al., 1998; Liu, 1999). In this study, HGF behaves more like a protective signal. Lower expression of HGF sensitizes γ-synuclein over-expressing LS 174T towards DOX toxicity, therefore, promoting apoptosis.

Upon binding to its only receptor, c-MET, the activated signalling system plays an essential role in tumourigenesis (Bottaro et al., 1991; Ma et

al., 2007). Paracrine effect of HGF was observed where over-expression of c-MET protein in colorectal cancers along with HGF, promotes the metastatic potential of cancer cells (Hiscox et al., 1997; Otte et al., 2000), while inhibition of HGF was reported to prevent colorectal metastasis (Wen et al., 2004). However, currently, the mechanism whereby HGF/c-MET leads to cell survival and apoptosis in cancer cells has not been well-understood. In this study, the suppression of intracellular signalling by low level of HGF occurs possibly through STAT family proteins phosphorylation and MAPK pathways regulations.

In HGF/c-MET/STAT pathway, STATs are initially present in inactive forms in the cytoplasm. Upon c-MET activation, Janus kinases (JAK) become activated and phosphorylated which leads to the phosphorylation of STAT proteins at their tyrosine residues. The phosphorylated STAT proteins translocate to the nucleus after homodimering and heterodimering to activate gene expression of specific DNA sequences (Akira, 1999). Another protein, Src is also activated down-stream to activation of c-MET, which will lead to STAT phosphorylation (Sam et al., 2007). However, Src is not being expressed in LS 174T cells (Trainer et al., 1988). JAK was also not a part of the phospho-kinase assay. Therefore, the JAK/STAT activation pathway could not be confirmed. This study has given the evidence that phosphorylations of STAT-2, STAT-5a and 5b, and STAT 6 could give impact to the DOX-induced cell death. STAT phosphorylation can contribute to the alteration in the genetic program of cells, most likely through increased proliferation and cell survival. The role of STATs in oncogenesis involves up-regulation of anti-

apoptotic gene (Bcl-xl, Mcl-1), cell cycle regulators (cyclins D1/D2, c-Myc), and angiogenesis factor (VEGF) which inhibit apoptosis, promote cell-cycle progression and tumourigenic transformation (Buettner et al., 2002). The down-regulation of STAT protein family in this study therefore could sensitize γ-synuclein over-expressing LS 174T towards DOX-induced apoptosis.

MAPK kinase pathways can also be activated through c-MET activation (Goyal et al., 2013). Three groups of MAPKs include p38α, JNKs and ERKs had been involved in the apoptosis pathway induced by DOX and regulated by γ-synuclein. HGF-induced phosphorylation of p38 is well-documented (Awasthi and King, 2000; Recio and Merlino, 2002; Coltella et al., 2006) and there are evidences suggesting that activation of the RAS protein and its downstream effectors can activate ERKs which is essential in mediating the biological effects of HGF (Hartmann et al., 1994; Potempa and Ridley, 1998; Paumelle et al., 2000). MSK has been shown to be the common substrate to p38α and ERKs (Siebel et al., 2013) and MSK was reported to be able to mediate ERK- or p38-induced phosphorylation of CREB at S133 (Wiggin et al., 2002). Apart from that, JNK and p38 MAPK pathways are required in HGF-induced cell biological responses including proliferation, differentiation, and migration (Su et al., 2012). Activated JNKs can phosphorylate and activate c-Jun, which subsequently mediate cell growth, proliferation, differentiation, growth arrest, or cell death (Leventaki et al., 2014). Therefore, the activation of three MAPKs members will result in activation of common ultimate transcription factors, CREB and c-Jun. The inhibition of JNK in lymphoma cells has been shown to lead to apoptosis and

cell cycle arrest at the G2/M phase (Gururajan et al., 2005). c-Jun inhibition also had been proved to reduce cellular migration and invasion of mammary tumour (Jiao et al., 2010). The interaction between JNK and anti-apoptotic protein, Bcl-2 through phosphorylation was reported to be able to mediate prolonged cell survival (Deng et al., 2001). Taken into the account of the pathways regulated by HGF, it can be concluded that low transduction of survival and proliferation signal as a result of down-regulation of HGF paracrine effect results in down-regulation of CREB and c-Jun, therefore, contributing to pro-apoptotic characteristic of γ-synuclein in LS 174T.

5.9 Future Studies and Implications

Future studies might be directed to investigate the novel functional interactions between γ-synuclein and the intracellular signalling proteins considering the involvement of HGF/MAPK and HGF/STAT signalling pathways in modulating the apoptosis cascade and the involvement of CREB in invasive property of LS 174T carcinogenesis. The pro-apoptotic property of γ-synuclein could also be confirmed by referring to the expression of specific apoptotic markers expressed down-stream to the said pathways. Furthermore, it would be important to determine whether the dual pro-invasive and pro-apoptotic properties of human γ-synuclein have *in vivo* relevance. A more detailed cell cycle analysis could be done to assess the effects of γ-synuclein expression on cell cycle progression by referring to cyclin and cyclin-dependent kinase expressions and activity levels. Moreover, studies might be performed to elucidate the exact in-depth mechanisms in connection to the

pro-invasive property of γ-synuclein in relation to MMP expression and activities. Other studies may be also directed to look into angiogenesis aspects of γ-synuclein as angiogenesis is required for invasive tumour growth and metastasis. This can be done by using chick embryo chorioallantoic membrane as an *in vivo* model and the ability of γ-synuclein to influence the expression of angiogenesis factors can be screened using a commercial angiogenesis antibody array or ELISA kit. Further investigation should be carried out to reveal whether γ-synuclein regulates the mitochondrial complex activity in LS 174T cells and also assess the involvement of anti-oxidant mechanism enzymes by examining the activity levels of superoxide dismutase, catalase, and glutathione peroxidase. These further investigations are important to give a better insight into the biological functions of γ-synuclein in the carcinogenesis of colorectal adenocarcinoma.

As γ-synuclein over-expression has been proved to be involved in colorectal adenocarcinoma cell invasion which leads to cancer progression and its cell pro-cytotoxic/pro-apoptotic property under stressful environment, the possibility of using γ-synuclein as a potential therapeutic target for colorectal adenocarcinoma drug therapy could be raised. The recruitment of multiple drugs in treating colorectal adenocarcinoma over-expressing γ-synuclein has not been experimented. It is also likely that γ-synuclein can be developed into a colorectal adenocarcinoma histological biomarker for diagnosis and prognosis purposes. This can be done by referring to its extracellular secretion level and mechanism in LS 174T cells and clinical colorectal adenocarcinoma tissue samples.

Last but not least, the relationship between γ-synuclein and other synuclein members has to be investigated in LS 174T colorectal adenocarcinoma cell to give a better idea on how synucleins could co-operatively modulate the activities of LS 174T.

CHAPTER 6

CONCLUSION

In summary, the finding of the present studies indicated that γ-synuclein could promote invasiveness of LS 174T cells while it has no noticeable effect on HEK 293 cells. The conclusion was made based on the observations that γ-synuclein over-expressing LS 714T had higher motility in scratch wound assay when compared with untransfected cells and mock transfectant. LS 174T-γsyn tended to scatter in cell scattering assay, while its ability to form lamellipodia and filopodia was enhanced in spreading assay. Enhanced adhesion of LS 174T-γsyn to fibronectin and collagen in adhesion assay and significant higher proliferation rate measured by MTT assay showed that γ-synuclein was able to increase extracellular matrix interaction and promoted anchorage-dependent proliferation of LS 174T. Higher invasiveness of LS 174T-γsyn was confirmed by enhanced invasion to the bottom of the basement membrane in Millipore® QCM™ Invasion Assay. Parallel with other findings, the current findings further supported the current literature that γ-synuclein was able to promote invasion and possibly metastasis, particularly in colorectal adenocarcinoma. It had been shown that γ-synuclein exerted its invasive effect in LS 174T through CREB phosphorylation.

Soft agar colony assay that was done to measure the anchorage-independent growth of cells showed low tumourigenic ability of LS 174T-

γsyn. Later, it was found the condition was due to anoikis promotion ability of γ-synuclein. This opened the window to investigate the effect of γ-synuclein in LS 174T cell death. LS 174T over-expressing γ-synuclein was shown to be more susceptible to DOX and VIN treatments as compared with mock transfectant and wild type cells, possibly through the decrease of the HGF expression which in turn suppressed the activation of MAPKs and STAT phosphorylations. The pro-cytotoxic effect of γ-synuclein against cancer drugs-mediated cell death was via an apoptotic mechanism. This study further drew attention to the anti-oxidant role of γ-synuclein during oxidative stress-induced cell death. MTT cell viability assay revealed that γ-synuclein sensitised LS 174T towards H_2O_2-induced cell death. DCFH-DA based assay demonstrated that γ-synuclein could act as a free radical promoter during oxidative stress and these further supported the pro-apoptotic effect of γ-synuclein.

In evaluating the possibility to use γ-synuclein mAb as the therapeutic means in colorectal adenocarcinoma, γ-synuclein mAb was found to be able to attenuate the basal growth of LS 174T. However, the pro-apoptotic characteristic of γ-synuclein was reversed by mAb treatment, thus suggesting that the concurrent mAb and DOX treatments should be avoided and only cancer-drug treatment should be recommended in targeting LS 174T over-expressing γ-synuclein protein.

These results suggested that γ-synuclein conferred both pro-invasive and pro-apoptotic effects in LS 174T colorectal adenocarcinoma but has no

noticeable effect in HEK 293 which is non-cancerous human embryo kidney cells. Based on the findings, it can be concluded that γ-synuclein might have numerous important implications for colorectal carcinogenesis. Although the mechanism involved in the pro-invasive and pro-apoptotic characteristics were not pointed specifically in this study, the results provided a stimulus for further studies on the role of γ-synuclein in the colorectal cancer biology not just in the invasive and apoptotic aspects, but also in other hallmarks of cancer like replicative potential and angiogenesis.

REFERENCES

Abeliovich, A. et al., 2000. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron,* 25 (1), pp. 239-252.

Abercrombie, M., Heaysman, J. E. and Pegrum, S. M., 1970. The locomotion of fibroblasts in culture. III. Movements of particles on the dorsal surface of the leading lamella. *Experimental Cell Research,* 62 (2), pp. 389-398.

Ahmad, M., Attoub, S., Singh, M. N., Martin, F. L. and El-Agnaf, O. M., 2007. Gamma-synuclein and the progression of cancer. *FASEB Journal,* 21 (13), pp. 3419-3430.

Akira, S., 1999. Functional roles of STAT family proteins: lessons from knockout mice. *Stem Cells,* 17 (3), pp. 138-146.

Amsterdam, A. et al., 2012. Differential staining of gamma synuclein in poorly differentiated compared to highly differentiated colon cancer cells. *Oncology Reports,* 27 (5), pp. 1451-1454.

Arjonen, A., Kaukonen, R. and Ivaska, J., 2011. Filopodia and adhesion in cancer cell motility. *Cell Adhesion & Migration,* 5 (5), pp. 421-430.

Awasthi, V. and King, R. J., 2000. PKC, p42/p44 MAPK, and p38 MAPK are required for HGF-induced proliferation of H441 cells. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 279 (5), pp. L942-L949.

BDGP Resources, 2014. pOTB7 Vector [Online]. Available at: http://www.fruitfly.org/about/methods/pOTB7vector.html. [Accessed: 28 August 2014].

Bienert, G. P. et al., 2007. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *The Journal of Biological Chemistry,* 282 (2), pp. 1183-1192.

Bienert, G. P., Schjoerring, J. K. and Jahn, T. P., 2006. Membrane transport of hydrogen peroxide. *Biochimica et Biophysica Acta,* 1758 (8), pp. 994-1003.

Bosman, F. T. and Stamenkovic, I., 2003. Functional structure and composition of the extracellular matrix. *The Journal of Pathology,* 200 (4), pp. 423-428.

Bottaro, D. P. et al., 1991. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science,* 251 (4995), pp. 802-804.

Bruening, W. et al., 2000. Synucleins are expressed in the majority of breast and ovarian carcinomas and in preneoplastic lesions of the ovary. *Cancer,* 88 (9), pp. 2154-2163.

Brunner, A. and Tzankov, A., 2007. The role of structural extracellular matrix proteins in urothelial bladder cancer (review). *Biomarker Insights,* 2, pp. 418-227.

Bryan, T. M. and Cech, T. R., 1999. Telomerase and the maintenance of chromosome ends. *Current Opinion in Cell Biology,* 11 (3), pp. 318-324.

Buchman, V. L., Adu, J., Pinon, L. G., Ninkina, N. N. and Davies, A. M., 1998a. Persyn, a member of the synuclein family, influences neurofilament network integrity. *Nature Neuroscience,* 1 (2), pp. 101-103.

Buchman, V. L. et al., 1998b. Persyn, a member of the synuclein family, has a distinct pattern of expression in the developing nervous system. *The Journal of Neuroscience,* 18 (22), pp. 9335-9341.

Buettner, R., Mora, L. B. and Jove, R., 2002. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clinical Cancer Research,* 8 (4), pp. 945-954.

Burre, J. et al., 2010. Alpha-synuclein promotes SNARE-complex assembly *in vivo* and *in vitro*. *Science,* 329 (5999), 1663-1667.

Cancer Research Initiative Foundation, 2013. Status of Cancer in Malaysia [Online]. Available at: http://carif.com.my/?page_id=3872. [Accessed: 29 January 2015].

Chae, I. G. et al., 2014. Generation of ROS by CAY10598 leads to inactivation of STAT3 signaling and induction of apoptosis in human colon cancer HCT116 cells. *Free Radical Research,* 48 (11), pp. 1311-1321.

Chambers, A. F., Groom, A. C. and MacDonald, I. C., 2002. Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews Cancer,* 2 (8), pp. 563-572.

Chambers, A. F. and Matrisian, L. M., 1997. Changing views of the role of matrix metalloproteinases in metastasis. *Journal of the National Cancer Institute,* 89 (17), pp. 1260-1270.

Chen, C. L., Chan, P. C., Wang, S. H., Pan, Y. R. and Chen, H. C., 2010. Elevated expression of protein kinase C delta induces cell scattering upon serum deprivation. *Journal of Cell Science,* 123 (Pt 17), pp. 2901-2913.

Chen, J. et al., 2013a. The correlation of synuclein-gamma and matrix metalloproteinase 9 in breast cancer. *Zhonghua Wai Ke Za Zhi,* 51 (7), pp. 641-644.

Chen, J. et al., 2012. Neural protein gamma-synuclein interacting with androgen receptor promotes human prostate cancer progression. *BMC Cancer,* 12, pp. 593.

Chen, W. T., 1989. Proteolytic activity of specialized surface protrusions formed at rosette contact sites of transformed cells. *Journal of Experimental Zoology,* 251 (2), pp. 167-185.

Chen, X. H. et al., 2013b. Type 1 insulin-like growth factor receptor monoclonal antibody (HX-1162) treatment for liver cancer. *OncoTargets and Therapy,* 6, pp. 527-530.

Chen, Y. et al., 2014. Shikonin inhibits prostate cancer cells metastasis by reducing matrix metalloproteinase-2/-9 expression via AKT/mTOR and ROS/ERK1/2 pathways. *International Immunopharmacology,* 21 (2), pp. 447-455.

Chhabra, E. S. and Higgs, H. N., 2007. The many faces of actin: matching assembly factors with cellular structures. *Nature Cell Biology,* 9 (10), pp. 1110-1121.

Chiarugi, P. and Giannoni, E., 2008. Anoikis: a necessary death program for anchorage-dependent cells. *Biochemical Pharmacology,* 76 (11), pp. 1352-1364.

Choong, C. J. and Say, Y. H., 2011. Neuroprotection of alpha-synuclein under acute and chronic rotenone and maneb treatment is abolished by its familial Parkinson's disease mutations A30P, A53T and E46K. *Neurotoxicology,* 32 (6), pp. 857-863.

Christofori, G. and Semb, H., 1999. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends in Biochemical Sciences,* 24 (2), pp. 73-76.

Circu, M. L. and Aw, T. Y., 2010. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radical Biology and Medicine,* 48 (6), pp. 749-762.

Clayton, D. F. and George, J. M., 1998. The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends in Neurosciences,* 21 (6), pp. 249-254.

Coltella, N. et al., 2006. p38 MAPK turns hepatocyte growth factor to a death signal that commits ovarian cancer cells to chemotherapy-induced apoptosis. *International Journal of Cancer,* 118 (12), pp. 2981-2990.

Condeelis, J. and Segall, J. E., 2003. Intravital imaging of cell movement in tumours. *Nature Reviews Cancer,* 3 (12), pp. 921-930.

Damiano, J. S. et al., 2013. Neutralization of prolactin receptor function by monoclonal antibody LFA102, a novel potential therapeutic for the treatment of breast cancer. *Molecular Cancer Therapeutics,* 12 (3), pp. 295-305.

Davidson, W. S., Jonas, A., Clayton, D. F. and George, J. M., 1998. Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. *The Journal of Biological Chemistry,* 273 (16), pp. 9443-9449.

Deng, X. et al., 2001. Novel role for JNK as a stress-activated Bcl2 kinase. *The Journal of Biological Chemistry,* 276 (26), pp. 23681-23688.

Devi, T. S., Singh, L. P., Hosoya, K. and Terasaki, T., 2011. GSK-3beta/CREB axis mediates IGF-1-induced ECM/adhesion molecule expression, cell cycle progression and monolayer permeability in retinal capillary endothelial cells: Implications for diabetic retinopathy. *Biochimica et Biophysica Acta,* 1812 (9), pp. 1080-1088.

Fan, S. et al., 1998. Scatter factor protects epithelial and carcinoma cells against apoptosis induced by DNA-damaging agents. *Oncogene,* 17 (2), pp. 131-141.

Fan, Y. et al., 2006. Beta-synuclein modulates alpha-synuclein neurotoxicity by reducing alpha-synuclein protein expression. *Human Molecular Genetics,* 15 (20), pp. 3002-3011.

Fernandez-Garcia, B. et al., 2014. Expression and prognostic significance of fibronectin and matrix metalloproteases in breast cancer metastasis. *Histopathology,* 64 (4), pp. 512-522.

Fewou, S. N. et al., 2012. Anti-ganglioside antibody internalization attenuates motor nerve terminal injury in a mouse model of acute motor axonal neuropathy. *The Journal of Clinical Investigation,* 122 (3), pp. 1037-1051.

Fidler, I. J., 1990. Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. *Cancer Research,* 50 (19), pp. 6130-6138.

Flatmark, K., Maelandsmo, G. M., Martinsen, M., Rasmussen, H. and Fodstad, O., 2004. Twelve colorectal cancer cell lines exhibit highly variable growth and metastatic capacities in an orthotopic model in nude mice. *European Journal of Cancer,* 40 (10), pp. 1593-1598.

Frantz, C., Stewart, K. M. and Weaver, V. M., 2010. The extracellular matrix at a glance. *Journal of Cell Science,* 123 (Pt 24), pp. 4195-200.

Frisch, S. M. and Ruoslahti, E., 1997. Integrins and anoikis. *Current Opinion in Cell Biology,* 9 (5), pp. 701-706.

Fung, K. M., Rorke, L. B., Giasson, B., Lee, V. M. and Trojanowski, J. Q., 2003. Expression of alpha-, beta-, and gamma-synuclein in glial tumors and medulloblastomas. *Acta Neuropathologica,* 106 (2), pp. 167-175.

Gao, X. et al., 2013. ROS mediate proapoptotic and antisurvival activity of oleanane triterpenoid CDDO-Me in ovarian cancer cells. *Anticancer Research,* 33 (1), pp. 215-221.

Geback, T., Schulz, M. M., Koumoutsakos, P. and Detmar, M., 2009. TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. *Biotechniques,* 46 (4), pp. 265-274.

George, J. M., 2002. The synucleins. *Genome Biology,* 3 (1), pp. REVIEWS3002.

GLOBUCAN 2012, 2012a. Estimated Incidence, Mortality and Prevalence Worldwide in 2012 [Online]. Available at: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx. [Accessed: 31 January 2014].

GLOBUCAN 2012, 2012b. Colorectal Cancer Estimated Incidence, Mortality and Prevalence Worldwide in 2012 [Online]. Available at: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx?cancer=colorectal. [Accessed: 31 January 2014].

Goyal, L., Muzumdar, M. D. and Zhu, A. X., 2013. Targeting the HGF/c-MET pathway in hepatocellular carcinoma. *Clinical Cancer Research,* 19 (9), pp. 2310-2318.

Graham, F. L., Smiley, J., Russell, W. C. and Nairn, R., 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *Journal of General Virology,* 36 (1), pp. 59-74.

Green, D. R. and Reed, J. C., 1998. Mitochondria and apoptosis. *Science,* 281 (5381), pp. 1309-1312.

Gregersen, N. and Bross, P., 2010. Protein misfolding and cellular stress: an overview. *Methods in Molecular Biology,* 648, pp. 3-23.

Guo, J. et al., 2007. Neuronal protein synuclein gamma predicts poor clinical outcome in breast cancer. *International Journal of Cancer,* 121 (6), pp.1296-1305.

Gupta, A., Godwin, A. K., Vanderveer, L., Lu, A. and Liu, J., 2003a. Hypomethylation of the synuclein gamma gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. *Cancer Research,* 63 (3), pp. 664-673.

Gupta, A., Inaba, S., Wong, O. K., Fang, G. and Liu, J., 2003b. Breast cancer-specific gene 1 interacts with the mitotic checkpoint kinase BubR1. *Oncogene,* 22 (48), pp. 7593-7599.

Gururajan, M. et al., 2005. c-Jun N-terminal kinase (JNK) is required for survival and proliferation of B-lymphoma cells. *Blood,* 106 (4), pp. 1382-1391.

Halliwell, B. and Cross, C. E., 1994. Oxygen-derived species: their relation to human disease and environmental stress. *Environmental Health Perspectives,* 102 (Suppl 10), pp. 5-12.

Hanahan, D. and Weinberg, R. A., 2000. The hallmarks of cancer. *Cell,* 100 (1), pp. 57-70.

Hanahan, D. and Weinberg, R. A., 2011. Hallmarks of cancer: the next generation. *Cell,* 144 (5), pp. 646-674.

Hartmann, G., Weidner, K. M., Schwarz, H. and Birchmeier, W., 1994. The motility signal of scatter factor/hepatocyte growth factor mediated through the receptor tyrosine kinase met requires intracellular action of Ras. *The Journal of Biological Chemistry,* 269 (35), pp. 21936-21939.

Hashimoto, M. et al., 2002. alpha-Synuclein protects against oxidative stress via inactivation of the c-Jun N-terminal kinase stress-signaling pathway in neuronal cells. *The Journal of Biological Chemistry,* 277 (13), pp. 11465-11472.

Hashimoto, M., Rockenstein, E., Mante, M., Mallory, M. and Masliah, E., 2001. beta-Synuclein inhibits alpha-synuclein aggregation: a possible role as an anti-parkinsonian factor. *Neuron,* 32 (2), pp. 213-223.

Hazan, R. B., Phillips, G. R., Qiao, R. F., Norton, L. and Aaronson, S. A., 2000. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. *The Journal of Cell Biology,* 148 (4), pp. 779-790.

Hibi, T. et al., 2009. Synuclein-gamma is closely involved in perineural invasion and distant metastasis in mouse models and is a novel prognostic factor in pancreatic cancer. *Clinical Cancer Research,* 15 (8), pp. 2864-2871.

Himes, R. H., Kersey, R. N., Heller-Bettinger, I. and Samson, F. E., 1976. Action of the vinca alkaloids vincristine, vinblastine, and desacetyl vinblastine amide on microtubules *in vitro*. *Cancer Research,* 36 (10), pp. 3798-802.

Hiscox, S. E., Hallett, M. B., Puntis, M. C., Nakamura, T. and Jiang, W. G., 1997. Expression of the HGF/SF receptor, c-met, and its ligand in human colorectal cancers. *Cancer Investigation,* 15 (6), pp. 513-521.

Hua, H. et al., 2009. Up-regulation of gamma-synuclein contributes to cancer cell survival under endoplasmic reticulum stress. *The Journal of Pathology,* 217 (4), pp. 507-515.

Huijbers, I. J. et al., 2010. A role for fibrillar collagen deposition and the collagen internalization receptor endo180 in glioma invasion. *PLOS ONE,* 5 (3), pp. e9808.

Hunt, C. R. et al., 1998. Genomic instability and catalase gene amplification induced by chronic exposure to oxidative stress. *Cancer Research,* 58 (17), pp. 3986-3992.

Inaba, S. et al., 2005. Synuclein gamma inhibits the mitotic checkpoint function and promotes chromosomal instability of breast cancer cells. *Breast Cancer Research and Treatment,* 94 (1), pp. 25-35.

International Agency for Research on Cancer, 2014. World Cancer Factsheet. World Cancer Burden (2012) [Online]. Available at: http://publications.cancerresearchuk.org/downloads/product/CS_REPORT_WORLD.pdf. [Accessed: 31 January 2014].

Invitrogen, 2010. pcDNA™ 3.1 Directional TOPO® Expression Kit [Online]. Available at: https://tools.lifetechnologies.com/content/sfs/manuals/pcdna3_1dv5histopo_man.pdf. [Accessed: 31 January 2014]

Ishida, M. et al., 2007. Involvement of cAMP response element-binding protein in the regulation of cell proliferation and the prolactin promoter of lactotrophs in primary culture. *American Journal of Physiology Endocrinology and Metabolism,* 293 (6), pp. E1529-E1537.

Iwaki, H. et al., 2004. Diagnostic potential in bladder cancer of a panel of tumor markers (calreticulin, gamma -synuclein, and catechol-o-methyltransferase) identified by proteomic analysis. *Cancer Science,* 95 (12), pp. 955-961.

Jakes, R., Spillantini, M. G. and Goedert, M., 1994. Identification of two distinct synucleins from human brain. *FEBS Letters,* 345 (1), pp. 27-32.

Jarvelainen, H., Sainio, A., Koulu, M., Wight, T. N. and Penttinen, R., 2009. Extracellular matrix molecules: potential targets in pharmacotherapy. *Pharmacological Reviews,* 61 (2), pp. 198-223.

Jenco, J. M., Rawlingson, A., Daniels, B. and Morris, A. J., 1998. Regulation of phospholipase D2: selective inhibition of mammalian phospholipase D isoenzymes by alpha- and beta-synucleins. *Biochemistry,* 37 (14), pp. 4901-4909.

Ji, H. et al., 1997. Identification of a breast cancer-specific gene, BCSG1, by direct differential cDNA sequencing. *Cancer Research,* 57 (4), pp. 759-764.

Jia, T., Liu, Y. E., Liu, J. and Shi, Y. E., 1999. Stimulation of breast cancer invasion and metastasis by synuclein gamma. *Cancer Research,* 59 (3), pp. 742-747.

Jiang, H. et al., 2007. Parkinson's disease genetic mutations increase cell susceptibility to stress: mutant alpha-synuclein enhances H2O2- and Sin-1-induced cell death. *Neurobiology of Aging,* 28 (11), pp. 1709-1717.

Jiang, Y., Liu, Y. E., Goldberg, I. D. and Shi, Y. E., 2004. Gamma synuclein, a novel heat-shock protein-associated chaperone, stimulates ligand-dependent estrogen receptor alpha signaling and mammary tumorigenesis. *Cancer Research,* 64 (13), pp. 4539-4546.

Jiang, Y. et al., 2003. Stimulation of estrogen receptor signaling by gamma synuclein. *Cancer Research,* 63 (14), 3899-3903.

Jiao, X. et al., 2010. c-Jun induces mammary epithelial cellular invasion and breast cancer stem cell expansion. *The Journal of Biological Chemistry,* 285 (11), pp. 8218-8226.

Jun, E. J., Kim, H. S. and Kim, Y. H., 2007. Role of HGF/c-Met in serum-starved ARPE-19 cells. *Korean Journal of Ophthalmology,* 21 (4), pp. 244-250.

Kauppila, S., Stenback, F., Risteli, J., Jukkola, A. and Risteli, L., 1998. Aberrant type I and type III collagen gene expression in human breast cancer *in vivo*. *The Journal of Pathology,* 186 (3), pp. 262-268.

Kim, E. M., Elliot, J. J., Hobson, P. and O'Hare, E., 2009. Effects of intrahippocampal NAC 61-95 injections on memory in the rat and attenuation with vitamin E. *Progress in Neuro-Psychopharmacology & Biological Psychiatry,* 33 (6), pp. 945-951.

Lavedan, C., 1998. The synuclein family. *Genome Research,* 8 (9), pp. 871-880.

Lavedan, C. et al., 1998. Identification, localization and characterization of the human gamma-synuclein gene. *Human Genetics,* 103 (1), pp. 106-112.

Lee, B. R. and Kamitani, T., 2011. Improved immunodetection of endogenous alpha-synuclein. *PLOS ONE,* 6 (8), pp. e23939.

Lee, F. J., Liu, F., Pristupa, Z. B. and Niznik, H. B., 2001. Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. *FASEB Journal,* 15 (6), pp. 916-926.

Leventaki, V. et al., 2014. c-JUN N-terminal kinase (JNK) is activated and contributes to tumor cell proliferation in classical Hodgkin lymphoma. *Human Pathology,* 45 (3), pp. 565-572.

Li, A. E. et al., 1999. A role for reactive oxygen species in endothelial cell anoikis. *Circulation Research,* 85 (4), pp. 304-310.

Li, Z. et al., 2004. Overexpression of synuclein-gamma in pancreatic adenocarcinoma. *Cancer,* 101 (1), pp. 58-65.

Liang, B. et al., 2013. Synuclein-gamma suppression mediated by RNA interference inhibits the clonogenicity and invasiveness of MCF-7 cells. *Oncology Letters,* 5 (4), pp. 1347-1352.

Lim, G. C., 2002. Overview of cancer in Malaysia. *Japanese Journal of Clinical Oncology,* 32 (Suppl 1), pp. S37-S42.

Lippa, C. F., Schmidt, M. L., Lee, V. M. and Trojanowski, J. Q., 2001. Alpha-synuclein in familial Alzheimer disease: epitope mapping parallels dementia with Lewy bodies and Parkinson disease. *Archives of Neurology,* 58 (11), pp. 1817-1820.

Liu, C. et al., 2012. Elevated serum synuclein-gamma in patients with gastrointestinal and esophageal carcinomas. *Hepatogastroenterology,* 59 (119), pp. 2222-2227.

Liu, H. et al., 2005. Loss of epigenetic control of synuclein-gamma gene as a molecular indicator of metastasis in a wide range of human cancers. *Cancer Research,* 65 (17), pp. 7635-7643.

Liu, J. et al., 2000. Transcriptional suppression of synuclein gamma (SNCG) expression in human breast cancer cells by the growth inhibitory cytokine oncostatin M. *Breast Cancer Research and Treatment,* 62 (2), pp. 99-107.

Liu, Y., 1999. Hepatocyte growth factor promotes renal epithelial cell survival by dual mechanisms. *American Journal of Physiology,* 277 (4), pp. F624-F633.

Liu, Y. E. et al., 2007. Chaperoning of estrogen receptor and induction of mammary gland proliferation by neuronal protein synuclein gamma. *Oncogene,* 26 (14), pp. 2115-2125.

Lu, A., Zhang, F., Gupta, A. and Liu, J., 2002. Blockade of AP1 transactivation abrogates the abnormal expression of breast cancer-specific gene 1 in breast cancer cells. *The Journal of Biological Chemistry,* 277 (35), pp. 31364-31372.

Lu, P., Weaver, V. M. and Werb, Z., 2012. The extracellular matrix: a dynamic niche in cancer progression. *The Journal of Cell Biology,* 196 (4), pp. 395-406.

Ma, P. C. et al., 2007. Downstream signalling and specific inhibition of c-MET/HGF pathway in small cell lung cancer: implications for tumour invasion. *British Journal of Cancer,* 97 (3), pp. 368-377.

Machesky, L. M., 2008. Lamellipodia and filopodia in metastasis and invasion. *FEBS Letters,* 582 (14), pp. 2102-2111.

Maroteaux, L., Campanelli, J. T. and Scheller, R. H., 1988. Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. *The Journal of Neuroscience,* 8 (8), pp. 2804-2815.

Martindale, J. L. and Holbrook, N. J., 2002. Cellular response to oxidative stress: signaling for suicide and survival. *Journal of Cellular Physiology,* 192 (1), pp. 1-15.

Matsumoto, K. and Nakamura, T., 1996. Emerging multipotent aspects of hepatocyte growth factor. *The Journal of Biochemistry,* 119 (4), pp. 591-600.

Mayr, B. and Montminy, M., 2001. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nature Review Molecular Cell Biology,* 2 (8), pp. 599-609.

Melnikova, V. O., Mourad-Zeidan, A. A., Lev, D. C. and Bar-Eli, M., 2006. Platelet-activating factor mediates MMP-2 expression and activation via phosphorylation of cAMP-response element-binding protein and contributes to melanoma metastasis. *The Journal of Biological Chemistry,* 281 (5), pp. 2911-2922.

Merlino, G., 1994. Regulatory imbalances in cell proliferation and cell death during oncogenesis in transgenic mice. *Seminars in Cancer Biology,* 5 (1), pp. 13-20.

Meza-Junco, J., Au, H. J. and Sawyer, M. B., 2011. Critical appraisal of trastuzumab in treatment of advanced stomach cancer. *Cancer Management and Research,* 3, pp. 57-64.

Mills, E. M. et al., 1998. Nerve growth factor treatment prevents the increase in superoxide produced by epidermal growth factor in PC12 cells. *The Journal of Biological Chemistry,* 273 (35), pp. 22165-22168.

Mitchison, T. J. and Cramer, L. P., 1996. Actin-based cell motility and cell locomotion. *Cell,* 84 (3), pp. 371-379.

Morgan, J. et al., 2009. Synuclein-gamma (SNCG) may be a novel prognostic biomarker in uterine papillary serous carcinoma. *Gynecologic Oncology,* 114 (2), pp. 293-298.

Nakajo, S. et al., 1990. Purification and characterization of a novel brain-specific 14-kDa protein. *Journal of Neurochemistry,* 55 (6), pp. 2031-2038.

Nakajo, S., Tsukada, K., Omata, K., Nakamura, Y. and Nakaya, K., 1993. A new brain-specific 14-kDa protein is a phosphoprotein. Its complete amino acid sequence and evidence for phosphorylation. *European Journal of Biochemistry,* 217 (3), pp. 1057-1063.

National Cancer Registry Malaysia, 2006. Malaysian Cancer Statistics – Data and Figure Peninsular Malaysia 2006 [Online]. Available at: http://www.moh.gov.my/images/gallery/Report/Cancer/MalaysiaCancerStatistics_2006.pdf. [Accessed: 31 December 2014].

Ninkina, N. N., Privalova, E. M., Pinon, L. G., Davies, A. M. and Buchman, V. L., 1999. Developmentally regulated expression of persyn, a member of the synuclein family, in skin. *Experimental Cell Research,* 246 (2), pp. 308-311.

Nobes, C. D. and Hall, A., 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell,* 81 (1), pp. 53-62.

Nogueira-Pedro, A. et al., 2013. Hydrogen peroxide (H_2O_2) induces leukemic but not normal hematopoietic cell death in a dose-dependent manner. *Cancer Cell International,* 13 (1), pp. 123.

Oaks, A. W. and Sidhu, A., 2011. Synuclein modulation of monoamine transporters. *FEBS Letters,* 585 (7), pp. 1001-1006.

Oort, P. J. et al., 2008. Gamma-synuclein is an adipocyte-neuron gene coordinately expressed with leptin and increased in human obesity. *Journal of Nutrition,* 138 (5), pp. 841-848.

Oskarsson, T., 2013. Extracellular matrix components in breast cancer progression and metastasis. *Breast,* 22 (Suppl 2), pp. S66-S72.

Otte, J. M. et al., 2000. Functional expression of HGF and its receptor in human colorectal cancer. *Digestion,* 61 (4), pp. 237-246.

Pan, Z. Z., Bruening, W., Giasson, B. I., Lee, V. M. and Godwin, A. K., 2002. Gamma-synuclein promotes cancer cell survival and inhibits stress- and chemotherapy drug-induced apoptosis by modulating MAPK pathways. *The Journal of Biological Chemistry,* 277 (38), pp. 35050-35060.

Pan, Z. Z., Bruening, W. and Godwin, A. K., 2006. Involvement of RHO GTPases and ERK in synuclein-gamma enhanced cancer cell motility. *International Journal of Oncology,* 29 (5), pp. 1201-1205.

Panneerselvam, M. et al., 2013. Molecular dynamic simulations of the tubulin-human gamma synuclein complex: structural insight into the regulatory mechanism involved in inducing resistance against Taxol. *Molecular Biosystems,* 9 (6), pp. 1470-1488.

Paoli, P., Giannoni, E. and Chiarugi, P., 2013. Anoikis molecular pathways and its role in cancer progression. *Biochimica et Biophysica Acta,* 1833 (12), pp. 3481-3498.

Park, J. Y. and Lansbury, P. T., Jr., 2003. Beta-synuclein inhibits formation of alpha-synuclein protofibrils: a possible therapeutic strategy against Parkinson's disease. *Biochemistry,* 42 (13), pp. 3696-3700.

Parmar, N., Wetton, N., Alvarado, S. and Kennedy, S., 2014. Profiling of human normal and cancer cell lines using phenotype microarray analysis. *FASEB Journal*, 28(1 Supplement), pp. 613–615.

Paumelle, R. et al., 2000. Sequential activation of ERK and repression of JNK by scatter factor/hepatocyte growth factor in madin-darby canine kidney epithelial cells. *Molecular Biology of the Cell,* 11 (11), pp. 3751-3763.

Perez, R. G. et al., 2002. A role for alpha-synuclein in the regulation of dopamine biosynthesis. *The Journal of Neuroscience,* 22 (8), pp. 3090-3099.

Potempa, S. and Ridley, A. J., 1998. Activation of both MAP kinase and phosphatidylinositide 3-kinase by Ras is required for hepatocyte growth factor/scatter factor-induced adherens junction disassembly. *Molecular Biology of the Cell,* 9 (8), pp. 2185-2200.

Rasola, A. et al., 2004. Hepatocyte growth factor sensitizes human ovarian carcinoma cell lines to paclitaxel and cisplatin. *Cancer Research,* 64 (5), pp. 1744-1750.

Recio, J. A. and Merlino, G., 2002. Hepatocyte growth factor/scatter factor activates proliferation in melanoma cells through p38 MAPK, ATF-2 and cyclin D1. *Oncogene,* 21 (7), pp. 1000-1008.

Ridley, A. J., 1994. Membrane ruffling and signal transduction. *Bioessays,* 16 (5), pp. 321-327.

Sakamoto, K. M. and Frank, D. A., 2009. CREB in the pathophysiology of cancer: implications for targeting transcription factors for cancer therapy. *Clinical Cancer Research,* 15 (8), pp. 2583-2587.

Sam, M. R., Elliott, B. E. and Mueller, C. R., 2007. A novel activating role of SRC and STAT3 on HGF transcription in human breast cancer cells. *Molecular Cancer,* 6, pp. 69.

Scarpino, S. et al., 1999. Hepatocyte growth factor (HGF) stimulates tumour invasiveness in papillary carcinoma of the thyroid. *The Journal of Pathology,* 189 (4), pp. 570-575.

Schaefer, L. and Schaefer, R. M., 2010. Proteoglycans: from structural compounds to signaling molecules. *Cell and Tissue Research,* 339 (1), pp. 237-246.

Schmidt, S. and Friedl, P., 2010. Interstitial cell migration: integrin-dependent and alternative adhesion mechanisms. *Cell and Tissue Research,* 339 (1), pp. 83-92.

Schoumacher, M., Goldman, R. D., Louvard, D. and Vignjevic, D. M., 2010. Actin, microtubules, and vimentin intermediate filaments cooperate for elongation of invadopodia. *The Journal of Cell Biology,* 189 (3), pp. 541-556.

Scott, A. M., Wolchok, J. D. and Old, L. J., 2012. Antibody therapy of cancer. *Nature Review Cancer,* 12 (4), pp. 278-287.

Seo, J. H. et al., 2002. Alpha-synuclein regulates neuronal survival via Bcl-2 family expression and PI3/Akt kinase pathway. *FASEB Journal,* 16 (13), pp. 1826-1828.

Shaywitz, A. J. and Greenberg, M. E., 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annual Review of Biochemistry,* 68, pp. 821-861.

Shen, J. C. et al., 2007. Inhibitor of growth 4 suppresses cell spreading and cell migration by interacting with a novel binding partner, liprin alpha1. *Cancer Research,* 67 (6), pp. 2552-2558.

Shen, P. H. et al., 2011. SNCG shRNA suppressed breast cancer cell xenograft formation and growth in nude mice. *Chinese Medical Journal,* 124 (10), pp. 1524-1528.

Siebel, A. et al., 2013. Contribution of S6K1/MAPK signaling pathways in the response to oxidative stress: activation of RSK and MSK by hydrogen peroxide. *PLOS ONE,* 8 (9), pp. e75523.

Singh, M., Sharma, H. and Singh, N., 2007a. Hydrogen peroxide induces apoptosis in HeLa cells through mitochondrial pathway. *Mitochondrion,* 7 (6), pp. 367-373.

Singh, M. and Singh, N., 2008. Induction of apoptosis by hydrogen peroxide in HPV 16 positive human cervical cancer cells: involvement of mitochondrial pathway. *Molecular and Cellular Biochemistry,* 310 (1-2), pp. 57-65.

Singh, V. K. et al., 2007b. Synuclein-gamma targeting peptide inhibitor that enhances sensitivity of breast cancer cells to antimicrotubule drugs. *Cancer Research,* 67 (2), pp. 626-633.

Sinha, K., Das, J., Pal, P. B. and Sil, P. C., 2013. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Archives of Toxicology,* 87 (7), pp. 1157-1180.

Small, J. V., 2010. Dicing with dogma: de-branching the lamellipodium. *Trends in Cell Biology,* 20 (11), pp. 628-633.

Small, J. V., Stradal, T., Vignal, E. and Rottner, K., 2002. The lamellipodium: where motility begins. *Trends in Cell Biology,* 12 (3), pp. 112-120.

Snyder, H. et al., 2005. beta-Synuclein reduces proteasomal inhibition by alpha-synuclein but not gamma-synuclein. *The Journal of Biological Chemistry,* 280 (9), pp. 7562-7569.

Sordella, R. et al., 2002. Modulation of CREB activity by the Rho GTPase regulates cell and organism size during mouse embryonic development. *Developmental Cell,* 2 (5), pp. 553-565.

Specht, C. G. et al., 2005. Subcellular localisation of recombinant alpha- and gamma-synuclein. *Molecular and Cellular Neuroscience,* 28 (2), pp. 326-334.

Spillantini, M. G., Divane, A. and Goedert, M., 1995. Assignment of human alpha-synuclein (SNCA) and beta-synuclein (SNCB) genes to chromosomes 4q21 and 5q35. *Genomics,* 27 (2), pp. 379-381.

Spillantini, M. G. et al., 1997. Alpha-synuclein in Lewy bodies. *Nature,* 388 (6645), pp. 839-840.

Stefani, M. and Rigacci, S., 2013. Protein folding and aggregation into amyloid: the interference by natural phenolic compounds. *International Journal of Molecular Sciences,* 14 (6), pp. 12411-12457.

Steller, H., 1995. Mechanisms and genes of cellular suicide. *Science,* 267 (5203), pp. 1445-1449.

Su, Y. et al., 2012. JNK/P38 mitogen-activated protein kinase used for hepatocyte growth factor-induced proliferation, differentiation, and migration in human dental papilla cells. *Journal of Endodontics,* 38 (9), pp. 1207-1213.

Sung, Y. H. and Eliezer, D., 2006. Secondary structure and dynamics of micelle bound beta- and gamma-synuclein. *Protein Science,* 15 (5), pp. 1162-1174.

Sung, Y. H. and Eliezer, D., 2007. Residual structure, backbone dynamics, and interactions within the synuclein family. *Journal of Molecular Biology,* 372 (3), pp. 689-707.

Surgucheva, I., McMahon, B. and Surguchov, A., 2006. Gamma-synuclein has a dynamic intracellular localization. *Cell Motility and the Cytoskeleton,* 63 (8), pp. 447-458.

Surgucheva, I., Sharov, V. S. and Surguchov, A., 2012. Gamma-Synuclein: seeding of alpha-synuclein aggregation and transmission between cells. *Biochemistry,* 51 (23), pp. 4743-54.

Surgucheva, I. and Surguchov, A., 2008. Gamma-synuclein: cell-type-specific promoter activity and binding to transcription factors. *Journal of Molecular Neuroscience,* 35 (3), pp. 267-271.

Surgucheva, I. G., Sivak, J. M., Fini, M. E., Palazzo, R. E. and Surguchov, A. P., 2003. Effect of gamma-synuclein overexpression on matrix metalloproteinases in retinoblastoma Y79 cells. *Archives of Biochemistry and Biophysics,* 410 (1), pp. 167-176.

Surguchov, A., 2013. Synucleins: are they two-edged swords?. *Journal of Neuroscience Research,* 91 (2), pp. 161-166.

Surguchov, A., Palazzo, R. E. and Surgucheva, I., 2001. Gamma synuclein: subcellular localization in neuronal and non-neuronal cells and effect on signal transduction. *Cell Motility and the Cytoskeleton,* 49 (4), pp. 218-228.

Tabner, B. J., El-Agnaf, O. M., German, M. J., Fullwood, N. J. and Allsop, D., 2005. Protein aggregation, metals and oxidative stress in neurodegenerative diseases. *Biochemical Society Transactions,* 33 (Pt 5), pp. 1082-1086.

Tacar, O., Sriamornsak, P. and Dass, C. R., 2013. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. *Journal of Pharmacy and Pharmacology,* 65 (2), pp. 157-170.

Taherian, A., Li, X., Liu, Y. and Haas, T.A., 2011. Differences in integrin expression and signaling within human breast cancer cells. *BMC Cancer*, 11, pp. 293.

Takahashi, T., Yamaguchi, T., Kitamura, K., Noguchi, A. and Honda, M., 1992. Monoclonal antibody-drug conjugate therapy for the patients with colorectal cancer. *The Tohoku Journal of Experimental Medicine,* 168 (2), pp. 371-374.

Thomas, P. and Smart, T. G., 2005. HEK293 cell line: a vehicle for the expression of recombinant proteins. *Journal of Pharmacological and Toxicological Methods,* 51 (3), pp. 187-200.

Thornberry, N. A. and Lazebnik, Y., 1998. Caspases: enemies within. *Science,* 281 (5381), pp. 1312-1316.

Tom, B. H. et al., 1976. Human colonic adenocarcinoma cells. I. Establishment and description of a new line. *In Vitro,* 12 (3), pp. 180-91.

Trainer, D. L. et al., 1988. Biological characterization and oncogene expression in human colorectal carcinoma cell lines. *International Journal of Cancer,* 41 (2), pp. 287-296.

Trojanowski, J. Q. and Lee, V. M., 1998. Aggregation of neurofilament and alpha-synuclein proteins in Lewy bodies: implications for the pathogenesis of Parkinson disease and Lewy body dementia. *Archives of Neurology,* 55 (2), pp. 151-152.

Ueda, K. et al., 1993. Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America,* 90 (23), pp. 11282-11286.

Vu, T. H. and Werb, Z., 2000. Matrix metalloproteinases: effectors of development and normal physiology. *Genes & Development*, 14, pp. 2123-2133.

Wang, X., Martindale, J. L., Liu, Y. and Holbrook, N. J., 1998. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. *Biochemical Journal,* 333 (Pt 2), pp. 291-300.

Weiner, G. J., 2007. Monoclonal antibody mechanisms of action in cancer. *Immunologic Research,* 39 (1-3), 271-278.

Wen, J., Matsumoto, K., Taniura, N., Tomioka, D. and Nakamura, T., 2004. Hepatic gene expression of NK4, an HGF-antagonist/angiogenesis inhibitor, suppresses liver metastasis and invasive growth of colon cancer in mice. *Cancer Gene Therapy,* 11 (6), pp. 419-430.

Wersinger, C. and Sidhu, A., 2003. Differential cytotoxicity of dopamine and H_2O_2 in a human neuroblastoma divided cell line transfected with alpha-synuclein and its familial Parkinson's disease-linked mutants. *Neuroscience Letters,* 342 (1-2), pp. 124-128.

Wiechen, K. and Dietel, M., 1995. c-erbB-2 anti-sense phosphorothioate oligodeoxynucleotides inhibit growth and serum-induced cell spreading of P185c-erbB-2-overexpressing ovarian carcinoma cells. *International Journal of Cancer,* 63 (4), pp. 604-608.

Wiggin, G. R. et al., 2002. MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. *Molecular Cell Biology,* 22 (8), pp. 2871-2881.

Wilding, C. et al., 2014. gamma-Synuclein antibodies have neuroprotective potential on neuroretinal cells via proteins of the mitochondrial apoptosis pathway. *PLOS ONE,* 9 (3), pp. e90737.

Windisch, M. et al., 2002. Development of a new treatment for Alzheimer's disease and Parkinson's disease using anti-aggregatory beta-synuclein-derived peptides. *Journal of Molecular Neuroscience,* 19 (1-2), pp. 63-69.

World Health Organization, 2014. *Cancer* [Online]. Available at: http://www.who.int/mediacentre/factsheets/fs297/en/. [Accessed: 31 December 2014].

Wu, K. et al., 2013. Expression of synuclein gamma indicates poor prognosis of triple-negative breast cancer. *Medical Oncology,* 30 (3), pp. 612.

Wu, K. et al., 2007. Expression of neuronal protein synuclein gamma gene as a novel marker for breast cancer prognosis. *Breast Cancer Research and Treatment,* 101 (3), pp. 259-267.

Wu, K. et al., 2003. Stage-specific expression of breast cancer-specific gene gamma-synuclein. *Cancer Epidemiology, Biomarkers and Prevention,* 12 (9), pp. 920-925.

Xiang, W. et al., 2013. Oxidative stress-induced posttranslational modifications of alpha-synuclein: specific modification of alpha-synuclein by 4-hydroxy-2-nonenal increases dopaminergic toxicity. *Molecular and Cellular Neuroscience,* 54, pp. 71-83.

Yang, Z. et al., 2007. Up-regulation of gastric cancer cell invasion by Twist is accompanied by N-cadherin and fibronectin expression. *Biochemical and Biophysical Research Communications,* 358 (3), pp. 925-930.

Ye, Q. et al., 2010a. Demethylation of the gamma-synuclein gene CpG island in colorectal cancer and its clinical significance. *Zhonghua Wei Chang Wai Ke Za Zhi,* 13 (6), pp. 440-444.

Ye, Q. et al., 2009. Expression of gamma-synuclein in colorectal cancer tissues and its role on colorectal cancer cell line HCT116. *World Journal of Gastroenterology,* 15 (40), pp. 5035-5043.

Ye, Q. et al., 2013. Effects of gamma-synuclein on the tumorigenicity and metastasis of colon cancer SW1116 cells *in vitro* and *in vivo*. *Oncology Reports,* 30 (5), pp. 2161-2170.

Ye, Q. et al., 2014. Construction of gamma-synuclein eukaryotic expression vector and its effect on invasion and metastasis of colon cancer cell line SW1116 *in vitro*. *Zhonghua Wei Chang Wai Ke Za Zhi,* 17 (1), pp. 36-40.

Ye, Q. et al., 2010b. Expression of alpha-, beta- and gamma-synuclein in colorectal cancer, and potential clinical significance in progression of the disease. *Oncology Reports,* 23 (2), pp. 429-436.

Ye, Q. et al., 2008. Aberrant expression and demethylation of gamma-synuclein in colorectal cancer, correlated with progression of the disease. *Cancer Science,* 99 (10), pp. 1924-1932.

Zhang, H., Kouadio, A., Cartledge, D. and Godwin, A. K., 2011. Role of gamma-synuclein in microtubule regulation. *Experimental Cell Research,* 317 (10), pp. 1330-1339.

Zhang, N. Y., Tang, Z. and Liu, C. W., 2008. alpha-Synuclein protofibrils inhibit 26 S proteasome-mediated protein degradation: understanding the cytotoxicity of protein protofibrils in neurodegenerative disease pathogenesis. *The Journal of Biological Chemistry,* 283 (29), pp. 20288-20298.

Zhao, W. et al., 2006. Abnormal activation of the synuclein-gamma gene in hepatocellular carcinomas by epigenetic alteration. *Internatinal Journal of Oncology,* 28 (5), pp. 1081-1088.

Zhou, C. Q. et al., 2003. Down-regulation of gamma-synuclein in human esophageal squamous cell carcinoma. *World Journal of Gastroenterology,* 9 (9), pp. 1900-1903.

Zhou, Y., Inaba, S. and Liu, J., 2006. Inhibition of synuclein-gamma expression increases the sensitivity of breast cancer cells to paclitaxel treatment. *International Journal of Oncology,* 29 (1), pp. 289-295.

Zhu, G. G. et al., 1995. Immunohistochemical study of type I collagen and type I pN-collagen in benign and malignant ovarian neoplasms. *Cancer,* 75 (4), pp. 1010-1017.

Zhu, M., Qin, Z. J., Hu, D., Munishkina, L. A. and Fink, A. L., 2006. Alpha-synuclein can function as an antioxidant preventing oxidation of unsaturated lipid in vesicles. *Biochemistry,* 45 (26), pp. 8135-8142.

Zou, J., Fan, Y. J., Meng, Y. Q., Xu, H. and Fan, J. , 2012. An exploratory analysis of gamma-synuclein expression in endometrioid endometrial cancer. *BMJ Open,* 2 (2), pp. e000611.