Functional studies of the RBBP6 (retinoblastoma binding protein 6) gene and its related genes in breast and cervical cancer: a promising diagnostic and management assay for cancer progression

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Abstract

Overexpression of RBBP6 in cancers of the colon, lung and oesophagus makes it a potential target in anticancer therapy. This is especially important because it associates with the tumour suppressor gene p53, inactivation of which has been linked to over 50% of all cancer types. Cancer is an enormous burden of a disease globally. Today, more people die from cancer than HIV/AIDS, tuberculosis and malaria combined. And in females, breast and cervical malignancies remain the most common types. Currently, cervical cancer is the most diagnosed gynaecological cancer type, whose mortality rate is the highest in developing countries due to the asymptomatic nature of the disease coupled with inadequate cancer control tools and facilities. Breast cancer incidence rate has increased beyond that of lung cancer, making it the most common malignancy among women.

Breast and cervical tumour progression is partly as a result of p53 inactivation by overexpressed ubiquitous regulatory proteins that possess an E3 ligase activity. MDM2 and E6 viral oncoprotein have been shown to negatively regulate p53 in breast and cervical cancer, respectively. RBBP6 forms a member of such proteins since it has an E3 ligase activity and a RING finger-like domain. And therefore its overexpression in several malignancies makes it a potential target in cancer management. However, it is not clearly defined whether or not RBBP6 interaction with p53 promotes cancer progression and therefore serves as a potential biomarker. In this study we manipulated RBBP6 expression levels and treated cells with either camptothecin or GABA treatment in breast and cervical cancer cells to induce apoptosis or cell cycle arrest. Initially, the human cervical cancer tissue sections were stained with anti-RBBP6 mAb to evaluate its extent of expression in patients' specimens. We then followed on with silencing or

overexpression of RBBP6 and treatment with anticancer agents to evaluate how cells respond to combinational therapy. Furthermore, we evaluated the apoptotic response of breast cancer cell lines that display different p53 expression levels, to RBBP6 targeting and co-treatment with anticancer agents. Apoptosis induction was evaluated using confocal microscopy, flow cytometry with Annexin V staining as well as assessing the mitochondrial and caspase 3/7 activities. The cell cycle arrest was evaluated using flow cytometry through staining with propidium iodide.

RBBP6 was highly expressed in cervical cancer tissue sections that were in stage II and III of development. Overexpression of RBBP6 seemed to promote S-phase in cell cycle and cell proliferation whereas silencing triggered apoptosis. This observation predicts a proliferative role for RBBP6 in cancer progression rather than a cancer causing gene. The wt. p53-expressing cell lines (MCF-7, HeLa and SiHa) were more susceptible to apoptosis induction as opposed to the mt. p53-expressing MDA-MB-231. RBBP6 silencing led to a significant accumulation of p53 expression in MCF-7, HeLa and SiHa as compared to MDA-MB-231. These results implicate the distinctive p53 genotypic status of the cancer cell lines to play a major role in their responsiveness to RBBP6 targeting. Furthermore, co-treatment with camptothecin seemed to sensitize the cells to apoptosis induction rather than cell cycle arrest; whereas GABA co-treatments failed to substantially exhibit this phenotype. Sensitization of cells to camptothecin-induced apoptosis by RBBP6 silencing therefore suggests a promising tool for halting breast and cervical cancer progression.

Research Outputs

Research Articles (Original Publications)

Pontsho Moela, Mpho S. Choene and Lesetja R. Motadi. Silencing RBBP6 sensitizes breast cancer cells to *staurosporine-* and *camptothecin*-induced cell death. *Immunobiology*; 2014. ***219:513-601***.

Pontsho Moela and Lesetja R. Motadi. Retinoblastoma binding protein 6 (RBBP6): a potential biomarker for apoptosis induction in human cervical cancer cell lines. *OncoTargets and Therapy*; 2016. ***In press***.

Pontsho Moela and Lesetja R. Motadi. The differential RBBP6 (retinoblastoma binding protein 6) expression predicts p53-induced apoptosis in human breast cancer cell lines. *BMC Cancer*; 2016. ***Submitted.***

Chapters in Books

Pontsho Moela and Lesetja R. Motadi (2015) Apoptotic Molecular Advances in Breast Cancer Management, Cell Death - Autophagy, Apoptosis and Necrosis, Dr. Tobias Ntuli (Ed.), ISBN: 978-953-51-2236-4, InTech, DOI: **10.5772/61654**. Available from: http://www.intechopen.com/books/cell-death-autophagy-apoptosis-and-necrosis/apoptotic-molecular-advances-in-breast-cancer-management

Conference Outputs

Oral Presentations

Pontsho Moela and Lesetja R. Motadi. Retinoblastoma binding protein 6 (RBBP6): a potential biomarker for apoptosis induction in human cervical cancer cell lines. ***Annual MBRT (Molecular Biosciences Research Thrust) Research Day)***, Wits University, Johannesburg, December 2015

Pontsho Moela, Mpho S. Choene and Lesetja R. Motadi. Silencing RBBP6 sensitizes breast cancer cells to *staurosporine-* and *camptothecin*-induced cell death. ***5th Cross-Faculty Graduate Symposium***, Wits University, Johannesburg, August 2014

Poster Presentations

<u>Pontsho Moela</u> and Lesetja R. Motadi. Expression studies of RBBP6 (retinoblastoma binding protein 6) in cervical cancer suggest a role in apoptosis. ***7th Cross-Faculty Graduate Symposium***, Wits University, Johannesburg, March 2016

<u>Pontsho Moela</u>, Mpho S. Choene and Lesetja R. Motadi. Silencing RBBP6 sensitizes breast cancer cells to *staurosporine-* and *camptothecin*-induced cell death. ***European Molecular Biology Organisation (EMBO) Lecture Course***, Wits University, Johannesburg, June 2015

<u>Pontsho Moela</u>, Mpho S. Choene and Lesetja R. Motadi. Silencing RBBP6 sensitizes breast cancer cells to *staurosporine-* and *camptothecin*-induced cell death. ***SASBMB (South African Society of Biochemistry and Molecular Biology) Congress***. Goudini Spa, Cape Town, June 2014

<u>Pontsho Moela</u>, Mpho S. Choene and Lesetja R. Motadi. Silencing RBBP6 sensitizes breast cancer cells to *staurosporine-* and *camptothecin*-induced cell death. ***AACR (American Association for Cancer Research) Annual Meeting***. San Diego Convention Centre, CA, April 2014

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Dedication

I dedicate this thesis to my late father Reuben Majelane Sihlangu and my late younger sister Malebo Moela

"Education is the great engine of personal development. It is through education that the daughter of a peasant can become a doctor, that the son of a mine worker can become the head of the mine, that a child of a farm worker can become the president of a great nation. It is what we make out of what we have, not what we are given, that separates one person from another"

- ***Nelson Mandela***

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Abbreviations

AIDS	Acquired immune deficiency syndrome
AP	Alkaline phosphate
Apaf-1	Apoptosis protease-activating factor-1
ATM	Ataxia telangiectasia mutated
ATP	Adenine triphosphate
ATPases	Adenine triphosphatase
Bad	Bcl-x$_L$/Bcl-2-associated death protein
Bax	Bcl-2-associated death protein
BCA	Bicinchoninic acid
Bcl-2	B cell leukaemia-2
Bid	B cell leukaemia lymphoma-2
Bp	Base pair
BRCA1/2	Breast cancer 1/2 gene
BSA	Bovine serum albumin
Caspase	Cysteine aspartic-specific proteases
CCD	Charge-coupled device
CDK	Cyclin dependent kinase
CD4	Cluster of differentiation 4
cDNA	Complementary DNA
C. elegans	Caenorhabditis elegans
CFS	Chromosomal fragile sites
CI	Cell index
CMV	Cytomegalovirus
CNS	Central Nervous System
CPT	Camptothecin
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DISC	Death signalling complex
DMEM	Dulbecco's modified medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleotides
DWNN	Domain with no name
dsRNA	Double-stranded RNA
E.coli	Escherichia coli
ELISA	Enzyme-Linked Immunosorbent Assay
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ETC	Electron transport chain
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain
Fas	Fibroblast-associated
FasL	Fas Ligand
FDA	Food and Drug Administration
FBS	Foetal bovine serum
FIGO	International federation of gynecology and obstetrics
FITC	Fluoresceine-isothiocyanate
GABA	Gamma butyric acid
GFP	Green fluorescent protein
HAART	Highly active antiretroviral therapy
Hdm2	Human double minute 2
HeLa	Henrietta Lacks
HER2	Human Epidermal growth factor receptor 2
HIER	Heat-induced epitope retrieval
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HR	High Risk
HR-HPV	High-risk HPV
HRP	Horseradish peroxidase
H & E	Haematoxylin and Eosine
H + L	Heavy chain + Light chain

ICAD	Inhibitor of caspase activated DNase
IC50	Inhibitory concentration (half maximal)
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IP	Immunoprecipitation
Kb	Kilobase
kDa	Kilo Dalton
l	Litre
LEEP	Loop electrosurgical excision procedure
MCF-7	Michigan Cancer Foundation-7
MDM2	Murine Double Minute 2
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
MTS	3-(4,5-Dimethylthiazol-2-Yl)-2,5-(3-carboxymethoxyphenyl)-2
Mt.	Mutant
MW	Molecular Weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NHL	Non-hodgkin lymphoma
NMR	Nuclear magnetic resonance
NP-40	Nonident P-40
OMM	Outer mitochondrial membrane
OM	Optical microscopy
FM	Fluorescence microscopy
p21	Cyclin-dependent kinase inhibitor 1
P2P-R	Proliferation potential protein-related
P53/TP53	Protein 53/tumor protein 53
PACT	P53-associated cellular protein testis-derived
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction

PERP	p53 apoptosis effector related to PMP-22
PI	Propidium Iodide
PIER	Proteolytic-induced epitope retrieval
PLD	Phospholipase D
pRB	Retinoblastoma protein
pRBBP6	RBBP6 cDNA construct
PS	Phosphatidylserine
PTEN	Phosphate and tension gene
PTGS	Post-transcriptional gene silencing
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
Rb	Retinoblastoma
RBBP6	Retinoblastoma binding protein 6
RBQ-1	Q protein 1
RING	Really Interesting New Gene
RIPA	Radioimunoprecipitation assay
RISC	RNA-induced silencing complex
RLU	Relative light unit
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RTCA	Real time cell analyser
RT-PCR	Reverse transcription PCR
SCC	Squamous cell carcinoma
SDEV	Standard deviation of the mean
SDS	Sodium dodecyl sulphate
SIL	Squamous intraepithelial lesions
siRBBP6	Short interfering RBBP6
siRNA	Short interfering RNA
ssRNA	Single-stranded RNA
TB	Tuberculosis

TBST	Tris-buffered saline and tween 20
TMA	Tissue microarray slide
TNF	Tumour necrosis factor
TP53	Tumour Protein 53
TRAIL	TNF-related apoptosis-inducing ligand
UPP	Ubiquitin Proteasomal Pathway
V	Volts
VEGFR	Vascular endothelial growth factor receptor
VIA	Visual inspection via acetic acid
VLP	Virus-like particles
WHO	World Health Organisation
WST	Water-soluble Tetrazolium salts
Wt.	Wild-type
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5 carboxanimide
ΔCP	Delta cross point
ΔΨm	Mitochondrial Membrane Potential
μg	Micro gram

CHAPTER ONE - Introduction and Literature Review

1.1.   Introduction

Second to cardiovascular diseases, cancer claims more lives globally than (Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS), Tuberculosis (TB) and Malaria combined (Iyoke and Ugwu, 2013). Previously, gynaecological cancers including breast cancer have been reported to account for only 11% of all female-related cancer cases (Ma and Yu, 2006). However, in a recent report by the Cancer Research UK (2013), breast cancer incidence is the highest of all cancers overtaking lung cancer. Cervical cancer remains the only gynaecological cancer that can be detected at an early stage using the Pap test while similar cancers are still reliant on symptoms. This results in poor statistical reporting of the diseases especially in developing and poor countries due to lack of adequate cancer control tools (Cancer Research UK, 2013; Iyoke and Ugwu, 2013).

The cause of these cancers is not thoroughly understood; however deregulation of tumour suppressor genes has been adequately shown to play a central role in cancer development where mutation and inactivation of p53 tumour suppressor accounts for over 50% of all human cancers (Haupt *et al.*, 2016). For example, in cervical cancer wt. p53 is negatively regulated by the E6 oncoprotein which has been shown to mediate its degradation via the ubiquitin proteasome pathway, whereas in breast cancer MDM2 is responsible for the negative regulation of p53 (Yuan *et al.*, 2016; Haupt *et al.*, 2016). Recently, RBBP6 has been shown to negatively regulate p53 (Li *et al.*, 2007, Mbita *et al.*, 2012; Chen *et al.*, 2013; Moela *et al.*, 2014). These recent reports have drawn our attention as to whether RBBP6 can be used as early detection marker for

gynaecological cancers. Therefore the present study focuses on investigating the involvement of RBBP6 in breast and cervical cancer as a potential cancer biomarker.

1.2. Cervical Cancer

ervical cancer is the most common cancer type amongst women second to breast cancer ,worldwide, with over 80% of cases arising from women in developing countries (Siegel *et al.*, 2014; Richter, 2013; Snyman, 2013). Ninety-nine percent of cervical cancers arise from persistent infection of the cervix by the sexually transmissible human papilloma viruses (HPVs), making HPV infection a prerequisite in the pathogenesis of cervical cancer (Hu *et al.*, 2015; Fernandes and Araujo, 2013; Crosbie *et al.*, 2013; Denny, 2010; Bosch *et al*, 2002; Schiffman and Brinton, 1995 and Munoz *et al.*, 1992). Therefore, cervical cancer is preventable through targeting the HPV virus (Botha and Richter, 2015; Richter, 2013; Snyman, 2013; Sherris *et al.*, 2001). Two groups of HPV have been classified, those that infect keratin-rich skin surfaces resulting in common warts and those that infect the mucosa of the mouth, trachea, respiratory and mostly the anogenital tract (Fernandes and Araujo, 2013). Certain strains of mucosal HPVs may cause common genital warts whereas other types are implicated in the development of premalignant intraepithelial lesions, a major risk factor of invasive cervical cancer development (Fernandes and Araujo, 2013).

1.2.1. Current Status of Cervical Cancer in Southern Africa

On a worldwide scale, cervical cancer is highest in Eastern, Western and Middle Africa, followed by central America, south-central Asia and Melanesia (Arbyn *et al.*, 2011). Moreover, sub-Saharan Africa has been reported to have an estimated 667 000 cervical cancer diagnoses in 2008 and a 78% mortality rate mostly due to poor socio-economic conditions, competing health needs and incomplete infrastructure (Denny, 2010). This makes cervical cancer prevention and

treatment more important in South Africa and other developing countries (Denny, 2010). About 38.7 incidence rates have been reported in southern Africa as a whole, where an incidence rate of 61.6 was reported in Lesotho, 58.9 in Swaziland, 30.4 in Botswana and 22.2 in Namibia (Denny, 2010).

Data on cervical cancer for South Africa has been unavailable since 1999 due to poor maintenance of pathology-based cancer registry until recently where it was estimated that 1 in 41 South African women screen positive for cervical cancer and approximately 8 deaths are reported daily (Botha and Richter, 2015; Snyman, 2013; Denny, 2010). In terms of race, cervical cancer is more prevalent in black women over the age of forty (Snyman, 2013). The lifetime risk of cervical cancer is higher in black women from developing countries (1 in 43) when compared to that in white women (1 in 94) and this discrepancy may be as a result of the reflected better socio-economic status and more frequent screening practices among the white community (Botha and Richter, 2015; Crosbie *et al.*, 2013; Moodely, 2009). These alarming statistics potentiate the need to implement new and efficient cervical cancer management strategies.

1.2.2. Cervical Cancer in the Context of HIV

It is highly important to understand the incidence of cervical cancer in the context of Human Immunodeficiency Virus (HIV) since it remains a serious pandemic more especially in South Africa. A South African statistical release of 2013 reported that an estimated 5.6 million population size of adults and children are living with HIV, 68% of which is women (Adler *et al.*, 2014; Tshifularo *et al.*, 2013). Research shows that even though HIV positive women have a reduced AIDS mortality due to access to antiretroviral therapy, the incidence rate of cervical cancer is most likely to be increased as a result of the HIV pandemic (Reddy and Frantz, 2011). This is because it has been noted that the longer an HIV positive woman lives, the higher the

chances of her developing cervical cancer due to a compromised immune system (Tshifularo *et al.*, 2013; Chirenje, 2005; Denny, 2010). And as a result, large numbers of women living with HIV have been diagnosed with cervical cancer (Tshifularo *et al.*, 2013; Chirenje, 2005; Denny, 2010). Both HPV and HIV are sexually transmitted diseases that present without any immediate visible symptoms (Tshifularo *et al.*, 2013). However, the difference between a woman who has oncogenic HPV infection and one who has HIV infection is that the HIV infected woman commonly shows invasive cancer ten years earlier than the woman who has early stage cervical lesions and that is due to excessive immunosuppression by HIV (Bomela and Stevens, 2009).

1.2.3. Biological Mechanism of HPV Infection

Oncogenic viral infections such as hepatitis viruses (B/C) and human HPV are responsible for up to 20% cancer deaths in low and middle-income countries (deMartel, 2012). HPV, which accounts for 99% of cervical cancer cases, is a minute viral particle of approximately 50-60 nm in diameter that lacks an envelope and contains a closed circular genome protected by a network of capsid proteins named L1 and L2 (Fernandes *et al.*, 2013). HPV is classified as mucosal and non-mucosal. Of the mucosal types, high-risk types and the probable high-risk types may cause cancer whilst the low-risk types cause genital warts. The high-risk types include HPV 16, 18, 45, 31, 33, 52, 58 and 35 among others (Munoz *et al.*, 1992; Munoz *et al.*, 2003).

Cervical infection by HPV takes advantage of the exposed basement membrane of the cervix following chemical or mechanical trauma of the epithelium as a result of sexual intercourse or direct contact of infected mucosal surfaces of the genitalia (Bosch *et al.*, 2002; Munoz *et al.*, 1992). The viral particle is therefore able to secrete viral proteins that are capable of executing internalisation of the virus into the host cells (Munoz *et al.*, 1992). L1 is a major capsid protein

responsible for first interaction of viral particle to the extracellular matrix of the epithelial cell surface receptors known as heparin sulphate proteoglycans, which are located on the regions of the exposed basement membrane (Bosch *et al.*, 2002).

The host's repair mechanism induces migration of basal keratinocytes over the basement membrane in order to cover the wound (Munoz *et al.*, 1992). However this triggers conformational changes in the L1 capsid protein which exposes a previously hidden region of the L1 that interacts with basal keratinocytes that have migrated over the wounded basement membrane, thus kick-starting the infection process (Munoz *et al.*, 1992). This is followed by internalisation of the viral particle via the keratinocyte surface receptor with a subsequent entry of the viral genome into the nucleus (Fernandes *et al.*, 2013).

The life cycle of HPV inside the host cell is divided into two infection stages, namely the maintenance phase and the proliferation-dependant phase. The purpose of the maintenance phase is to delay viral antigen expression and replication inside the basal undifferentiated cells to locations that are less likely to have a high host immune response (Munoz *et al.*, 1992). The virus does this by maintaining a low copy number of the viral genome which is kept in tight regulation by viral expression of the E1 and E2 proteins which are responsible for recruiting cellular DNA polymerases necessary for DNA replication and the E6 and E7 proteins for modulation of cell cycle regulators in order to maintain a long-term infection (Bosch *et al.*, 2002; Fernandes *et al.*, 2013).

During the differentiation-dependant phase, the E6 and E7 viral proteins regulate the viral replication ability in differentiated cells by supressing transcription of the p53 and retinoblastoma protein (pRB) cellular proteins thus abrogating the host cells' potential to initiate cell cycle arrest, prompting uncontrolled cell proliferation (Bosch *et al.*, 2002; Fernandes *et al.*, 2013). This type of HPV interference with p53 and Rb functioning is similar to that of RBBP6 functioning which has been shown to bind p53 and Rb thus leading to cell cycle inactivation and subsequently increased cell proliferation. Possibly this may suggests a mechanism by RBBP6 and HPV in the development cervical cancer. Human papilloma viruses use this infection tactic of reducing viral protein expression in undifferentiated cells and intensifying it in differentiated cells in order to escape the host immune response (Fernandes *et al.*, 2013). The clearance of high-risk HPV (HR HPV) by the immune system occurs one to two years after infection. However some women (~10%) fail to clear HPV infection and this results in a persistent infection that leads to the development of lesions that favour the progression to malignancy, the most important risk factor for cervical cancer (Fernandes *et al.*, 2013).

1.2.4. Other Risk Factors of Cervical Cancer

Besides HPV infection, cervical cancer aetiological factors include long-term use of oral or hormonal contraceptives, parity and other sexually transmitted diseases such as herpes simplex virus subtype 2 (Holmes *et al.*, 2009). A minimum of five years is sufficient to place users at risk of developing cervical cancer and the risk increases far more after ten years of oral contraceptive usage. This relationship between long-term usage of oral or hormonal contraceptives and cervical cancer development is however not extensively assessed, leaving room for confirmation of these findings in a more diverse population.

The risk of cervical cancer development has also been shown to increase in HPV-positive women with high parity, i.e. women with more than seven full-term pregnancies have a four-fold higher risk than nulliparous women. Tobacco use has also been associated with increasing risk of cervical cancer as seen in HPV-positive current smokers presenting with a three-fold higher risk than HPV-positive non-smokers (Holmes *et al.*, 2009). However the biological mechanism behind smoking and cervical cancer development is not fully understood.

Human immunodeficiency virus (HIV) is another sexually transmitted disease that is believed to be a potential causal factor of cervical cancer (Keller, 2015; De Flora and La Maestra, 2015; Ebrahim *et al.*, 2016; Konopnicki *et al.*, 2016). This current research suggests that suppression of the immune system by the HIV makes patients highly susceptible to persistent HPV infection which might increase the prevalence of cervical intraepithelial neoplastic lesions and a rapid progression to invasive cervical cancer. HPV/HIV co-infection results in dysfunction of cellular and hormonal arms of the local and systemic immune system and a subsequent disease progression (Keller, 2015). HIV-infected women with decreased CD4 counts show a two-fold increase in the prevalence of squamous intraepithelial lesions (SIL) when compared to women with higher counts meaning higher immunosuppression results in high HPV viral load (Keller, 2015; Konopnicki *et al.*, 2016). Highly active antiretroviral therapy (HAART) is a combination of multiple drugs that act on different targets of HIV in an attempt to control HIV infection (De Flora and La Maestra, 2015; Ebrahim *et al.*, 2016). These drugs have proven to be so successful that in many parts of the world HIV has become a chronic condition in which progression to AIDS has become increasingly rare.

1.2.5. Prevention, Screening and Treatment of Cervical Cancer

Primary prevention of cervical cancer previously relied on conventional strategies such as abstinence, mutual monogamy of virgins and use of condoms which proved insufficient as seen by alarming figures of cervical cancer incidence in developing countries (Sawaya and Smith-McCune, 2016; Mariani *et al.*, 2015). Recently, a breakthrough in the development of two commercially available vaccines against high-risk HPV type 16 and 18 gave new promise for primary prevention strategies (Smith *et al.*, 2015). Cervarix® is a bivalent injection suspension that prevents infection of HPV 16 and 18 types and Gardasil® is a quadrivalent injection suspension against HPV type 6, 11, 16 and 18 (Panatto *et al.*, 2015).

The mechanism of action by these two vaccines relies on the use of L1 proteins of HPV which form bodies of virus-like particles (VLPs) that function by inducing neutralising serum antibodies. These are meant to pass through the membrane across the cervical epithelium in concentrations that are high enough to interact with virus particles and prevent infection (Panatto *et al.*, 2015). The vaccines are safe, highly immunogenic and vaccination lasts for up to 6.5 years and therefore highly efficient; however at a cost of three hundred and sixty US dollars per three injection doses these are the most expensive vaccines available (Isidean *et al.*, 2015).

Even though it is too late for women already infected with HR-HPV strains, these vaccinations suggest that future generation women might enter their sexually active years protected from infection by HPV 16 and 18 if administered in young girls (Handler *et al.*, 2015; Kash *et al.*, 2015). Modelling research predicts that 70% vaccine coverage in young girls between ages 9-12

might reduce cervical cancer life-time risk by up to 45%. Consequently in March 2014, a vaccination program was launched in South Africa, targeting about 550 000 young girls entering grade 4 of their primary level of school (Palmer *et al.*, 2014). In a study performed by Moodely *et al.* (2013) it has been shown that the overall uptake of the Gardasil vaccine among 9-12 year old school girls in Kwa-Zulu Natal, South Africa, was as high as 99.7%, 97.9% and 97.8% for all first, second and third doses, respectively, demonstrating a highly successful implementation of HPV vaccination among young school girls (Moodely *et al.*, 2013).

Cervical cancer screening is a strategy used to detect the cervical cancer-causing precursors, namely pre-invasive lesions and early-stage invasive lesions in order to inhibit progression of the disease into invasive cervical cancer (Richter, 2013). A traditional method referred to as cervical cytology which involves collection of cells from the cervical canal onto a glass microscope slide or into a liquid medium for screening is the first technique that has led to a marked reduction in the incidence rate of cervical cancer in the early 1980's (ACOG Practice Bulletin 45, 2003). Also known as Papanicolaou technique, Pap smear screening has proven successful however discrepancies such as human errors in sampling and data interpretation as well as the requirement for high level of medical infrastructure each year of screening have contributed to low sensitivity of the technique.

HPV-based screening offers more advantages over cervical cytology such as high clinical sensitivity and excellent negative predictive value (Richter, 2013). This screening procedure focuses on detecting HPV DNA in squamous epithelial cells of the cervix and sample collected

is similar to that in conventional screening. Recently a few techniques have been developed to detect mRNA of the E6 and E7 proteins that form part of HPV genome profile.

Screening guidelines for South Africa are that every woman should take three pap smears in a lifetime at 10 year intervals from the age of 30 and internationally, guidelines allow 30 year old women and above to combine HPV screening with cervical cytology (Kitchener *et al.*, 2013). Affordable screening methods in resource-restricted environments have been implemented, such as the visual inspection using 3-5% acetic acid (VIA) which uses naked eye examination aided by a bright light source (Nalliah *et al.*, 2015; Sankaranarayanan *et al.*, 2009; Denny 2010; Louie *et al.,* 2009). The VIA works by detecting aceto-white or yellow areas in the cervical transformation zone, which serve to indicate cervical abnormalities and possible pre-cancerous lesions (Nalliah *et al.*, 2015; Sankaranarayanan *et al.*, 2009). This method has been advocated as the most inexpensive screening technique that provides immediate results and therefore offers a good alternative screening method for women in developing countries (Nalliah *et al.*, 2015; Sankaranarayanan *et al.*, 2009).

Screen-positive women normally undergo a pre-therapy procedure known as colposcopy which is a method used to diagnose and evaluate the extent of lesion on the cervix (Richter, 2012). This is followed by a treatment method, either cryotherapy or loop electrosurgical excision procedure (LEEP) (Louie *et al.,* 2009). In cryotherapy, abnormal tissue on the cervix is subjected to extreme cold that freezes the tissue until it gradually disappears allowing the cervix tissue to

heal. Unlike cryotherapy, LEEP functions to remove the abnormal cervical tissue using a low-voltage electrified wire loop (Louie *et al.,* 2009).

Although very effective in reducing cervical cancer as evidenced by high successful rates, most of the current methods are highly expensive thus making cervical cancer management in developing areas very restricted. This therefore calls for a need to implement more diagnostic and treatment strategies that are cost-effective and therefore easily accessible in developing countries like South Africa. Recent research indicates that RBBP6 is highly expressed in several malignancies and it is responsible for promoting cell proliferation, making it a potential marker of cancer and thus a promising target for gene therapy against cancer (Moela *et al.*, 2014; Li *et al.*, 2007; Chen *et al.*, 2013; Mbita *et al.*, 2012)..

1.3. Breast Cancer

Breast cancer is classified into three subtypes based on the presence or absence of certain cancer cell receptors (Tinoco *et al.,* 2013). These include the hormone receptor (HR) subtype (estrogen and/or progesterone receptors) which accounts for nearly 60% of all breast cancer incidences, the oncogene human epidermal growth factor receptor 2 (HER2/neu) subtype of which 20% is observed in all breast cancer cases and the triple negative breast cancer (negative for the expression of estrogen, progesterone and HER2/neu receptors) which also constitute about 20% of all breast cancer cases (Tinoco *et al.,* 2013). The estrogen receptor positive breast cancers are the most common type of breast cancer because the estrogen and progesterone hormones are playing a primary role as transcription factors that mediate breast tumorigenesis, thus enabling recurrence of the disease even after an effective targeting of the estrogen receptor (ER) signalling

(Lim *et al.,* 2012). In addition to the receptor-associated subtypes of breast cancer, researchers have documented intrinsic molecular subtypes: Luminal A and B, and HER2-like subsets (Tinoco *et al.,* 2013); (Lim *et al.,* 2012). This heterogeneous nature of breast cancer implicates a need for patient-specific treatment, making breast cancer management a major challenge.

1.3.1. Breast Cancer Statistics

Breast cancer remains a female-related health problem on a global scale, accounting for over a million newly estimated cases that are still on the rise (Tao *et al.*, 2015; Jemal *et al.*, 2012). Breast cancer incidence rate differs much across the world and this remarkable variance is attributed to substantial regional differences in the prevalence and distribution of socio-economic factors (Jemal *et al.*, 2012). For example, a highest incidence rate of 99.4 per 100 000 people in North America alone was reported (WHO, 2013). Furthermore, moderately increasing incidence rates are reported in Europe, other parts of America as well as in Southern Africa (WHO, 2013).

Mortality rates amongst women were estimated at 69% worldwide (~519 000 deaths) in 2012 (Tao *et al.*, 2015; Jemal *et al.*, 2012). Breast cancer survival rate (number of people per 100 000 who die of breast cancer during a given time period) is lower in black women (75%) than in white women (89%) as a result of poor prognosis in black women (Tao *et al.*, 2015; Jemal *et al.*, 2012). This is because black women often present with late stage breast cancers with large and more aggressive tumours (Jemal *et al.*, 2012). The World Health Organisation statistics for the year 2012 indicate a steady increase of breast cancer cases worldwide where about 380 000 deaths are observed each year (WHO, 2013). In Africa, 92 600 breast cancer cases and 50 000 deaths were reported in 2008 statistics, with Southern African women showing highest breast cancer incidence rates when compared to women from all African countries (Jemal *et al.,* 2012).

1.3.2. Breast Cancer Risk Factors

In a study published by (Steiner, Klubert and Knutson, 2008), breast cancer risk factors are divided into non-modifiable and modifiable factors. Advanced age and female gender are the major non-modifiable risk factors associated with breast cancer. This is due to the fact that the patient's lifetime exposure to female hormones (progesterone and estrogen) associated with breast cancer would have increased significantly (Steiner *et al.,* 2008). Other important non-modifiable breast cancer risk factors are menarche before the age of 12, menopause after the age of 45, genetic mutations and family history (Steiner *et al.,* 2008).

In addition, women with first degree relatives demonstrate higher risk of developing breast cancer. Furthermore, the risk is much higher in women with first degree relatives who are diagnosed at a younger age compared to older aged first degree relatives (Nelson *et al.,* 2012). However, breast cancer cases arising from genetic mutations account for only about 5% and 60% of inherited breast cancers are as a result of mutations in tumour suppressor genes, BRCA1 and BRCA2 (breast cancer 1 and 2) (Steiner *et al.,* 2008). P53 is another tumour suppressor gene known to contribute not only to breast cancer development but most human cancers as well (Bai and Zhu, 2006; Dumitrescu and Cotarla, 2005). Functional p53 helps prevent cancer development through activation in response to a stress signal. This is usually followed by induction of cell cycle arrest in order to allow for DNA repair (Bai and Zhu, 2006). However the presence of p53 negative regulators compromises the cells' ability to correct for such alterations, thus leading to cancer development. Besides MDM2 and E6 oncoprotein, RBBP6 is also suspected to play a role in the negative regulation of p53 since it has been show to interact with

RBBP6 prior to degradation by the proteasome pathway (Li *et al.*, 2007). The restoration of active p53 by targeting RBBP6 is therefore important in the present study.

1.4.   The Cell Cycle

Cancer develops from mutations in tumour suppressor genes which lead to alterations in signalling pathways (Bai and Zhu, 2006). The cell cycle is the most vulnerable signalling pathway during tumorigenesis since it is responsible for cell proliferation. The most important function of the cell cycle is to make sure that DNA is faithfully replicated in the S-phase and that identical chromosomes are equally distributed to two daughter cells during the mitotic phase (DiPaola, 2002). However, during cancer development cells have the ability to progress through the cell cycle without being monitored due to deregulated cell cycle checkpoints. RBBP6 plays a role during cell division in which it has been shown to stabilize chromosomal fragile sites (CFSs) in order to accelerate cell cycle progression (Miotto *et al.*, 2014). Overexpression of RBBP6 in tumorigenesis might therefore be responsible for the uncontrolled cell proliferation. This makes functional studies of RBBP6 in relation to the cell cycle necessary.

There are four coordinated processes involved in eukaryotic cell cycle, namely cell growth, DNA replication process (or the interphase), the segregation of replicated chromosomes into two separate cells (or mitotic phase), and cell division (cytokinesis) (Figure 1) (Vermeulen *et al.*, 2003; Cooper, 2000). A typical human cell divides approximately every 24 hours and even though mitosis is the most dramatic stage of the cell cycle, it only lasts about an hour, while the interphase takes up about 95% of the cell cycle duration in which cell growth and DNA replication occur in preparation for cell division (Cooper, 2000).
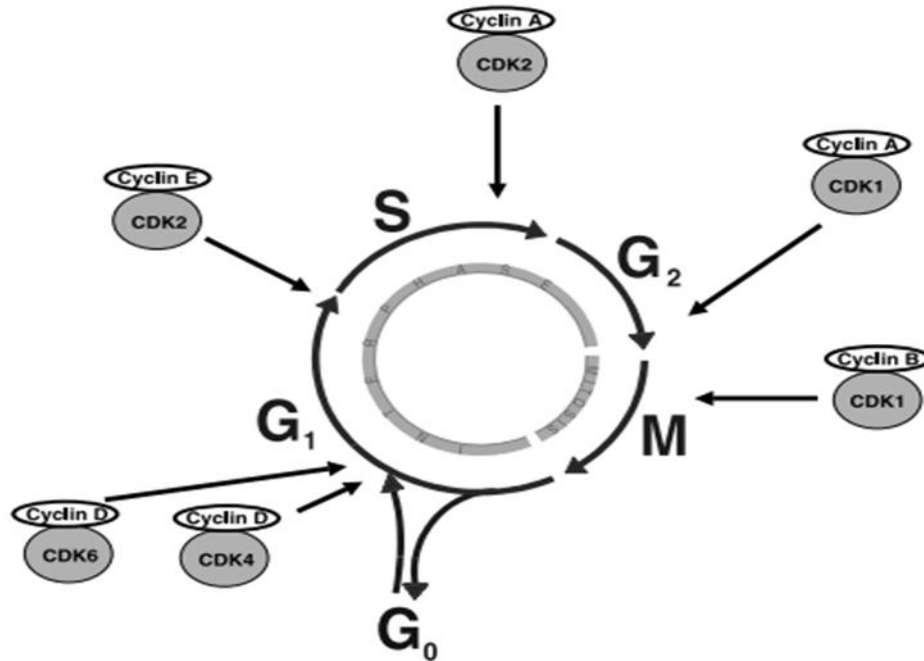
Figure 1: The stages of the cell cycle and the sites of regulatory cyclin/CDK complexes (Vermeulen *et al.,* 2003).

The interphase is made up of G1, S and the G2 phase and DNA replication takes place in the S-phase. The S-phase is preceded by the G1 phase where preparation of DNA synthesis takes place followed by G2 phase where preparation of cell division or mitosis occurs as shown in Figure 1 (Khodjakov and Rieder, 2009; Collins *et al.*, 1997; DiPaola, 2002). Depending on the type of extracellular stimuli, cells in G1 respond by either progressing to the S-phase or by entering a quiescent or resting phase called G0 where they do not undergo any form of cellular growth though they are metabolically active (Sherr, 1996; Vermeulen *et al.,* 2003).

1.4.1. Regulation of the cell cycle

Uncontrolled cell proliferation is the hallmark of cancer development mostly due to damage in genes that are responsible for the regulation of the cell cycle (Sherr, 1996). Serine/threonine protein kinase family members known as the cyclin-dependent kinases (CDKs) are the key

regulators of the transition from one cell cycle phase to another (Vermeulen *et al.,* 2003). Five CDKs are known to be activated during different stages of the cell cycle, i.e. CDK 4, 6 and 2 during G1, CDK 2 during the S-phase and CDK 1 during G2 and M-phase (Khodjakov and Rieder, 2009; Collins *et al*, 1997; DiPaola, 2002). The activated CDKs phosphorylate target proteins in order to induce downstream processes. CDKs are activated by cyclins which are required at different stages of the cell cycle. The type D cyclins (cyclin D1, D2, and D3) bind and activate CDK 4 and 6, and cyclin E and A bind to CDK 2. In addition to cyclin binding, CDKs are activated by phosphorylation at conserved serine/threonine regions and are counteracted by CDK inhibitors which bind to either CDKs alone or to CDK/cyclin complexes (Khodjakov and Rieder, 2009; Vermeulen *et al.,* 2003). These protein complexes ensure a tight regulation of cell cycle progression.

1.5.   Apoptosis

Apoptosis is a programmed cell death strategy that plays a central role in controlling the cell fate if DNA damage is not repaired. DNA can be damaged by stress signals such irradiation, reactive oxygen species (ROS) as well as other potential carcinogens, thus leading to cancer development (Ouyang *et al.*, 2012; Liu *et al.*, 2011). The cell responds to these signals by activating p53 tumour suppressor gene. The activated p53 in turn activates genes responsible for cell cycle arrest so as to allow for DNA repair, failure of which prompts the cell to undergo apoptosis (Debatin, 2004). Apoptosis, as first described by Kerr *et al.* (1972), is a form of programmed cell death characterized by cell morphological changes that include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation and a subsequent cell death (Ouyang *et al.,* 2012). Other forms of programmed cell death include autophagy and necrosis which are both distinguished from apoptosis by their morphological differences (Bialik

et al., 2010). The understanding of apoptosis has provided the basis for novel targeted therapies that can induce death in cancer cells or sensitize them to established cytotoxic agents and radiation therapy (Ghobrial *et al.*, 2005).

1.5.1. The Apoptotic Pathway

Apoptosis is comprised of two signalling pathways, namely the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic) (Wen *et al.,* 2012). Activation of the extrinsic pathway is initiated by binding of the Fas Ligand to the cell surface death receptor, Fas, a member of the TNF (tumour necrosis factor) receptor superfamily. Other members include the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor, Apo-2 and TNF-1 death receptors (Ghobrial *et al.*, 2005). The Fas-L-Fas (Fas ligand) complex forms a death inducing signalling complex (DISC) by aggregating with the Fas-associated death domain (FADD) adaptor protein and pro-caspase-8. This therefore leads to an active caspase-8 enzyme which subsequently activates pro-caspase-3, an effector caspase required for the execution of apoptosis (Ouyang *et al.*, 2012). In other cells, caspase-8 interacts with death modulators (e.g. Bid) of the intrinsic pathway which lead to the release of cytochrome c, a crosstalk between the extrinsic and the intrinsic pathways (Ghobrial *et al.,* 2005).
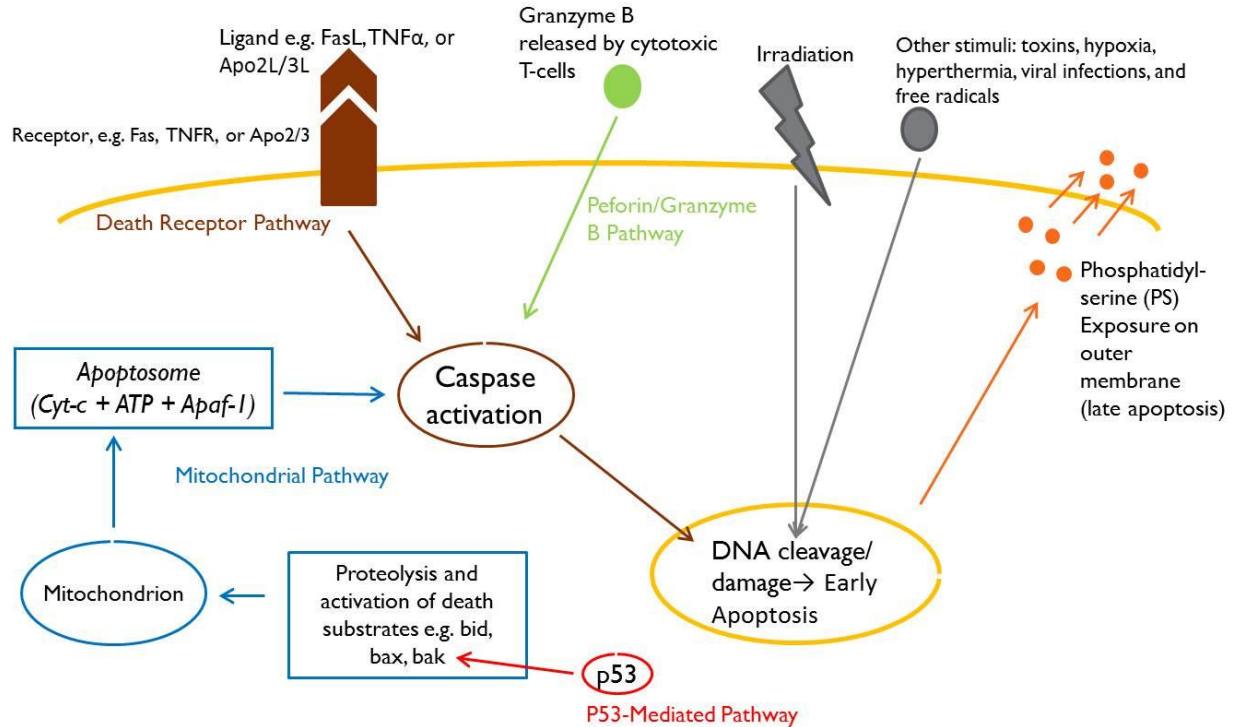
Figure 2: A simplified schematic diagram of both the intrinsic and extrinsic apoptotic pathways

The intrinsic pathway is regulated by the Bcl-2 family proteins which become activated by posttranslational modifications followed by translocation to the mitochondria in response to either extracellular or intracellular stimuli. This triggers the release of cytochrome c from the outer mitochondrial membrane into the cytoplasm. The cytoplasmic cytochrome c interacts with pro-caspase-9 and Apaf-1 molecule to form an apoptosome body which leads to an active caspase-9 responsible for cleaving pro-caspase-3 into an active caspase-3 necessary for the induction of apoptosis (Ouyang *et al.,* 2012; Ghobrial *et al.,* 2005).

1.5.2. Regulation of Apoptosis: Bcl-2 Family Proteins

The Bcl-2 family proteins, first identified in follicular non-Hodgkin lymphoma (NHL), are known to control apoptosis at the intrinsic pathway level and are highly expressed in many other cancers, therefore numerous anti-tumour drugs exerts their effect on the Bcl-2 proteins (Ghobrial

et al., 2005). This family of proteins is made up of the proapoptotic Bcl-2 proteins which promote apoptosis (Bax, Bak, Bad, Bcl-Xs, Bid and Bik) and the anti-apoptotic Bcl-2 proteins which suppress apoptosis (Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1) (Ghobrial *et al.*, 2005).

The proapoptotic members promote apoptosis by mediating the release of cytochrome c from mitochrondria and the anti-apoptotic members block apoptosis by inactivating the pro-apoptotic members (Ouyang *et al.,* 2012). Bax and Bak are termed BH3-only proteins which exert their pro-apoptotic effects using the BH3 molecule (Ghobrial *et al.*, 2005; Ouyang *et al.*, 2012). In response to cellular stress, these proapoptotic proteins undergo activation processes such as dephosphorylation and cleavage followed by movement to the mitochondria where they initiate cytochrome c release by permeabilizing the outer mitochondrial membrane (Ghobrial *et al.*, 2005; Ouyang *et al.*, 2012).

1.6.   Targeting Apoptosis in Cancer

The attached manuscript "**Apoptotic Molecular Advances in Breast Cancer Management**" below is a recently (2015) published chapter with a literature review on the importance of targeting apoptosis in breast cancer and tumorigenesis in general.

Apoptotic Molecular Advances in Breast Cancer Management

Pontsho Moela and Lesetja R. Motadi

Additional information is available at the end of the chapter

Abstract

Breast cancer is the most common cancer type amongst women, accounting for most female cancer deaths second to cervical cancer worldwide. It is, therefore, highly crucial to understand the molecular biology and explore other pathways involved in carcinogenesis in order to select appropriate treatment not only for breast cancer but for other cancers as well. Cancer progression is favoured by DNA damage and in most cases a consequent disruption of the apoptotic pathway, thus leading to uncontrolled cell proliferation. Therefore, current therapeutic strategies aim at targeting the apoptotic pathways in order to combat cancer. In this manuscript, we discuss the ways in which evasion of apoptosis during carcinogenesis occurs and the types of current therapeutic strategies as well as promising future approaches against breast cancer.

Keywords: Breast cancer, apoptosis, small molecules, p53, RBBP6

1. Introduction

The human body is composed of trillions of cells that behave and function to provide structure of the body, convert nutrients into energy and carry out specialised functions [1, 3]. Growing, dividing, differentiating and dying are the cells' behavioural mechanisms to maintain tissue homeostasis [3]. However, molecular disturbances that disrupt this balance may potentially lead to disease. Such molecular disturbances include mutations, among others, during which any change to the DNA sequence might result in abnormality in the cell or tissue [4]. With a population of more than a trillion cells, the human body is prone to mutations that may give one cell a selective advantage of growing and dividing more vigorously to become a growing mutant clone [4, 5]. Such mutations, in which a mutant clone of cells grows and divides out of control at an expense of neighbouring wild-type cell populations, serve as a prerequisite for the development of cancer [3].

Cancer is defined as uncontrolled cell proliferation that leads to the formation of abnormal cells and invasion of other adjacent tissues [1, 3-5]. The migration of cells from the origin of tumour to another part of the body is referred to as metastasis. Tumours can either be malignant or benign. While malignant tumours have the ability to invade surrounding tissue, benign tumours cannot invade other tissues and are therefore not as life-threatening [3]. Efficient treatment against malignant tumours is therefore necessary in cancer management. In this chapter, we discuss current anticancer strategies that are targeted on the apoptosis pathway in breast cancer management.

In order to understand breast cancer, it is necessary to understand the normal anatomy of the female breast [3, 20]. The female breast is made up of milk-producing glands called lobules which are connected to ducts that transport milk from the glands to the nipples. The ducts and lobules are surrounded by connective tissue, fatty tissue, blood vessels and lymphatic vessels. In most cases, breast cancer starts in cells surrounding the ducts or the lobules [23]. Metastatic breast cancer is as a result of migration of cancerous cells from ducts and/or lobules via lymphatic vessels to the lymph nodes of the lymphatic system [3, 20, 23].

Breast cancer is the most common cancer type amongst women accounting for many cancer deaths, second to cervical cancer. Risk factors of breast cancer are divided into non-modifiable and modifiable factors [39]. Advanced age, female gender, menarche before the age of 12, menopause after the age of 45, genetic mutations and family history are the major non-modifiable risk factors associated with breast cancer [6, 11, 26, 46, 56, 57]. Breast cancer risk factors that can be controlled include hormone replacement therapy, oral contraceptives, pregnancy, breast feeding and high breast density [31]. Behavioural and life-style risk factors associated with the development of breast cancer include poor diet, i.e. high fat, low vegetable/fruit, low fibre and high in simple carbohydrates; overweight and obesity; and decreasing physical activity [29, 39].

Nearly 80% of human breast cancers are hormone-positive (estrogen and progesterone), followed by human epidermal growth factor receptor 2 (HER2)-positive, then vascular endothelial growth factor (VEGF)-positive breast tumours [8, 9]. Targeting estrogen receptor (ER) pathway, VEGF and HER2 are the long-established breast cancer therapeutic approaches responsible for the improvements of breast cancer prevention and treatment. However, resistance to these endocrine and cell-growth-inhibiting treatments is the main drawback that reduces the benefits of these novel treatment approaches [8, 9, 20, 23]. It is therefore highly crucial to understand the molecular biology and explore other pathways involved in carcino-genesis in order to select appropriate treatment not only for breast cancer but for other cancers as well. In this chapter we discuss different ways of targeting apoptosis in breast cancer management.

2. Targeting apoptosis in breast cancer treatment

During the process of breast cancer progression, normal cells transform into malignant types as a result of genetic alterations [12]. This leads to dysregulation of cellular processes such as

angiogenesis, cell cycle and apoptosis [17]. Therefore, current therapeutic strategies aim at targeting these pathways, more especially apoptosis, in order to combat cancer [18]. Apoptosis is a form of programmed cell death in which cells are programmed to die if found to be cellular damaged [21, 25]. Apoptosis is made up of two major pathways called the death receptor pathway and the mitochondrial pathway, which are both propagated by a caspase cascade that ultimately leads to apoptosis induction [27, 34]. Evasion of apoptosis during carcinogenesis occurs by three distinct mechanisms: disrupted signalling of death receptors, loss of caspase activity as well as impaired balance between anti-apoptotic and pro-apoptotic proteins [14, 42, 50, 59]. Targeting the caspase cascade, Bcl-2 family proteins as well as other factors associated with apoptosis signalling have thus become the major strategy in anticancer therapeutics (table 1).

Reagent	Target	Technology	Function	Status
Apoptin	Caspases in the extrinsic pathway	Vector-based (adenoviral and virus vectors)	Caspase 3 and 8 activation	Preclinical
Flavipirodol, gossypol, depsipeptide, ABT-737, ABT-264, fenretinide, HA 14-1, GX15-070	Anti-Bcl-2 family proteins	Small molecule	Inhibit BCl-2 family proteins by reducing their expression	Phase I/II
ABT 737	Anti-apoptotic proteins	Small molecule	Inhibit expression of anti-apoptotic proteins such as Bcl-xL, Bcl-2 and Bcl-W	Phase I
Oblimersen Sodium	Anti-Bcl-2 targeted drug	Antisense	Bcl-2 antisense increases survival rates in chronic myeloid leukaemia patients when combined with chemotherapy	Phase II
ONYX-015 drug	p53-based gene therapy	Adenoviral	Genetically engineered adenovirus that has been modified to infect and lyse p53-deficient cells	Phase III
CD8^{+} cytotoxic T-lymphocytes (CTLs)	Tumour associated antigens (mutant p53)	Vaccine	Recognize TAA-derived peptides that are processed and presented on the tumours cell surface in association with MHC class I molecules, leading to killing of tumour cells	Phase I

Reagent	Target	Technology	Function	Status
Phikan083, CP-31398	p53-targeted	Small molecule	Restores p53 function by intercalating with p53-bound DNA and destabilising the p53-DNA interaction	Phase I/II
Tenovins, Nutlins, MI-219	p53-MDM2 interaction	Small Molecule	Interrupt the p53-MDM2 interaction to prevent inactivation of p53 by MDM2	Phase I/II
siMDM2, siE6/7, siBBP6	p53-MDM2, p53-E6, p53-RBBP6 interaction	Liposomal encapsulated synthetic siRNA	Interrupt p53 interaction with its negative regulators	Research

Table 1. Apoptosis-based anticancer drugs in development

2.1. Caspase-targeted therapy

Pathogenic as they are, disruptions in the apoptotic pathway provide compelling possible strategies for the treatment of breast cancer and other related types of cancers [59]. Therapeutic agents designed to re-establish the normal functioning of the apoptotic signalling pathways have the potential to get rid of over 50% of human cancers including breast cancer [34]. Novel drug discoveries in recent years have led to promising advances in the treatment of breast cancer as well as other cancers. For example, the caspase-targeting therapies that use small molecules to act as caspase activators have been identified [24, 32]. These small molecule caspase activators are pro-apoptotic due to their characteristic arginine-glycine-aspartate motif that enables them to directly convert non-active procaspase-3 into active caspase-3 thus leading to apoptosis induction.

Apoptotin is a caspase-based drug therapy that has the ability to induce caspase activity thus increasing apoptosis induction [32]. MCF-7 breast cancer cells completely lack the expression of caspase-3 due to frame-shift mutation in exon 3 of the caspase-3 gene [13]. As a result, caspase-based gene therapy that relies on caspase-3 gene delivery techniques in order to up-regulate caspase-3 expression in caspase 3-deficient breast cancers has been invented. In human liver tumorigenesis, caspase-3 gene therapy led to a significant increase in apoptosis and shrinkage in tumour size when combined with other chemotherapeutic drugs [13, 32]. Caspase-8 expression has also been found to be impaired due to hypermethylation in several cancer cells. In small cell lung carcinomas, demethylation treatments have been shown to sensitise these cancer cells to drug-induced apoptosis [32, 53].

2.2. Anti-Bcl-2 therapy

The mitochondrial pathway is down-regulated by the anti-apoptotic Bcl-2 family proteins [19, 22, 40, 43, 60]. Drug-based therapy using anti-Bcl-2 small molecules has led to a significant

induction of apoptosis in several cancers. Flavipirodol, gossypol, depsipeptide, ABT-737, ABT-264, fenretinide, HA 14-1 and GX15-070 are some of the small molecules that inhibit BCl-2 by reducing their expression [41, 45, 59]. Small molecules with the ability to mimic pro-apoptotic or anti-apoptotic BH3-only Bcl-2 family proteins in order to induce apoptosis have also been designed [2, 41]. This class of drugs that imitate BH3-only pro-apoptotic and anti-apoptotic Bcl-2 family proteins is referred to as BH3-only mimetic drugs [2, 35]. ABT 737 is one example of the BH3-only mimetics that has been shown to inhibit expression of anti-apoptotic proteins such as Bcl-xL, Bcl-2 and Bcl-W; and is showing promising results in clinical trials [2, 59]. The first anti-Bcl-2 targeted drug to enter clinical trials in leukemic patients is known as oblimersen sodium [41, 59]. This Bcl-2 antisense has been shown to increase survival rates in chronic myeloid leukaemia patients when combined with chemotherapy [41, 59].

2.3. p53-based gene therapy

The loss of p53 function is a common feature in almost all human cancer including breast cancer [37, 43, 47]. Because of this, there is a lot of interest in targeting p53 for anticancer therapeutic drugs [7, 10, 16, 55]. The first biological approach which is now widely used in targeting p53 is gene delivery of wild-type p53 into tumour cells using adenoviral or retroviral techniques [28, 48]. p53-based gene therapy is however not effective on its own in killing cancer cells and for this reason combinational therapies involving other modes of treatments in the presence of p53 therapy are being investigated [10, 55, 59].

For example, it was discovered that concurrent treatment using adenoviral-mediated wild-type p53 injection with ionising therapy significantly reduces tumour size in cancers of prostate, brain and spine as well as head and neck [28, 59]. Elimination of p53-defective cells using synthetic viruses designed to infect and kill cancer cells is another breakthrough in p53-based gene therapy [28, 48, 59]. One example is the ONYX-015 drug, which is a genetically engineered adenovirus that has been modified to infect and lyse p53-deficient cells [28]. Genetic alterations that take place in p53 during tumorigenesis can trigger the immune responses in both T- and B-cells [10]. This provides yet another interesting platform for p53-based anticancer therapy, and a number of p53-based vaccines are currently undergoing clinical trials [10, 59].

2.4. Small molecule approach in p53-based drug therapy

In comparison to large biological drugs that are present with complex structures, small molecular drugs are organic compounds designed to be extremely low in molecular weight and are made up of well-defined chemical structures that enable them to pass through the cell membrane when taken orally. A further advantage of small molecule drugs over biologics is that they are stable, mostly non-immunogenic and it is easy to characterise their molecular composition and heterogeneity. The mode of action for small molecules relies on their binding to specific biopolymers such as proteins and nucleic acids and act as effectors to alter function or activity of the specific biopolymer.

In cancer, small molecules are used to restore mutated proteins back to their wild-type forms and induce activity of proteins responsible for elimination of tumorigenic cells [30]. In p53-based drug therapy, several small molecules that can restore the function of mutated p53 have been investigated. One example of a small molecule drug known is CP-31398; which has been shown to restore p53 function by intercalating with p53-bound DNA and destabilising the p53-DNA interaction [30]. Another small molecule called Phikan083, which is a derivative of carbazole, has been identified as one of the small molecules that has the ability to restore mutant p53 too. The most advanced of these small molecules are those that act by interrupting the p53-MDM2 interaction which is responsible for the inactivation of wild-type p53 [51, 52, 54]. These include the nutlins, tenovins and the MI-219 [52]. MDM2 acts as a negative regulator of p53 by binding to and inactivating the function of p53. This activity results in the loss of p53-mediated apoptosis in cancer cells, thus promoting carcinogenesis. While the MI-219 small molecular drugs are responsible for the destabilisation of the MDM2-p53 interactions in order to selectively induce apoptosis and inhibit apoptosis, nutlins disrupt the MSM2-p53 complex and selectively induce senescence [51, 52, 54].

2.5. siRNA-based p53 therapy

There are certain cancers with no mutations in p53 but in which non-mutated p53 might be down-regulated by certain p53 negative regulators [30]. In these cancers, development of specific siRNAs for silencing of the negative regulatory genes is often used to activate p53 [10, 30, 55]. MDM2 E3 ligase and the viral E6 protein are two extensively studied negative regulators of p53 that are associated with cancer progression [51, 52, 54].

Under normal cellular conditions, p53 tumour suppressor gene is kept under tight regulation by the MDM2-p53 auto-regulatory feedback loop [36, 51, 52]. In response to stress stimuli such as DNA damage or radiation, activated p53 interacts with genes responsible for the induction of cell cycle arrest or apoptosis (figure 1) [36]. During cancer development, the interaction between p53 and MDM2 mediates p53 interaction with the ring finger domain of the MDM2 ubiquitin ligase for degradation of the p53 tumour suppressor protein [54]. This event compromises the occurrence of cell cycle arrest and p53-mediated apoptosis and facilitates abnormal cell proliferation[52]. The use of MDM2-specific siRNA to disrupt the p53-MDM2 interaction in breast cancer cells has been shown to induce apoptosis, inhibit cell proliferation and lead to decreased tumour size [36, 51, 52, 54].

The E6 viral protein is another thoroughly studied p53 negative regulator in HPV (human papillomavirus)-related cancers such as anogenital, cervical, head and neck cancers. During HPV infection, E6 protein expression increases in order to facilitate HPV replication and viral integration into the host cell. The E6 protein achieves this outcome by using its E3 ligase Hect domain to bind to and degrade the cellular tumour suppressor proteins p53 and pRB, thus abrogating the host cells' potential to initiate cell cycle arrest and apoptosis. Therapeutic strategies to disrupt E6-p53 interactions in the form of antisense and siRNA application specific to E6 viral protein have received the most attention in HPV-related cancer therapeutics [10, 30, 55].

A third ubiquitous protein suspected to be yet another negative regulator of active p53 especially in breast cancer progression is known as retinoblastoma binding protein 6 (RBBP6)
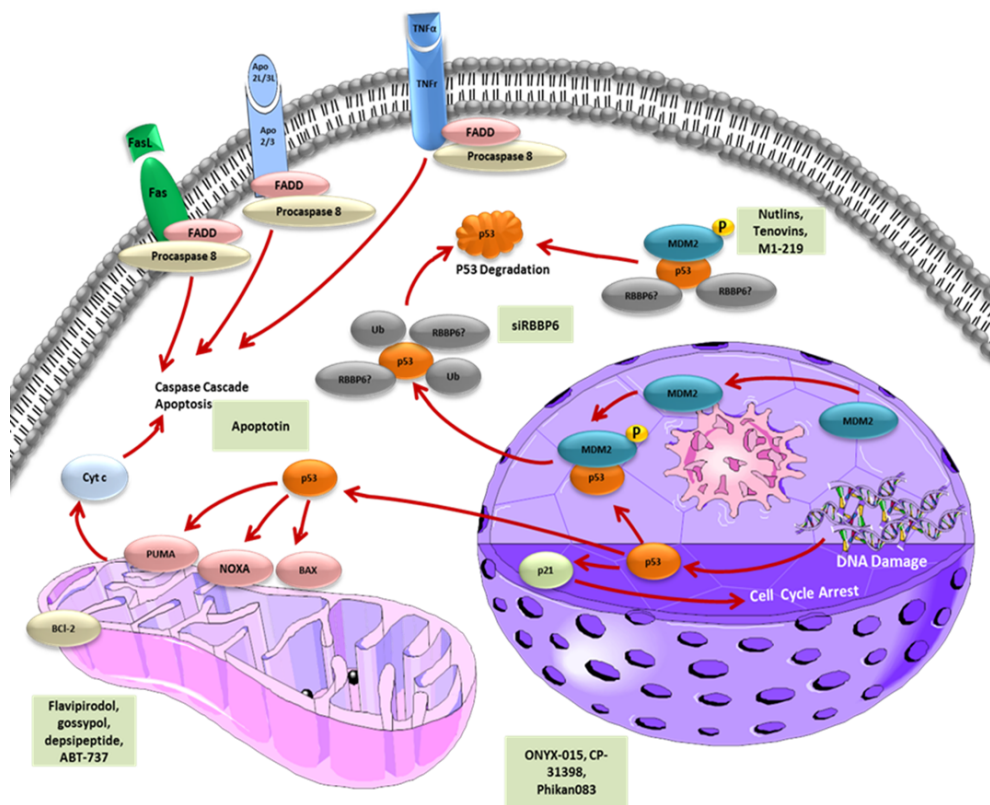
Figure 1. A simplified diagrammatic representation of the apoptotic signalling pathway and p53 negative regulation by MDM2 and another ubiquitous protein (RBBP6) suspected to be involved in p53 degradation. Current drugs that target different points of the apoptotic pathways are highlighted in light-green

[36]. RBBP6 is a 250kDa protein that has been shown to interact with and possibly lead to the degradation of p53 tumour suppressor gene since it possesses an E3 ligase activity [44, 49]. Its mRNA codes for a p53-binding domain as well as other domains known as DWNN domain, zinc finger domain and a ring finger domain, which are responsible for the ubiquitous nature of RBBP6 [44]. Besides the p53 domain, which is only present in human RBBP6, the above-mentioned domains are conserved in about all eukaryotic organisms such as humans, plants, protozoa, fungi, microsporidia and the single-celled parasite *Encephalitozoon cuniculi* [44]. RBBP6 is a spliced-associated protein and therefore exists in different other homologues known as PACT and P2P-R [44, 58].

A critical insight into the role played by RBBP6 in certain cancers via p53 has been elucidated [15]. Transfection of lung cancer cells with siRBBP6 led to a decrease in RBBP6 expression whereas sip53 transfection led to an increase in RBBP6 expression and, according to this study, RBBP6 may be involved in the degradation of p53 thereby enhancing abnormal cell prolifer-

ation [38]. In one study, it was demonstrated that down-regulation of the PACT homologue of RBBP6 in mice induces embryonic lethality with a consequent accumulation of p53 and a widespread apoptosis [33]. In addition to identifying PACT as a negative regulator of p53, further discoveries suggest that PACT knockdown enhances p53-Hdm2 interaction thus reducing p53 poly-ubiquitination by RBBP6 [33].

In recent studies, it was found that silencing RBBP6 gene in MCF-7 and CAMA-1 cells led to p53 up-regulation and sensitised the breast cancer cells to apoptosis induction [36]. Concurrent treatment of these cells with apoptosis-inducing agents, camptothecin or staurosporine, further increased apoptosis induction [36]. Furthermore, up-regulation of bax as a result of the co-treatment provided early insights into the possible mechanism behind the observed apoptosis [36]. Taken together, it is suspected that RBBP6 silencing may be responsible for the identified p53 up-regulation in breast cancer and other cancers and that the observed apoptosis is more likely p53-dependent; however, further *in vivo* investigations would validate these observations.

3. Conclusions

Taken all together, it is evident that anticancer therapeutics primarily depend on apoptosis pathway activation in breast cancer and several other cancers. However, a few milestones still need to be reached with regard to this novel anti-tumour molecular approach. For example, most of the experimentally studied apoptosis-inducing regimens in breast cancer cells have not reached the clinical stages. Another important factor that needs to be addressed in apoptosis-targeted therapy is to determine whether the observed cytotoxicity of breast cancer cells in experimental settings is comparable in clinical settings. Moreover, understanding tumour biology of individual cancer patients can help select therapeutic interventions that are highly specific to a presented tumour. Nonetheless, the link between apoptosis and tumori-genesis has been thoroughly investigated in breast cancer and has led to lots of promising strategies that attempt to eradicate cancer cells by targeting the apoptosis signalling pathway.

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1.7.   Retinoblastoma Binding Protein 6 (RBBP6)

RBBP6 is a 250kDa protein that has been shown to interact with p53 and pRB tumour suppressor genes (Pugh *et al*., 2006; Simons *et al*., 1997; Gao *et al*., 2002; Sakai *et al*, 1995). Also known as RBQ-1 (RB-binding Q-protein), PACT (p53-Associated Cellular protein Testis-derived) or P2P-R (Proliferation Potential protein-Related), the RBBP6 mRNA consists of three main types of domains known as DWNN domain, zinc finger domain and RING finger domain, which are conserved in all eukaryotic organisms including humans, plants, protozoa, fungi, *microsporidia* and the single-celled parasite *Encephalitozoon cuniculi* as shown in Figure 3 (Pugh *et al*., 2006). In higher eukaryotes (human and mouse) and insects (D. *melanogaster* and C. *elegans*) RBBP6 has been found to be larger in size due to the presence of two additional domains known as p53-binding domain and pRB-binding domain located towards the C-terminus of the 1792 amino acid-long peptide (Pugh *et al*., 2006).
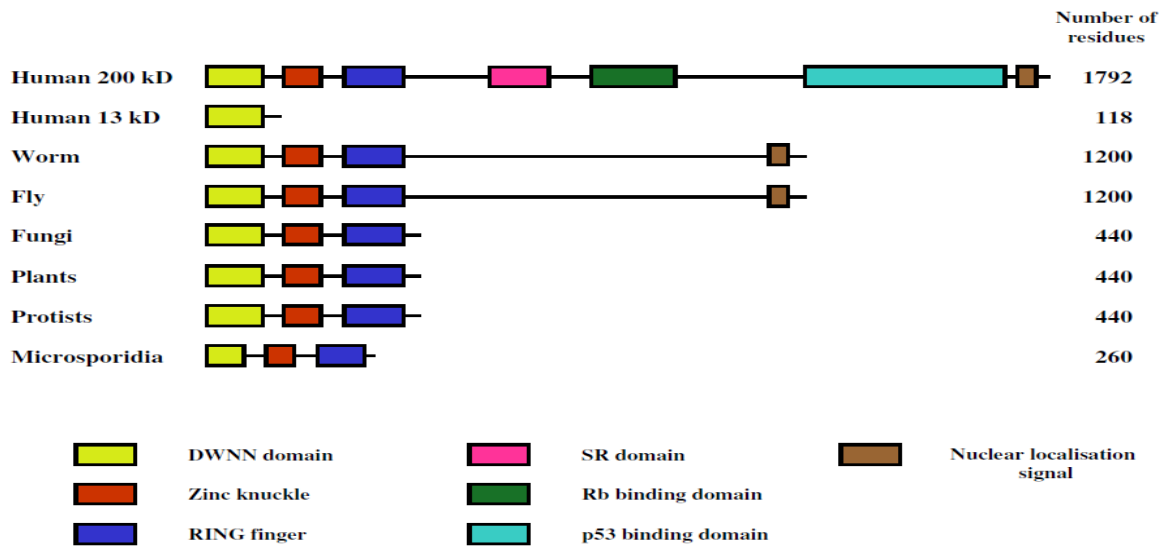
Figure 3: Domain structure of RBBP6 in eukaryotic organisms. DWNN, Zinc knuckle and RING finger domains are found in the complete genome of all eukaryotes whereas the SR domain, p53 domain and the pRB domain are only found in human genome (Pugh *et al*., 2006).

The DWNN (Domain With No Name) is a highly conserved domain located from residue 1-81 on the N-terminus of the RBBP6 protein. It is present in all three RBBP6 isoforms, with isoform 3 consisting entirely of the DWNN domain. The region from 1-81 amino acid residues on RBBP6 was confirmed by recombinant expression studies in *E. coli*, and the heteronuclear NMR technique was used to determine the structure of DWNN domain. Comparison of the DWNN structure to a protein database using a tool called Dali Server confirmed high similarity between DWNN and human ubiquitin domains, a finding that confers DWNN as a domain that has an ubiquitin-like function. A double glycine region at the C-terminus of ubiquitin-like proteins serves as a recognition motif for proteolysis and the presence of this GG-motif (glycine-glycine motif) at the same location in both human and mouse DWNN domain further implicated DWNN as an ubiquitin-like protein (Pugh *et al*., 2006).

RING finger-like domains (or zinc knuckles) are small independently-folded cysteine/histidine-rich motifs of widely diverse structure and function that fold into a secondary structure made up of antiparallel β-sheets and an α-helix joined together by long peptide loops (Pugh *et al*., 2006; Liew *et al*., 2000; Mulaudzi *et al*., 2007; Laity, Lee and Wright, 2001). Independent folding of the RING finger motif is made possible by its ability to interact with two Zn^{2+} ions via cysteine and histidine residues, forming a stabilized zinc finger structure as shown in Figure 4 (Liew *et al*., 2000; Laity *et al.,* 2001). The diverse functions of zinc finger domains as a result of their structural diversity ranges from cell growth, differentiation and ubiquitination (Liew *et al*., 2000; Mulaudzi, 2007).
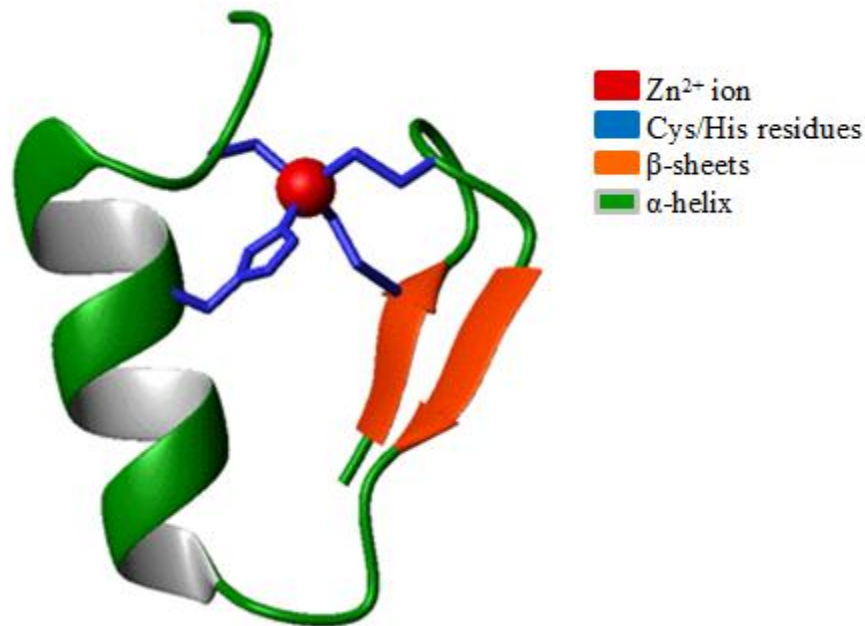
Figure 4: Solution structure of zinc finger-like domain, FOG family protein U-shaped mediate interaction. The structure is made up of antiparallel β-sheets (orange) and α-helices (green and grey), one histidine and three cysteine residues (blue) joined together by long peptide loops (green) (Liew *et al*., 2000).

1.7.1. RBBP6 Homologues

Retinoblastoma binding protein 6 (RBBP6) was isolated by direct screening of human cDNA clones using pRB as a probe (Saijo *et al*., 1995). The 140 kDa RBQ-1 protein lacks 34 amino acids which correspond to a region on exon 11 of the full length RBBP6 gene and this is as a result of alternative splicing. Alternative splicing is a process in which gene expression results in a single gene coding for multiple proteins that will contain differences in their biological functions. RBBP6 has been shown to be a splicing-associated protein and another truncation of the RBBP6 protein was isolated using p53 as a probe by screening a mouse testis expression library, which encodes a 250 kDa protein named PACT (Simons *et al*., 1997). PACT has been shown to interact with p53 using a C' terminal lysine-rich domain and introduction of two mutations in this region led to a loss of this function (Simons *et al*., 1997). Using precipitation

experiments it was also shown that PACT can interact with both cellular p53 and pRB. Another PACT homologue was discovered from a cDNA encoding a P2P-R protein that had 1 exon responsible for encoding 34 amino acids demolished. This 250 kDa protein was isolated using antibodies against P2P-R protein and RB1 precipitation using glutathione-s-transferase. This provided evidence that the P2P-R cDNA encodes a protein domain that binds Rb1 (Witte and Scott, 1997).

The RBBP6 name was approved for the full length gene. Characterisation of the 16p12.2 gene locus on human cDNA suggests that RBBP6 has three major transcripts of 6.1, 6.0, and 1.0 kb in size named isoform 1, 2, and 3, respectively, as a result of alternative splicing. They appear in the GeneBank under the accession numbers NP_008841, NP_061173 and NP_116015, respectively. RBBP6 has since been known as a 250 kDa protein that interacts with both pRB and p53 tumour suppressor genes in human and mouse (Pugh *et al*., 2006).

1.7.2. RBBP6 Expression in Cancer

Complementary DNA microarray analysis showed that RBBP6 is highly expressed in oesophageal cancer cells than in normal cells where it has a role in promoting cell proliferation of oesophageal cancer cells (Yoshitake *et al*., 2004). A critical insight into the level of expression of RBBP6 in colon cancer has been elucidated by Chen *et al*. (2013). The study found that RBBP6 was highly expressed in colon tumorous tissues coupled with accumulation of mutant TP53. Chen *et al*. (2013) further demonstrated that RBBP6 overexpression alone or in combination with mutant TP53 accumulation leads to recurrent cancer and poor survival rate. Similarly, in a study performed by Dlamini *et al*. (2005) it has been shown that RBBP6

expression is high in squamous tumours that are well-differentiated and that RBBP6 expression is directly proportional to proliferation. They also demonstrated the occurrence of apoptosis around islands of such tumours, stipulating it to be involved in fighting invading tumours as there was no visible apoptosis in tissues with little or no RBBP6 expression (Dlamini *et al*., 2005). Similarly, expressional experiments performed by Motadi *et al*. (2011) showed that RBBP6 mRNA and its protein products were significantly expressed in subtypes of lung cancer.

Transfection of lung cancer cells with siRBBP6 led to a decrease in RBBP6 expression whereas sip53 transfection led to an increase in RBBP6 expression. This suggests that RBBP6 may be involved in the degradation of p53 thereby enhancing cell proliferation (Motadi *et al*., 2011). In a study performed by Li *et al*. (2007) it was demonstrated that down-regulation of the PACT homologue of RBBP6 in mice induces embryonic lethality with a consequent accumulation of p53 and a widespread apoptosis. In addition to showing that PACT might be a negative regulator of p53, findings by Li *et al*. (2007) further suggest that PACT-knockdown enhances p53-Hdm2 interaction thus reducing p53 poly-ubiquitination by RBBP6. In a recent study we further demonstrated using breast cancer cell line models (MCF-7 and CAMA-1) that indeed down-regulation of RBBP6 using RNA interference leads to p53 accumulation and a notable enhancement of apoptosis (Moela *et al*., 2014). We also demonstrated that further down-regulation of RBBP6 was observed following co-treatment with camptothecin; however the study still warrants further investigation into the mechanism responsible for the further down-regulation of RBBP6 as a result of camptothecin. Furthermore, up-regulation of bax as a result of the co-treatment provided early insights into the possible mechanism of the observed apoptosis, (Moela *et al*., 2014). Conversely, data presented by Gao and Scott (2003) indicates that

overexpression of P2P-R, another homologue of RBBP6, promotes camptothecin-induced cell death as well as cell cycle arrest in MCF-7 cell line. These findings (Li *et al*., 2007; Moela *et al*., 2014; Gao and Scott, 2003) may appear contradictory. However taken altogether they suggest that RBBP6 is involved in p53 regulation and apoptosis, and may therefore serve as an important marker in cancer therapy development.

1.7.3. RBBP6 Role in p53 Regulation

Ubiquitin-like DWNN domain and RING finger-like domain presence give RBBP6 the ability to function through some ubiquitin-like modification. It is thus associated with the execution of p53 degradation via its p53-binding domain following inhibition of the tumour suppressor gene by MDM2, thus facilitating cell proliferation. Little is known however, about the mechanism by which RBBP6 degrade the p53 tumour suppressor protein (Li *et al*., 2007; Ntwasa, 2008; Pretorius *et al*., 2013). The first human cDNA clones of the p53 tumour suppressor gene which codes for a protein of 393 amino acid were first isolated in the early 1980's and it was named so because it elicits a band size of 53kDa in agarose gel electrophoresis (Nag *et al.*, 2013). P53 is a tumour suppressor gene responsible for the regulation of cell cycle arrest, DNA repair and apoptosis and it is highly mutated in several cancers thus leading to deregulation of the cell cycle and apoptosis impairment (Ghobrial *et al*., 2005).

Following nuclear stress such as radiation, p53 activates p21 gene which is responsible for the induction of cell cycle arrest during which damaged DNA is allowed to undergo repair. However, if damage is irreversible, p53 activates pro-apoptotic Bcl-2 protein members in order to induce apoptosis although the mechanism by which p53 induces apoptosis is not well

understood. Oncogenes and tumour suppressor genes maintain the balance between cell growth and cell death and p53 is one of the well-studied tumour suppressor genes that plays a role in the development and homeostasis of cells and tissues (Jiang *et al*, 2013). In addition to its downstream effectors such as cyclin dependent kinase inhibitor p21, pro-apoptotic bax and tumour necrosis factor receptors (Fas and/or Apo1), p53 transcriptionally activates its negative regulator, MDM2, in an auto-regulatory feedback loop (Jiang *et al*., 2013; deRoziere *et al*., 2000).

MDM2 was established as an oncogene in a study which demonstrated that overexpression of the MDM2 gene rendered rodent fibroblasts tumorigenic in nude mice (Mendez *et al*, 2009). The MDM2 gene codes for a 491 amino acid protein and it contains two transcriptional promoter elements, one of which is p53-dependent (Mendez *et al*, 2009). Also known as a family member of the E3 ubiquitin ligases, MDM2 directly interacts with p53 to mediate its ubiquitination (Jiang *et al*, 2013). Following its expression, MDM2 translocates from the nucleus to the cytoplasm where it mediates the degradation of p53 and other targets (Mendez *et al*, 2009). In most cancers, the mutated p53 or the function of wild-type p53 is inhibited by the MDM2 oncogene resulting in the impairment of tumour suppressive p53-pathways such as apoptosis and cell cycle arrest (Nag *et al*, 2013).

1.8.   Rationale/Hypothesis

Previous RBBP6 expressional studies have shown that RBBP6 localization is widespread in a number of squamous cell carcinomas including those of the lung, colon and oesophagus; suggesting that the protein may serve as an important diagnostic marker and a promising target

for treatment of squamous cell carcinomas. Limited evidence however exists on the involvement of RBBP6 in the progression of breast and cervical cancer. Cancer is an enormous burden of a disease globally and today more people die from cancer than HIV/AIDS, tuberculosis and malaria combined. Currently, cervical cancer is the most common cancer; accounting for over 60% of the gynaecological cancer burden in developing countries (Sankaranarayanan *et al.,* 2009) and breast cancer remains the leading cause of cancer related deaths in women. RBBP6 has been shown to be highly expressed in several cancers, and its ability to interact with p53 tumour suppressor has drawn attention in evaluating its potential as a cancer biomarker. P53 is a crucial regulatory protein that triggers cellular responses such as DNA repair, cell cycle arrest, senescence and apoptosis in response to cellular stress. In most human cancers, however, the TP53 pathway is often found to be defective, either by mutations or through deregulation by its negative regulators. In cervical cancer and certain breast cancer subtypes, p53 is found to be inactivated rather than mutated and RBBP6 is suspected to be one of such negative regulators of p53.

In terms of cervical cancer, extensive research of the main causative agent of cervical cancer has evidently singled out HPV as the main cause (Fernandes *et al.*, 2013; Denny, 2010; Bosch *et al.*, 2002). During the differentiation-dependant phase, the E6 viral protein regulates the viral replication in differentiated cells by supressing transcription of active p53 cellular protein thus abrogating the host cells' ability to initiate cell cycle arrest, prompting uncontrolled cell proliferation. It is suspected that the viral protein achieves this p53 suppression via the proteasomal pathway in which it acts as an E3 ligase that functions to ubiquitinate protein molecules for proteasomal degradation (Fernandes *et al.*, 2013; Denny, 2010; Bosch *et al.*,

2002). This mechanism is similar to the one involving RBBP6 in which it interacts with TP53 via its p53-binding domain. Research has shown that RBBP6 plays a role as a scaffold protein to promote the assembly of the p53/TP53-MDM2 complex, resulting in increased MDM2-mediated ubiquitination and degradation of p53/TP53. Therefore, this suggests that RBBP6 may function as a negative regulator of p53/TP3, thus leading to both apoptosis and cell growth (Pugh *et al*., 2006; Motadi *et al*., 2011).

Therefore in this study we propose that RBBP6 mediates TP53 inactivation in the same manner as MDM2 in breast cancer and HPV E6 protein in cervical cancer since these proteins possess the activity of E3 ligases and have also been shown to interact with TP53. Our hypothesis is that RBBP6 expression may be high in cervical cancer as is in breast cancer. In addition, that further up-regulation of this gene in both cancers would lead to poor prognosis whereas down-regulation might slow down breast and cervical cancer progression. We have therefore asked the following question: what is the expression profile of RBBP6 in cervical cancer and how would manipulation of this gene affect cervical as well as breast cancer progression? We therefore aim to manipulate the expression of RBBP6 in breast and cervical cancer cell lines and co-treat with chemotherapeutic agents in order to sensitise apoptosis induction.

CHAPTER TWO - Research Aim and Objectives

2.1. Aim

The main aim of this study was to manipulate RBBP6 gene expression and evaluate its effects in cervical and breast cancer progression. Targeted therapy works best in combination with chemotherapy which is why we further aim to analyse the effect of RBBP6 targeting in combination with anticancer agents camptothecin and γ-Aminobutyric acid (GABA) on cancer progression. We also aim to understand the relationship between RBBP6, p53 and p53-mediated apoptotic genes and possibly outline their mechanism of action relative to apoptosis at a transcriptional level. Understanding these relationships may confer RBBP6 as an important diagnostic marker of cervical and breast cancer.

2.2. Objectives

- To localize RBBP6 in cervical cancer tissue microarray slides using immunohistochemistry in order to understand RBBP6 distribution in cervical cancer in the context of intact tissue.

- To overexpress and silence RBBP6 gene in adherent breast and cervical cancer cell lines (HeLa, SiHa, MCF-7 and MDA-MB-231) using an expression vector and siRNA technology, respectively, and quantify the level of RBBP6 expression using qPCR, immunoprecipitation/western blotting, FACS analysis and confocal microscopy

- To analyse the effect of RBBP6 targeting (overexpression and silencing) on cell proliferation using xCELLigence real time cell analyser (RTCA)

- To analyse the effect of RBBP6 targeting (overexpression and silencing) and co-treatment on apoptosis by measuring plasma membrane integrity, mitochondrial ATP

content and caspase activity and on cell cycle by quantifying DNA content in the cell cycle checkpoints (G0/G1 and G2/M) and the S-phase

- To quantify the expression of p53, bax, bak1, bad, bcl-2, caspase-3 and caspase-8 apoptotic genes in response to RBBP6 gene manipulation and co-treatment using quantitative PCR.

- To analyse the effect of RBBP6 overexpression and silencing on the expression of p53 at a protein level using western blotting

CHAPTER THREE - Materials and Methods

3.1.   Materials

3.1.1. Tissue Sections

Formalin-fixed paraffin-embedded human cervical cancer tissue microarray slides (TMAs) were purchased from Cybrdi (USA) with ethics clearance number M140801. The TMAs consisted of 20 cases of both squamous cell carcinoma and adenocarcinoma that were diagnosed between stages I to III in Caucasian and African-American women aged between 25 and 51.

3.1.2. Cell lines

Breast and cervical cancer cell lines as well as a non-tumorigenic cell line were used in this study as a source of mRNA and protein, and for studying growth and morphological changes after co-treatment. The MCF-7 breast cancer cell line was used as a wild-type p53-expressing cell line and the highly metastatic MDA-MB-231 as a mutant p53-expressing cell line. The adenocarcinoma-derived HeLa and the squamous cell carcinoma-derived SiHa cervical cancer cell lines, both of which express wild-type p53 were used as cell line models for cervical cancer studies. MRC-5 lung fibroblasts were treated as normal cells and all cell lines were obtained from ATCC (USA).

3.1.3. Primers and Antibodies

Pre-designed and validated IDT PrimeTime® qPCR primers for rbbp6, TP53, bax, bak1, bad, bcl-2, caspase-3 and caspase-8 were purchased from Whitehead Scientific (Pty) Ltd. GFP (green fluorescent protein) tagged mouse (IgG2a) monoclonal primary antibody and goat anti-mouse IgG (H+L) secondary antibody with alexa fluor® 488 conjugate were obtained from Life Technologies™. Mouse monoclonal primary antibody against the full length RBBP6 was

purchased from Cell Signalling Technology®. Anti-mouse secondary antibody with HRP-conjugate and p53 mouse monoclonal primary antibody were from Santa Cruz Biotechnology®, Inc.

3.1.4. RNAi Oligos

Silencing was achieved by the use of Ambion's *Silencer*® Select Pre-designed siRNAs supplied by Life Technologies™, which target the full length RBBP6. Delivery of the Ambion's *Silencer*® Select Pre-designed short interfering RNAs into the mammalian adherent cells was achieved by the use of the lipid-based siPORT™ *NeoFX*™ transfection agent also manufactured by Ambion®.

3.1.5. Expression Vector

TrueORF™ pCMV6-AC-GFP (cytomegalovirus 6) cDNA clone containing open reading frame of RBBP6 transcript variant 3 (NM_032626.5) sequence was purchased from Origene Co. for transient transfection of cell lines. The pCMV6 expression vector contained ampicillin as an antibiotic selection marker and was tagged to green fluorescent protein (GFP) tag at the carboxy-terminus which allowed positive identification of RBBP6-overexpressing cells (Figure 5). Transfection of cells with the RBBP6 construct was obtained using Lipofectamine® 3000 transfection reagent purchased from Life Technologies™.
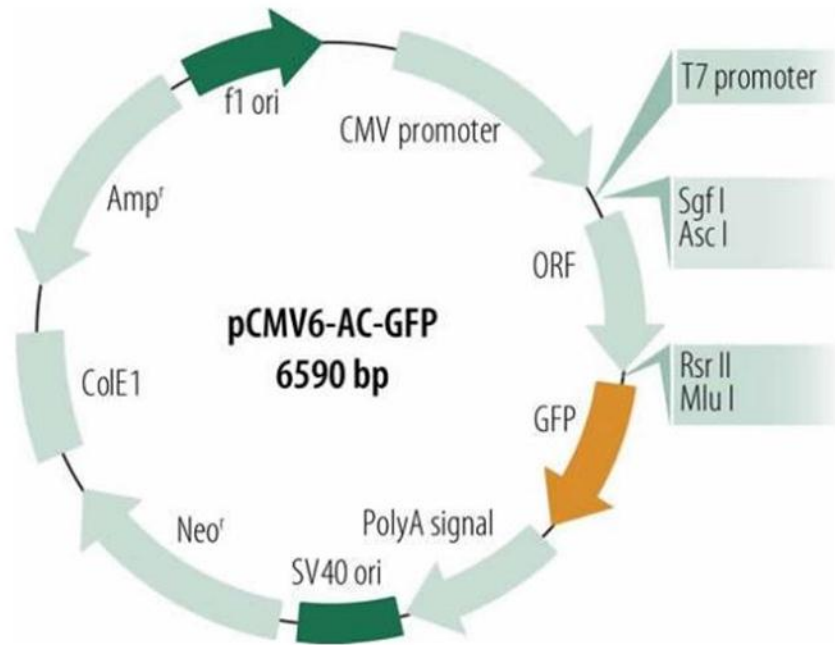
Figure 5: An example of a typical vector map for pCMV6-AC-GFP with antibiotic selection markers Ampr (confers selection in *E. coli*) and Neor (confers selection in mammalian cells), origins of replication SV40 ori (mammalian), ColE1 (bacterial) and f1 ori (filamentous phage), CMV promoter region, ORF insert region, restriction enzyme sites (Sgf I, Asc I, Rsr II, and Mlu I) and the GFP (green fluorescent protein) tag region.

3.1.6. γ-Aminobutyric acid (GABA)

Chosen as one of the anticancer agents in this study, GABA (Sigma®) is known as a major inhibitory neurotransmitter in adult central nervous system (CNS) which acts on $GABA_{A/C}$ ionotropic and $GABA_B$ metabotropic receptors. Binding to $GABA_{A/C}$ receptors leads to an increase in chloride ion conductance thus restoring the cell resting membrane potential. As an inhibitory neurotransmitter GABA is therefore used clinically as a dietary supplement to reduce anxiety and enhance sleep. As an anticancer agent, it is suspected to be involved in modulation of proliferation, differentiation and migration of cancer cells as shown by a recent study (Chen *et*

al., 2013) where migration of human liver cancer cells was reduced in the presence of GABA even though its mechanism of action is not yet fully discovered.

3.1.7. Camptothecin

Camptothecin (Calbiochem®) is a plant extract isolated from *Camptotheca acuminata* tree which is originally cultivated in most regions of China where it is used for anticancer treatment in Chinese traditional medicine. It is classified as a quinoline alkaloid that exerts its cytotoxic effects by the induction of apoptosis. Camptothecin binds to DNA topoisomerase I complex to form a ternary structure that stabilizes the complex to block religation of DNA thus causing DNA strand breaks that result in apoptosis (Nieves-Neira and Pommier 1999). Clinical trials on this apoptosis-inducing agent revealed low solubility and adverse drug reactions which led to the synthesis of more soluble and beneficial camptothecin derivatives which demonstrate good activity against tumours of the lung, breast, colorectal and ovary (Nieves-Neira and Pommier 1999). Examples are *irinotecan hydrochloride* and *topotecan* which have been clinically approved by the FDA and are clinically used in anticancer therapy (Arakawa *et al.*, 2009).

3.2. Methods

3.2.1. Immunohistochemistry (IHC)

Principle

Immunohistochemistry is a technique used to demonstrate distribution and location of proteins in tissue sections and although less sensitive quantitatively compared to western blot and ELISA it enables observation of processes in the context of intact tissue (Taylor, 2014; Ramos-Vara, 2011). Protein of interest is detected by the use of specific antibodies that recognize the target protein inside the tissue section and visualisation is accomplished by the use of chromogenic

enzymes conjugated on the secondary antibody to cleave the substrate and create a coloured precipitation at the location of the protein (Taylor, 2014; Ramos-Vara, 2011). Alternatively, fluorescent dyes are used to enable detection of target protein in which a fluorophore conjugated to a secondary antibody can be visualised using fluorescent microscopy (Valli *et al*., 2009).

Sample preparation for IHC is very important for the maintenance of cell morphology, tissue architecture and the antigenicity of target epitope. To obtain this one needs proper tissue collection, fixation and sectioning. Fixation helps prevent the excised tissue from autolysis, necrosis, protects antigenicity and helps increase resistance of cellular components from tissue processing. Paraformaldehyde is a commonly used fixative however different antigens prefer different fixation methods and for this reason a wide variety of fixatives exists for a particular type of antigen. A robust and optimized fixation protocol is therefore a critical step in IHC. This is then followed by tissue imbedding in paraffin medium or by freezing in order to preserve morphology and give the tissue support during microtomy, a process used to section tissues (Ramos-Vara, 2011).

This is then followed by tissue sectioning using microtome or cryostat instrument, mounting of tissue slices onto a slide, dehydration using alcohol washes of increasing concentrations, followed by clearing of dehydrating agents using xylene. Fixation can lead to cross-linking which masks epitopes and can lead to restricted antigen-antibody binding and this makes it necessary to include additional step called antigen retrieval that involves pre-treating the sections with heat or protease. The proteolytic-induced epitope retrieval (PIER) method makes use of enzymes such as pepsin, trypsin, or proteinase k to restore binding of the antibody to its epitope

and the heat-induced epitope retrieval (HIER) method uses heat from a variety of sources such as water bath, microwave, autoclave, pressure cooker or steamer to unmask epitopes.

For cytoplasmic target of epitopes, permeabilization will be necessary following antigen retrieval and solvents like methanol and acetone or detergents like NP-40, triton or tween-20 are commonly used. Before immunostaining, blocking with sera is essential to block unspecific absorbance to tissue. Factors commonly blocked include protein-protein unspecific binding, biotin unspecific binding (if using biotinylated secondary antibody), peroxidase if using HRP-linked secondary antibody, alkaline phosphatase blocking if using AP chromogenic substrate, or autofluorescence blocking if using fluorescent label for detection.

Technique

Paraffin-embedded formalin-fixed cervical cancer TMAs were firstly heated in an oven at 65°C for 1 hour followed by deparaffinization and rehydration steps in which slides underwent a series of washes with xylene twice for 5 minutes each. This was then followed by ethanol rinses of decreasing concentrations for 5 minutes each, twice per concentration of 100%, 95%, 70%, 50%, 30%, then 5 minutes washes with H_2O and wash buffer on a shaker. Antigen retrieval was done at 80°C for 5 minutes at low microwave setting in 0.1M sodium citrate, pH 6.0. The sections were then allowed to cool to room temperature for about 20 minutes followed by a 2 minutes wash with wash buffer containing 1% BSA (bovine serum albumin). Hydrogen peroxidase was then inactivated using 3% hydrogen peroxide at room temperature followed by 2 minutes washing with TBST containing 1% BSA. Primary antibody was diluted in 1% BSA per recommendation on data sheet followed by application of diluted primary antibody to the tissue

sections and an overnight incubation in a humidified chamber at 4˚C. This was then followed by 3 minute washes with TBST three times on a shaker, then incubation with secondary antibody for 1 hour at room temperature. The tissue sections were then incubated with freshly prepared substrate according to manufacturer's protocol (ABC Staining System®, Santa Cruz Biotechnology) followed by coverslip mounting of slides with xylene-based medium and allowed to completely dry before visualization and imaging with light microscope.

3.2.2. Cell culture routine

Human monolayer normal and cancer cell lines were grown in HyClone® DMEM/High Glucose growth medium supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic (penicillin/streptomycin) and routinely maintained at 37°C in a 5% CO_2 incubator. The cells were fed every second day of the week by replacing old growth medium with equal amount of fresh growth medium after rinsing twice with 2ml PBS. The cells were sub-cultured when at near-confluency by trypsinization at least once per week and excess sub-cultures were preserved at $\geq$ -80°C in DMEM/High Glucose growth medium supplemented with 10% FBS and 20% DMSO (dimethyl sulfoxide).

3.2.3. RBBP6 Protein Overexpression

Principle

Most biopharmaceutical drugs, vaccines and clinical reagents are based on recombinant protein overexpression for use in diagnoses, prevention and therapeutics. The first recombinant protein, insulin, was approved by the FDA in the early 1980s and ever since then hundreds of recombinant therapeutics has been FDA-approved (Kamionka, 2011). The process of recombinant protein production in mammalian cells depends on the delivery of a cDNA clone into the host cell nucleus using a liposomal transfection reagent, where it becomes transcribed

and translocated into the cytoplasm for translation into r-protein of interest (Aricescu *et al*., 2006; Condreay *et al*., 1999). In the present section we overexpressed RBBP6 protein by transient transfection (Tom *et al*., 2008) using the expression vector described in section 3.1.5 above.

Technique

Briefly, fifty thousand $(5X10^4)$ cells were plated in a 6-well plate 18-24 hours before transfection to obtain 50-70% confluency. A mixture of cDNA clone/transfection reagent in a 1:3ratio was then prepared and incubated at room temperature for 10 minutes to allow DNA uptake by the transfection reagent. The cDNA clone/transfection reagent mixture was then added drop-wise into each well that already contained 500 µl of culture medium followed by gentle back-and-forth side-to-side rocking of the 6-well plate in order to achieve an even distribution of the transfection mixture before incubating for 18-24 hours at 37˚C.

3.2.4. RNA interference (RNAi)

Principle

RNA interference (RNAi), also called post-transcriptional gene silencing (PTGS), is a eukaryotic regulatory mechanism that plays an important role in directing development and suppressing parasitic gene expression. It uses small interfering RNA (siRNA) molecules to degrade specific mRNA and silence gene activity (Aagaard and Rossi, 2007). The RNAi pathway is initiated by cleavage of long double-stranded RNA by DICER enzyme into short interfering double-stranded RNA molecules of about 20 nucleotides in length.

These short interfering RNA molecules have a characteristic 5'phosphate and a two nucleotide 3'overhang which allows them to be recognised by the RNAi machinery that eventually leads to degradation of target mRNA (Meister and Tuschl, 2004). The short double-stranded siRNA separates into two single-stranded RNA (ssRNA) named the guide strand and the passenger strand. The passenger strand becomes degraded and the guide strand associates with the RNA-induced silencing complex (RISC) where it specifically base pairs with complementary target mRNA. This induces catalytic cleavage of the target gene by a component of the RISC complex known as Argonaute enzyme (Aagaard and Rossi, 2007 and Meister and Tuschl, 2004).

Introduction of artificial siRNAs has been adopted as a tool to target and inactivate gene expression both *in-vitro* and *in-vivo*. Most human diseases such as cancer, viral infections, autoimmune diseases, and genetic disorders are associated with alterations in one or more genes and therefore this makes the RNAi intervention an attractive strategy for gene-related therapeutics (Aagaard and Rossi, 2007). In this study we used RNAi to silence RBBP6 in breast and cervical cancer cell lines.

Technique

Briefly, 70-80% confluent cells were transfected with 30nM siRNAs targeting RBBP6 over a 24 hour period and the adherent cells were trypsinized and resuspended in an antibiotic-free media. The cell suspension was then mixed with siRNA/transfection agent complex in a 24-well plate and incubated at 37°C for 24 hours. Post-transfection cells were exposed to 100μM GABA and 0.25μM camptothecin for additional 24 and 48 hours and then harvested for subsequent analysis.

3.2.5. RNA Extraction

Principle

RNA is used as a starting point for downstream processes such as reverse transcription real-time PCR (polymerase chain reaction), conventional PCR, array analysis and cDNA library constructions (Valasek and Repa, 2005; Ginzinger, 2002). However this single-stranded RNA is highly unstable with a very short half-life once extracted from cells or tissues. It is the ubiquitous presence of RNAses on all tissues (especially on skin, in blood, and in most bacteria and fungi in the environment) that make RNA easily degradable. High quality total RNA is therefore an absolute prerequisite in the performance of molecular techniques (Huggett *et al*., 2013; Tan and Yiap, 2009). Most RNA isolation procedures make use of RNAse inhibitory agents such as chaotropic ions (e.g. guanidine salt), sodium dodecylsulfate (SDS) denaturant or phenol-based compounds. The commercial RNA isolation kit used in this study relies on guanidine chaotropic salt.

Technique

Cells were transfected with siRBBP6 (short interfering RBBP6) and pRBBP6 (RBBP6 cDNA construct) construct for 48 hours and co-treated with camptothecin and GABA for additional 24 hours before RNA isolation. The general purification steps which were used in this study following co-treatments of nearly confluent cultured cells with Nucleospin® RNA II total RNA isolation kit (Separations Scientific) include: effective disruption of cells → denaturation of nucleoprotein complexes → inactivation of DNAses → washing steps for the removal of salts, metabolites and macromolecular cellular components → elution of pure RNA. The resulting RNA was quantified using the nanodrop technique and RNA purity was confirmed by ensuring

that A260/A280 ratio was >1.7 and the RNA integrity was confirmed by examination of the 18S and 28S RNA bands on agarose gel stained with ethidium bromide.

3.2.6. Reverse Transcription

Principle

In order to perform RT-PCR, the obtained RNA template must first be converted into a cDNA by the use of reverse transcriptase enzyme. In this study we performed reverse transcription using the ImProm-II™ Reverse Transcription System commercial kit manufactured by Promega.

Technique

Cells were transfected with siRNAs and pRBBP6 construct for 48 hours and co-treated with camptothecin and GABA for additional 24 hours before RNA isolation. Sequence specific primers together with the obtained total RNA template formed major components of the PCR cocktail used in the synthesis of cDNA. The RNA reverse transcription was performed according to the manufacture's detailed protocol using the Multigene Gradient Thermal Cycler.

3.2.7. Real Time RT-PCR

Principle

Quantitative PCR was performed to check whether the transfection of cells was successful and to study gene expression changes following co-treatment of cells (Huggett *et al*., 2013; Tan and Yiap, 2009). The total mRNA transcribed into complementary cDNA was amplified and quantified a thermal light cycler (Roche® Light Cycler 480). Quantitative PCR differs from conventional PCR in that instead of detecting and quantifying amplified DNA at the end of the reaction, the amount of PCR product is measured at each cycle in real time (Valasek and Repa, 2005; Ginzinger, 2002). Measurement of the amount of DNA at each cycle is accomplished by

the use of fluorescent dyes capable of intercalating each of the newly synthesized double-stranded DNA to produce a signal that's directly proportional to the amount of PCR product. Commonly used fluorescent dye or probes include SYBR Green, TaqMan, Molecular Beacons, Scorpions, etc. and in our study we chose to use SYBR Green fluorescent dye due to its advantages, i.e. it is inexpensive, easy to use and highly sensitive (Valasek and Repa, 2005; Ginzinger, 2002).

Technique

SYBR® Green JumpStart Taq ReadyMix (SIGMA®) dye was used to quantify the amount of gene expression from cDNA synthesized from total RNA that was isolated from cultured cells exposed to 48 hour transfection with 30nM siRBBP6 and 1μg pRBBP6 and co-treatment with GABA and camptothecin. The qPCR was performed in a 20μl reaction mixture containing 2100ng/ul cDNA, SYBR Green, forward and reverse primers under the following conditions: 36 cycles of 94°C for 35s, 59°C for 45s, and 72°C for 45s.

3.2.8. Western Blot and Immunoprecipitation

Western Blotting

Principle

Western blot analysis continues to be a routine technique in most laboratories to determine the level of expression of proteins. This protein analysis technique was introduced by Towbin *et al*. (1979). The term blotting refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane (Harper *et al*., 1990). The first step in a western blotting procedure is to separate the macromolecules using gel electrophoresis. Following electrophoresis, the separated molecules are transferred or blotted onto a second

matrix, generally a nitrocellulose or polyvinylidene fluoride (PVDF) membrane (Osborne and Brooks, 2006; Kurien and Scofield, 2006). Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. The transferred protein is complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic or fluorogenic precipitate on the membrane for colorimetric or fluorometric detection, respectively.

The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a by-product (Alegria-Schaffer *et al*., 2009). The light output can be captured using film, a CCD camera or a phosphor-imager that is designed for chemiluminescent detection (Haan and Behrmann, 2007). Whatever substrate is used, the intensity of the signal should correlate with the abundance of the antigen on the blotting membrane. Detailed procedures of a western blot vary widely. One common variation involves direct vs. indirect detection. With the direct detection method, the primary antibody that is used to detect an antigen on the blot is also labeled with an enzyme or a fluorescent dye. This detection method is not widely used as most researchers prefer the indirect detection method. In the indirect method, a primary antibody is added first to bind to the antigen. This is followed by a labeled secondary antibody that is directed against the primary antibody (Osborne and Brooks, 2006; Kurien and Scofield, 2006). Labels include biotin, fluorescent probes such as fluorescein or rhodamine, and enzyme conjugates such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) (Scofield, 2006). In this study, the indirect method was used since it offers more advantages over the direct method, i.e. it has increased sensitivity, a wide variety of labeled

secondary antibodies are available commercially and immunoreactivity of the primary antibody is not affected by labeling.

Technique

Whole cell protein was extracted using RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 10% sodium SDS, and 3 μl/ml aprotinin and 5μg/ml leupeptin in PBS, pH 7.4). Seventy two hours post-transfection and co-treatment with either GABA or camptothecin, cells were washed twice with cold PBS then resuspended in 500μl RIPA buffer and collected by scraping. The total protein was then separated from cell debris by centrifugation at 14000 rpm for 15 minutes followed by quantification with Pierce® BCA Protein Assay Kit. The protein was heated at 95°C for 5 minutes and 30μg was loaded per well for electrophoretic separation in 30% acrylamide/bis (37.5:1) gel preparation at 100V for 1hour. The protein was transferred onto a nitrocellulose membrane using wet electro-transfer method overnight at 30V followed by incubation with primary antibody after 1 hour of blocking with 5% non-fat milk buffer. The weak light signal produced by HRP-linked secondary antibody was detected and enhanced using the Pierce® ECL Western Blotting Chemiluminescence Substrate and the blots were imaged by the CCD-based ChemiDoc™ MP system.

Immunoprecipitation

Principle

Immunoprecipitation is a procedure that results in the enrichment of a specific protein from a heterogeneous mixture, cell lysate or culture supernatant (Chan *et al*., 2015). This enrichment is accomplished by binding the protein of interest with a specific antibody, followed by precipitation of the immune complexes with Protein G or Protein A immobilized onto beads such

as agarose (Moser *et al*, 2009; Lal *et al*, 2005). The precipitated immune complexes are then denatured and resolved by SDS-PAGE (polyacrylamide gel electrophoresis) for further analysis. Immunoprecipitation can be used to confirm the identity of a protein, to quantify expression levels, to study the biochemical characteristics such as protein:protein interactions, or to evaluate posttranslational modifications (Chan *et al.*, 2015; Moser *et al.*, 2009; Lal *et al.*, 2005). In this study immunoprecipitation was used to purify our protein of interest which was overexpressed in the cells by the use of RBBP6-containing construct labelled with GFP reporter gene. Cells transfected with the pRBBP6 construct were lysed and the cell lysates were precipitated with anti-GFP monoclonal antibody and the antibody-antigen complex was extracted from the sample using protein G-coupled agarose beads before western blot analysis as described above.

Technique

Following 48 hour transfection of cells with the pRBBP6 construct, culture medium was removed and cells were washed with ice-cold PBS followed by harvesting of approximately 5×10^6 cells by scrapping; which were then centrifuged at 400xg for 10 minutes at 4°C. After the second wash, the supernatant was completely removed and pellet resuspend in 1mL of ice-cold RIPA buffer containing protease and phosphatase inhibitors. The cell lysate was then gently vortexed and then transferred to a fresh 1.5 mL tube and placed the tube on ice for 30 minutes with occasional mixing. Another centrifugation of the cell lysate followed at 10,000xg for 15 to 30 minutes at 4°C then supernatant was carefully collected without disturbing the pellet and transferred to a clean tube. The protein concentration was determined by BCA assay. To pre-clear the lysate, immobilized protein G bead slurry was resuspended by gentle vortexing and 10 µl of the prepared protein G slurry was added to 100 µL of cell lysate (~1×10^6 cells or ~100 µg

protein) then incubated on a rotator for 30 to 60 minutes at 4°C. The mixture was centrifuged at 2,500xg for 2 to 3 minutes at 4°C and the supernatant was transferred to a fresh 1.5 mL tube. An amount of 1μg GFP antibody was added to the pre-cleared cell lysate then incubated at 4°C overnight on a rotator followed by addition of at least 10 μL of protein G slurry pre-equilibrated in the corresponding IP (immunoprecipitation) buffer/Lysis buffer then incubated overnight at 4°C on a rotator to capture the immune complexes. The tubes were then centrifuged at 2,500xg for 30 seconds at 4°C followed by careful removal of the supernatant. The beads were then washed three to five times with 500 μl of ice-cold lysis buffer then centrifuged to pellet the beads in between each wash. After the last wash, supernatant was carefully aspirated and 1X Laemmli sample buffer was added to the bead pellet followed by vortexing for 10 minutes and centrifugation at 10,000xg for 5 minutes to pellet the beads. Supernatant was then collected carefully for subsequent loading onto an SDS-PAGE gel which preceded western blot analysis.

3.2.9. xCELLigence System

Principle

Conventional methods used to assess cytotoxicity of certain compounds administered in cultured cells include the use of tetrazolium salts such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), which reduces into insoluble purple formazan crystals by NAD(P)H-dependent enzymes present in viable cells (Jurisic and Bumbasirevic, 2008; Larson and Worzella, 2005). Other related tetrazolium salts include XTT, MTS and the WSTs (water-soluble tetrazolium salts). To date, tetrazolium salts have since been the most widely used assays in cell biology for measuring cell viability, proliferation and cytotoxicity, and for this reason they serve as the gold standard for comparing modern techniques that provide similar outputs (Jurisic and Bumbasirevic, 2008; Larson and Worzella, 2005). However, these end-point qualitative

methods are labor-intensive as they present with multiple-step protocols that might greatly lead to inconsistencies among the end-points (Limame *et al.*, 2012). Furthermore, results obtained from these conventional assays are in the form of fluorescent intensity, absorbance spectra or luminescence, markers which are often prone to interferences that might cause them to deform thus leading to unreliable data. Implementation of new technologies capable of non-invasively monitoring physiological parameters of the cell in real time without compromising the quality of results is therefore necessary (Larson and Worzella, 2005).

Real time cell analyzer (RTCA) named *xCELLigence system™* is a recent technique launched by Roche Applied Sciences in collaboration with ACEA Biosciences (Ke *et al.*, 2011; Scrace *et al.*, 2013). This label-free assay allows the possibility for real time measurement of cellular responses and potentially eliminates most of the aforementioned short-comings of end-point assays. The *xCELLigence system™* uses specially designed microtiter 16-well E-plates that contain interdigitated gold microelectrodes at the base of each well (Ke *et al.*, 2011; Scrace *et al.*, 2013). This technique non-invasively monitors the adhesion, spreading and/or proliferation of adherent cells using electrical impedance as a read out in the form of cell index (CI). An electric field is created between the electrodes at the bottom of the wells by the application of a low voltage in the presence of a buffer or cell culture medium. Introduction of adherent cells into the medium can impede the existing current flow and this impedance is displayed as the previously mentioned CI algorithm which is defined as $CI = Rn - \frac{Rb}{Rb}$ where Rn is impedance of the well with cells and Rb is background impedance of the well with cell culture medium only (Scrace *et al.*, 2013).

In addition to being able to assess responses of cells in their physiological state, the *xCELLigence system*™ can reveal changes in cell morphology and growth (Scrace *et al.*, 2013). The assay also provides IC_{50} (inhibitory concentration) values that are time-dependent thus being more informative compared to single IC_{50} end-points in conventional assays. Another advantage of *xCELLigence system*™ is that it is cost-effective since it does not require additional reagents as in end-point methods.

Technique

Before cells were seeded, 16-well E-plates containing antibiotic-free medium were imposed to current flow on the xCELLigence instrument to record background readings. 1×10^5 cells were then seeded in each well of the E-plates simultaneously with transfection agents siRBBP6 and pRBBP6. The E-plates were connected back on current flow supplied by the xCELLigence instrument that was placed in a 37°C incubator and the experiment was allowed to run for 24 hours before further treatment with GABA and camptothecin. The experiment was continued for an additional 48 hours. Cell Index values were recorded at 15 minute interval sweeps until the end of the experiment under the following xCELLigence parameters: [1st step: 1 sweep, 1 minute interval, 00:00:39 total time; 2nd step: 100 sweeps, 15 minute interval, 24:45:39 total time; 3rd step: 100 sweeps, 15 minute interval, 49:30:39 total time].

3.2.10. Flow Cytometry

Flow cytometry enables measurement of optical and fluorescence characteristics of single cells (or any other particle, including nuclei, microorganisms, chromosome preparations, and latex beads) (Adan *et al.*, 2016; Wlodkowic *et al.*, 2009; Williams, 2004). Physical properties, such as

size (represented by forward angle light scatter) and internal complexity (represented by right-angle scatter) can resolve certain cell populations. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells (Adan *et al.*, 2016). When labelled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths (Wlodkowic *et al.*, 2009). The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths (or "colors"), allows several cell properties to be measured simultaneously (Williams, 2004). Commonly used dyes include propidium iodide, phycoerythrin, and fluorescein, although many other dyes are available (Wlodkowic *et al.*, 2009). The present study employed the use of flow cytometry in order to 1) detect and measure apoptosis, 2) to analyse cell cycle and 3) to quantify the expression of an intracellular protein of interest.

AnnexinV FITC/PI Staining

Principle

Apoptosis is a physiological process which occurs normally during embryonic development as well as in maintenance of tissue homeostasis, and is turned off during abnormal growth of cells or in cancer (Liu *et al.*, 2011; Ouyang *et al.*, 2012; Wen *et al.*, 2012). The apoptotic process is characterized by certain morphological features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus and internucleosomal cleavage of DNA. Loss of plasma membrane asymmetry and attachment are the earliest features of apoptosis. In early apoptotic cells, the membrane phospholipid phosphatidyl serine (PS) is

translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment.

Annexin V, a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, binds to the exposed PS (Adan *et al.*, 2016; Wlodkowic *et al.*, 2009; Williams, 2004). Annexin V may be conjugated to fluorochromes including FITC. This format retains its high affinity for PS and thus acts as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, AnnexinV FITC staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation.

AnnexinV FITC staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore staining with AnnexinV FITC is typically used in conjugation with vital dyes such as propidium iodide (PI) to allow identification of early apoptotic cells (PI negative, AnnexinV FITC positive). Viable cells with intact membrane exclude PI, whereas the membranes of the dead and damaged cells are permeable to PI (Newbold *et al.*, 2014). Viable cells are considered AnnexinV FITC and PI negative, cells that are in early apoptosis are considered AnnexinV FITC positive and PI negative, and cells that are in late apoptosis are considered both AnnexinV FITC and PI positive.

Technique

Cultured cells were transfected with siRBBP6 and pRBBP6 construct for 48 hours and co-treated with camptothecin and GABA for additional 24 hours. The co-treated cells were then

trypsinized, transferred to 15 ml tubes, pelleted for 2 minutes at 1500 rpm and resuspended in

100µl 1X binding buffer (annexin V-FITC Apoptosis Detection Kit, Abcam®) at a concentration

of $1x10^4$ cells/ml. The cell solution was then transferred into 1ml tubes and 5µl of FITC Annexin

V and 5µl of PI were added. This was followed by gentle vortexing and incubation for 15

minutes at room temperature in the dark. 400µl 1X binding buffer was then added to each tube

and the cell solutions were analysed by flow cytometry within 1 hour.

Cell Cycle Analysis

Principle

Cell cycle analysis is a well-established method that enables the identification of cell distribution

during the various phases of the cell cycle by quantitatively measuring nuclear DNA content of a

cell using flow cytometry (Huang *et al.*, 2013). Four distinct phases can be recognized in a

proliferating cell population: the G1, S- (DNA synthesis phase), G2- and M-phase (mitosis)

(Khodjakov and Rieder, 2009; Collins *et al.*, 1997; DiPaola, 2002). At different stages of the cell

cycle, cell nuclei contain different amounts of DNA. For example, after receiving signals for

proliferation, diploid cells exit the resting state Gap 0 (G0) phase and enter the Gap 1 (G1)

phase. At this stage, the diploid cells maintain their ploidy by retaining two complete sets of

chromosomes (2N). As the cells enter the synthesis (S) phase, DNA replication starts, and in this

phase, cells contain varying amounts of DNA. The DNA replication continues until the DNA

content reaches a tetraploid state (4N) with twice the DNA content of the diploid state.

Tetraploid cells in the G2 phase start preparing for division and enter the mitosis (M) phase when

the cells divide into two identical diploid (2N) daughter cells. The daughter cells continue on to

another division cycle or enter the resting stage (G0 phase) (DiPaola, 2002).

Based on DNA content alone, the M phase is indistinguishable from the G2 phase, and G0 is indistinguishable from G1. Therefore, when based on DNA content, cell cycle is commonly described by the G0/G1, S, and G2/M phases (Khodjakov and Rieder, 2009). Damage to DNA often triggers the cell to interrupt the cell cycle at G0/G1 and G2/M checkpoints in order to prevent progression to the next phase; however during cancer development the cell machinery fails to arrest cells at these checkpoints thus leading to uncontrolled cell proliferation. Experimentally, a fluorescent dye that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei (Khodjakov and Rieder, 2009). The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA (Khodjakov and Rieder, 2009). The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Propidium iodide (PI) was used as a DNA-binding fluorescent dye in this study in order to analyse the effect of RBBP6 expression and co-treatment with camptothecin or GABA on cell cycle in cancer cells.

Technique

Following 48 transfection with either siRBBP6 or pRBBP6 and 24-hour co-treatment with camptothecin or GABA, old medium was discarded and cells were washed twice with 2 ml cold PBS, followed by trypsinization for 2-3 minutes in order to detach cells from the 6-well plates. Trypsin was deactivated by the addition of 2 ml fresh DMEM followed by transfer of cells into 1.5 ml tubes and centrifugation at 2000 rpm in order to collect the pellet, which was further rinsed twice with cold PBS. This was then followed by fixation and permeabilization of the cell's

plasma membrane using 500 µl ice cold methanol for 1 hour at -20 °C. The cells were then collected by centrifugation at 2000 rpm and then rinsed twice with ice cold 1 ml PBS before adding 300µl of PI containing RNase (Life Technologies[®]) and vortexed for 30seconds followed by 30 minutes incubation in the dark. The cell suspension was then analysed using the Acuri C6 flow cytometer (BD Biosciences, USA).

FACS Analysis

Principle

The availability of fluorescent proteins now allows real-time monitoring of gene expression and protein localization. Many applications for these proteins require the ability to analyze and sort expressing cells accurately and quantitatively using a fluorescence-activated cell sorter (FACS). In particular we demonstrate the utilization of FACS to isolate populations of cells that express the GFP from those that do not express the protein of interest.

Technique

Cells were seeded in the presence of pRBBP6 for 72 hours in 6-well plates followed by harvesting using 1 ml trypsin for 2-3 minutes after rinsing cells twice with PBS. After the last wash, supernatant was discarded and pulse vortexed the sample to completely dissociate the pellet. Typically about 100 µL residual volume remained. Cells were then fixed by adding 100 µL of BD Fixation Buffer then pulse vortexed again. Followed by incubation of tubes in the dark at room temperature for 20-60 minutes. Without washing, 2 mL of 1X BD permeabilization buffer was added to each tube. Samples were then centrifuged at 300-400 rpm at room temperature for 5 minutes, then supernatant was discarded. The cell pellet was then resuspended

in 2 mL of 1X permeabilization buffer then centrifuged at 300-400 rpm at room temperature for 5 minutes. Pellet was then resuspended in 100 µL of 1X BD permeabilization buffer. Anti-GFP primary antibody for detection of intracellular RBBP6 antigen was added to cells and incubated in the dark at room temperature for 20-60 minutes. 2 mL of 1X BD permeabilization buffer was added to each tube followed by centrifugation of samples at 300-400xg at room temperature for 5 minutes. Alexa Fluor-488-conjugated secondary antibody was added to cells and further incubated in the dark for 20-60 minutes followed by addition of 2 mL flow cytometry staining buffer to each tube. Samples were then centrifuged at 300-400rpm at room temperature for 5 minutes, then supernatant was discarded. Stained cells were then resuspended in an appropriate volume of flow cytometry staining buffer and samples were analysed on a flow cytometer.

3.2.11. Confocal Microscopy

Principle

Confocal microscopy is an imaging technique in which a beam of incoming light (the excitation beam) is focused through the microscope objective on a small spot inside the specimen where the same objective gathers the reflected or fluorescent light coming back from the same specimen (Que *et al.*, 2015; Sanderson *et al.*, 2015; Coling and Kachar, 2001). In contrast to conventional light microscopy, this light is projected (like a slide projector) and not directly viewed. The advantages of confocal microscopy over conventional one is that it captures images of high resolution and contrast and it does not bring about artifacts induced by conventional microscopy such as cell shrinkage, loss of fat, etc (Que *et al.*, 2015; Sanderson *et al.*, 2015; Coling and Kachar, 2001). Immunocytochemistry is one of the most powerful ways of asking where in an organism a particular protein/molecule is expressed (Ramos-Vara, 2011). It is used to anatomically localize the presence of a specific protein antigen in cells by use of fluorescent dyes

or a specific primary antibody that binds to it. In terms of immunostaining, the primary antibody is bound to a secondary antibody conjugated to a fluorophore in order to allow visualisation of the protein under a fluorescence microscope. Fluorescent dyes are usually added to cells at an optimum dilution followed by incubation in the dark prior to imaging (Sanderson *et al.*, 2015). In this study, the Olympus BX 63 OM/FM confocal microscopy was used to visualise the overexpression of RBBP6 following transfection with an expression vector and to detect apoptosis induction using annexinV/FITC/DAPI in cell lines (Hollville and Martin, 2016).

Technique

Cells were grown on coverslips inserted in 6 well plates to attach overnight in the presence of siRBBP6 and pRBBP6 and then treated with the desired concentration of camptothecin (0.25μM) or GABA (100μM). The cells were then allowed for further 24 hour incubation. For apoptosis detection, the cells were washed with cold PBS, stained with annexin V-FITC/DAPI for 30 minutes in the dark followed by fixation for 30 minutes with 4% formaldehyde. For protein expression analysis, fixed cells were permeabilized using 0.01% triton-X100 and then immunostained with anti-GFP primary antibody for one hour in the dark after blocking for non-specific binding with 1% BSA blocking buffer. The cells were then rinsed 4X with cold PBS followed by incubation for another one hour with secondary antibody conjugated to alexa fluor-488 fluorescent dye in order to enable visualization of GFP-positive cells. The cells were imaged at 10X magnification using Olympus BX63 confocal fluorescence microscope.

3.2.12. Caspase 3/7 Activity

Principle

Caspases are a group of cysteine protease enzymes which play an essential role in apoptosis (Parrish *et al.*, 2015). Caspases were first identified in *C. elegans* when the CED-3 was found in close homology with the mammalian interleukin-1-converting enzyme and that overexpression of the enzyme induced apoptosis (Parrish *et al.*, 2015; Nicholson and Thornberry, 1997). Caspases are divided into two types, namely the initiator caspases (caspase 1,2,8,9 and 10) and the effector caspases (caspases 3, 6, 7 and 14). During apoptosis, the initiator caspases cleave the inactive pro-forms of effector caspases, thereby activating them (Parrish *et al.*, 2015; Preaudat, 2002). Effector caspases in turn cleave other protein substrates within the cytoplasm and the nucleus thus executing apoptosis. During cancer development however, transforming cells have the ability to avoid apoptosis and therefore continue to proliferate (Preaudat, 2002). Anticancer therapeutic strategies therefor focus on restoring the apoptotic pathway and caspases are some of the potential targets in anticancer therapeutics (Parrish *et al.*, 2015). Endpoint assays that detect activity of effector caspases allow for the confirmation of whether or not apoptosis has occurred and in this case apoptosis was measured by analysing the activity of effector caspases 3 and 7. The following protocol was adopted from Promega's Caspase-Glo® 3/7 Assay kit which relies on the cleavage of luminogenic caspase-3/7 substrate by active caspases 3 and 7 present in the cell sample. Addition of the luminogenic caspase-3/7 substrate results in cell lysis followed by caspase cleavage of the substrate and generation of a glow-type luminescent signal which is proportional to caspase activity.

Technique

Before starting the assay, caspase-Glo® 3/7 reagent was prepared by transferring the contents of the caspase-Glo® 3/7 buffer bottle into the amber bottle containing caspase-Glo® 3/7 substrate

followed by swirling and inverting the contents until the substrate was thoroughly dissolved to form the reagent. The reagent was then allowed to equilibrate to room temperature for 30 minutes. Briefly, cells ($5x10^4$ cells/well) were seeded in the presence of either siRBBP6 or pRBBP6 (expression vector) on a white-walled 96-well microplate and treated with camptothecin or GABA for 24 hours. The well plates containing cells were then removed from the incubator and allowed to equilibrate to room temperature followed by the addition of 100µl Caspase-Glo® 3/7 reagent to each well containing 100µl of blank, negative control cells or treated cells in culture medium. The contents of the wells were then gently mixed using a plate shaker at 300–500rpm for 30 seconds followed by incubation at room temperature for 1 hour. Luminescence was then measured using a plate-reading Glomax®-96 microplate luminometer. The assay was performed in triplicates and the results were presented as mean of Relative Light Units (RLU).

3.2.13. Mitochondrial ATP Depletion Assay

Principle

Total levels of cellular ATP can be used to assess cell viability, cell proliferation and cytotoxicity of a wide range of compounds and biological response modifiers (Hansen *et al*., 2015; Gergely *et al*., 2002). While the levels of cellular ATP serve as a hallmark of cell viability, proliferation, cytotoxicity and cell death under standard culture conditions, they are not representative of the mitochondrial bioenergetic state of the cell (Tokarz and Blasiak, 2014; Lu *et al*., 2000; Goldin *et al.*, 2007). Cultured cells can survive in the absence of an active electron transport chain by supplying all their ATP demands through glycolysis. However, when cells are cultured with non-fermentable carbon substrates such as galactose, they require an active electron transport chain (ETC) to supply all their ATP demand. Under these conditions, damage to the mitochondrial

bioenergetic state of the cell will lead to depletion of cellular ATP, which can be easily measured by luminescence as a decrease in total levels of ATP measured. The assay is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. The emitted light is proportional to the ATP concentration inside the cell. The reaction can be summarized as follows:

$$ATP + D\text{-}Luciferin + O_2 \xrightarrow[\text{Luciferase}]{Mg^{2+}} Oxyluciferin + AMP + PPi + CO_2 + Light$$
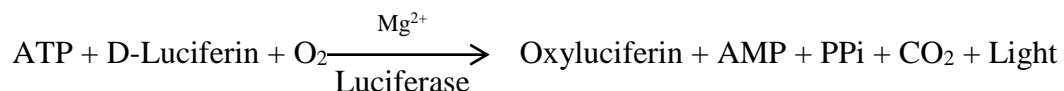
This luminescence assay allows irreversible inactivation of ATP degrading enzymes (ATPases) during the lysis step, ensuring that the luminescent signal obtained truly corresponds to the endogenous levels of ATP. The major advantages of the luminescence assay include long luminescence signal since half-life of the luminescent signal is greater than 5 hours and therefore a special luminometer with injectors is not required; results are obtained in less than 30 minutes, the assay is simple to perform, i.e. there are no separation steps and there are only 2 reagent additions, no cell harvesting or centrifugation are required, it is highly sensitive with a wide linear dynamic range of approximately from 1pM to 1µM of ATP (Tokarz and Blasiak, 2014; Lu *et al*., 2000; Goldin *et al*., 2007).

Technique

Twenty five microlitres of $1x10^4$ cells per well were plated in wells of white luminometer plate containing glucose-free, galactose enriched growth medium in the presence of transfection agents (siRBBP6 or pRBBP6) for 48 hours. Cells were then treated with equal amount of 0.25µM camptothecin or 100µM GABA diluted in a glucose free media supplemented with 10mM galactose. Before starting with the assay, the ATP detection reagent was prepared by

thawing the ATP detection buffer in a 37°C water bath and the ATP detection substrate at room temperature. 10ml of ATP detection buffer was then transferred to the amber bottle containing the ATP detection substrate to reconstitute the lyophilized enzyme/substrate mixture in order to make up a 2X ATP detection reagent. The reagent was then mixed by vortexing and swirling the contents to obtain a homogeneous solution and equilibrated to room temperature for 5-10 minutes prior to use. ATP detection reagent was added at a 1:1 ratio to each well at 2, 4, 6, 8, and 18 hour intervals following a 24 hour treatment with camptothecin and GABA. The plate was mixed by orbital shaker (500–700rpm) for 1–5 minutes and then incubated for 30 min at room temperature before luminescence was measured using GLOMAX (Promega, USA). The assay was conducted in triplicates and ATP levels were reported as a mean of relative light units (RLU) normalized to percentage of control at each time point.

3.3.   Statistical analysis

The results of each series of experiments (performed in triplicates unless otherwise stated) are expressed as the mean values ± standard deviation of the mean (SD). Levels of the statistical significance were calculated using the paired student t-test when comparing two groups. Statistical significance was considered according to the following p-values: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

The attached email below serves as proof that the manuscript "**Retinoblastoma binding protein 6 (RBBP6): a potential biomarker for apoptosis induction in human cervical cancer cell lines**" as detailed in the following chapter (4), has been accepted for publication and is currently in press.

CHAPTER FOUR - Retinoblastoma binding protein 6 (RBBP6): a potential biomarker for apoptosis induction in human cervical cancer cell lines

4.1. Introduction

Cancer is an enormous burden of a disease globally (Ma and Yu, 2006). Today, more people die from cancer than HIV/AIDS, tuberculosis and malaria combined. Currently, cervical cancer is the most common, accounting for over 60% of the gynaecological cancer burden in developing countries (Sankaranarayanan *et al.*, 2009). RBBP6 has been shown to be highly expressed in several cancers, and its ability to interact with p53 tumour suppressor has drawn attention in evaluating its potential as a cancer biomarker. P53 is a crucial regulatory protein that triggers cellular responses such as DNA repair, cell cycle arrest, senescence and apoptosis in response to cellular stress. In most human cancers, however, the TP53 pathway is often found to be defective, either by mutations or through deregulation by its negative regulators. In cervical cancer, p53 is found to be inactivated rather than mutated and RBBP6 is suspected to be one of such negative regulators of p53.

Extensive research on the cause of cervical cancer has evidently singled out human papillomaviruses (HPVs) as the main causative agent (Fernandes, 2013; Denny, 2010; Bosch *et al.*, 2002; Schiffman, 1995; Munoz *et al.*, 1992). During the differentiation-dependant phase, the E6 viral protein regulates the viral replication in differentiated cells by supressing transcription of active p53 cellular protein thus abrogating the host cells' ability to initiate cell cycle arrest, prompting uncontrolled cell proliferation (Fernandes, 2013; Bosch *et al.*, 2002). It is suspected that the viral protein achieves this p53 suppression via the proteasomal pathway in which it acts as an E3 ligase that functions to ubiquitinate protein molecules for proteasomal degradation. This

mechanism is similar to the one involving RBBP6 in which it interacts with TP53 via its p53-binding domain. Research has shown that RBBP6 plays a role as a scaffold protein to promote the assembly of the p53/TP53-MDM2 complex, resulting in increased MDM2-mediated ubiquitination and degradation of p53/TP53. Therefore, this suggests that RBBP6 may function as a negative regulator of p53/TP3, thus leading to both apoptosis and cell growth (Pugh *et al.*, 2006; Motadi *et al.*, 2011). So in this study we propose that RBBP6 mediates TP53 inactivation in the same manner as HPV viral proteins since both proteins possess the activity of E3 ligases and have also been shown to interact with TP53. We therefore aim to manipulate the expression of RBBP6 in cervical cancer cell lines and co-treat with chemotherapeutic agents in order to sensitise apoptosis induction.

4.2.   Results

This section describes results obtained from both human cervical cancer tissue specimens and cell lines HeLa, SiHa and MRC-5. TMA slides were immunostained with full length RBBP6 mAb for analysis of localization and spread of the protein in cervical cancer tissues. This was followed by manipulation at gene level by both silencing and up-regulation. Successful transfections prompted further analysis of any possible effect RBBP6 might have on growth of the cell lines in question. Annexin V staining, mitochondrial ATP levels and activity of caspase 3/7 were used to detect and confirm apoptosis induction following RBBP6 targeting alone or in combination with anticancer agents camptothecin and GABA. mRNA quantification of key apoptotic genes including p53 were quantified for further investigation and western blot was used for analysis at protein level.

4.2.1. Immunohistochemical staining in human cervical cancer tissues sections

Before altering the expression of RBBP6 in cell lines, it is important to understand its expression profile in cervical cancer patients. We employed the use of immunohistochemical staining of human cervical cancer tissue sections where haematoxylin and eosin (H&E) staining procedure was first used to examine histopathology of the tissue in order to understand the manifestations of the disease before immunostaining with monoclonal anti-mouse primary antibody raised against RBBP6. Haematoxylin stains the nuclear components including nucleoli and chromatin material whereas eosin stains the cytoplasmic components such as muscle fibres, red blood cells, collagen, etc. (Fischer *et al.*, 2008). Tissue microarray (TMA) slides consisting of 20 cases of both squamous cell carcinoma (SCC) and adenocarcinomas were diagnosed relative to adjacent

normal cervix tissue in a mix of Caucasian and African American females aged between 25 and 51.

In terms of histopathology, H&E staining revealed early invasive phenotypes of cancer in some patients where neoplastic cells appeared to have penetrated through the basement membrane separating the epithelium from connective tissue, into the underlying stroma (Figure 6, panel A). Panel A shows non-keratinizing invasive squamous cell carcinoma which appears as infiltrating network of neoplastic lymphocytes and plasma cells with intervening stroma. Well-differentiated keratinizing SCCs are shown (Figure 6 A) as characteristic whorls of polygonal epithelial cells containing central nests of keratin pearls. In the less prevalent adenocarcinoma as shown in Figure 6 (panel G), well-differentiated columnar cells form abnormal glandular elements that seem to project into the endocervix.

Immunostaining of RBBP6 in paraffin-embedded formalin-fixed TMAs followed by treatment with the chromogenic DAB (3,3'-diaminobenzidine) substrate, which enabled visualization of our protein of interest revealed localization mostly in the cytoplasm of early invasive squamous cells as shown by the brown precipitates indicated by the arrow (Figure 6, panel B). Colorization of the DAB product was even higher in both the cytoplasm and the nucleus of non-keratinizing SCC (Figure 6, panel B). However, expression was only confined to the keratinizing areas in the well-differentiated squamous cell carcinoma (Figure 6, panel B). Out of the 20 cases analyzed, only two diagnoses were categorized as adenocarcinomas and similar to SCC, they also stained positive for RBBP6 as shown by the intensely chromogenic columnar cells in the well-differentiated phenotype (Figure 6, panel B).
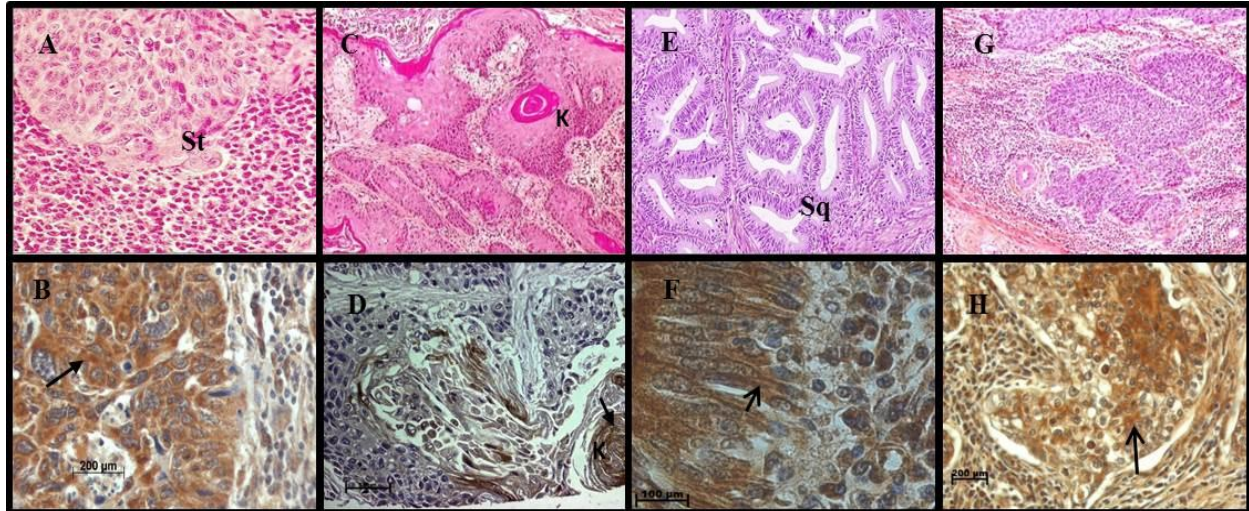
Figure 6: Expression of RBBP6 protein in subtypes of cervical cancer.

(A) Early invasive stromal squamous cell carcinoma (SCC), (C) Keratinizing well-differentiated SCC, (E) well-differentiated adenocarcinoma and (G) Non-keratinizing invasive squamous carcinoma (B) Expression of RBBP6 in the stromal region of the cancer. (D) RBBP6 expression in well-differentiated RBBP6 expression in non-keratinized tissue of the cervical cancer showing over expression of RBBP6 in the cytoplasm of the cells. The sections were taken at 40X squamous cell carcinoma with poor expression in most cell but higher expression around the keratin regions (k). (F) Expression of RBBP6 in squamous well-differentiated cancer cell 100X magnification (H).

4.2.2. Confirmation of gene silencing and up-regulation

In order to continue with our analysis of the role played by RBBP6 in cervical cancer progression, we had to verify if our silencing and overexpression was successful. RBBP6 overexpression was confirmed using immunoprecipitation of GFP reporter gene. In HeLa cell line, transfection efficiency was significantly high ($P < 0.001$) with GFP expression of ~98% band intensity (Figure 7 A). A second distinct yet smaller band is detected in pCMV6-AC-GFP+ cell lysate of HeLa cells which might be indicative of one of the RBBP6 spliced variants. Similarly, GFP expression was significant ($P < 0.05$) in SiHa cell lysates, with a band intensity of 87% (Figure 7 B). Western blotting and qPCR were used to confirm gene silencing at both mRNA and protein levels. RBBP6 mRNA was successfully silenced in both HeLa and SiHa cell lines, with nearly 90% and 80% knockdown, respectively (Figure 7 C). Quantification at protein level further confirmed the observed gene knockdown (Figure 7 C). Approximately 72.9% and 53.4 % up-regulation was recorded in HeLa and SiHa cells, respectively, compared to non-transfected controls where only 0.4% auto-fluorescence was observed in both cell lines according to FACS analysis which was further confirmed by fluorescence microscopy (Figure 8; Figure 9).
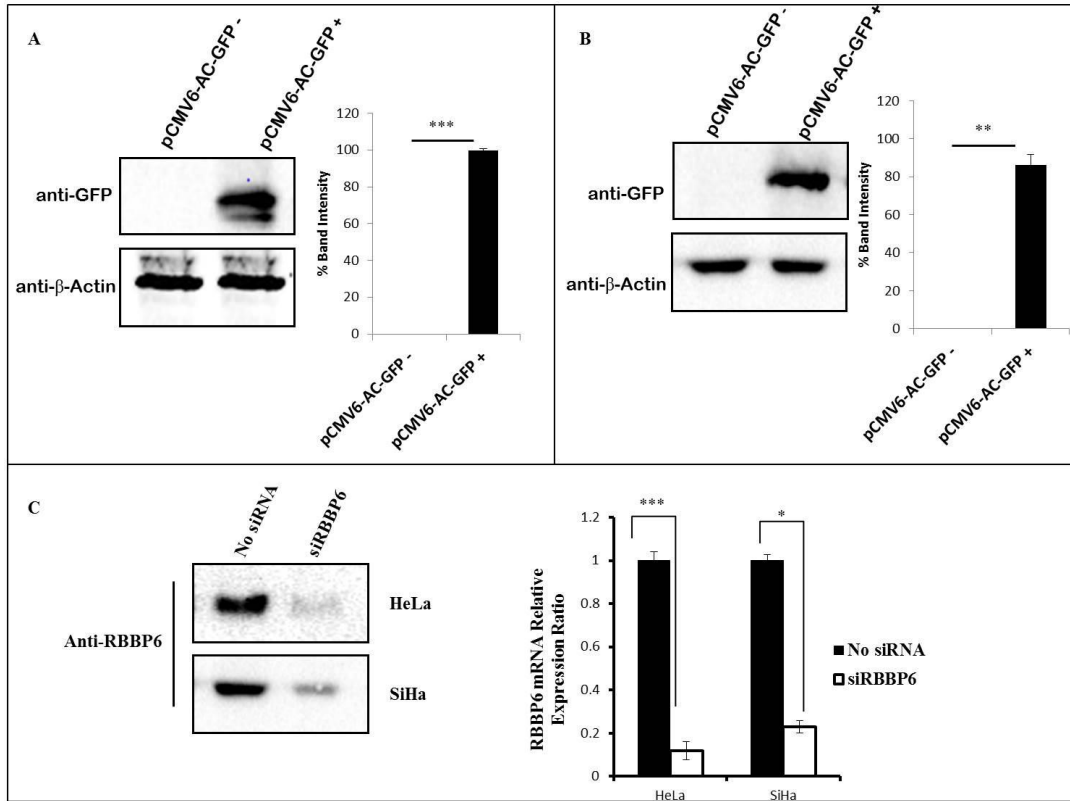
Figure 7: Confirmation of successful gene silencing and up-regulation of RBBP6-GFP tagged cDNA. (A) Shows successful overexpression of RBBP6 tagged with GFP in HeLa cell line, (B) shows successful overexpression of RBBP6-GFP tagged in SiHa cells and (C) showing successful silencing of RBBP6 in both HeLa and SiHa cell lines.
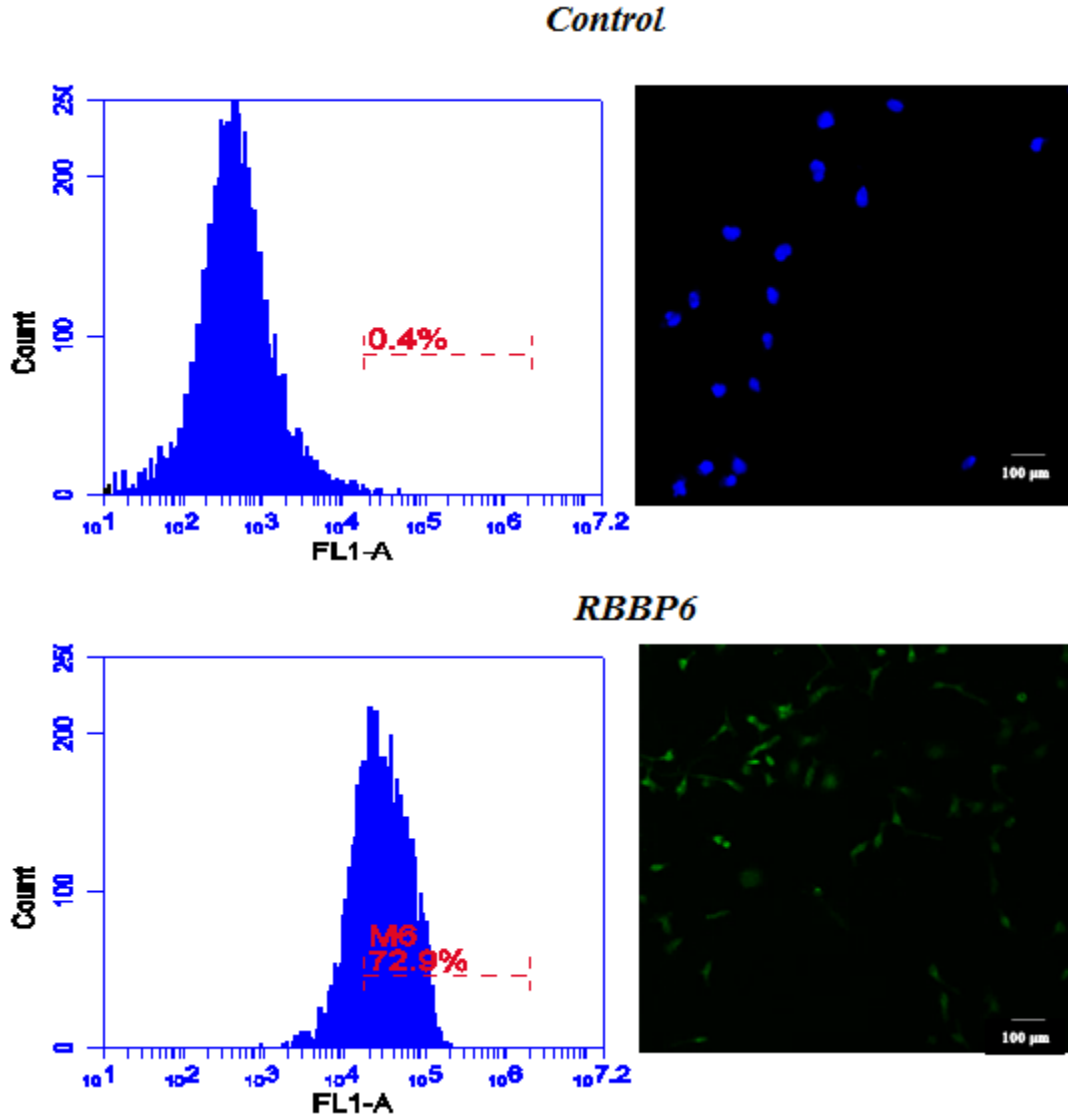
Figure 8: FACS analysis and immunocytochemistry of HeLa cells.

Stained with 0.2 μg/ml mouse monoclonal anti-GFP antibody and labelled with Alexa Fluor-488 goat anti-mouse secondary antibody 48-hours post-transfection. Cells were fixed using 4% formaldehyde and permeabilized using 0.01% triton X-100 before immunostaining. Counterstaining was obtained using DAPI. Histograms represent FACS profiles of RBBP6 expression, i.e. *top left*: control cells that were not transfected and *bottom left*: cells transfected with the plasmid (*pCMV6-AC-GFP)*. Fluorescence microscopy images (*top and bottom right*) show cytoplasmic/nuclear localisation of RBBP6.
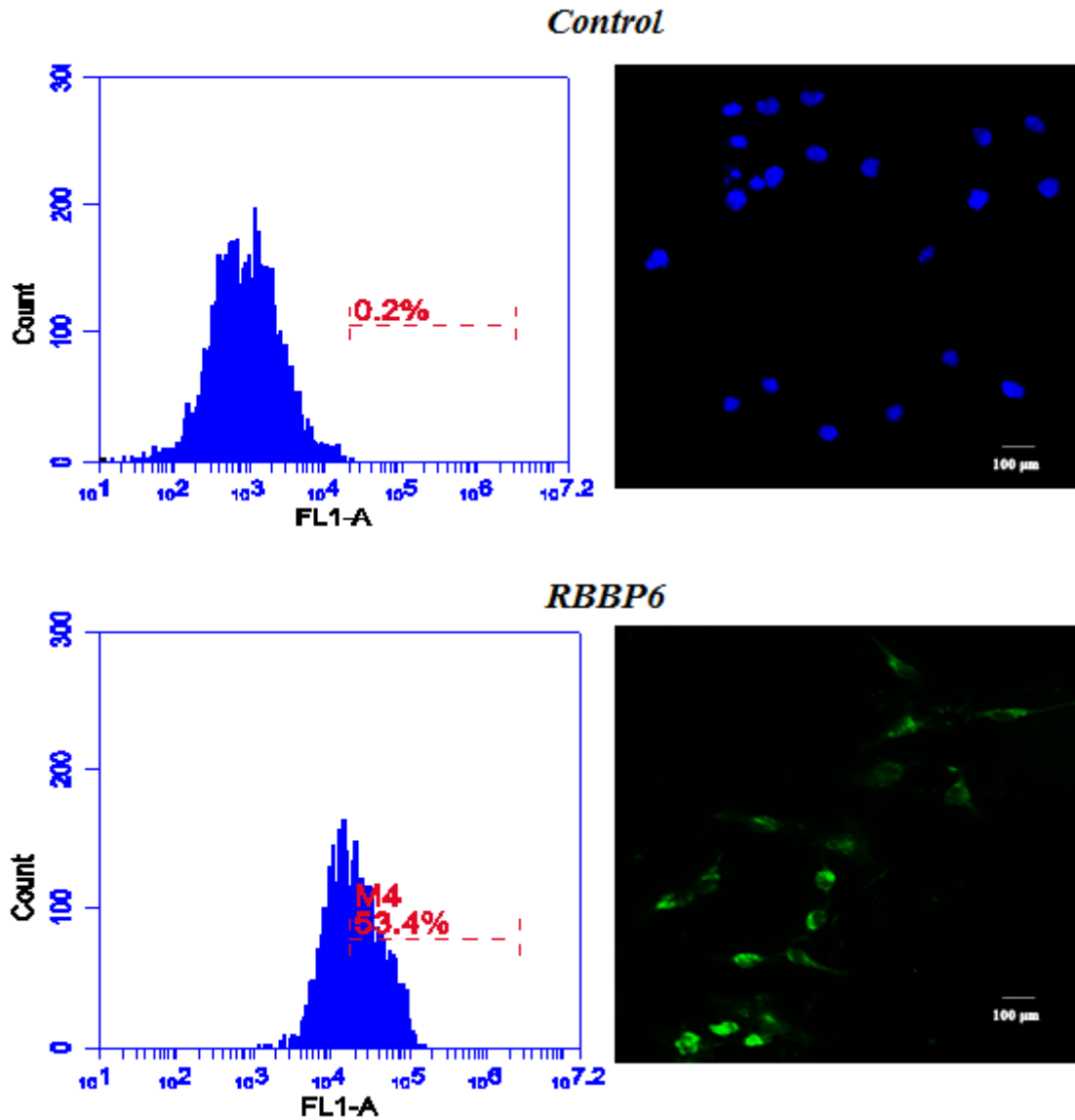
Figure 9: FACS analysis and immunocytochemistry of SiHa cells.

Stained with 0.2 μg/ml mouse monoclonal anti-GFP antibody and labelled with Alexa Fluor-488 goat anti-mouse secondary antibody 48-hours post-transfection. Cells were fixed using 4% formaldehyde and permeabilized using 0.01% triton X-100 before immunostaining. Counterstaining was obtained using DAPI. Histograms represent FACS profiles of RBBP6 expression, i.e. *top left*: control cells that were not transfected and *bottom left*: cells transfected with the plasmid (*pCMV6-AC-GFP*). Fluorescence microscopy images (*top and bottom right*) show cytoplasmic/nuclear localisation of RBBP6.

**4.2.3. The effect of RBBP6 overexpression and silencing on cell proliferation**

The objective of this section was to monitor the effect of RBBP6 overexpression and silencing on cell proliferation in both the normal and the tumorigenic cell lines using xCELLigence system. The xCELLigence system uses specially designed microtiter 16-well plates that contain interdigitated gold microelectrodes at the bottom of each well to non-invasively monitor the viability of cultured cells using electrical impedance as a read out in the form of cell index (CI) (Ke *et al.*, 2011). The cells were monitored over a period of about 3 days in the presence of transfection agents, i.e. the RBBP6 expression vector (pRBBP6) and siRBBP6 oligonucleotide. Growth curves were normalised to the cell index (CI) at 24 hours just before the effects of either gene silencing or overexpression on cell proliferation was monitored.

HeLa growth curve, which is presented in Figure 10, showed a significant reduction in cellular growth 24 hours post siRBBP6 transfection. RBBP6 (red curve) overexpression promoted growth of HeLa cells, as shown by the continued proliferation of cells transfected with pRBBP6 (Figure 10). In SiHa cells, RBBP6 silencing steadily reduced growth rate to 1.0 cell index, whereas RBBP6 overexpressing cells maintain higher cell index throughout the remaining 32 hours of the entire experiment (Figure 11). In siRBBP6 and siRBBP6+Camptothecin, there was significant reduction in HeLa and SiHa cell indices while GABA and siRBBP6+GABA could not reduce cell index (Figure 12). MRC-5 cell line did not show any change in cell index. In MRC-5, pRBBP6 significantly increased cell index or cell proliferation (Figure 13).

Figure 10: Cell growth of HeLa cells was analyzed using the xCELLigence system

It relies on the generation of electrical impedance as cell growth by 16-well plates coated with a gold microelectron covering at the base of each well. The y-axis shows normalised cell index or cell adhesion over a period of about 72 hours (x-axis). The growth patterns highlighted in green show cells that are not treated. Those highlighted in coral show control cells populations treated with transfection reagent only whereas red and magenta growth curves show cells that are overexpressing RBBP6 (transfected with pRBBP6) and cells that were silenced, respectively.
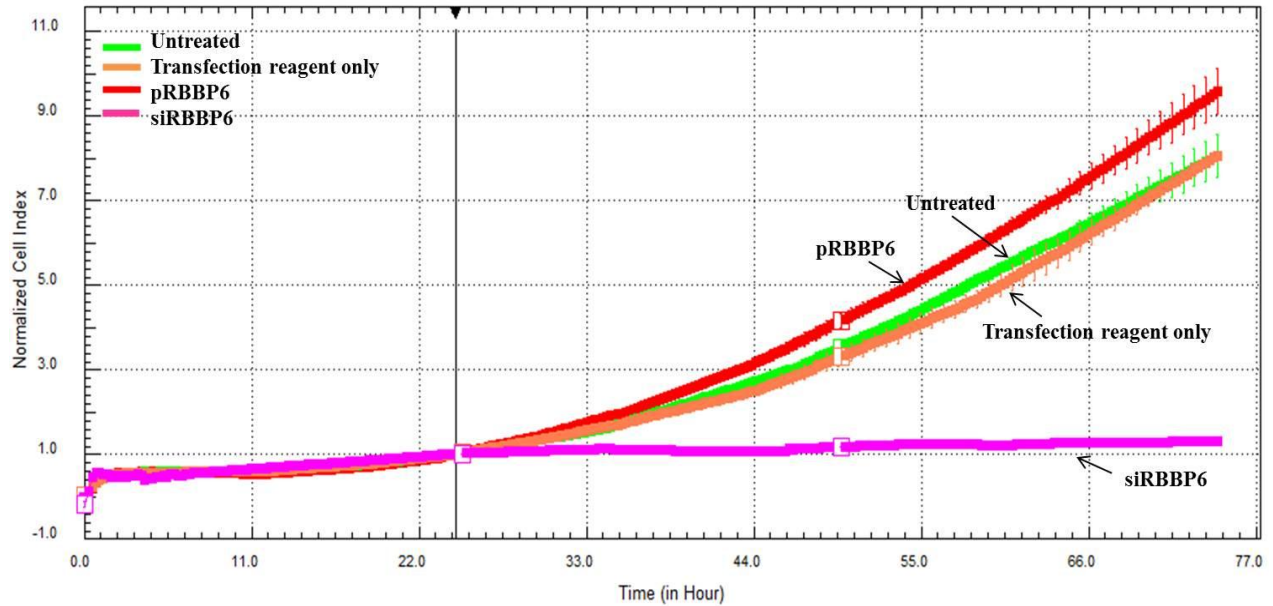
Figure 11: Cell growth of SiHa cells was analyzed using the xCELLigence system

It relies on the generation of electrical impedance as cell growth by 16-well plates coated with a gold microelectron covering at the base of each well. The y-axis shows normalised cell index or cell adhesion over a period of about 72 hours (x-axis). The growth patterns highlighted in green show cells that are not treated. Those highlighted in coral show control cells populations treated with transfection reagent only whereas red and magenta growth curves show cells that are overexpressing RBBP6 (transfected with pRBBP6) and cells that were silenced, respectively.
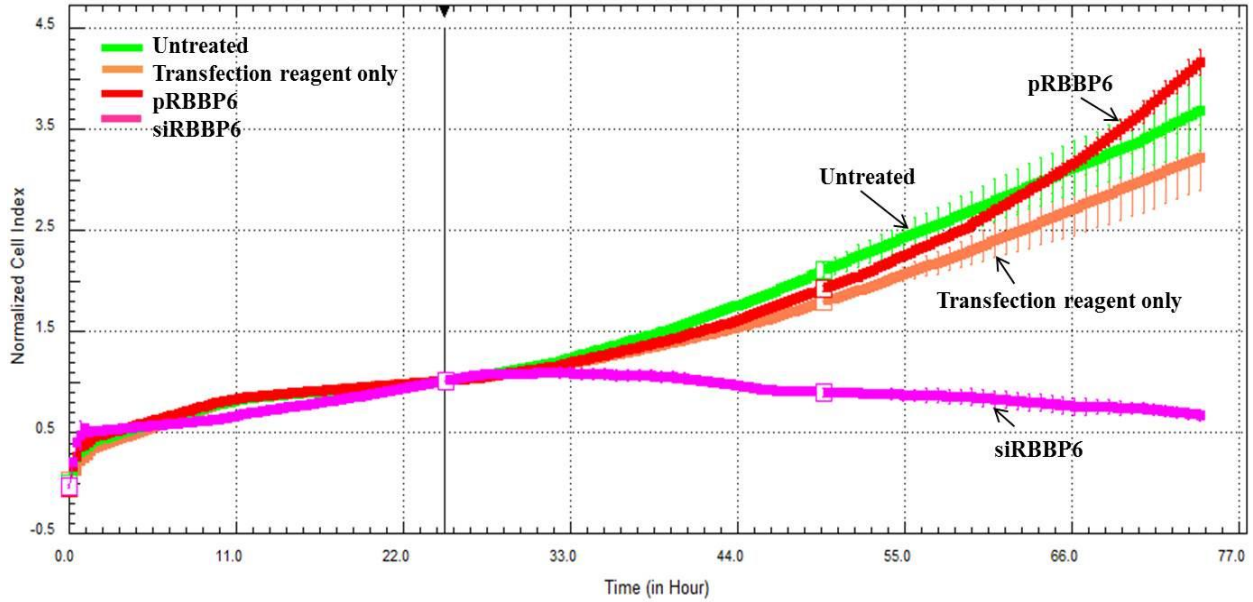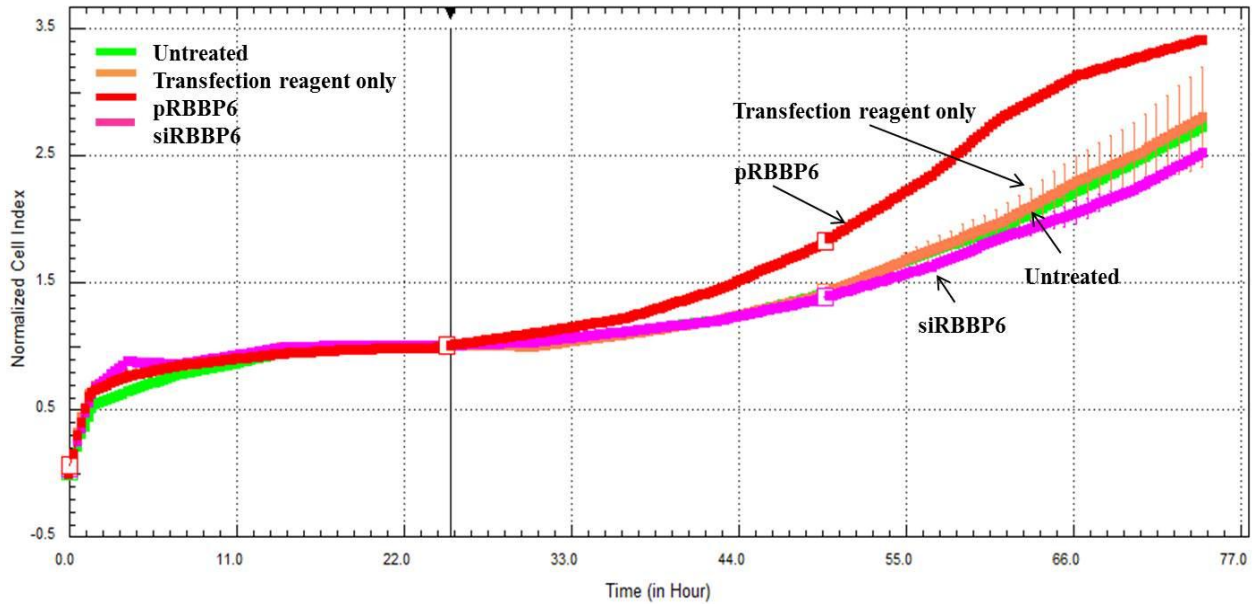
Figure 12: Cell growth of MRC-5 cells was analyzed using the xCELLigence system

It relies on the generation of electrical impedance as cell growth by 16-well plates coated with a gold microelectron covering at the base of each well. The y-axis shows normalised cell index or cell adhesion over a period of about 72 hours (x-axis). The growth patterns highlighted in green show cells that are not treated. Those highlighted in coral show control cells populations treated with transfection reagent only whereas red and magenta growth curves show cells that are overexpressing RBBP6 (transfected with pRBBP6) and cells that were silenced, respectively.
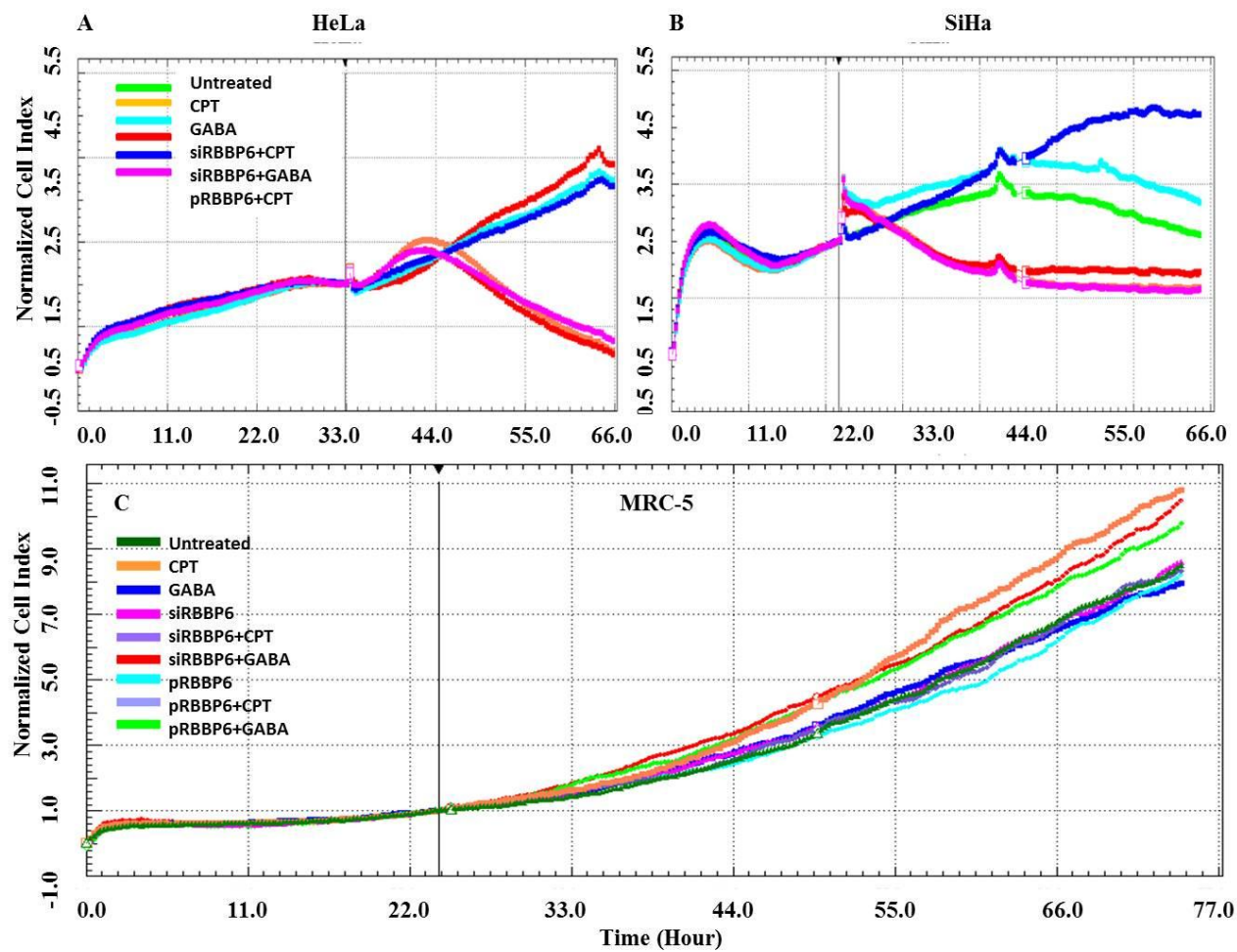
Figure 13: Growth of SiHa (B), HeLa (A) and MRC-5 (C) cells analysed using xCELLigence system.

Camptothecin and siRBBP6 treatment shows significant cell growth inhibition.

4.2.4. Apoptosis Detection

Apoptosis is an energy-dependent form of programmed cell death commonly characterised by distinct morphological changes and condensation of the chromatin network (Liu *et al*., 2011; Ouyang *et al*., 2012; Wen *et al*., 2012). Under normal physiological conditions, a cell maintains an asymmetric distribution of phospholipids in the outer and inner membrane leaflets with phosphatidylserine (PS) facing the cytoplasmic leaflet. During early apoptosis induction this membrane asymmetry is lost resulting in the exposure of PS on the outer membrane leaflet which enables recognition and a subsequent removal of the dying cell by phagocytes. This phenomenon provides platform for the novel annexin V apoptosis detection assay described by Andree *et al*. (1990) which is based on the measurement of PS exposure on the outer membrane leaflet using the high affinity binding of annexin V peptide to the phospholipid. AnnexinV-FITC staining combined with the DNA intercalating PI (propidium iodide) agent make flow cytometry an effective technique to distinguish between necrosis, early and late apoptosis in cultured cells. In this section, we measured apoptosis following transfection of HeLa, SiHa, and MRC-5 cell lines with siRBBP6 or pRBBP6 and co-treatment with either camptothecin or GABA using flow cytometry.

Flow cytometry detection of apoptosis

Analysis by flow cytometry revealed that the percentage of apoptotic cells following transfection with siRBBP6 was significantly increased in most cell lines, with 48% apoptosis in HeLa and 46% in SiHa ($p<0.05$) (Figure 14). Treatment with only camptothecin and GABA led to a significantly increased apoptosis induction in HeLa (52% and 29%, respectively) and SiHa cells (44% and 32%, respectively). Co-treatment of siRBBP6 and camptothecin significantly

increased apoptosis in HeLa and SiHa cells to 62% and 49% respectively with a change of 18% and 17% increments, respectively due to combination (Figure 14). However, no increase in apoptosis was observed in HeLa and SiHa cells that were co-treated with siRBBP6 and GABA (Figure 14).

Transfection with pRBBP6 induced minimum amount of apoptosis in both cell lines, with 18% and 14% apoptosis in HeLa and SiHa, respectively (Figure 14). Co-treatment of pRBBP6 and camptothecin or GABA reduced apoptosis by 30% or 16% in HeLa cells and by 12% or 19% in SiHa (Figure 14). Apoptosis induction in MRC-5 fibroblast was fairly low following treatment with camptothecin or GABA (16% and 12%, respectively) as compared to the one observed in tumorigenic cell lines (Figure 15). Only 0.2% apoptosis was observed in untreated MRC-5 cells. Silencing MRC-5 with siRBBP6 induced 13% apoptosis whereas up-regulation of RBBP6 induced 0.1% apoptosis (Figure 15). Combination with camptothecin led to 19% and 13% apoptosis induction following silencing and up-regulation, respectively (Figure 15). There was no significant change in apoptosis following combination of siRBBP6 and GABA (11%) or pRBBP6 with GABA (18%) when compared to GABA treatment only (12%) (Figure 15). No significant necrosis was observed in response to all treatments across all cell lines as shown in Figure 16, Figure 17 and Figure 18.
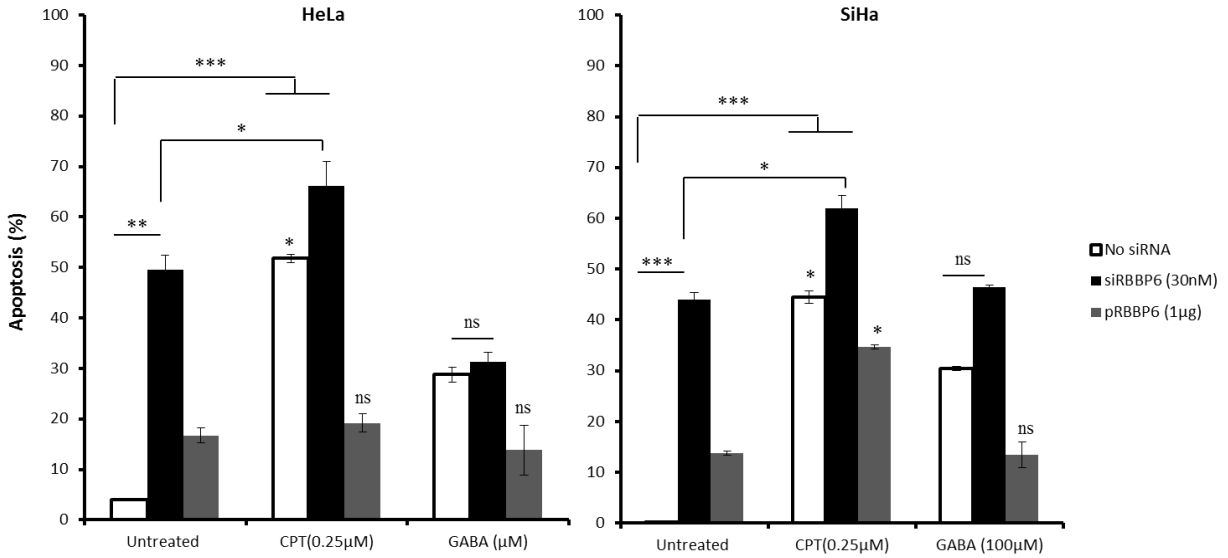
Figure 14: Statistical analysis of flow cytometry-obtained apoptosis (%) in HeLa and SiHa cell lines. T-test was used to generate p-values in order to compute the difference between treated and untreated scores. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$ and ns indicates $p \geq 0.05$.
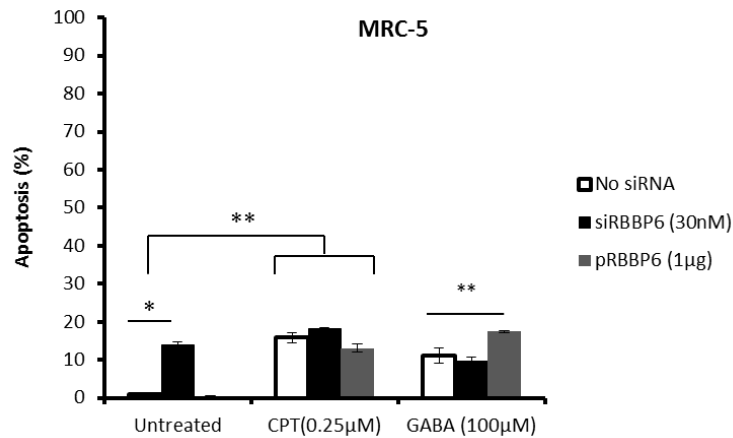
Figure 15: Statistical analysis of flow cytometry-obtained apoptosis (%) in MRC-5 cell line. T-test was used to generate p-values in order to compute the difference between treated and untreated scores. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$ and ns indicates $p \geq 0.05$.
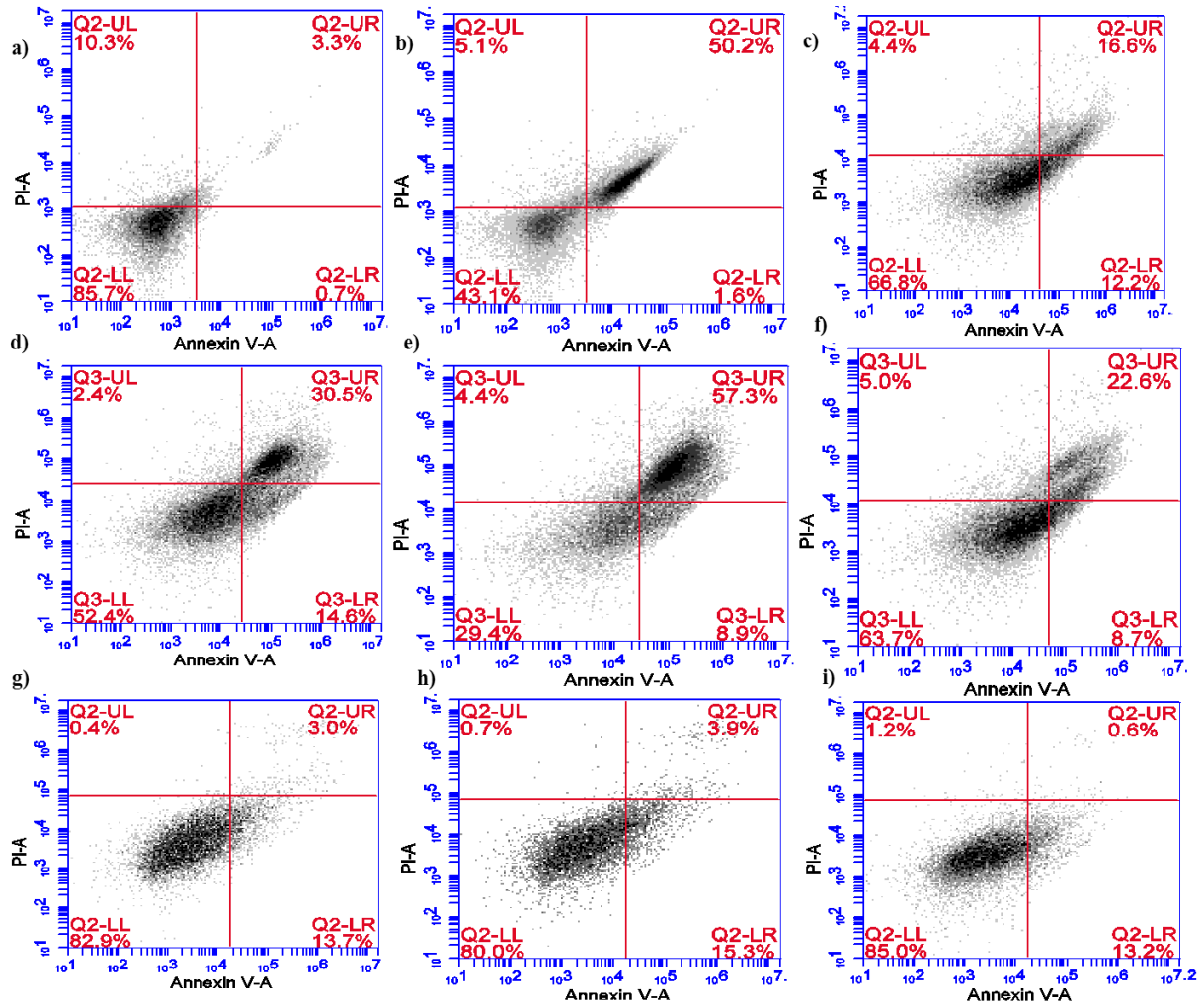
Figure 16: Apoptosis of HeLa cells was analyzed using flow cytometer with annexin V and PI staining. Upper left quadrants show necrotic cells which stain positive for PI and negative for annexin V, upper right quadrants show cells undergoing late apoptosis with positive staining for both PI and annexin V. Lower right quadrants show cells undergoing early apoptosis and are staining positive for annexin V and negative for PI; and lastly the lower left quadrants show viable cells. (a) Untreated. (b) Camptothecin, (c) GABA, (d) siRBBP6, (e) siRBBP6+camptothecin, (f) siRBBP6+GABA, (g) pRBBP6, (h) pRBBP6+camptothecin and (i) pRBBP6+GABA.
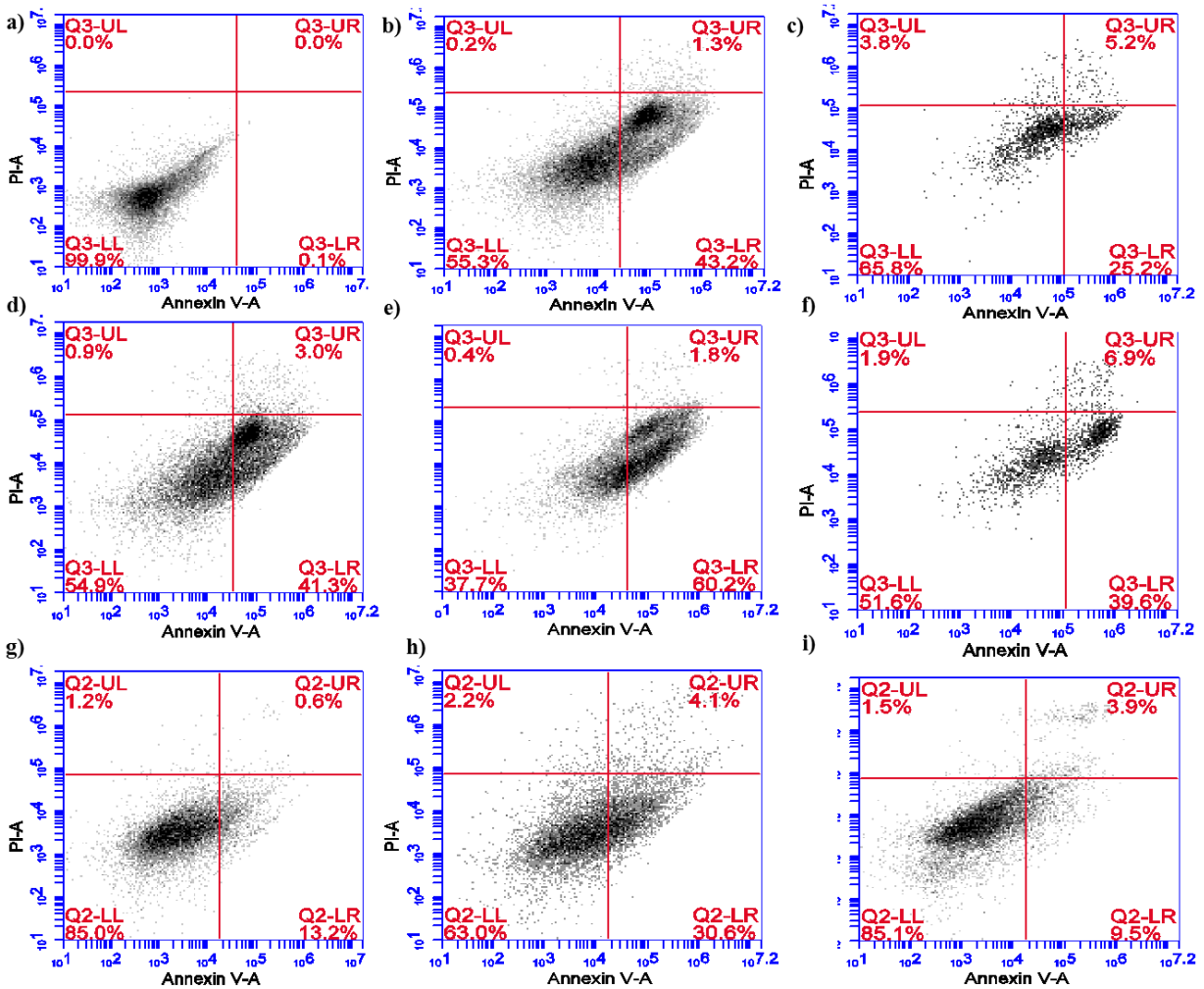
Figure 17: Apoptosis of SiHa cells was analyzed using flow cytometer with annexin V and PI staining. (a) Untreated. (b) Camptothecin, (c) GABA, (d) siRBBP6, (e) siRBBP6+camptothecin, (f) siRBBP6+GABA, (g) pRBBP6, (h) pRBBP6+camptothecin and (i) pRBBP6+GABA.
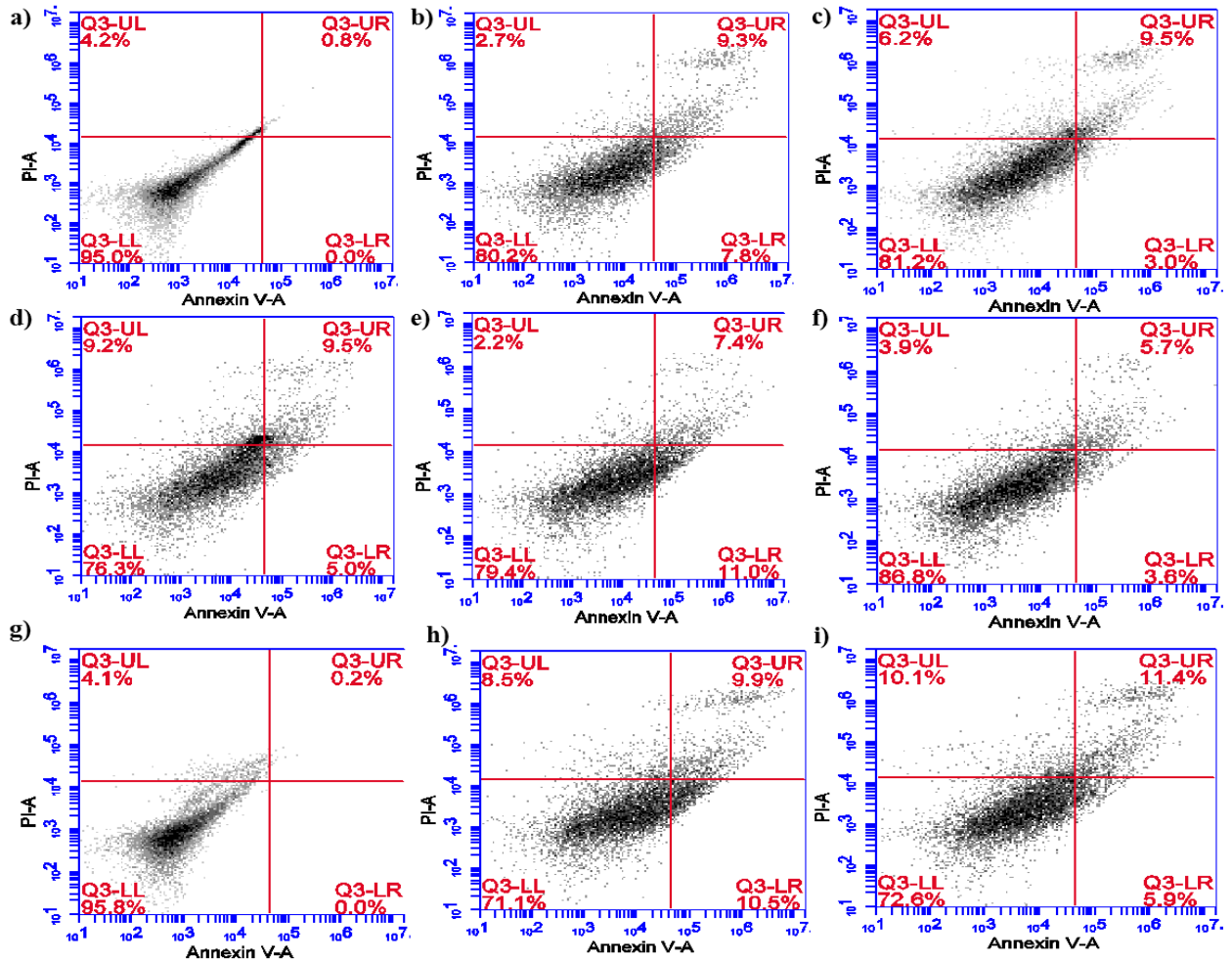
Figure 18: Apoptosis of MRC-5 cells was analyzed using flow cytometer with annexin V/PI staining.

(a) Untreated. (b) Camptothecin, (c) GABA, (d) siRBBP6, (e) siRBBP6+camptothecin, (f) siRBBP6+GABA, (g) pRBBP6, (h) pRBBP6+camptothecin and (i) pRBBP6+GABA.

Microscopic detection of apoptosis

As mentioned in the previous section, externalization of PS, which is a marker of early apoptosis, as well as changes in nuclear morphology, were used to analyse the observed apoptosis. These events were imaged using confocal fluorescence microscopy in the presence of DAPI and annexin V-FITC staining to confirm the event of apoptosis in intact attached cells. In HeLa cells, green fluorescence signal which is indicative of apoptosis induction was observed in both siRBBP6 and camptothecin treatment, whereas combinational treatment further indicated increased apoptosis induction (Figure 19). GABA treatment alone produced a weaker fluorescence signal; however combination with siRBBP6 led to higher fluorescence intensity (Figure 19).

pRBBP6, alone or in combination with camptothecin or GABA, produced weaker fluorescence signal compared to siRBBP6 combination (Figure 19). Changes in cell morphology were observed in treated cells compared to untreated cells, where nuclei appeared to remain intact and round in shape in untreated cell (blue stain) whereas cells undergoing apoptosis had adopted irregular shapes (Figure 19). RBBP6 silencing led to a subtle apoptosis induction in SiHa cells as shown by the weak fluorescence signal; however combination with camptothecin led to a much higher PS externalization and loss in cell morphology (Figure 20). Unlike GABA treatment alone, combination with siRBBP6 produced a visibly strong fluorescence signal coupled with loss of cell shape in SiHa cells (Figure 20). Co-treatment of pRBBP6 with either camptothecin or GABA failed to produce any visible green fluorescence signal in SiHa cells, however cell shrinkage is especially observed in pRBBP6+GABA co-treatment (Figure 20).

HeLa

| Untreated | 0.25 µM Camptothecin (24 hr.) | 100 µM GABA (24 hr.) |

Figure 19: Microscopic analysis of Annexin V stained HeLa cells.

The Annexin V positive cells are indicated by the green dye as a representation of apoptotic cells. It is visible from the staining above that siRBBP6 (panel D), siRBBP6+camptothecin (panel E) and siRBBP6+GABA (panel F) were able to induce apoptosis in HeLa at 10X magnification.

SiHa

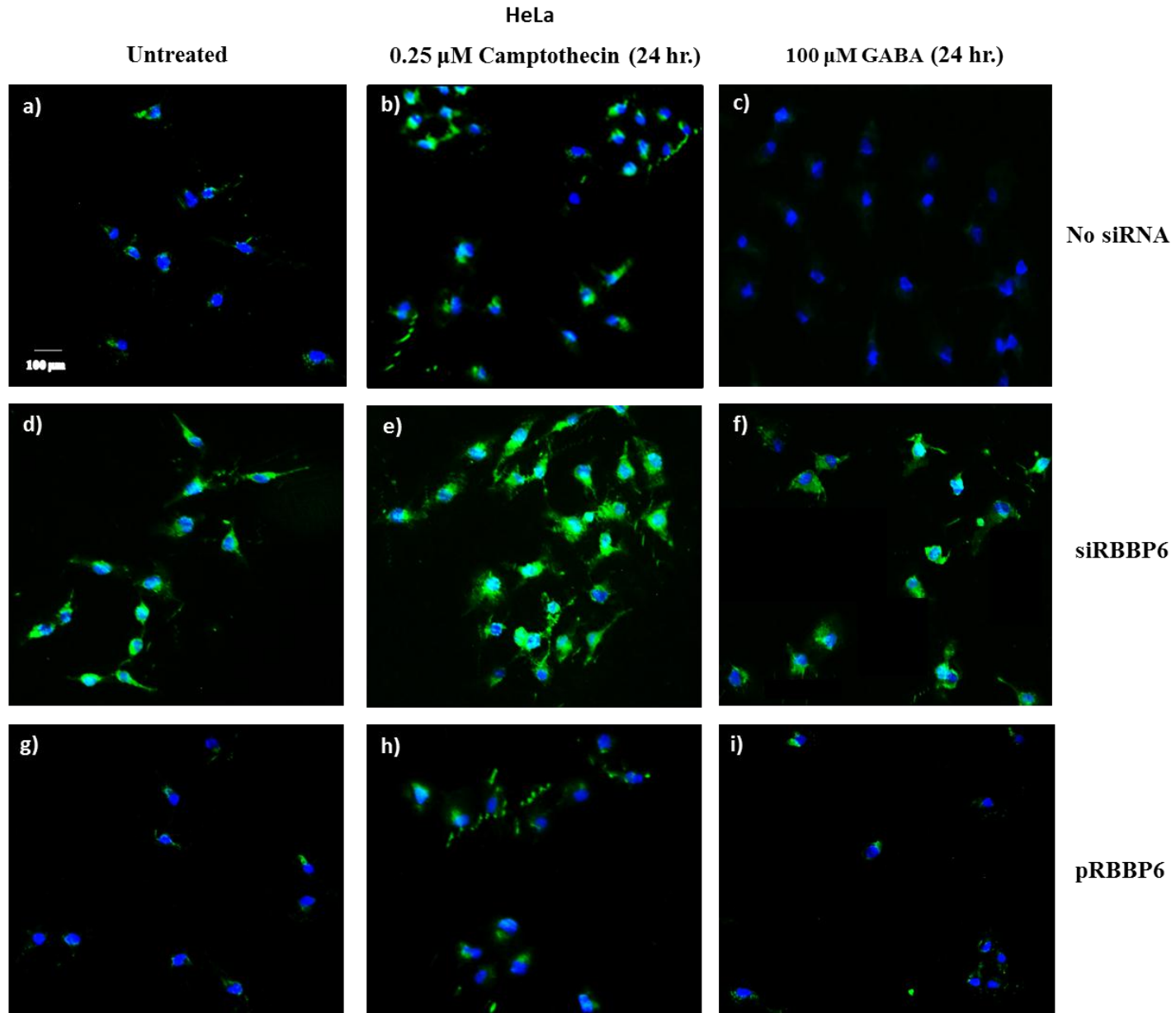| **Untreated** | **0.5 µM Camptothecin (24 hr.)** | **100 µM GABA (24 hr.)** | |
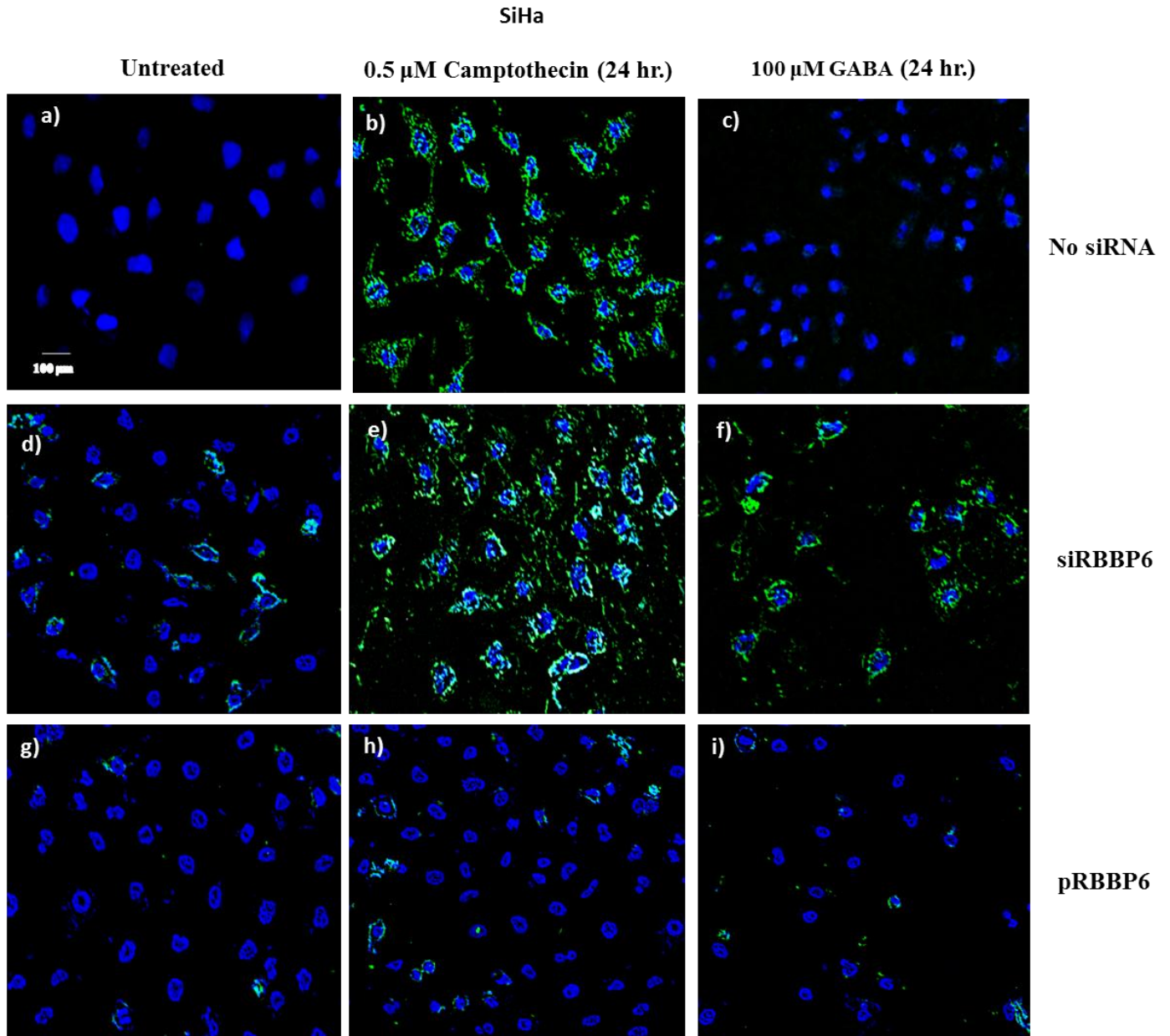
Figure 20: Microscopic analysis of Annexin V stained SiHa cells.

The Annexin V positive cells are indicated by the green dye as a representation of apoptotic cells. It is visible from the staining above that siRBBP6+camptothecin (panel E) and siRBBP6+GABA (panel F) were able to induce apoptosis in SiHa cells at 10X magnification.

4.2.5. Analysis of Caspase activity and mitochondrial ATP

Flow cytometry and fluorescence microscopy confirmed that the observed reduction in cell viability was as a result of apoptosis. In this section, caspase activity and mitochondrial ATP measurement were performed to further confirm the observed apoptosis induction and to determine whether or not the observed apoptosis was as a result of the activation of mitochondrial pathway. Caspase 3/7 activity was significantly increased in both HeLa (4100 RLU) and SiHa (3800 RLU) cells transfected with siRBBP6 in comparison to untreated cells (~400 RLU) (Figure 21). Camptothecin alone or combined with siRBBP6 further increases caspase activity relative to either untreated or siRBBP6 transfected in both HeLa and SiHa cells (Figure 21).

In HeLa cells, co-treatment of siRBBP6 and camptothecin significantly sensitizes the activity of caspase 3/7 to 8200 RLU in comparison to siRBBP6-only (4100 RLU) or camptothecin only (5900 RLU). Caspase activity is sensitized from 3800 RLU in siRBBP6-only to 7200 RLU in siRBBP6+camptothecin in SiHa cells however no significant increase is observed between camptothecin-only and siRBBP6+camptothecin (Figure 21). No significant activation of caspase 3/7 was observed in pRBBP6-only or pRBBP6 combination with either camptothecin or GABA in both HeLa and SiHa cell lines (Figure 21). No significant caspase activity was observed in normal fibroblasts (MRC-5) except in cells transfected with siRBBP6 where a statistically significant caspase activity (308 RLU) was induced (Figure 22).
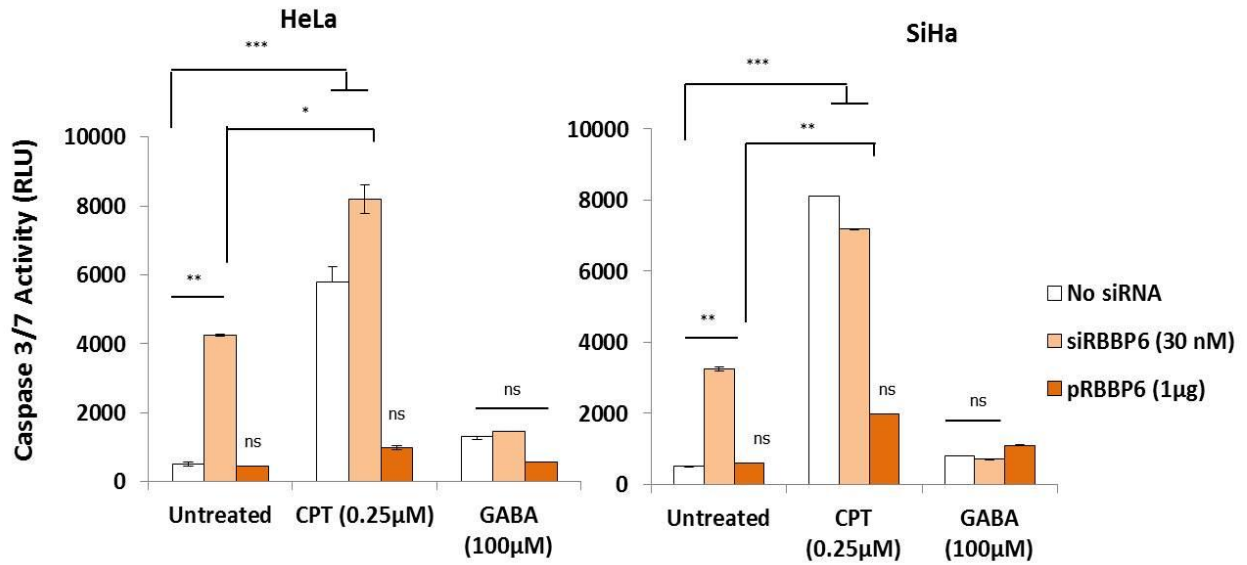
Figure 21: Caspase 3/7 activity analysis of Hela and SiHa cells.

Caspase activity was increased in siRBBP6 treated only and in co-treatment with Camptothecin.
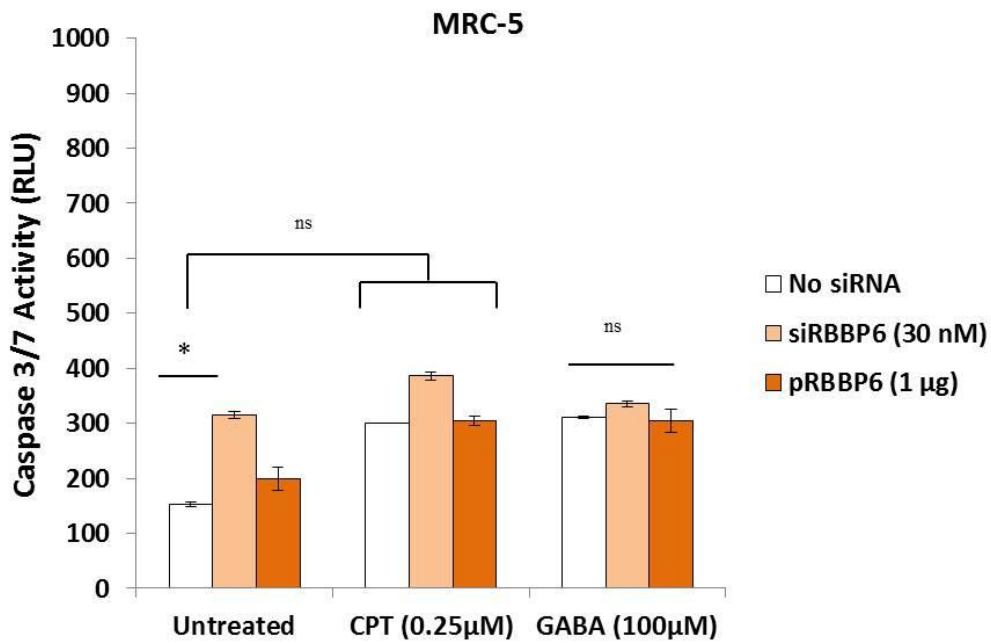
Figure 22: Caspase 3/7 activity analysis of MRC-5 cells.

Mitochondrial ATP depletion is a good indicator of outer membrane permeabilization due to activated pro-apoptotic proteins responsible for the activation of the intrinsic pathway. Following transfection with either siRBBP6 or pRBBP6 for 24 hours, mitochondrial ATP depletion was measured at two hours intervals post co-treatment with either camptothecin or GABA. In both HeLa and SiHa cell lines, six hours after treatment with siRBBP6-only or co-treated with siRBBP6+camptothecin ATP production decreased to below 50 RLU (Figure 23). No ATP depletion was observed in cells co-treated with siRBBP6+GABA in both HeLa and SiHa cells. HeLa and SiHa cells treated with either pRBBP6+camptothecin or pRBBP6+GABA continued to show increase in ATP generation throughout the entire experiment (Figure 23). No ATP depletion was observed in MRC-5 fibroblasts except in camptothecin and siRBBP6+GABA treatments where it decreased for 2 hours before continuing to increase for the remainder of the experiment (Figure 24).
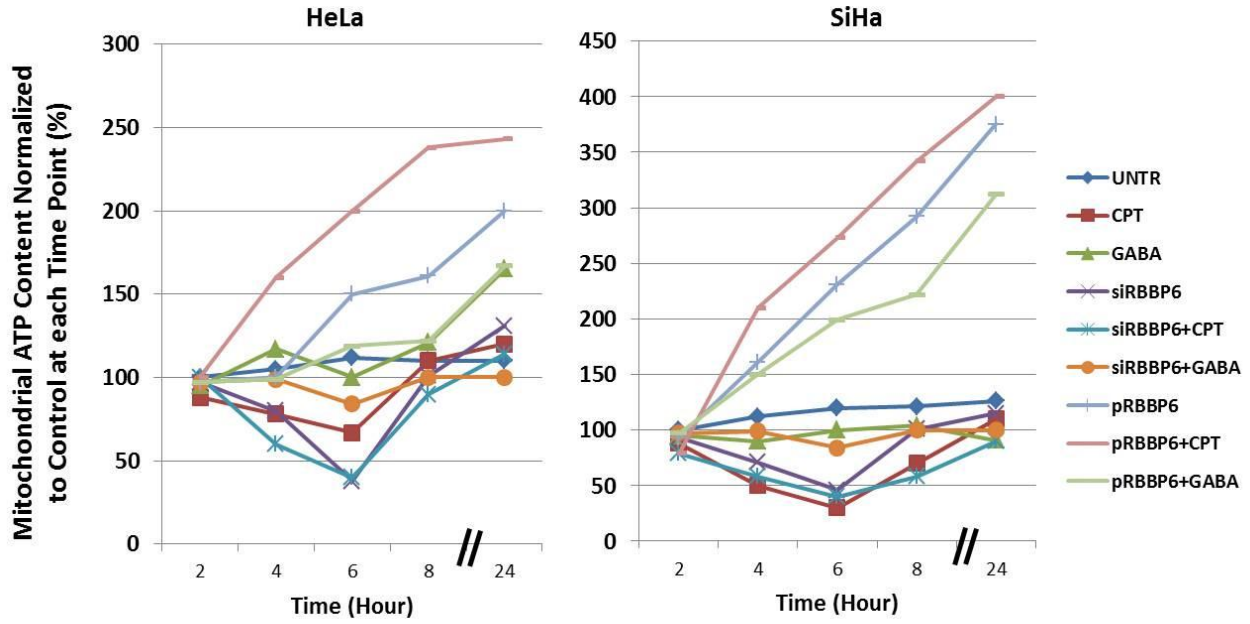
Figure 23: Mitochondrial ATP analysis in HeLa and SiHa cells at different time interval in hours.

The assay was conducted in triplicate and ATP levels were reported at two hour intervals as a mean of

relative light units (RLU) normalized to percentage of control at each time point.
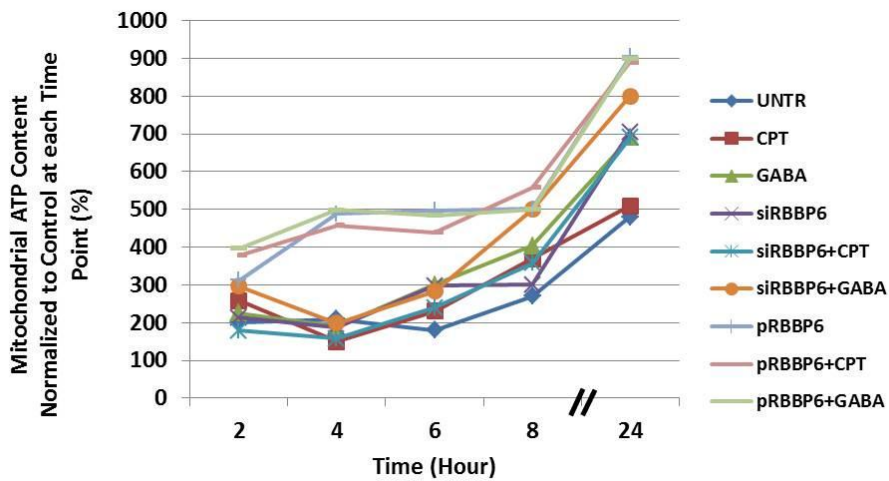
Figure 24: Mitochondrial ATP analysis in MRC-5 cells at different time intervals in hours.

The assay was conducted in triplicates and ATP levels were reported as a mean of relative light units

(RLU) normalized to percentage of control at each time point.

4.2.6. Analysis of cell cycle arrest

The cell cycle is a set of organized and monitored events responsible for proper cell division and proliferation. It is divided into three sequential phases that go from quiescence (G0 phase) to DNA replication (S phase) and then cell division (M phase); with G0/G1 and G2/M sensor mechanisms serving as checkpoints that function to prevent transition to the next phase until the previous one is complete (Khodjakov and Rieder, 2009; Collins *et al.*, 1997). Cell proliferation is an essential mechanism for cell growth and development; however disturbances in regulatory proteins responsible for keeping the cell cycle in check often lead to cancer development (Khodjakov and Rieder, 2009; Collins *et al.*, 1997). During cancer progression cells evade the cell cycle checkpoints and continue to proliferate uncontrollably (Khodjakov and Rieder, 2009; Collins *et al.*, 1997). In this section we analyze the induction of cell cycle arrest following treatment with siRBBP6+camptothecin, siRBBP6+GABA, pRBBP6+camptothecin and pRBBP6+GABA by measuring DNA content at G0/G1, G2/M checkpoints and the S-phase using flow cytometry.

Camptothecin and GABA significantly induced G0/G1 cell cycle arrest in HeLa cells by 68.65% and 61.65%, respectively (Figure 25). Percentage of cells in the S-phase decreases in camptothecin (9.75%) and GABA (9.5%) relative to untreated (14.8%). G2/M arrest in untreated cells was at 17.9%, however following treatment with camptothecin it decreased slightly to 13.5% while in GABA remained the same at 16.55%. RBBP6 gene silencing alone or in combination with either camptothecin or GABA led to a significant induction of cell cycle arrest at the G0/G1 checkpoint in both HeLa and SiHa cells (Figure 25). In HeLa cells, siRBBP6 induced 67.25% G0/G1 arrest whereas pRBBP6 induced about 48.65%. In HeLa cells treated

with siRBBP6+camptothecin and siRBBP6+GABA, G0/G1 populations remained unchanged at 67.9% and 59.55% respectively. The opposite was true in pRBBP6+camptothecin and pRBBP6+GABA were populations in G0/G1 arrest were reduced to 37.25% and 38.1% in HeLa. The percentage of HeLa cells undergoing DNA replication in the S-phase was slightly reduced in siRBBP6 at 10.85%, 8.25% in siRBBP6+camptothecin and 6.7% in siRBBP6+GABA relative to untreated cells which recorded 14.8% (Figure 25). The cells in the S-phase were increased from 14.8% in untreated to 30.9% in pRBBP6, 21.25% in pRBBP6+camptothecin and 27.6% in pRBBP6+GABA treatments (Figure 25). For G2/M, there was no significant change in cell populations following treatment will all anticancer agents and transfections. However, there was a slight reduction of cells in G2/M arrest from 20.15% in siRBBP6-only to 16.2% in siRBBP6+camptothecin and to 15.75% in siRBBP6+GABA (Figure 25). Similar trend was also recorded in pRBBP6 treatments where cells in G2/M arrest were reduced from 18.75% in pRBBP6-only to 13.15% in pRBBP6+camptothecin. However there was an increase in cell percentage to 22.9% in pRBBP6+GABA (Figure 25).

In SiHa cell line, camptothecin and GABA induced a significant amount of populations in G0/G1 cell cycle arrest by 66.3% and 58.5%, respectively (Figure 25). Percentage of cells in the S-phase remained unchanged in camptothecin (3.55%) and slightly increased in GABA (9.45%) treatment relative to untreated (4.4%). Cells in G2/M arrest in camptothecin treated cells increased slightly from 26.45% in untreated to 30.35% and no significant change in GABA (27.1%). G0/G1 arrest of SiHa cells showed no significant change in siRBBP6+camptothecin at 69% and siRBBP6+GABA at 66.8% relative to siRBBP6-only which was 70.25% (Figure 25). Treatment with pRBBP6, pRBBP6+camptothecin and pRBBP6+GABA induced G0/G1 arrest of

48.65%, 37.25% and 38.1%, respectively of SiHa cells (Figure 25). Populations in the S-phase remained lower at 4.4% in untreated, 3.55% in camptothecin, 9.44% in GABA, 4.55% in siRBBP6, 3.75% in siRBBP6+camptothecin and 7.2% in siRBBP6+GABA, however a significant increase was observed in pRBBP6 at 17.4%, 16.5% in pRBBP6+camptothecin and 14.6% in pRBBP6+GABA (Figure 25). Cells in G2/M arrest remained almost unchanged across all treatments, i.e. untreated (26.45%), camptothecin (30.35%), GABA (27.1%), siRBBP6 (24.6%), siRBBP6+camptothecin (25.8%), siRBBP6+GABA (18.9%), pRBBP6 (18.75%), pRBBP6+camptothecin (13.15%) and pRBBP6+GABA (22.9%) (Figure 25).
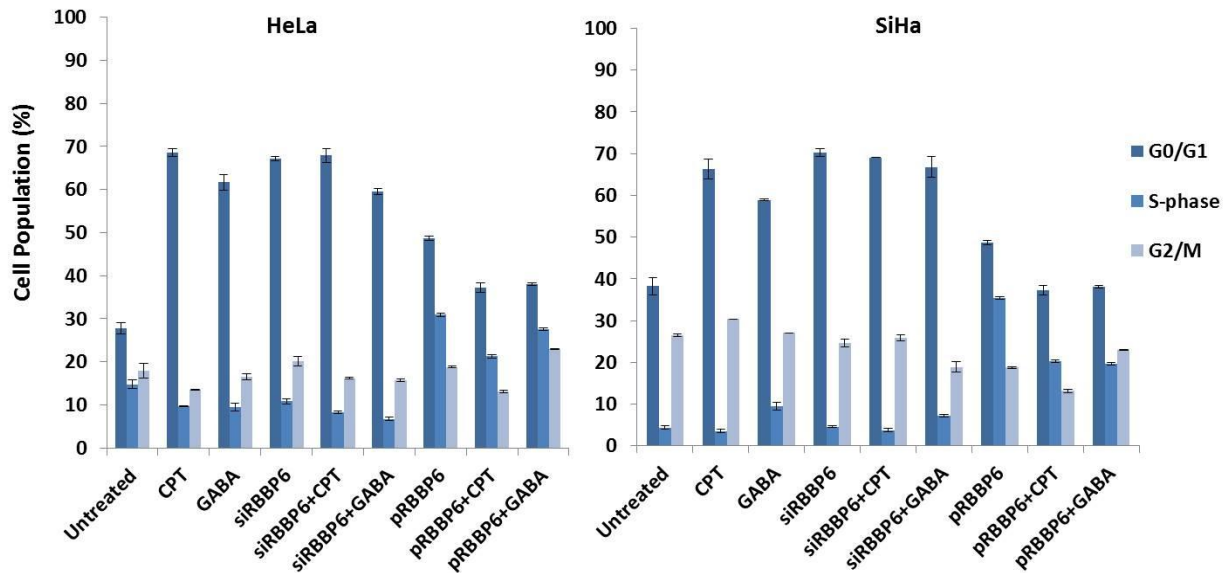
Figure 25: Cell cycle analysis in HeLa, SiHa cell lines. Note increase in S-phase in pRBBP6 cells.

In normal lung fibroblasts (MRC5) induction of G0/G1 arrest was observed however there was no significant change following different treatments (Figure 26). Cells in G0/G1 arrest was recorded as 53.85% in untreated, 59.55% in camptothecin, 50.2% in GABA, 50.55% in siRBBP6, 55.35% in siRBBP6+camptothecin, 50.95% in siRBBP6+GABA, 55.5% in pRBBP6,

57.75% in pRBBP6+camptothecin and 57.6% in pRBBP6+GABA. Slight changes were observed in cell populations in the S-phase with camptothecin treatment at 19.55% in comparison to untreated at 13.85%. There was a decrease of cell populations, however to 10.2% in GABA, 10.55% in siRBBP6 and 10.95% in siRBBP6+GABA. The cells in S-phase were increased to 15.4% in pRBBP6, 17.75% in pRBBP6+camptothecin and 16% in pRBBP6+GABA. G2/M arrest populations remained around 30% across all treatments, with 32.75% in untreated, 33.55% in camptothecin, 25.75% in GABA, 37.8% in siRBBP6, 38.75% in siRBBP6+camptothecin, 38.7% in siRBBP6+GABA, 38.65% in pRBBP6, 27.25% in pRBBP6+camptothecin and 28.1% in pRBBP6+GABA (Figure 26).
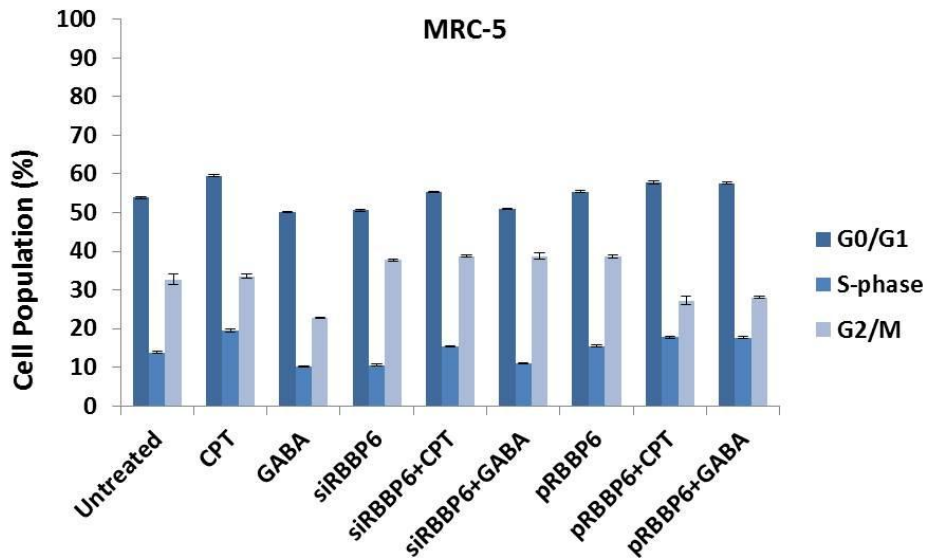
Figure 26: Cell cycle analysis in MRC-5 cell line.

4.2.7. Quantification of genes involved in apoptosis

The apoptotic phenotypes observed by microscopic detection in treated cells prompted us to evaluate if there were any possible changes in the gene expression at mRNA level that might be involved in inducing apoptosis that was observed in our study. qPCR was used to analyse mRNA expression levels of both pro- and anti-apoptotic genes in response to RBBP6 gene silencing and overexpression. Key regulatory genes that were quantified include the transcription factor TP53, which is involved in cell cycle and apoptosis, pro-apoptotic bax, bak1, bad, caspase-3 and caspase-8 and the anti-apoptotic Bcl-2. Relative gene expression was calculated using REST 2009 software (version 2.0.13) which computes expression ratios based on real time PCR efficiency and delta crossing points (ΔCP) of unknown samples versus a untreated control.

As expected, RBBP6 was down-regulated by a mean factor of 0.095 following silencing of HeLa cells with 30nM siRNA. Whereas transfection with 1µg pRBBP6 led to the up-regulation of RBBP6 by a mean factor of 16.000 (Figure 27). TP53 expression was significantly increased by a mean factor of 42.518 in response to RBBP6 silencing while the expression of pro-apoptotic genes was observed to be increased following silencing of RBBP6. That is, bax was up-regulated by a mean factor of 6.727, bad by 2.099 and caspase-3 by 7.037; however change in the expression of bak1 was not significant. The death receptor pathway-associated caspase-8 was down-regulated by a mean factor of 0.428 following RBBP6 silencing as well as the pro-survival Bcl-2 at 0.402. Overexpression of RBBP6 (16.000) had no significant impact on TP53 expression including bax, bak1, bad and bcl-2. It was however interesting to notice change in the expression of caspases in response to RBBP6 overexpression where caspase-3 was up-regulated by a mean factor of 1.414 whereas caspase-8 was down-regulated by 0.250. Treatment with

camptothecin led to the up-regulation of bak1, bad and caspase-3 by mean factors of 3.482, 4.691 and 6.916, respectively, whereas other genes were not significantly changed. GABA resulted in no significant change in the expression of RBBP6 including the apoptosis regulatory genes TP53, bax, bak1, bad, caspase-3, caspase-8 and bcl-2. Regulation of apoptotic genes in cells treated with GABA remained unchanged, with the exception being the death receptor associated caspase 8 (1.5-fold increase) and the effector caspase-3 (3-fold increase).

Combination of siRBBP6 with camptothecin increased the overexpression of TP53 synergistically by a mean factor of 75.061 (Figure 28). Bax expression increased from a ratio of 6 in silencing-only to a 20-fold expression in camptothecin+siRBBP6 combination. The mitochondria-associated bad mRNA level was increased by a 4-fold change with a mean factor of 5.296; however bak1 did not change relative to its control group (Figure 27). Effector caspase-3 mRNA increased additively by a mean factor of 14.723 in response to siRBBP6+camptothecin co-treatment as compared to camptothecin- or silencing-only treatments. Caspase-8, which remained relatively unchanged in camptothecin treatment and slightly reduced in RBBP6 silencing, was significantly increased by a mean factor of 10.126. Unlike in silencing- and camptothecin-only where Bcl-2 was down-regulated, this mRNA increased by atleast 2-folds when the two treatments were combined. GABA treatment in the presence of RBBP6 silencing led to no change in bax, bak1, bad, caspase-3 and -8 and a slight decrease in Bcl-2 expression by a mean factor of 3. Furthermore, combination with RBBP6 overexpression led to a slight down-regulation of TP53 and no significant changes in other genes.
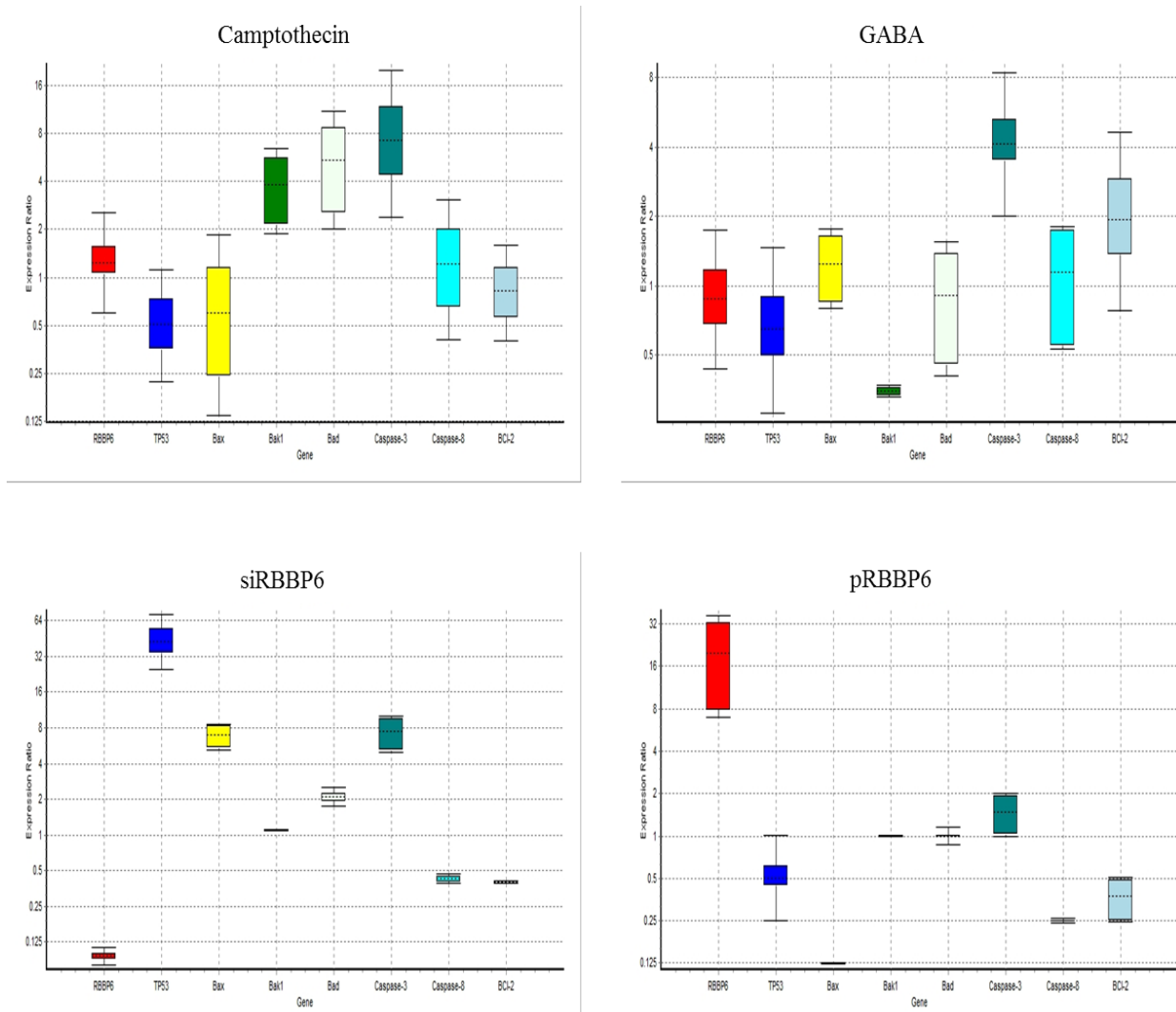
Figure 27: Relative quantification of gene expression in HeLa cell line was performed using qPCR. Whisker box plots with expression ratio on the y-axis and gene type on the x-axis indicate quantification of mRNA isolated from cells treated with camptothecin (top left), GABA (top right), siRBBP6 (bottom left) and pRBBP6 (bottom right). Gene expression is depicted by the colour coded whisker bars in the following manner: RBBP6 (red), TP53 (blue), bax (yellow), bak1 (light green), bad (grey), caspase-3 (dark green), caspase-8 (cyan) and Bcl-2 (light blue).

Figure 28: Relative quantification of gene expression in HeLa cell line was performed using qPCR. The cells were treated with siRBBP6+camptothecin (top left), siRBBP6+GABA (top right), pRBBP6+camptothecin (bottom left) and pRBBP6+GABA (bottom right).

In SiHa cell line, treatment with camptothecin had no significant impact on the expression of RBBP6 and TP53, whereas expression of bax changed over 32-folds (Figure 29). Bak1 increased by a factor of 9.849 and caspase-3 by 6.916 and no significant change was observed in the expression of caspase-8 and Bcl-2 following treatment with camptothecin. GABA treatment had no effect on the expression of RBBP6, TP53, bad and Bcl-2 genes. However; the mitochondria-

associated bax increased slightly by mean factor of 3.891 and caspase-8 by 31.233, whereas bak1 was down-regulated by a factor of 0.015. RBBP6 silencing led to a 2-fold increase in the expression of TP53 coupled with bax up-regulation by a factor of about 16. Similarly, caspase-3 expression increased by 4-folds and caspase-8 by ~13-folds following RBBP6 silencing, whereas bak1 expression decreased by a factor of less than 0.001. The change in the expression of bad and Bcl-2 was however insignificant. RBBP6 over-expressing cells led to a slight decrease in TP53 expression (0.63-fold change) and bax (0.125-fold change) whereas bak1 remained unchanged. Similarly, RBBP6 overexpression decreased bad by a mean factor of 0.500, caspase-3 by 0.044 and caspase-8 by 0.500; however Bcl-2 was increased by a mean factor of 1.414.

Combination of silencing with camptothecin led to a decrease in RBBP6 expression by a mean factor of 0.095 (Figure 30). TP53, bax, bad and caspase-3 were up-regulated by mean factors 42.518, 6.727, 2.099 and 7.037; however change in bak1 expression was insignificant. Caspase-8 and Bcl-2 were down-regulated by mean factors 0.428 and 0.402 in response to co-treatment with siRBBP6+camptothecin. Co-treatment with siRBBP6 and GABA decreased the expression of RBBP6 by a mean factor of 0.044 coupled with TP53 up-regulation by a mean factor of 2.828 and no significant effect on the expression of bax, caspase-3 and caspase-8. (Figure 30). Bak1, Bad and Bcl-2 were down-regulated by 0.500, 0.250 and 0.435 mean factors, respectively, following co-treatment with siRBBP6+GABA. Treatment with camptothecin in RBBP6 overexpressing cells had no additive effect on the expression of RBBP6 (Figure 30). TP53 and Bax were down-regulated by mean factors 0.250 and 0.105, respectively and Bak1 expression did not change significantly. Bad and Bcl-2 were down-regulated by mean factors 0.049 and 0.319; caspase-3 and caspase-8 up-regulated by 2.828 and 2.828, respectively.

Combination of pRBBP6 with GABA increased the expression of RBBP6 by 69.071 and down-regulated TP53 by 0.429. Bax, bak1 and Bcl-2 remained relatively unchanged whereas bad, caspase-3 and caspase-8 were down-regulated by 0.098, 0.250 and 0.500, respectively.

Figure 29: Relative quantification of gene expression in SiHa cell line was performed using qPCR. The cells treated with camptothecin (top left), GABA (top right), siRBBP6 (bottom left) and pRBBP6 (bottom right).
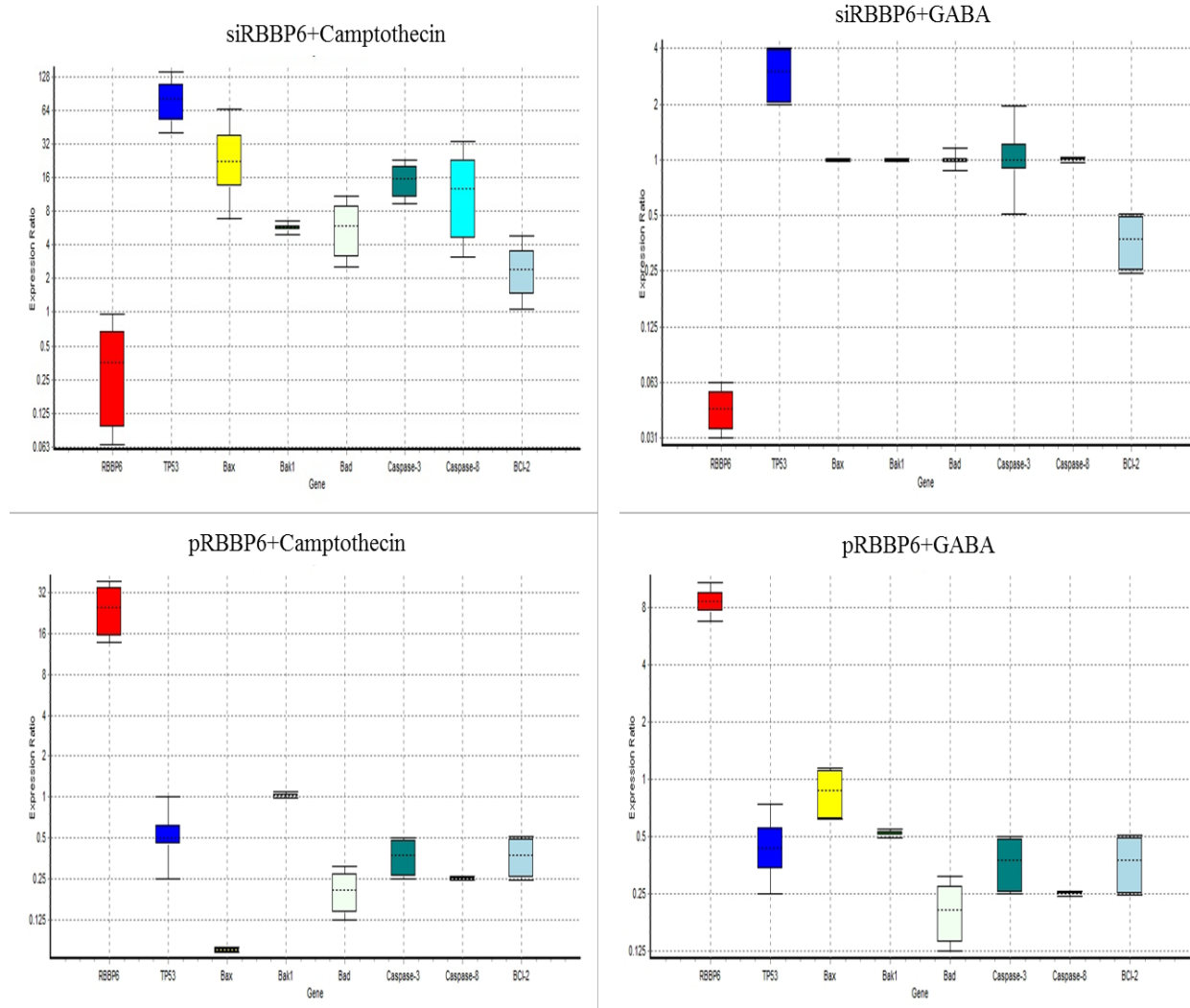
Figure 30: Relative quantification of gene expression in SiHa cell line was performed using qPCR. The cells were treated with siRBBP6+camptothecin (top left), siRBBP6+GABA (top right), pRBBP6+camptothecin (bottom left) and pRBBP6+GABA (bottom right).

No significant changes were observed in gene expression following treatment with camptothecin and GABA in normal lung fibroblasts (Figure 31). RBBP6 silencing (0.518) led to an increase in caspase-8 expression by 1.414 and had no significant effect on the expression of other genes. Similarly, no significant changes were observed in the expression of all genes in response to RBBP6 up-regulation (2.462). siRBBP6+camptothecin reduced RBBP6 expression by a mean factor of 0.154, whilst siRBBP6+GABA reduced RBBP6 and bak1 by 0.330 and 0.500, respectively. Furthermore, siRBBP6+GABA increased gene expression of bax by 1.414 (Figure

32). RBBP6 was up-regulated by a mean factor of 5.856 in response to co-treatment with pRBBP6+camptothecin, and no significant effect was observed on the expression of other genes. Co-treatment with pRBBP6+GABA increased RBBP6 expression by a mean factor of 3.053 and bax by a mean factor of 1.809.
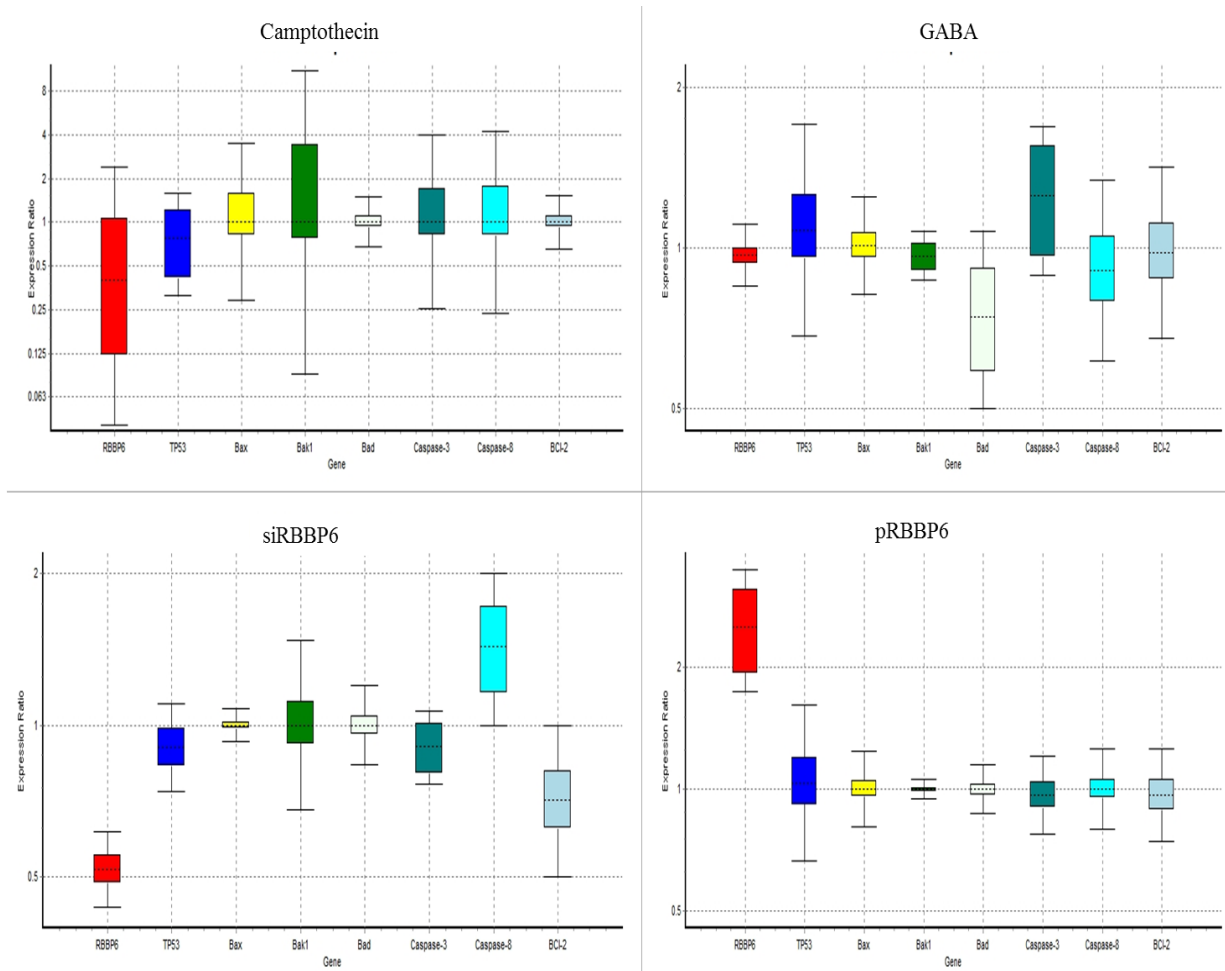
Figure 31: Relative quantification of gene expression in MRC-5 cell line was performed using qPCR. The cells were treated with camptothecin (top left), GABA (top right), siRBBP6 (bottom left) and pRBBP6 (bottom right).
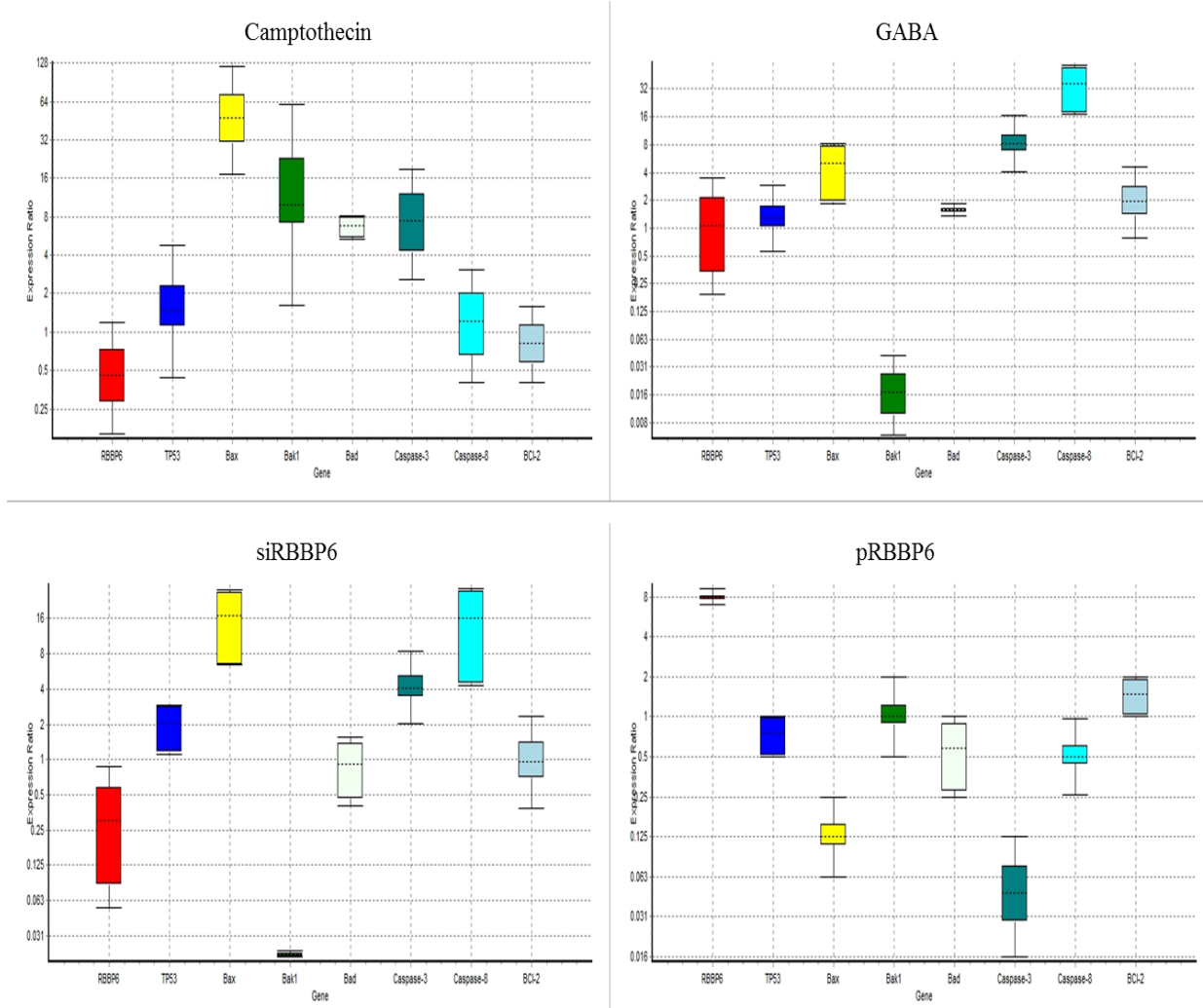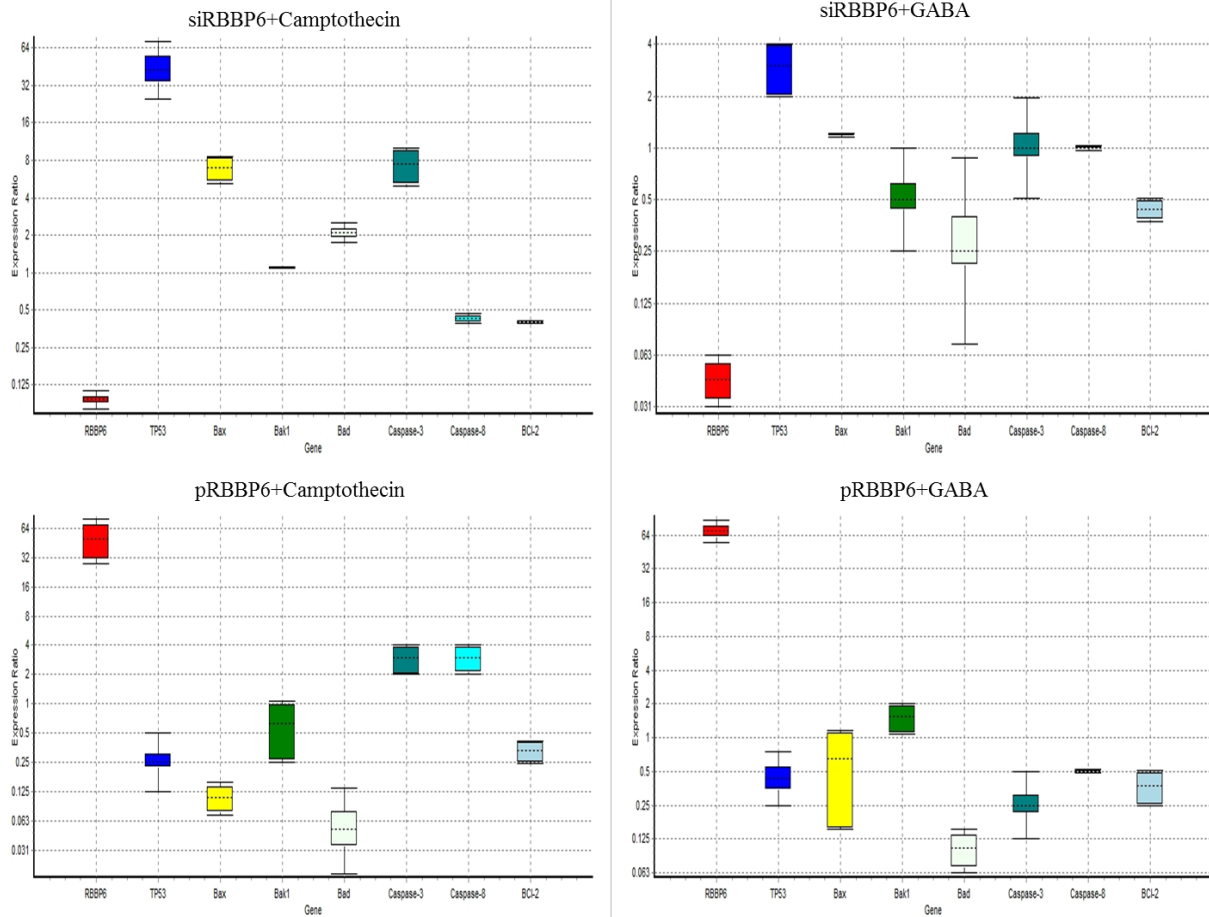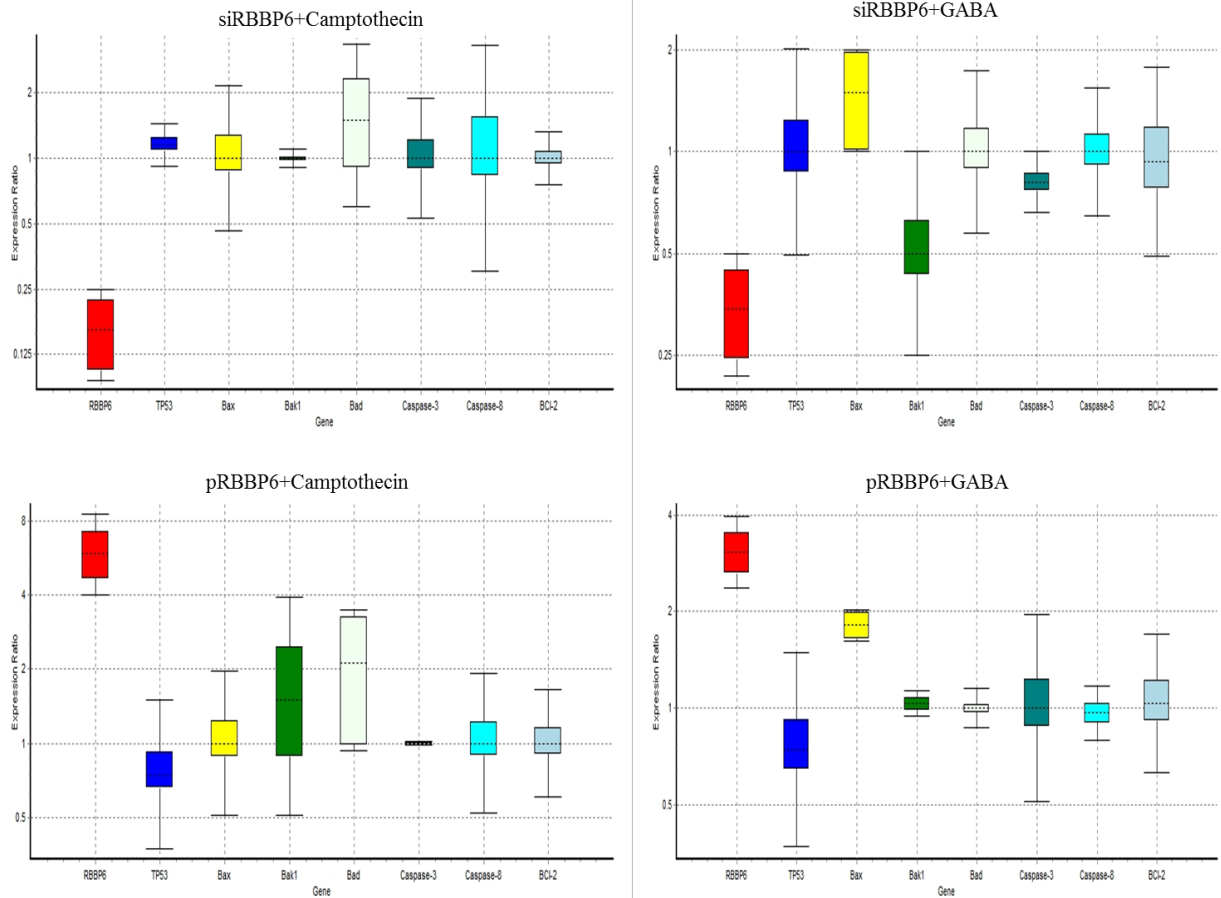
Figure 32: Relative quantification of gene expression in MRC-5 cell line was performed using qPCR. The cells were treated with siRBBP6+camptothecin (top left), siRBBP6+GABA (top right), pRBBP6+camptothecin (bottom left) and pRBBP6+GABA (bottom right).

4.2.8. RBBP6 and TP53 protein expression: analysis of the relationship

The relationship between RBBP6, MDM2 and TP53 in cancer development continues to be the focus for the betterment of cancer drug discovery (Pugh *et al*., 2006). Having shown at the mRNA level that RBBP6 silencing up-regulation was successful, it was necessary to further confirm the success at a protein level because in most cases mRNA is later translated by the cell machinery into functional protein and to analyze the effect of RBBP6 on MDM2 and TP53. In this study, western blotting was employed to analyze protein expression following RBBP6 silencing and up-regulation in combination with camptothecin and GABA treatments. Briefly, cells were transfected for 48 hours with siRBBP6 and pRBBP6 and then treated with either camptothecin or GABA for a further 24 hours, followed by whole cell protein extraction and analysis.

In HeLa cell line MDM2 expression remained unchanged across all treatments in comparison to untreated, and RBBP6 silencing led to the up-regulation of TP53 on its own and in combination with camptothecin treatment (Figure 33). No significant change was observed in TP53 expression between GABA-only and GABA+siRBBP6, however both treatment up-regulated the protein in comparison to untreated. RBBP6 over-expression was especially high in pRBBP6+camptothecin and pRBBP6+GABA co-treatments and TP53 expression showed to decrease in these treatments. In SiHa cell line, the observation suggests that RBBP6 up-regulation or silencing did not have a significant effect on the expression of MDM2, however silencing alone or in combination with GABA led to the up-regulation of TP53 especially in siRBBP6+camptothecin co-treatment as well as in GABA treatment. RBBP6 overexpression led to a decrease in the expression of TP53, on its own or in combination with both GABA and camptothecin.
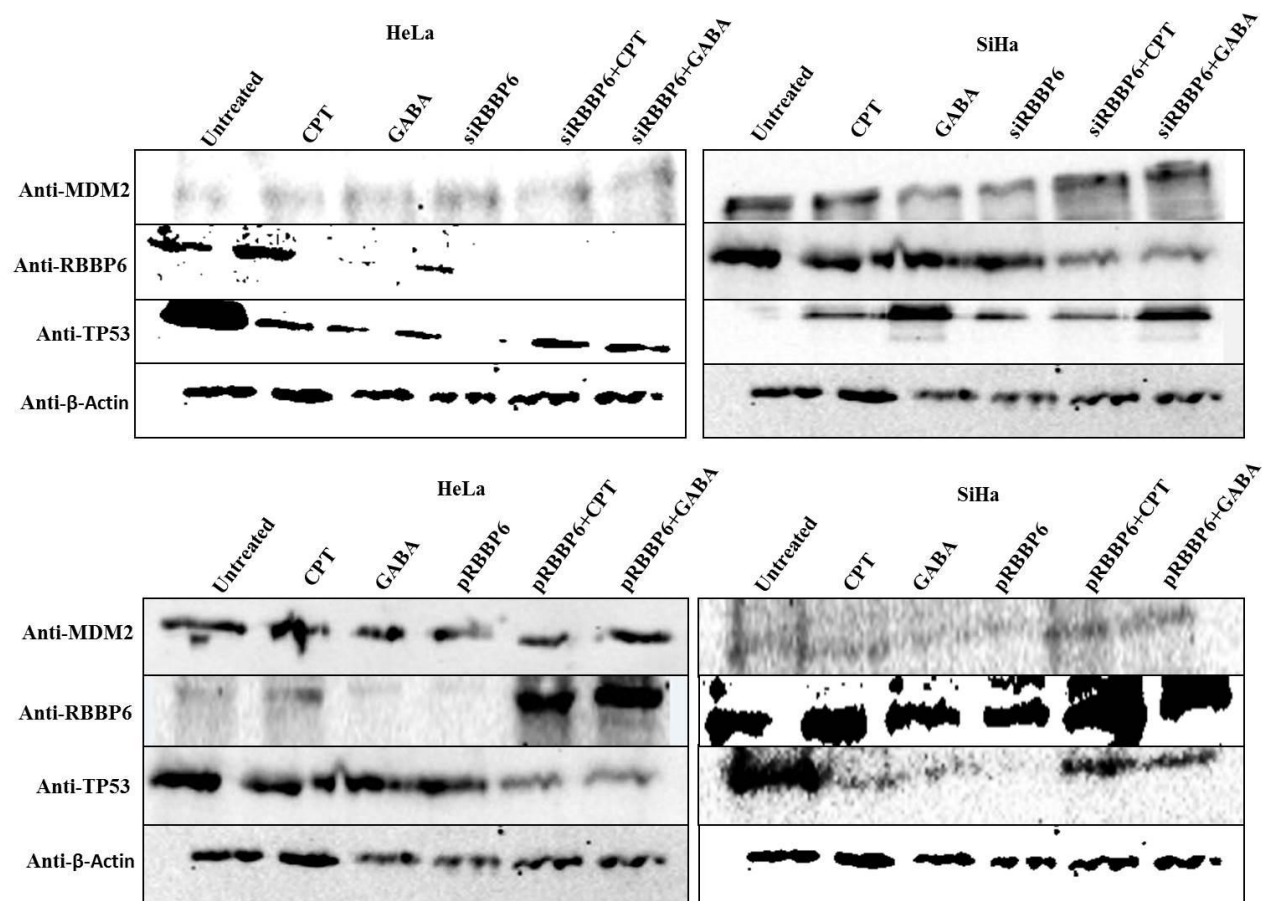
Figure 33: Western blot analysis of HeLa cells treated with pRBBP6, GABA and Camptothecin.

The electrophoretogram suggests down-regulation of RBBP6 results in upregulation of TP53 while MDM2 remains unchanged.

4.3. Discussion

RBBP6 localization is widespread in a number of squamous cell carcinomas including those of the lung, colon and esophagus; suggesting that the protein may serve as an important diagnostic marker and a promising target for the treatment of cancer. In this manuscript we describe how overexpression and silencing of RBBP6 combined with potential anticancer agents like GABA and camptothecin sensitize cervical cancer cells to apoptosis. These are the first result that attempt to characterize the role of RBBP6 in cancer development and progression.

It is generally known that the main physiological mechanism by which cancer cells can be successfully targeted and eradicated is by induction of apoptosis or cell cycle arrest, yet the precise mechanisms behind failed apoptosis induction in cancer cells is still unclear (Motadi *et al*., 2007; Bianco *et al*., 2005; Zhang *et al*., 2004). In cervical cancer, HPV is said to inactivate TP53 through E6 ligase resulting in cancer progression (Ajay *et al*., 2012). However, it remains unclear which other non-viral genes assist to degrade TP53. It is also known that RBBP6 has both TP53 and Rb binding domains together with DWNN and RING finger domains, which are associated with protein degradative function. In order to clarify the role of RBBP6 in cervical cancer, immunolabelling staining was performed on paraffin fixed tissues of which the results are shown above (Figure 6). Expression of RBBP6 was observed in all cancers from non-keratinized to keratinized and from adenocarcinoma to squamous carcinoma. The result further showed less expression in RBBP6 in the poorly-differentiated tumours as compared to well-differentiated tissues suggesting that RBBP6 expression is higher in proliferating cells; which further supports our hypothesis that it is a cell proliferating gene (Figure 6). Similar results were

reported in our earlier research where we observed overexpression of RBBP6 in lung cancer and by Chen *et al*. (2013) who also reported over expression of RBBP6 in colon cancer.

RBBP6 manipulation was successful in both silencing and up-regulation in which we reported a knockdown of up to 75% while up-regulation was increased to 90% (Figure 7). This was further confirmed by immunocytochemistry where GFP-expressing cells which are directly proportional to RBBP6 overexpression were identified (Figure 8 and Figure 9). The up-regulation of RBBP6 is the first study to ever be conducted while silencing has been done before in other laboratories including ours on MCF-7 breast cancer cell line (Moela *et al.,* 2014. Silencing RBBP6 halted both HeLa and SiHa cell progression as shown by xCelligence (Figure 10 and Figure 11). All cancer cells treated with siRBBP6 24 hour post treatment showed reduced cell index, which is an indicator of live cells that are attached to the surface of the plate and proliferating. However, the same was not true for cells that had RBBP6 up-regulation since they continued to show a steady increase in cell index (Figure 12). Interestingly MRC-5 treated with pRBBP6 had a significant increase in cell index suggesting that RBBP6 might be causing them to proliferate. The results suggest a role for RBBP6 in cancer progression and present a potential biomarker.

We then went on to combine RBBP6 targeting with chemotherapeutic agents, camptothecin and GABA. GABA is a major inhibitory neurotransmitter in adult central nervous system and has been shown to inhibit tumour cell migration in liver cancer cells through binding to $GABA_B$ receptors (Chen *et al*., 2013; Young and Bordey, 2009; Zhang *et al*., 2004; Nemana *et al*., 2013). Activation of these receptors has been linked to inhibition of an enzyme called adenylate cyclase

which plays a role in promoting cell migration via the protein kinase A signaling cascade (Chen *et al*., 2013; Young and Bordey, 2009; Zhang *et al*., 2004; Nemana *et al*., 2013). Camptothecin is a plant-derived natural compound that shows high potency in inducing apoptosis by interacting with DNA strands breaks thus preventing the function of topoisomerase I during replication (Liu, 2000). Treatment with camptothecin and siRBBP6+camptothecin halted cell proliferation while cells treated with GABA continued to proliferate in a similar manner to those treated with pRBBP6 and pRBBP6+GABA. These results indicate that siRBBP6 together with camptothecin were able to prevent cell proliferation in cancer cells but did not have any impact on MRC-5 cell lines. These types of results are similar to those reported earlier in which similar trends were observed in mouse cells (Pugh *et al*., 2006). GABA on the other hand does not seem to play much role in cell growth inhibition in cancer cells.

In tumours expressing inactive TP53, inhibition of RBBP6/MDM2 has been proposed as biomarker for restoration of p53 activity (Basset-Seguin *et al*., 2008; Boatright and Salvesen, 2003). In this study we combine silencing/up-regulation of RBBP6 together with camptothecin and GABA to evaluate their potential to sensitize wild-type TP53-expressing cervical cancer cell line to apoptosis-induced cell death. Through flow cytometry we observed increased apoptosis rather than necrosis in cancer cells treated with siRBBP6 and those treated with siRBBP6+ camptothecin (Figure 14). However, GABA alone did not induce a significant amount of cell death but in combination with siRBBP6 it showed a slight reduction in apoptosis perhaps playing antagonistic role for siRBBP6. Similar results were previously reported in MCF-7 treated with camptothecin and siRBBP6.

pRBBP6, in combination or alone, induced minimum amount of apoptosis suggesting that it might be promoting cancer progression. Microscopic analysis showed disruption of nucleus and shrinkage of cells treated with siRBBP6, siRBBP6+camptothecin, siRBBP6+GABA and GABA confirming that the cell death identified in cell index and flow cytometry was as a results of apoptosis induction rather than necrosis (Figure 19 and Figure 20). And that the observations by xCELLigence assay that suggested little cell growth inhibition by GABA might be because some of the cells were caught in between stages especially early apoptosis. Of note is the 0.1 % apoptosis induction in MRC-5 cells treated with pRBBP6, which correlates with the observed increased cell index in xCELLigence assay thus suggesting and supporting our hypothesis that indeed RBBP6 promotes proliferation. The proliferation potential of RBBP6 was also shown in a study by Miotto *et al*. (2014) in which cancer progression was associated with spontaneous breakage of CFSs (common fragile sites); which are otherwise stabilized by un-deregulated RBBP6.

The caspases are a family of cysteine proteases that are the main effectors of apoptosis or programmed cell death (PCD) and their activation leads to characteristic morphological changes of the cell such as shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing (Choene and Motadi, 2012). All this characteristics were identified using microscopy following treatment with siRBBP6, siRBBP6+camptothecin, siRBBP6+GABA and to a lesser extend in GABA. Further analysis of caspase activity also confirmed that caspase3/7 was increased in siRBBP6 and siRBBP6+camptothecin treated cells. However, the same was not true in GABA treated or combinational treatment of GABA+siRBBP6 (Figure 21). Up-regulation of RBBP6 did not result in any significant increase in caspase activity. These types of

results were also reported in a study by Moela *et al*. (2014) in which camptothecin was reported to increase apoptosis in MCF-7 cancer cell line. In general, the overall apoptosis detection experiments support the idea that silencing RBBP6 in cancer cells and co-treating with camptothecin halt cell proliferation by redirecting them via apoptosis.

A central checkpoint of apoptosis is the activation of Caspase-9 by mitochondrial cytochrome c. The pro-survival proteins appear to protect mitochondria, since Bcl-2 prevents the release of cytochrome c (Hansen *et al*., 2015) Mitochondria are also a place for ATP synthesis that is also required for induction of apoptosis (Elmore, 2007). Decrease in mitochondrial ATP activity might mean that more and more ATP is utilized by cells to induce apoptosis. These are some of the results we observed in siRBBP6 and siRBBP6+camptothecin treated cells after 6 hours of treatment where ATP was reduced. This results correlate with all other results that we reported in this study that have shown siRBBP6 and siRBBP6+camptothecin being responsible for cell death by apoptosis (Figure 23). pRBBP6 alone or in combination with GABA did not show any positive impact on cancer cell line except to keep cells proliferating. All these hallmarks of apoptosis were supported by an increase in bax, caspase-3 and caspase-8 gene expression in all cancer cells treated with siRBBP6 and siRBBP6+camptothecin whereas the Bcl-2 anti-apoptotic gene remained unchanged thus resulting in an increased bax/bcl2 ratio. However, mitochondrial activity assay suggested that the apoptotic pathway induced was not entirely dependent on mitochondria since bak1 remained unchanged. Bax oligomerises with itself or with bak1 during apoptosis activation via mitochondria and allows it to release cytochrome c (Wan *et al*., 2006). On the other hand TP53 was also increased in all siRBBP6 treatments suggesting that it might

have played a role in translocating bax to the outer mitochondrial membrane in order to initiate intrinsic apoptosis.

Cell cycle control is a fundamental cellular process that governs cellular proliferation. The protein product of CDKN1A, p21, is an important regulatory protein within the cell cycle which is activated by TP53, and when cells over-express p21, the cell cycle is arrested in the G1 and G2 phases (Wan *et al.*, 2006). In this study we have looked at the cell cycle induction following all treatment and what we observed is that siRBBP6 alone was able to induce cell cycle arrest at G0/G1 while in combination with GABA, percentage cell cycle arrest was slightly reduced (Figure 25). In camptothecin-only treatment and siRBBP6+camptothecin combination, most HeLa cells were arrested at G0/G1, which suggested that the cells did not progress through to the cell cycle process but rather redirected to apoptosis. In contrast, SiHa cell populations accumulated in the G2/M phase, which suggested that some of the cells might have escaped apoptosis following treatment and were instead directed to undergo what looks like mitotic apoptosis. GABA and camptothecin alone did induce cell cycle arrest in all cell lines except in MRC-5 but at a lower percentage. pRBBP6 showed an increase in S-phase which further suggested that DNA replication was increased and cells were allowed to proliferate. These results suggest that cell cycle arrest was not the preferred mechanism of anti-proliferation in this experiment but rather apoptosis. And further suggest that GABA, either alone or in combination with siRBBP6, has little or no impact on cancer progression whereas camptothecin and siRBBP6 play a critical role in halting proliferation of cervical cancer cell lines (Figure 26).

4.4.   Summary

Taken all together, we discovered that RBBP6 is markedly up-regulated in squamous cervical carcinomas, including the less prevalent adenocarcinomas. And further investigations revealed that not only does it play a role in promoting cell proliferation, it is also associated with apoptosis. The fact that RBBP6-knockdown cells become sensitized to apoptosis induced by camptothecin coupled with cell cycle arrest implicates the gene as a potential target in cervical cancer management. The additive effect observed between siRBBP6 and camptothecin could be attributed to the mechanism of action of each. That is, it is well known that camptothecin damages and prevents DNA replication thus resulting in failed cell division whereas RBBP6 is said to work alongside telomerase in promoting cell division and cell proliferation. Based on this, combination of siRBBP6 and camptothecin seems to be effective in preventing cell proliferation and inducing apoptosis in HeLa and SiHa cell lines.

Accumulation of TP53 following RBBP6 silencing provides early insights into the possible interaction between RBBP6 and TP53 and whether or not RBBP6 plays a role in TP53 degradation and therefore this area of study leaves room for further investigations. The inability of GABA to show effectiveness in inducing apoptosis either on its own or in combination with RBBP6 targeting could be attributed to the fact that its mechanism of action focuses more on cell migration rather than apoptosis induction. Future investigations on the effects of GABA on cell migration and chemotaxis in cervical cancer would therefore be interesting.

The attached email below serves as proof that the manuscript "**The differential RBBP6 (retinoblastoma binding protein 6) expression predicts TP53-induced apoptosis in human breast cancer cell lines**" as detailed in the following chapter (5), has been submitted for publication and is currently under review.

CHAPTER FIVE - The differential RBBP6 (retinoblastoma binding protein 6) expression predicts TP53-induced apoptosis in human breast cancer cell lines

5.1. Introduction

Breast cancer remains a female-related health problem on a global scale, accounting for over a million newly estimated cases that are still on the rise (Tao *et al*., 2015). Uncontrolled cell growth and metastasis are considered hallmarks of not only breast tumorigenesis but most other cancers as well. These malignant transformations are due to mutations and/or inactivation of genes involved in the regulation of cell cycle and apoptosis (Buchegger *et al*., 2016; Tao *et al*., 2015). For example, TP53 is the most important tumour suppressor gene which has been found to be mutated in over 50% of most human cancer types (Bai and Zhu, 2006). In breast cancer, the frequency of TP53 mutations varies greatly between the heterogeneous subtypes, with basal-like breast cancers having the highest frequency whereas the luminal subtypes have been shown to generally express wt. p53 (Haupt *et al*., 2016).

Wild-type p53 is negatively regulated by MDM2, which inhibits its transcriptional activity and facilitates its nuclear transport thus triggering degradation via the ubiquitin proteasome pathway (UPP). Research has been done in which the TP53-MDM2 interaction has been successfully disrupted (Haupt *et al*., 2016). Another extensively studied ubiquitous protein that has been shown to negatively regulate wt. TP53 is called E6 oncoprotein in cervical cancers since it possesses an E3 ligase activity, an important function that's needed during UPP (Yuan *et al*., 2016). RBBP6 is yet another suspected de-regulator of wt. p53 due to its E3 ligase activity as

well as the presence of both p53 and RING finger-like domains (Pugh *et al*., 2006). However, more research still need to be done in order to identify RBBP6 as a negative regulator of wt. p53.

RBBP6 is a large multi-domain protein whose localization is widespread in a number of squamous cell carcinomas including those of the lung, colon, breast and oesophagus, which suggests its involvement in tumorigenesis (Li *et al*., 2007; Motadi *et al*., 2011; Chen *et al*., 2013; Moela *et al*., 2014). We have previously shown that silencing RBBP6 in breast cancer cell line expressing wt. p53 leads to accumulation of the TP53 gene followed by apoptosis induction (Moela *et al*., 2014). Furthermore, we have shown that camptothecin-induced apoptosis becomes sensitized in a RBBP6-deficient status (Moela *et al*., 2014). These observations prompted us to further analyse the effects of RBBP6 silencing in a breast cancer cell line model that does not express wt. p53 as this will enable us to identify whether or not there is a relationship between RBBP6 and TP53 expression in breast cancer.

The aim of this chapter was therefore to overexpress and silence RBBP6 gene expression in the mutant p53-expressing MDA-MB-231 breast cancer cell line and analyse its effects on cell proliferation, cell cycle and apoptosis. Furthermore, the effect of camptothecin and GABA anticancer agents in combination with RBBP6 targeting was investigated.

5.2.   Results

To achieve our aim, RBBP6 was silenced and overexpressed in MDA-MB-231 cells as well as in the wt. p53-expressing MCF-7 cell line for comparative purposes. Fluorescence microscopy, FACS analysis, immunoprecipitation/western blotting and qPCR were used to measure transfection efficiency of the RBBP6-containing expression vector or the siRBBP6 oligonucleotide. Furthermore, apoptosis-based assays were performed to check the effect of silencing, overexpression and co-treatment with GABA or camptothecin on breast cancer cell lines. The assays included Annexin V/PI staining, caspase activity and mitochondrial ATP. To monitor cell proliferation and the cell cycle, xCELLigence assay and PI staining were performed respectively.

5.2.1. RBBP6 transfection efficiency

RBBP6 expression was manipulated in different cell lines (MDA-MB-231, MCF-7 and MRC-5) by either overexpression with pCMV6-AC-GFP or by gene silencing. RBBP6-containing expression vector (pCMV6-AC-GFP) was used for overexpression and synthetic siRNA oligonucleotides specific for RBBP6 were used in gene silencing. During transient transfection, the introduced nucleic acid exists in the cell only for a limited period of time (~4 days) because it is not integrated into the genome. Therefore, cell lines were transiently transfected for a period of 48-72 hours before determining transfection efficiency.

RBBP6 overexpression

The pCMV6-AC-GFP expression vector was used to deliver the open reading frame of RBBP6 transcript variant 3 (NM_032626.5) into the cells and green fluorescent protein (GFP) tagged on the C-terminus of the vector was used to permit positive identification of cells transfected with

the plasmid. RBBP6 protein was significantly overexpressed 72 hours after transfection in all cell lines (Figure 34, Figure 35 and Figure 36). The expression of RBBP6 was directly proportional to significant overexpression of GFP in pCMV6-AC-GFP$^+$ cell lysate in comparison to the pCMV6-AC-GFP$^-$ lysate which shows 0% GFP expression in almost all cell lines (Figure 34, Figure 35 and Figure 36). GFP expression was significant ($P < 0.05$) in MCF-7 and MDA-MB-231 cell lysates, with band intensities of 92 and 90%, respectively (Figure 34 and Figure 35). In contrast, MRC-5 cell line showed only 21% overexpression of RBBP6 according to the densitometry quantification of band intensity in pCMV6-AC-GFP$^+$ lysate (Figure 36).

FACS analysis indicated RBBP6 up-regulation in MCF-7 and MDA-MB-231 cell lines with varying fluorescence signal of 88.9% and 47.1% respectively (Figure 37 and Figure 38). The observed positive staining was further confirmed by the strong GFP fluorescence signal which shows cytoplasmic localization of RBBP6 in both tumorigenic cell lines (Figure 37 and Figure 38). MRC-5 cells on the other hand showed a FACS profile of only 14.9% overexpression in pCMV6-AC-GFP$^+$ cells (Figure 39) which is significantly low compared to the observed overexpression in the tumorigenic cell lines. More so, the normal cell line shows more cytoplasmic localisation of RBBP6 in contrast to the MDA-MB-231 tumorigenic cell line, which showed stronger signal in both nuclear and cytoplasmic RBBP6 localization (Figure 39). No visible GFP fluorescence was observed in pCMV6-AC-GFP$^-$ controls across all cell lines.
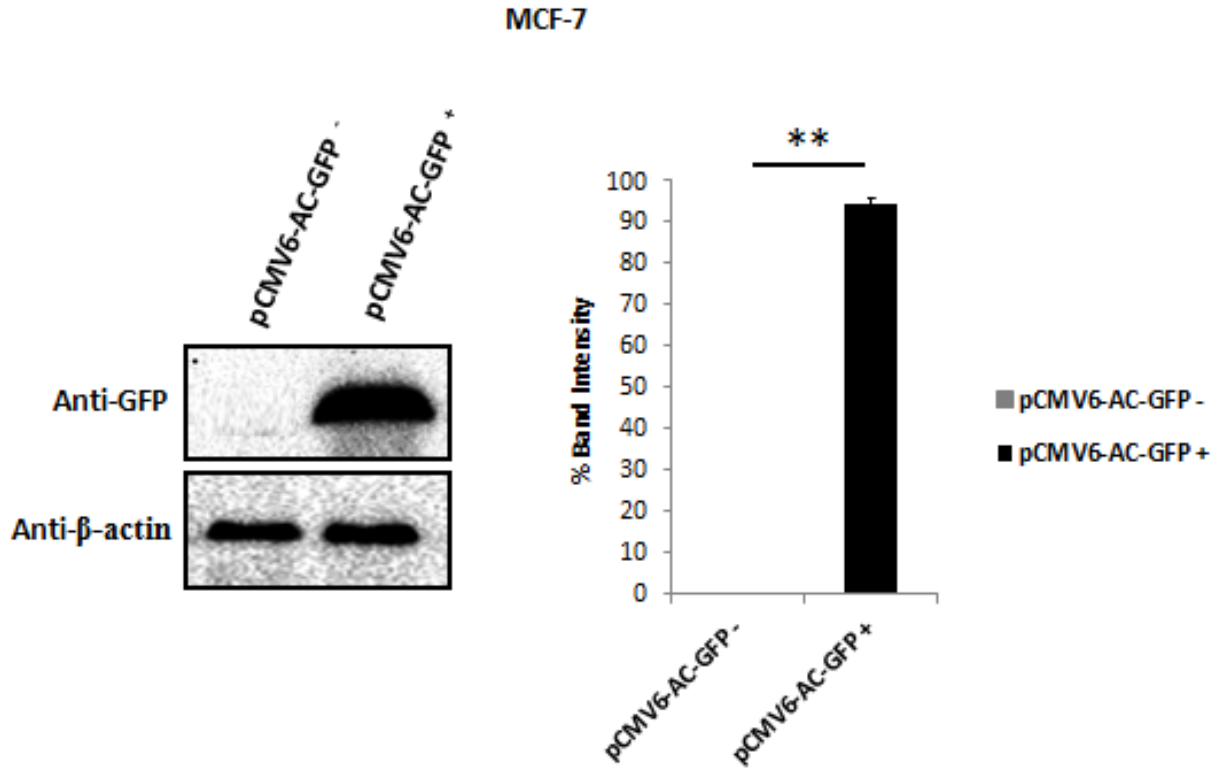
Figure 34: Overexpression of RBBP6 by transient transfection of MCF-7 cells with RBBP6-containing expression vector (*pCMV6-AC-GFP*). *Left*: IP-WB (immunoprecipitation-western blotting) electrophoretogram showing detection of RBBP6 expression which is directly proportional GFP. Expression was detected using anti-GFP monoclonal primary antibody and β-actin was chosen as the house-keeping gene. *pCMV6-AC-GFP (-)* indicates non-transfected cell lysates and *pCMV6-AC-GFP (+)* shows GFP expression in cell lysates transfected with the expression vector. *Right*: Corresponding densitometry quantification of band intensity acquired using the ChemiDoc MP software.
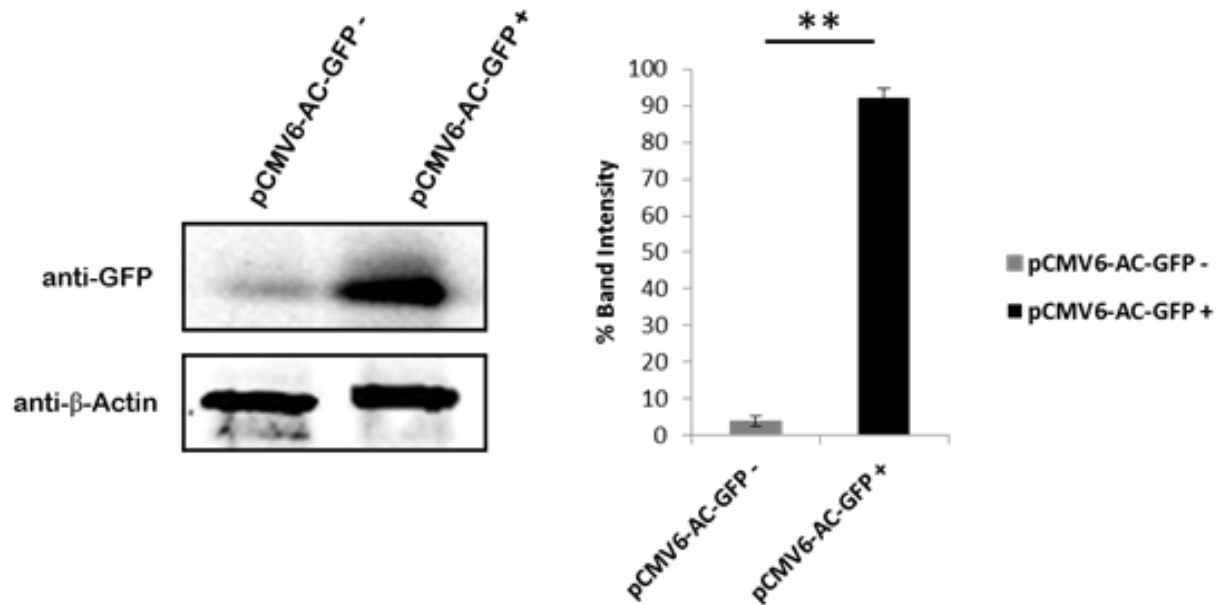
Figure 35: Overexpression of RBBP6 by transient transfection of MDA-MB-231 cells with RBBP6-containing expression vector (*pCMV6-AC-GFP*). *Left*: IP-WB electrophoretogram showing detection of RBBP6 expression which is directly proportional GFP. Expression was detected using anti-GFP monoclonal primary antibody and β-actin was chosen as the house-keeping gene. *pCMV6-AC-GFP (-)* indicates non-transfected cell lysates and *pCMV6-AC-GFP (+)* shows GFP expression in cell lysates transfected with the expression vector. *Right*: Corresponding densitometry quantification of band intensity acquired using the ChemiDoc MP software.
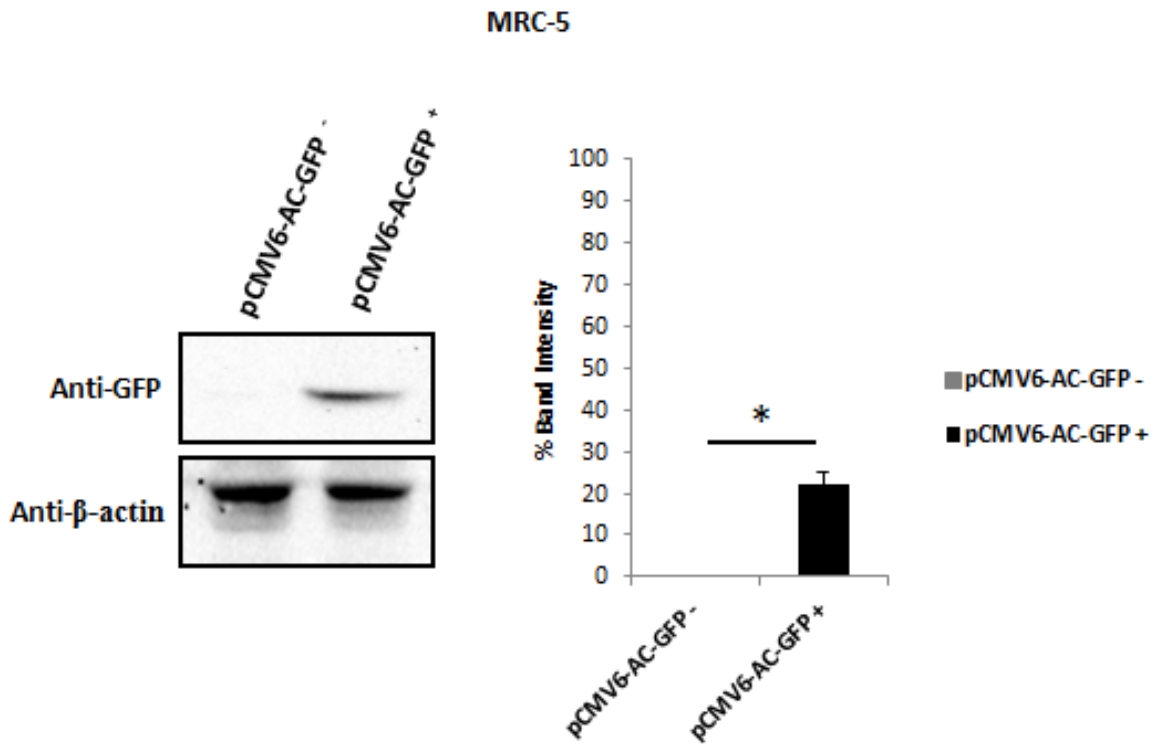
Figure 36: Overexpression of RBBP6 by transient transfection of MRC-5 cells with RBBP6-containing expression vector (*pCMV6-AC-GFP*). *Left*: IP-WB electrophoretogram showing detection of RBBP6 expression which is directly proportional GFP. Expression was detected using anti-GFP monoclonal primary antibody and β-actin was chosen as the house-keeping gene. *pCMV6-AC-GFP (-)* indicates non-transfected cell lysates and *pCMV6-AC-GFP (+)* shows GFP expression in cell lysates transfected with the expression vector. *Right*: Corresponding densitometry quantification of band intensity acquired using the ChemiDoc MP software.
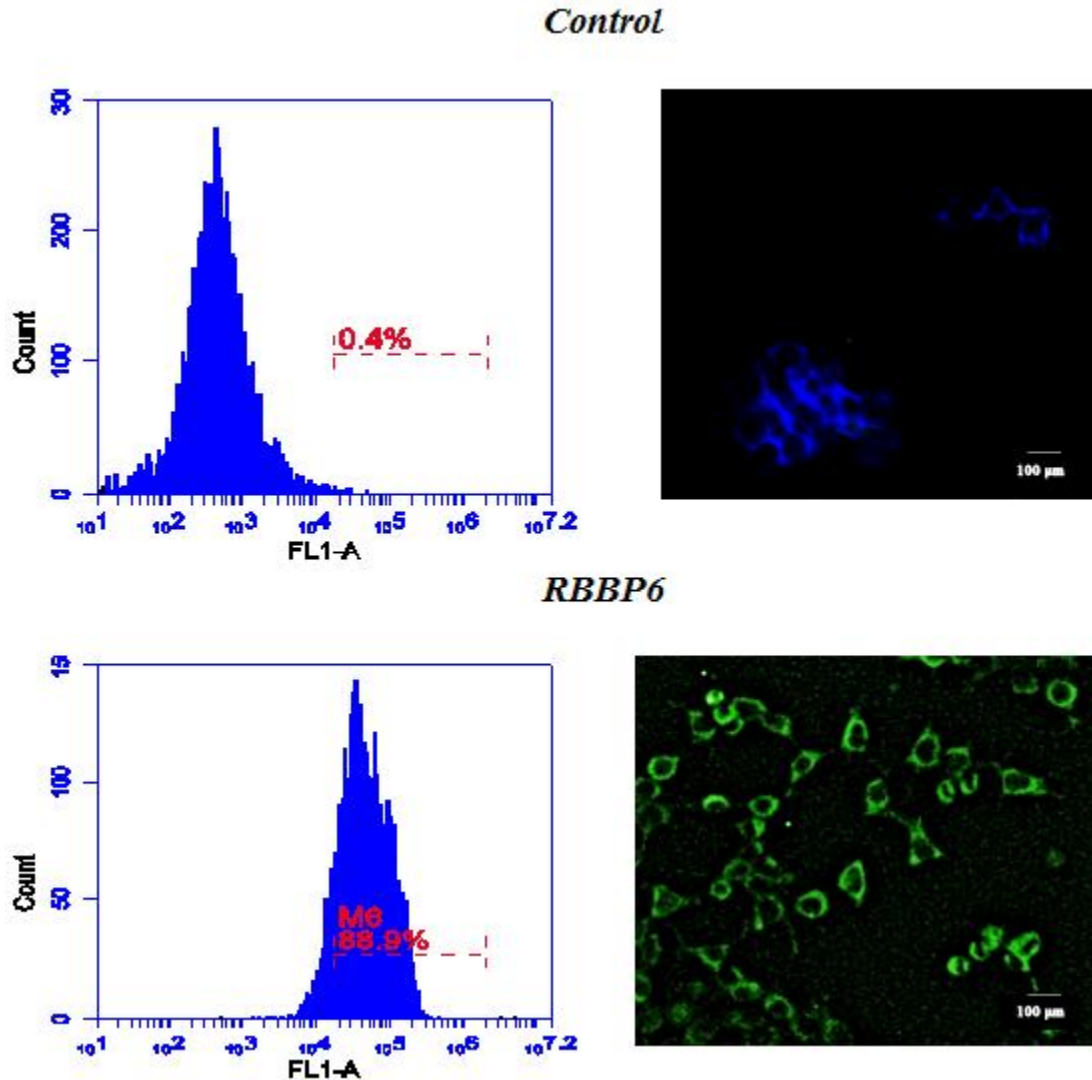
Figure 37: FACS analysis and immunocytochemistry of MCF-7 cells stained with 0.2 μg/ml mouse monoclonal anti-GFP antibody and labelled with Alexa Fluor-488 goat anti-mouse secondary antibody 48-hours post-transfection. Cells were fixed using 4% formaldehyde and permeabilized using 0.01% triton X-100 before immunostaining. Counterstaining was obtained using DAPI. Histograms represent FACS profiles of RBBP6 expression, i.e. *top left*: control cells that were not transfected and *bottom left*: cells transfected with the plasmid (*pCMV6-AC-GFP)*. Fluorescence microscopy images (*top and bottom right*) show cytoplasmic/nuclear localisation of RBBP6.
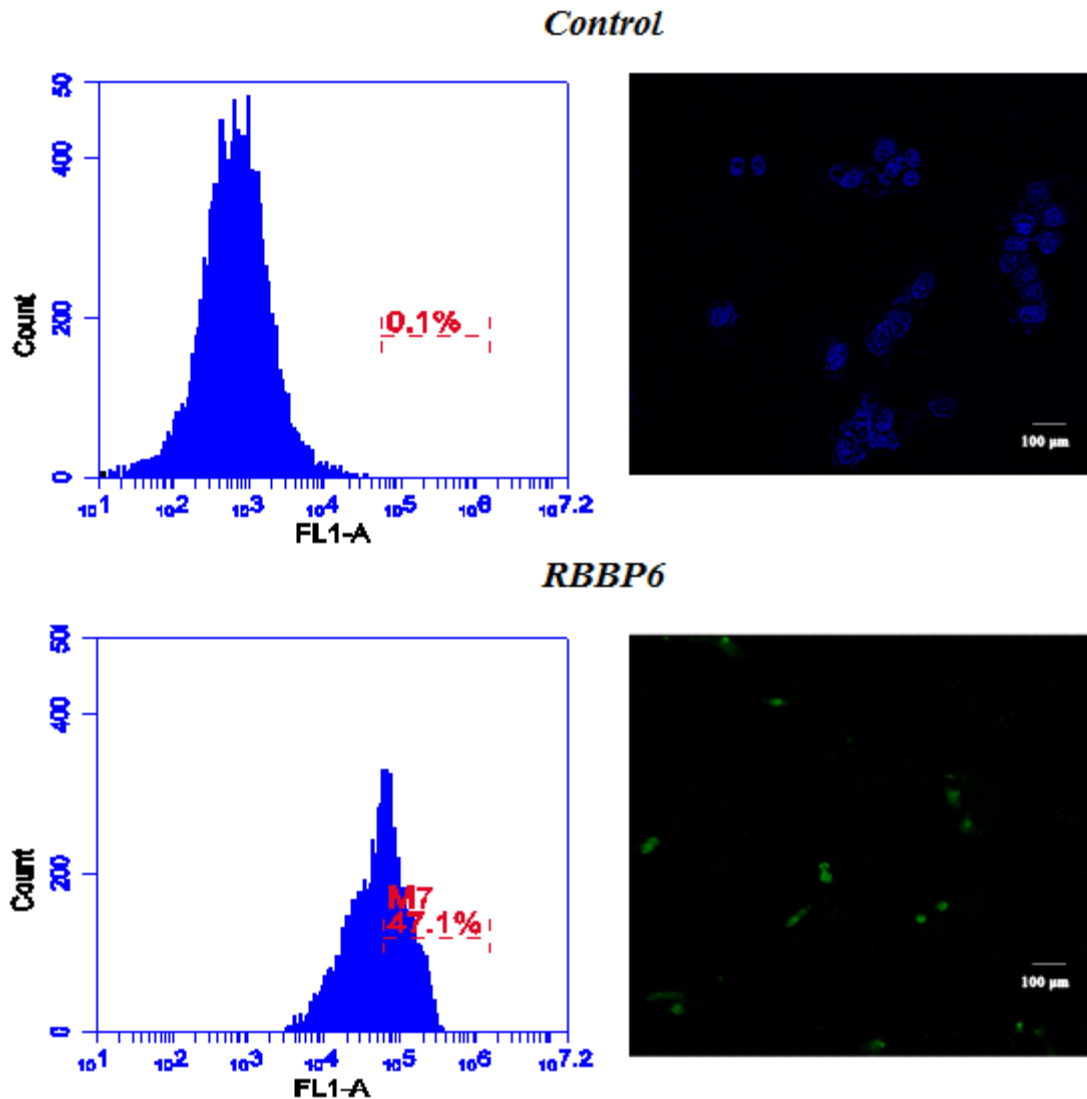
Figure 38: FACS analysis and immunocytochemistry of MDA-MB-231 cells stained with 0.2 µg/ml monoclonal anti-GFP antibody and labelled with Alexa Fluor-488 goat anti-mouse secondary antibody 48-hours post-transfection. Cells were fixed using 4% formaldehyde and permeabilized using 0.01% triton X-100 before immunostaining. Counterstaining was obtained using DAPI. Histograms represent FACS profiles of RBBP6 expression, i.e. *top left*: control cells that were not transfected and *bottom left*: cells transfected with the plasmid (*pCMV6-AC-GFP)*. Fluorescence microscopy images (*top and bottom right*) show cytoplasmic/nuclear localisation of RBBP6.
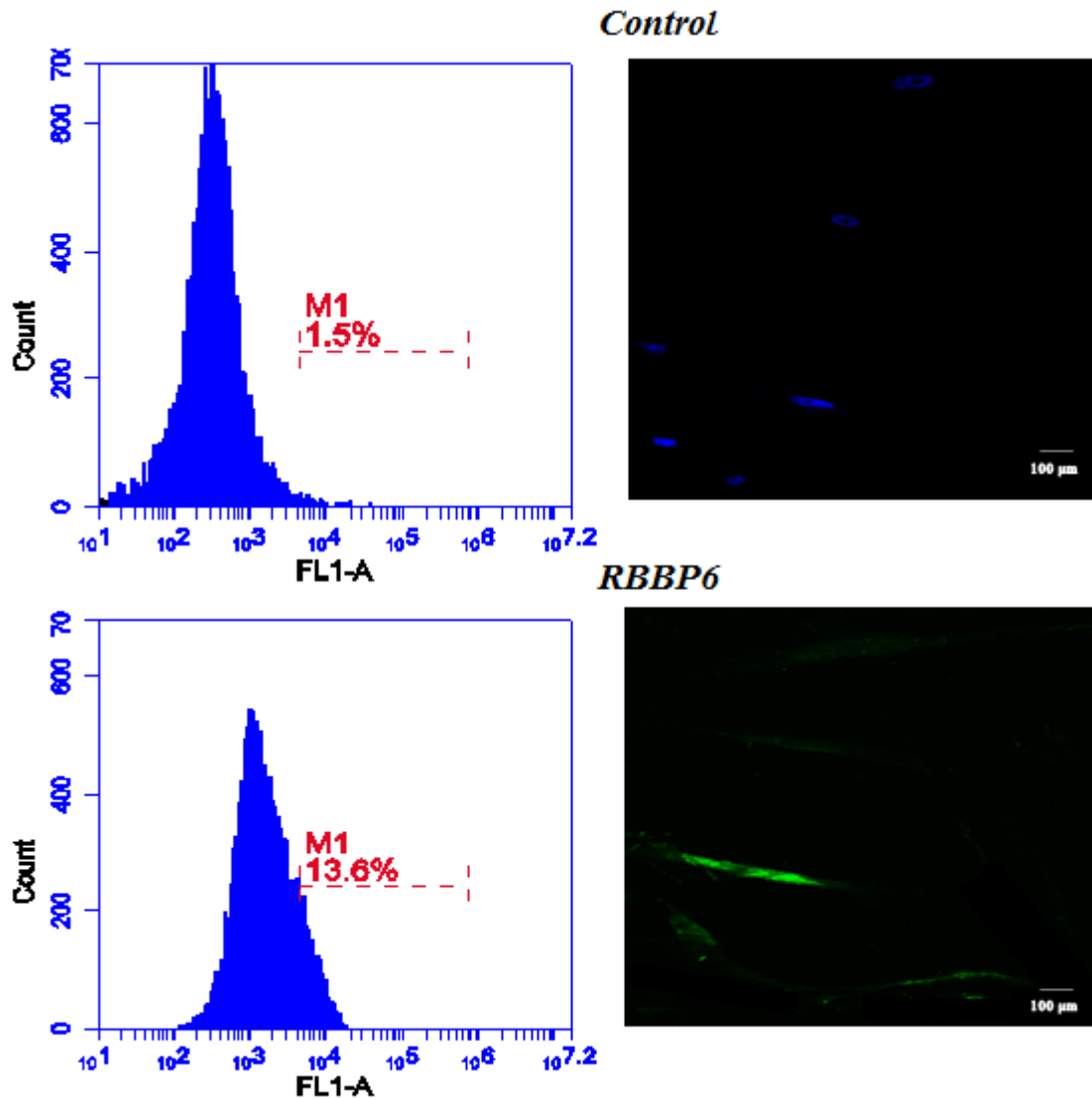
Figure 39: FACS analysis and immunocytochemistry of MRC-5 cells stained with 0.2 μg/ml mouse monoclonal anti-GFP antibody and labelled with Alexa Fluor-488 goat anti-mouse secondary antibody 48-hours post-transfection. Cells were fixed using 4% formaldehyde and permeabilized using 0.01% triton X-100 before immunostaining. Counterstaining was obtained using DAPI. Histograms represent FACS profiles of RBBP6 expression, i.e. *top left*: control cells that were not transfected and *bottom left*: cells transfected with the plasmid (*pCMV6-AC-GFP)*. Fluorescence microscopy images (*top and bottom right*) show cytoplasmic/nuclear localisation of RBBP6

RBBP6 silencing

Gene silencing by RNAi leads to a highly potent repression of gene expression mainly because it employs cellular machinery that effectively allows targeting of specific mRNA transcripts. In this study the RNAi technique was employed to down-regulate RBBP6 in breast cancer cells (MCF-7 and MDA-MB-231) as well as normal lung fibroblast (MRC-5). To check whether or not gene silencing was successful, qPCR which relies on reverse transcription of total RNA to synthesize complimentary DNA that serves as a template during real time PCR amplification of the genes of interest was used (Figure 40A). This was further confirmed by detection of RBBP6 protein expression using western blot analysis (Figure 40B). A statistically significant down-regulation of RBBP6 mRNA was achieved 48 hours post-transfection across all cell lines, with nearly 80% gene silencing in both MCF-7 and MDA-MB-231. Detection of RBBP6 at a protein level 72 hours post-transfection confirmed the observed gene knockdown. RBBP6 expression was down 70-90 % in cancer cell line (Figure 40A). In MRC-5 cell line RBBP6 expression was lowest as confirmed by the faint bands on the blot (Figure 40A), showing down-regulation of nearly 92% in RBBP6 mRNA expression (Figure 40B).

A

B
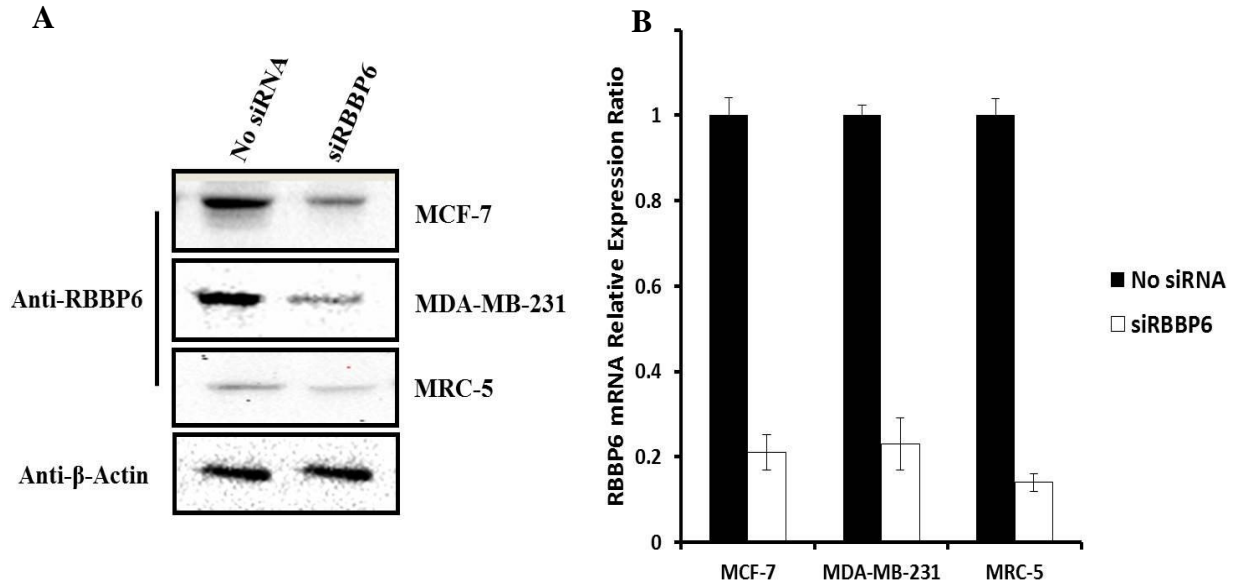
Figure 40: Transfection efficiency of RBBP6 gene silencing using siRNA technology in MCF-7, MDA-MB-231 and MRC-5 cell lines. A) Western blot detection of RBBP6 expression following 72 hour transfection. B) Relative quantification of gene expression was performed 48 hours post-transfection using qPCR. *No siRNA*: indicates negative control cells that were not transfected; *siRBBP6*: indicates cells transfected with oligonucleotides specific for RBBP6 and β-actin was used as a reference gene.

5.2.2. The effect of RBBP6 overexpression and silencing on cell proliferation

In order to analyse the effect of RBBP6 targeting on cell growth, the real time cell analysis (RTCA) assay was used. This voltage-based instrument depends on spreading and adhesion of cells to electron charged bottom of each well of a 16-well E-plate which produce electrical impedance (Ke *et al.*, 2011). The disturbance in current flow as a result of cell adhesion is represented by the logarithm CI which is directly proportional to cell proliferation. Cells were seeded simultaneously with transfection agents, siRBBP6 and pRBBP6, in 16-well E-plates and growth was monitored for a period of 72 hours using the RTCA machine.

Growth of MCF-7, MDA-MB-231 and MRC-5 cell lines was normalized at approximately 24 hours just before the effects of either gene silencing or overexpression on cell proliferation was observed (Figure 41, Figure 42 and Figure 43). In MCF-7 breast cancer cells, a rapid decrease in cell proliferation was evident (magenta) 24 hours later into silencing, whilst RBBP6-overexpressing cells (red) maintain the highest cell index throughout the last ~30 hours of the experiment (Figure 41). In contrast, the p53-mutated breast cancer cell line, MDA-MB-231; shows no significant difference in growth rate when compared to untransfected or negative control cells despite the induced RBBP6 overexpression (Figure 42). More so, RBBP6 silencing appears to have minor effect on proliferation of MDA-MB-231 cells since the cell index continues to increase although at a slower rate (magenta). In MRC5 there was no significant effect on cell proliferation/growth following transfection in both overexpression and silencing (Figure 43).
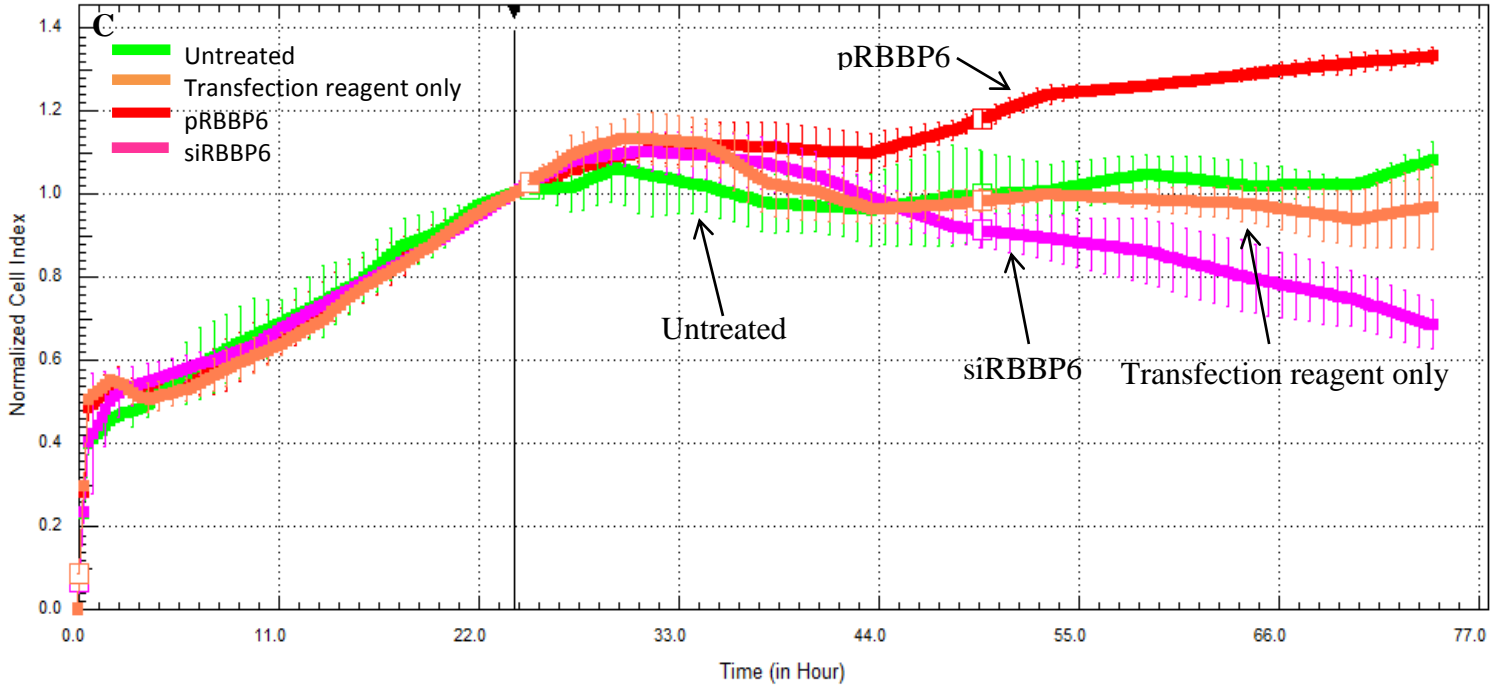
Figure 41: Cell growth of MCF-7 cells analyzed using the xCELLigence system

which relies on the generation of electrical impedance as cell growth by 16-well plates coated with a gold

microelectron covering at the base of each well. The y-axis shows normalised cell index or cell adhesion

over a period of about 72 hours (x-axis). The growth patterns highlighted in green show cells that are not

treated. Those highlighted in coral show control cell populations treated with transfection reagent only

whereas red and magenta growth curves show cells that are overexpressing RBBP6 (transfected with

pRBBP6) and cells that were silenced, respectively.
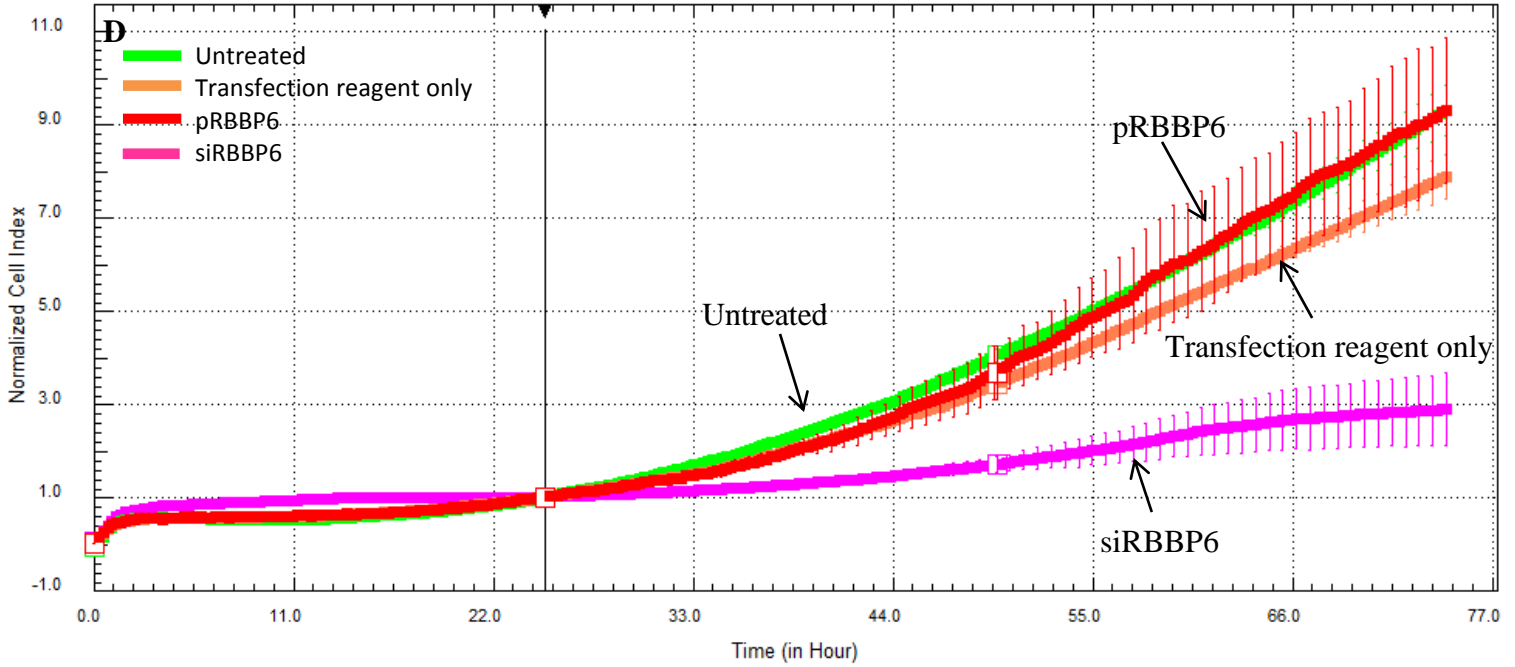
Figure 42: Cell growth of MDA-MB-231 cells analyzed using the xCELLigence system

which relies on the generation of electrical impedance as cell growth by 16-well plates coated with a gold

microelectron covering at the base of each well. The y-axis shows normalised cell index or cell adhesion

over a period of about 72 hours (x-axis). The growth patterns highlighted in green show cells that are not

treated. Those highlighted in coral show control cell populations treated with transfection reagent only

whereas red and magenta growth curves show cells that are overexpressing RBBP6 (transfected with

pRBBP6) and cells that were silenced, respectively.
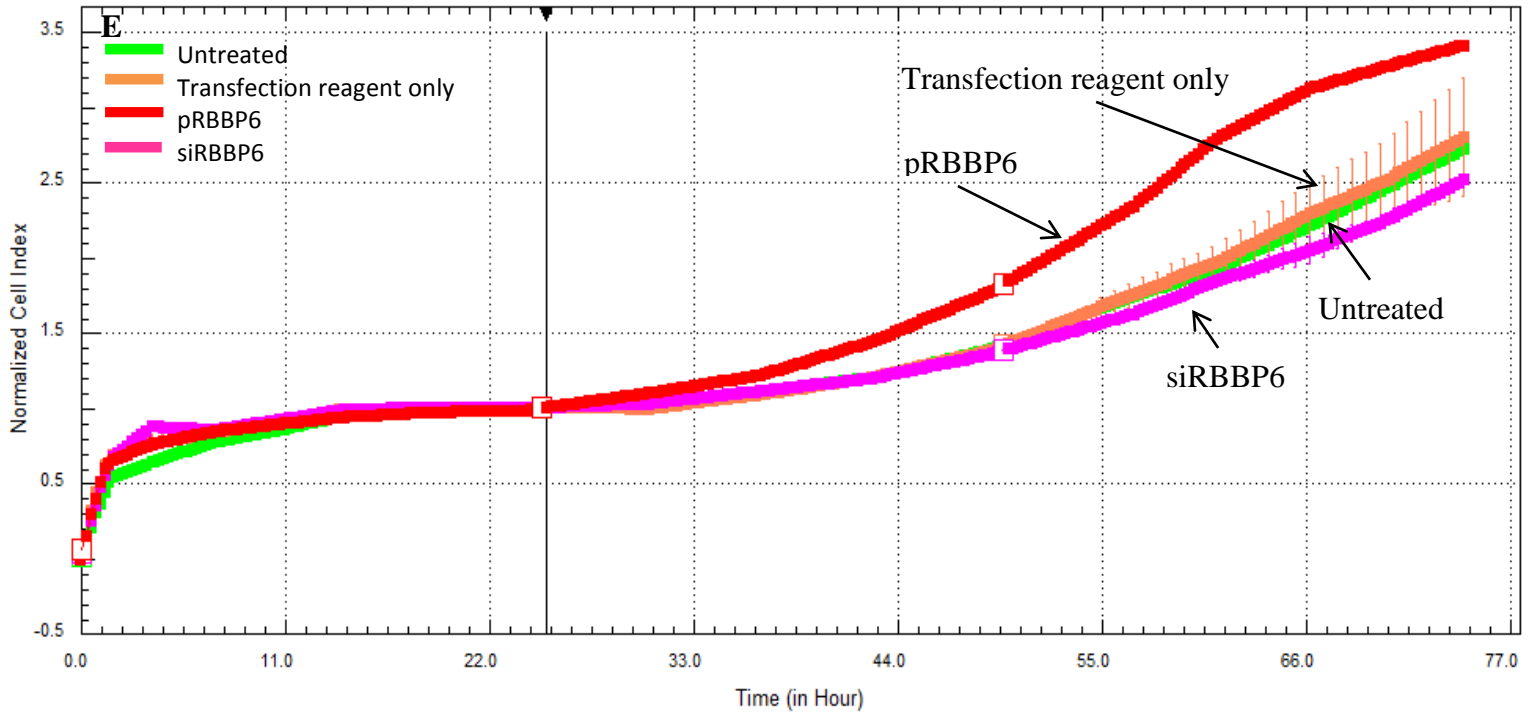
Figure 43: Cell growth of MRC-5 cells was analyzed using the xCELLigence system which relies on the generation of electrical impedance as cell growth by 16-well plates coated with a gold microelectron covering at the base of each well. The y-axis shows normalised cell index or cell adhesion over a period of about 72 hours (x-axis). The growth patterns highlighted in green show cells that are not treated. Those highlighted in coral show control cell populations treated with transfection reagent only whereas red and magenta growth curves show cells that are overexpressing RBBP6 (transfected with pRBBP6) and cells that were silenced, respectively.

5.2.3. Apoptosis Detection

Among other characteristics of apoptosis such as cell shrinkage, membrane blebbing, loss of adhesion with neighbouring cells, nuclear condensation and fragmentation, externalisation of phosphatidyl serine (PS) peptide outside the plasma membrane plays a central role in identifying apoptotic cells (Liu *et al*., 2011; Ouyang *et al*., 2012; Wen *et al*., 2012). This is because PS externalisation allows dying cell to be recognised by phagocytes for a subsequent removal. Synthetic peptides with high affinity for PS have therefore been developed in order to enable identification of apoptotic cells, and annexin V is the commonly used peptide in apoptosis assays (Andree *et al*., 1990). In flow cytometry and fluorescence microscopy detection of apoptosis, the peptide is usually tagged with a fluorescent dye, FITC, in order to enable positive identification of dying cells. And since PS externalisation enables detection of early apoptosis, it is usually combined with DNA binding dyes in order to allow for late apoptosis detection. Propidium iodide (PI) and DAPI (4',6-diamidino-2-phenylindole) are the commonly used DNA intercalating dyes during apoptosis assays. The present study made the use of annexin V/FITC, PI and DAPI fluorescent dyes in order to detect both early and late apoptosis using flow cytometer and fluorescence microscope, following transfections and co-treatments.

 Flow cytometry detection of apoptosis

Apoptosis was significantly increased following transfection with siRBBP6 in both tumorigenic cell lines, with 57% apoptosis in MCF-7, whereas in MDA-MB-231 cells, there was just 9% of cells undergoing apoptosis ($p<0.05$) (Figure 44). RBBP6 silencing significantly induced apoptosis in *camptothecin*-treated cells to 75% in MCF7 and 46.6 % in MDA-MB-231, as compared to that of GABA where there was no change in apoptosis induction in MCF7 and

MDA-MB-231 (Figure 44). Transfection with pRBBP6 induced minimum amount of apoptosis across both cell lines, with 12% and 17% apoptosis in MCF-7 and MDA-MB-231, respectively. Significant reduction in apoptosis following co-treatment with pRBBP6+camptothecin and pRBBP6+GABA was observed in MCF-7 (15% and 13%, respectively) and MDA-MB-231 (27% and 15%, respectively) (Figure 44).

Apoptosis induction in MRC-5 fibroblast was fairly low following treatment with camptothecin or GABA (16% and 12%, respectively) as compared to the one observed in tumorigenic cell lines (Figure 45). Only 0.2% apoptosis was observed in untreated MRC-5 cells. Silencing MRC-5 with siRBBP6 induced 13% apoptosis whereas up-regulation of RBBP6 induced 0.1% apoptosis (Figure 45). Combination with camptothecin led to 19% and 13% apoptosis induction following silencing and up-regulation, respectively (Figure 45). There was no significant change in apoptosis following combination of siRBBP6 and GABA (11%) or pRBBP6 with GABA (18%) when compared to GABA treatment only (12%) (Figure 45). Figure 46, Figure 47 and Figure 48 illustrate early (upper right quadrant) and late apoptosis (lower right quadrant) induction as well as the level of necrosis (upper left quadrant) in comparison to live cells (lower left quadrant) in all cell lines.
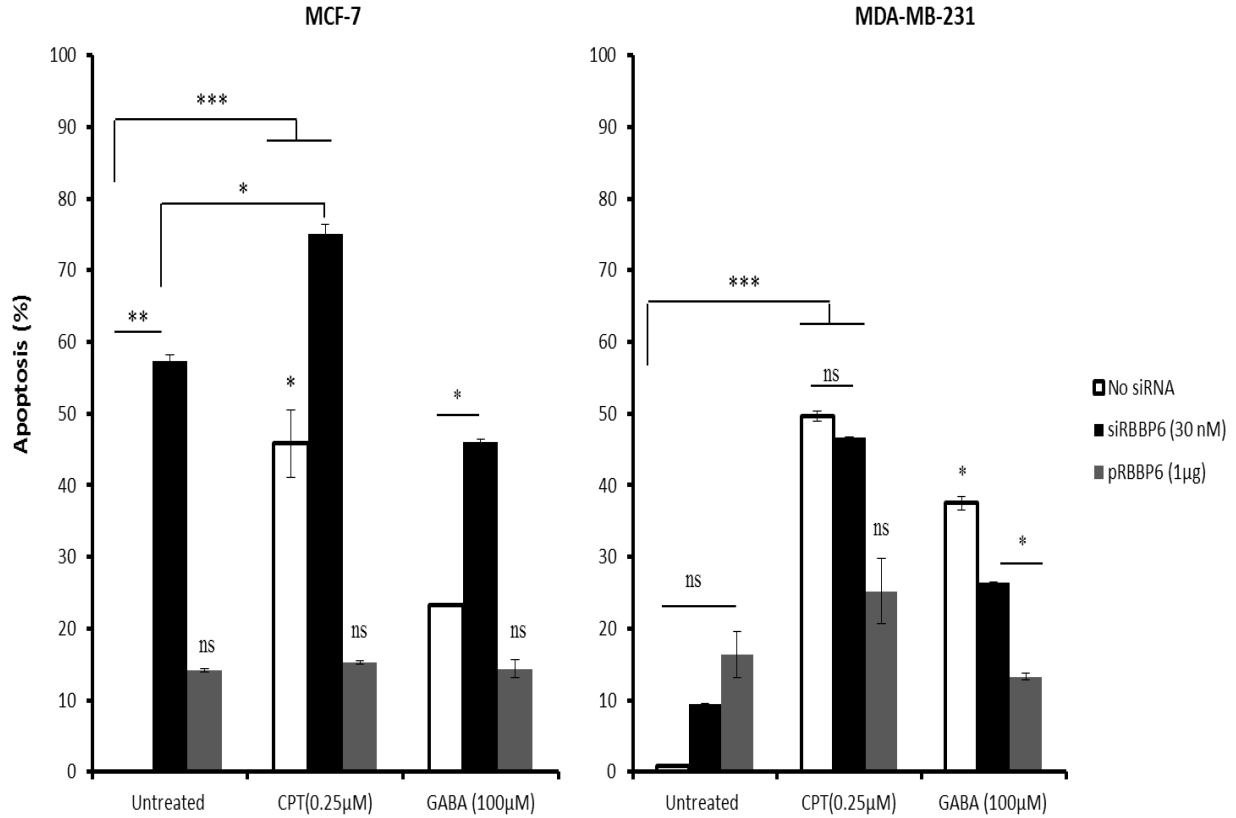
Figure 44: Statistical analysis of flow cytometry-obtained apoptosis (%) in MCF-7 and

MDA-MB-231. T-test was used to generate p-values in order to compute the difference between treated

and untreated scores. * indicates p ≤ 0.05, ** indicates p ≤ 0.01, *** indicates p ≤ 0.001 and ns indicates
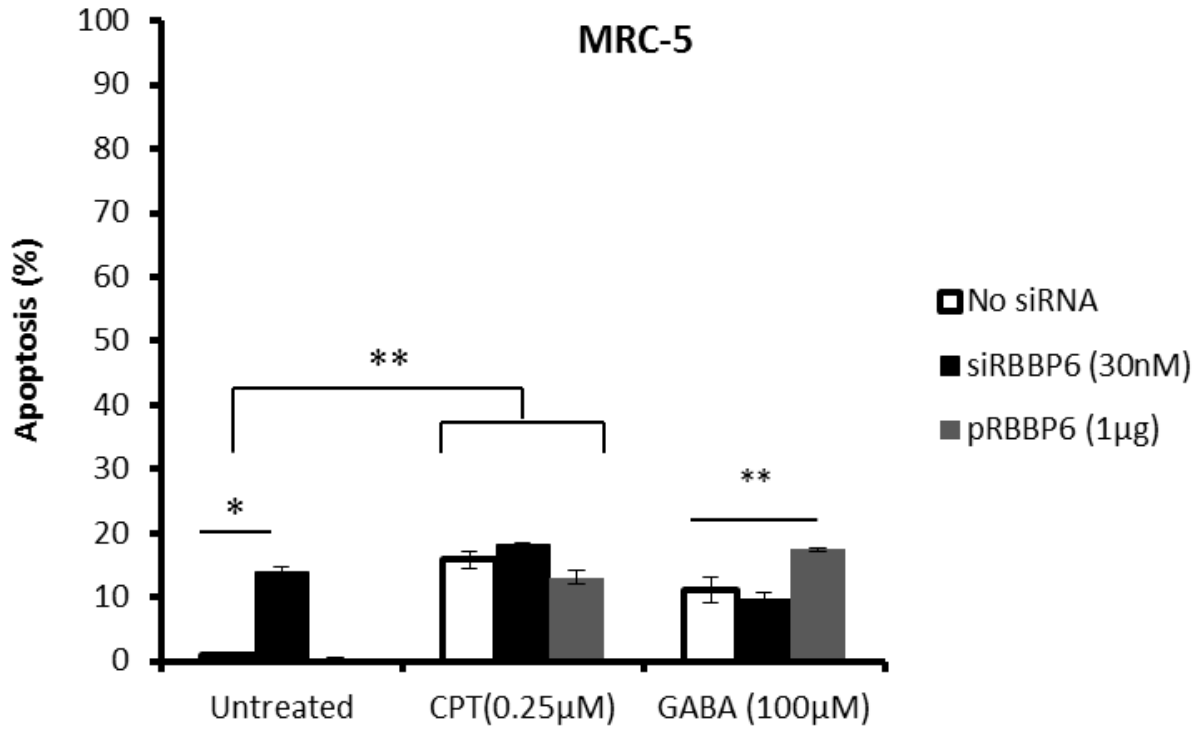
p ≥ 0.05.

Figure 45: Statistical analysis of flow cytometry-obtained apoptosis (%) in MRC-5 cell line.

T-test was used to generate p-values in order to compute the difference between treated and untreated

scores. * indicates $p \le 0.05$, ** indicates $p \le 0.01$, *** indicates $p \le 0.001$ and ns indicates $p \ge 0.05$.

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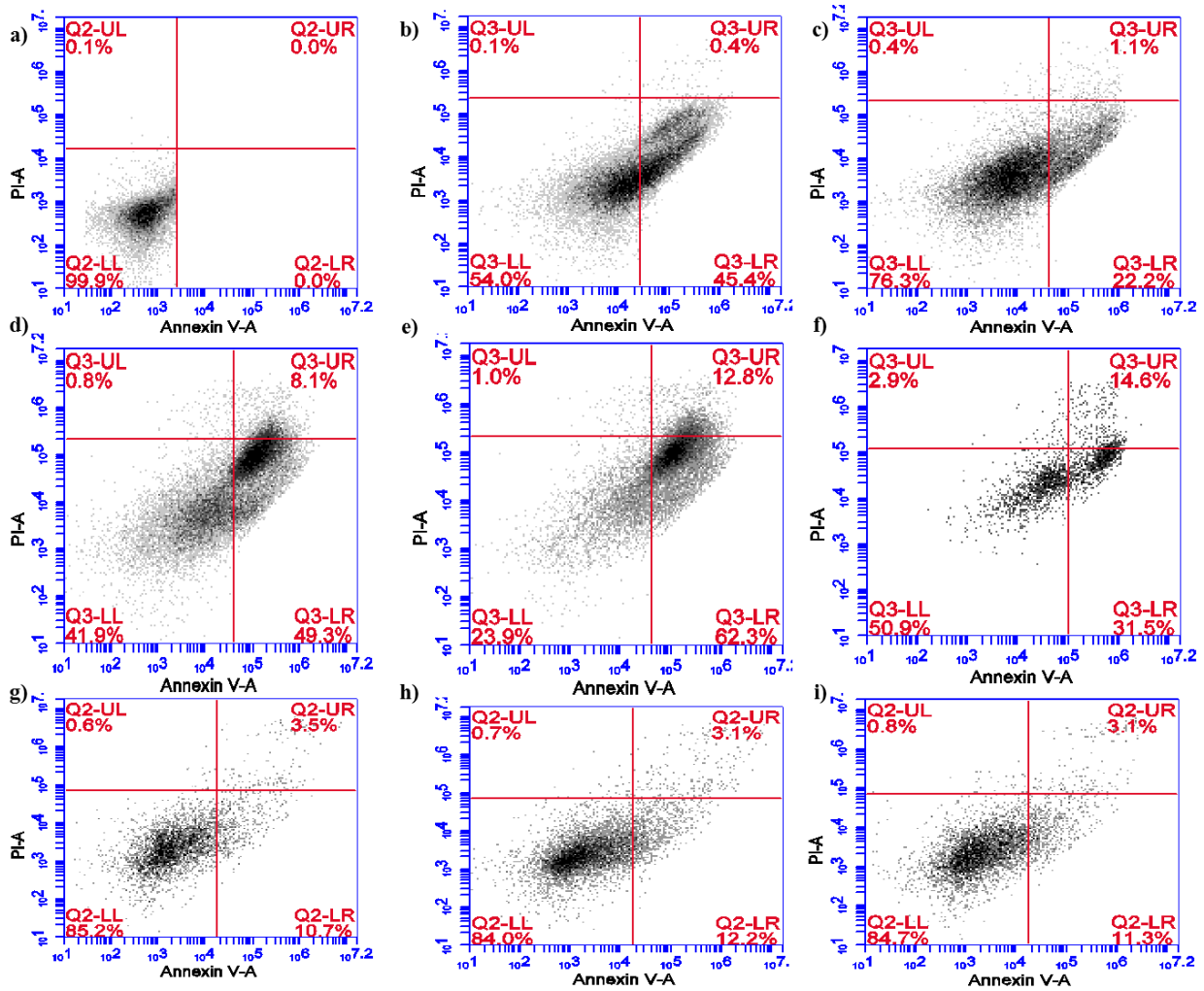
Figure 46: Apoptosis of MCF-7 cells was analyzed using flow cytometer with annexin V/PI staining.

(a) Untreated. (b) Camptothecin, (c) GABA, (d) siRBBP6, (e) siRBBP6+camptothecin, (f) siRBBP6+GABA, (g) pRBBP6, (h) pRBBP6+camptothecin and (i) pRBBP6+GABA.
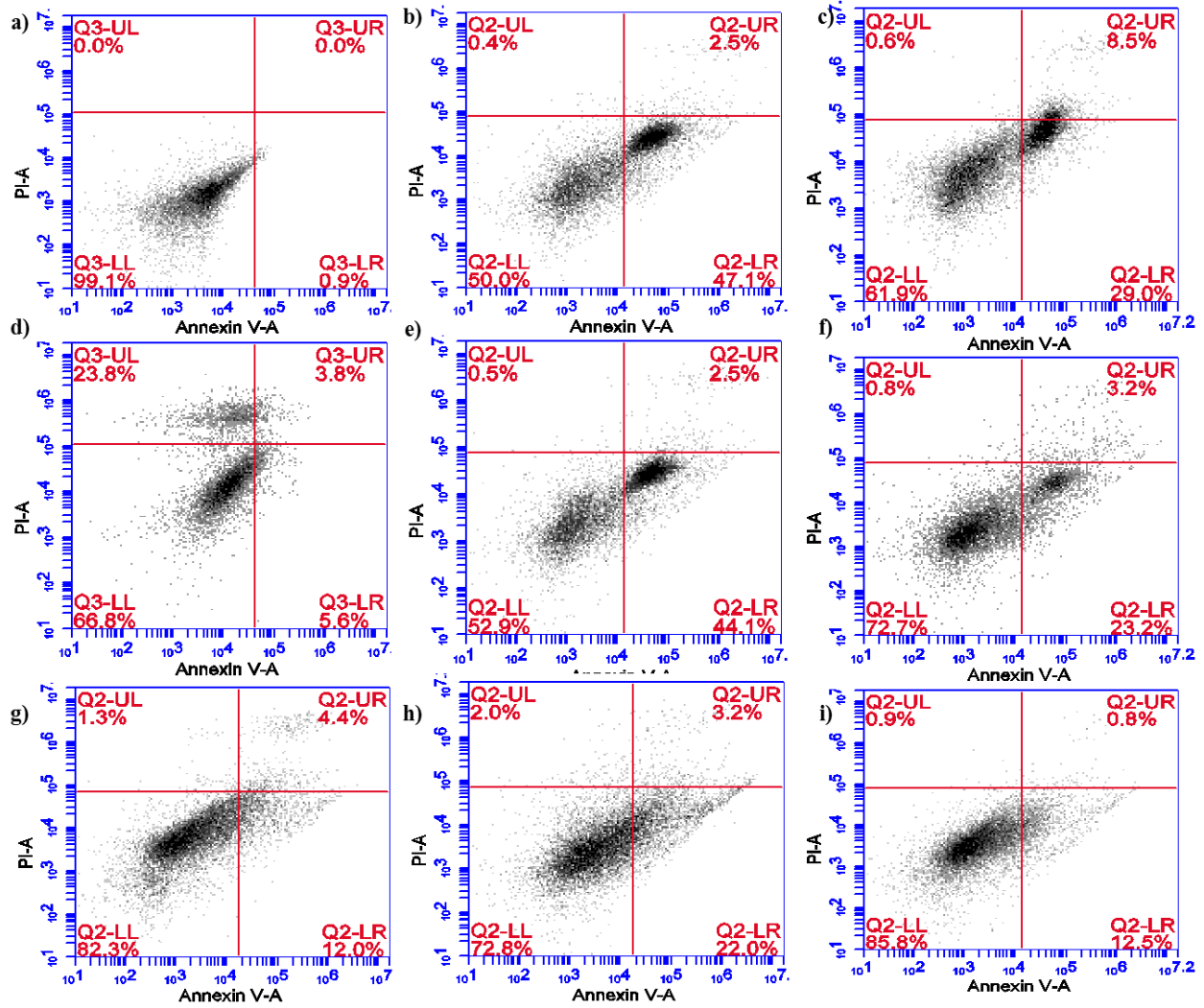
Figure 47: Apoptosis of MDA-MB-231 cells was analyzed using flow cytometer with annexin V/PI

(a) Untreated. (b) Camptothecin, (c) GABA, (d) siRBBP6, (e) siRBBP6+camptothecin, (f) siRBBP6+GABA, (g) pRBBP6, (h) pRBBP6+camptothecin and (i) pRBBP6+GABA.
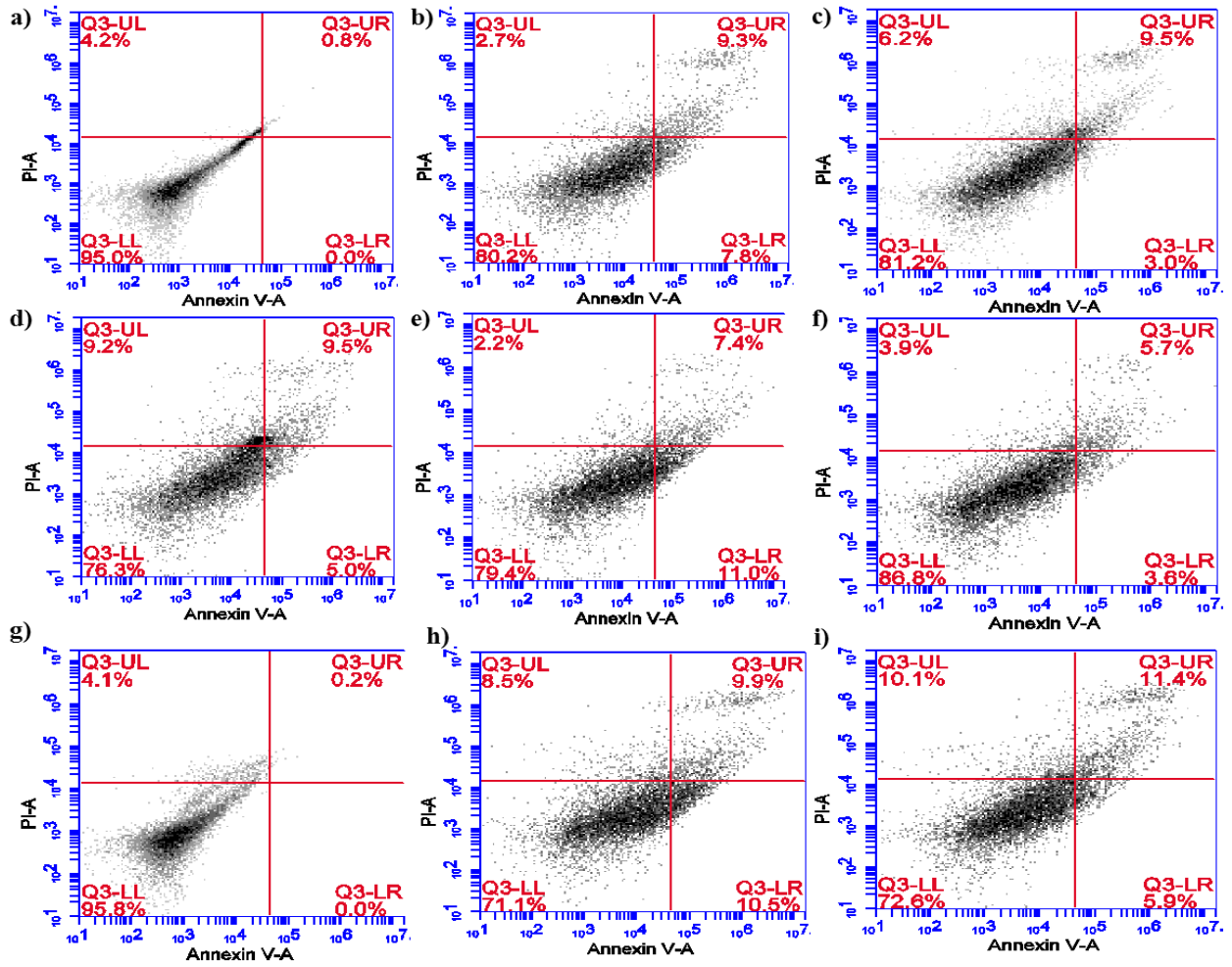
Figure 48: Apoptosis of MRC-5 cells was analyzed using flow cytometer with annexin V/PI staining. (a) Untreated. (b) Camptothecin, (c) GABA, (d) siRBBP6, (e) siRBBP6+camptothecin, (f) siRBBP6+GABA, (g) pRBBP6, (h) pRBBP6+camptothecin and (i) pRBBP6+GABA.

Microscopic detection of apoptosis

As mentioned in the previous section, externalization of phosphatidylserine peptide (PS), which is a marker of early apoptosis, as well as changes in nuclear morphology, was used to analyse the observed apoptosis. These events were imaged using confocal fluorescence microscope in the presence of DAPI and annexin V-FITC staining to confirm the event of apoptosis in intact attached cells. In MCF-7 cells, green fluorescence signal which is indicative of apoptosis induction was observed in both siRBBP6 and camptothecin treatment, whereas combinational treatment further indicated increased apoptosis induction (Figure 49). GABA treatment alone produced a weaker fluorescence signal; however combination with siRBBP6 led to higher fluorescence intensity (Figure 49). pRBBP6, alone or in combination with camptothecin or GABA, produced weaker fluorescence signal compared to siRBBP6 combination (Figure 49).

Changes in cell morphology were observed in treated cells compared to untreated cells, where nuclei appeared to remain intact and round in shape in untreated cell (blue stain) whereas cells undergoing apoptosis had adopted irregular shapes (Figure 49). RBBP6 silencing led to a subtle apoptosis induction in MDA-MB-231 cells as shown by the weak fluorescence signal; however combination with camptothecin led to a much higher PS externalization and loss in cell morphology (Figure 50). Similarly to GABA treatment alone, combination with siRBBP6 produced a weak fluorescence signal coupled with loss of cell shape in MDA-MB-231 cells (Figure 50). Co-treatment of pRBBP6 with either camptothecin or GABA failed to produce any visible green fluorescence signal in MDA-MB-231, however cell shrinkage is especially observed in pRBBP6+GABA co-treatment (Figure 50).

MCF-7

| Untreated | 0.25 µM Camptothecin (24 hr.) | 100 µM GABA (24 hr.) | |
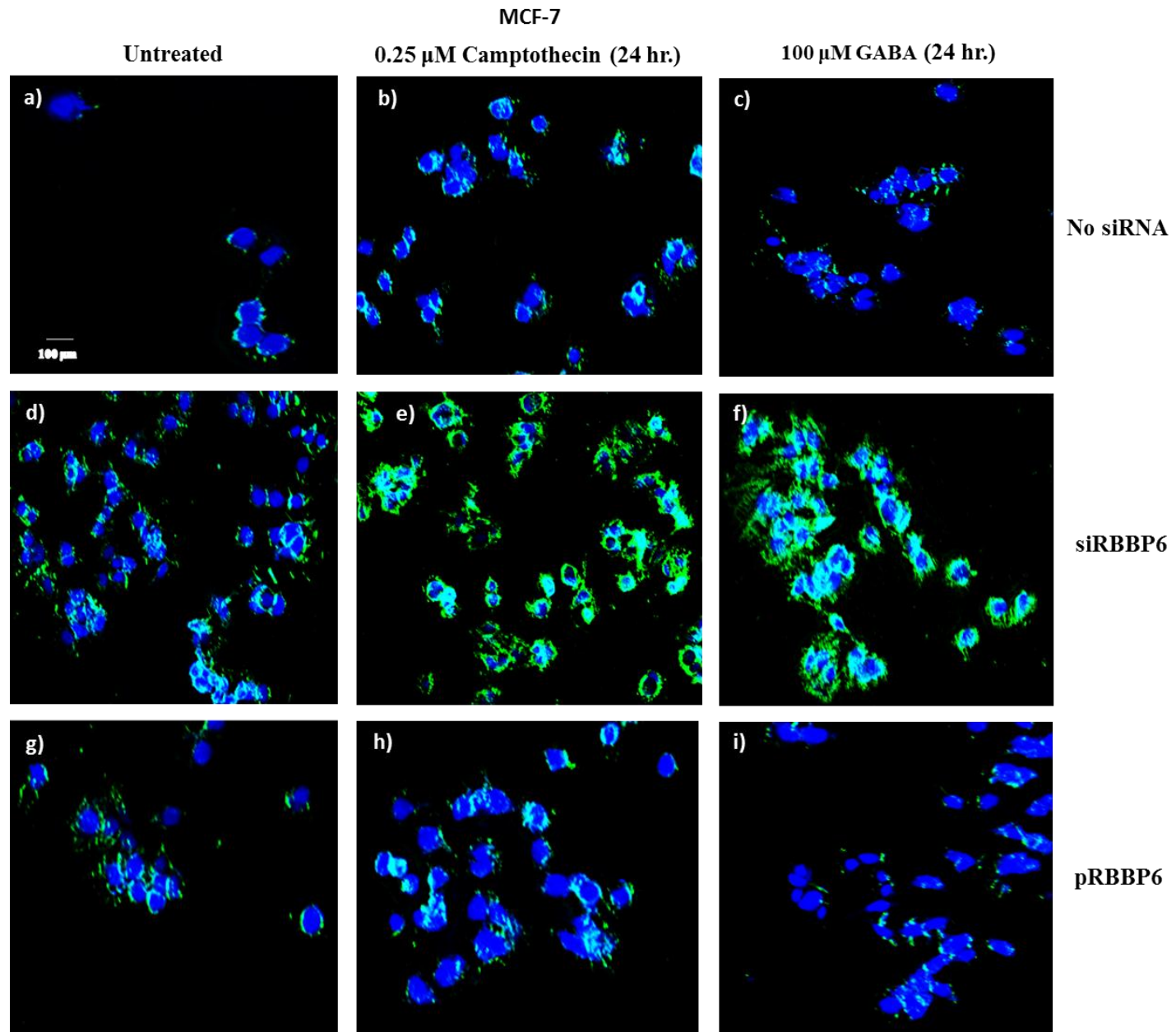
Figure 49: Microscopic analysis of Annexin V stained in MCF-7 cells.

The Annexin V positive cells are indicated by the green dye as a representation of apoptotic cells.

Apoptosis induction was high in siRBBP6+camptothecin (panel E) and siRBBP6+GABA (panel F).

MDA-MB-231

Untreated	0.5 µM Camptothecin (24 hr.)	100 µM GABA (24 hr.)	
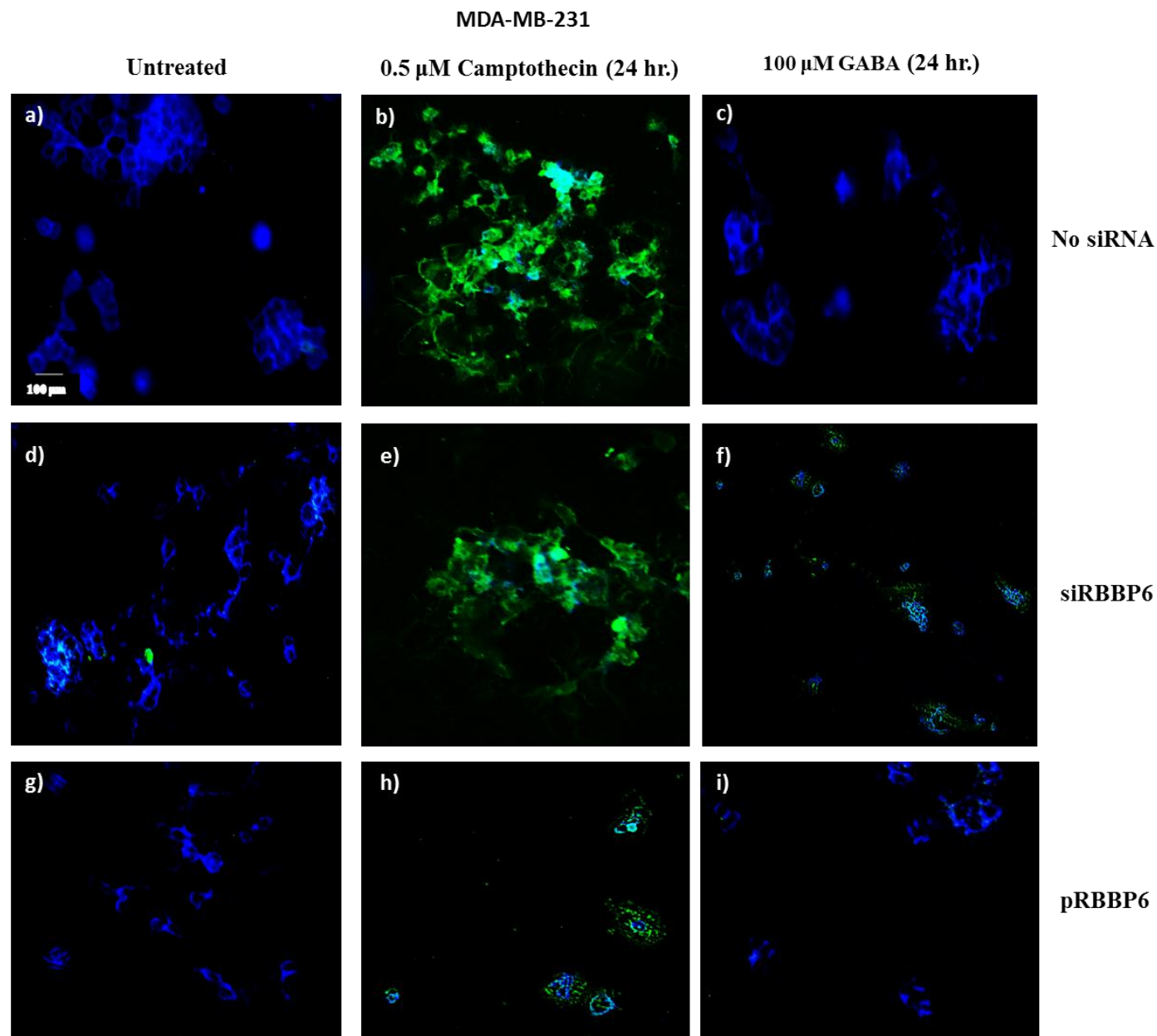
Figure 50: Microscopic analysis of Annexin V stained MDA-MB-231 cells.

The Annexin V positive cells are indicated by the green dye as a representation of apoptotic cells. The staining above shows visible apoptosis in camptothecin (panel B) and siRBBP6+camptothecin (panel E) at 10X magnification.

5.2.4. Analysis of caspase activity and mitochondrial ATP

Effector caspases 3 and 7 play an important role in executing apoptosis, and therefore their activation indicates a point of no return in apoptosis induction. Activation of these caspases depends on the release of cytochrome c from the outer mitochondrial membrane (OMM) following permeabilization by bcl-2 family pro-apoptotic proteins (Wen *et al*., 2012; Ouyang *et al*., 2012). In the cytosol, cytochrome c binds to apoptosis peptidase activating factor 1 (Apaf-1) and active caspase 9 in order to form an apoptosome complex which is responsible for the activation of the effector caspases (Wen *et al*., 2012; Ouyang *et al*., 2012). Active caspase 3 and 7 therefore trigger apoptosis by cleaving DNA in the nucleus. On the other hand, permeabilization of OMM leads to a decrease in mitochondrial transmembrane potential ($\Delta\Psi$m) which subsequently leads to ATP depletion (Gergely *et al*., 2002). Decreasing mitochondrial ATP is therefore another indicator of apoptosis induction which is why it was important for the present study to monitor ATP levels in response to treatment.

Caspase 3/7 activity was not significantly changed following transfection with either siRBBP6 or pRBBP6 in comparison to untreated cells in both MCF-7 and MDA-MB-231 cells (Figure 51). However camptothecin treatment increased caspase activity to 6000 RLU and 4000 RLU in MCF-7 and MDA-MB-231 cell lines, respectively. Co-treatment with siRBBP6 and camptothecin failed to sensitize caspase activity as well in MCF-7 cells (3800 RLU); however a significant increase was observed in MDA-MB-231 cells (4100 RLU) (Figure 51). pRBBP6, alone or in combination with either camptothecin or GABA led to a non-significant increase in caspase 3/7 activity in both MCF-7 and MDA-MB-231 cells (Figure 51). No significant caspase activity was observed in normal fibroblasts (MRC-5) except in cells transfected with siRBBP6 where a statistically significant caspase activity (308 RLU) was induced (Figure 52).
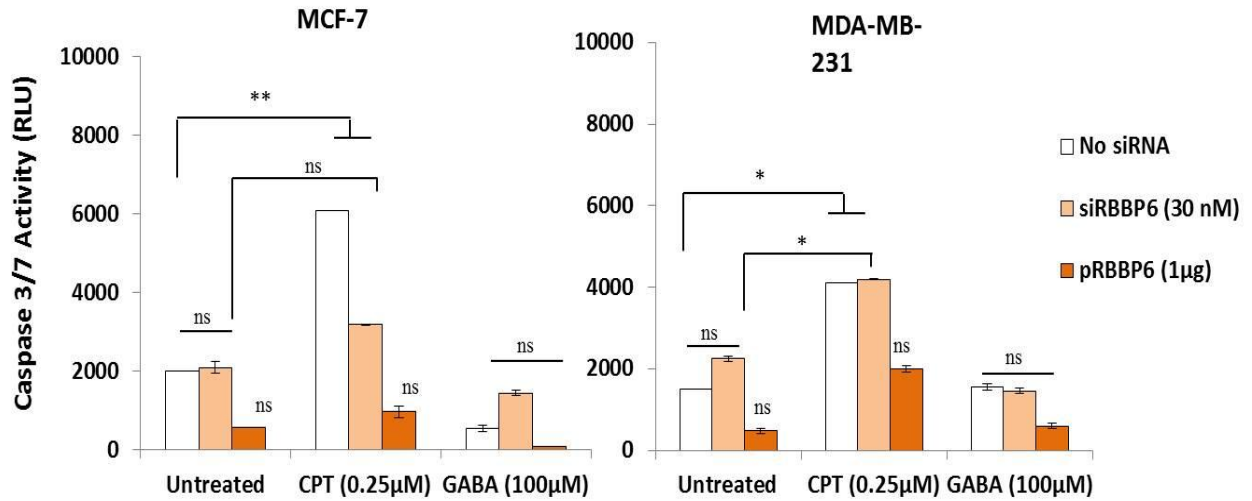
Figure 51: Caspase 3/7 activity analysis of MCF-7 and MDA-MB-231 cells.

Caspase activity was increased in co-treatment with Camptothecin and siRBBP6 in MDA-MB-231.
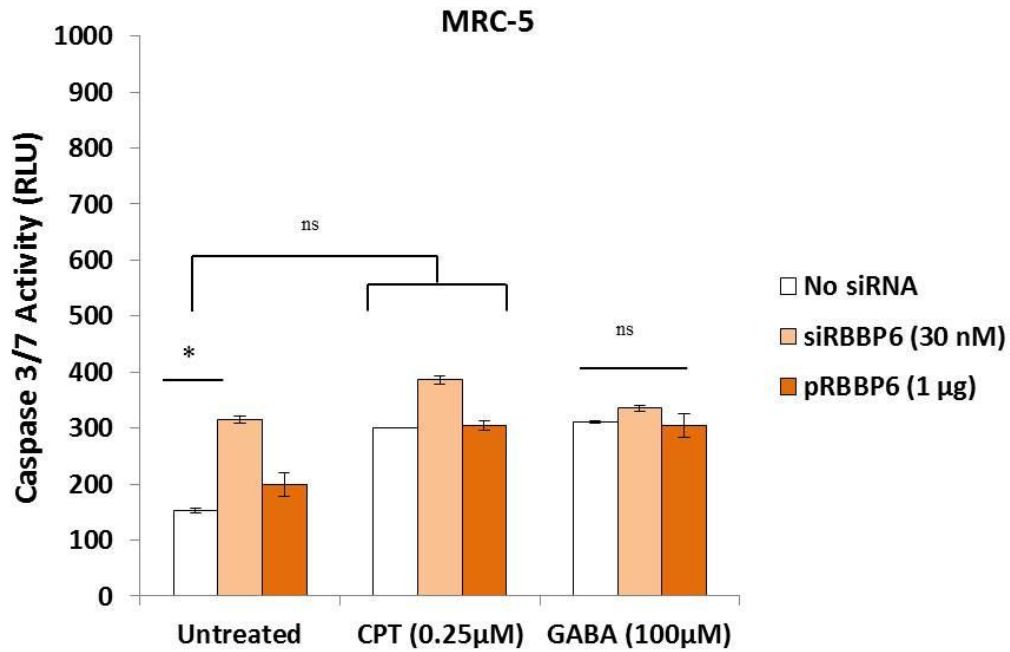
Figure 52: Caspase 3/7 activity analysis of MRC-5 cells.

Mitochondrial ATP depletion is a good indicator of outer membrane permeabilization due to activated pro-apoptotic proteins responsible for the activation of the intrinsic pathway. Following

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transfection with either siRBBP6 or pRBBP6 for 24 hours, mitochondrial ATP depletion was measured at two hours intervals post co-treatment with either camptothecin or GABA. In MCF-7 cells treated with siRBBP6, camptothecin and siRBBP6+camptothecin, ATP depletion continued steadily for 8 hours at the value of between 30 RLU before recovering back to 110 RLU 24 hours later (Figure 53). ATP continued to decrease throughout the entire experiment in MDA-MB-231 cell transfected with siRBBP6 whereas it remains constant below 50 RLU in siRBBP6+camptothecin treatment (Figure 53). pRBBP6, alone or in combination with camptothecin or GABA led to an increase in ATP production in both MCF-7 and MDA-MB-231 cells. However, in MDA-MB-231 cells, ATP level drops back to ~150 RLU where it remained constant throughout the experiment. No ATP depletion was observed in MRC-5 fibroblasts except in camptothecin and siRBBP6+GABA treatments where it decreased for 2 hours before continuing to increase for the remainder of the experiment (Figure 54).
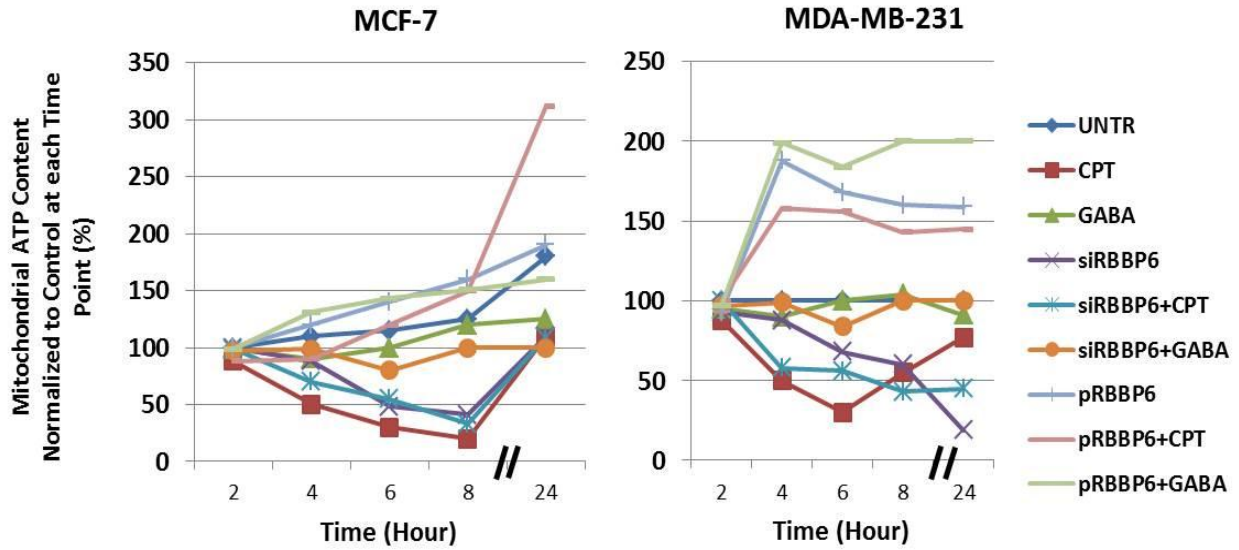
Figure 53: Mitochondrial ATP analysis in MCF-7 and MDA-MB-231 cells at different time intervals.
The assay was conducted in triplicate and ATP levels were reported as a mean % of relative light units
(RLU) normalized to control at each time point.
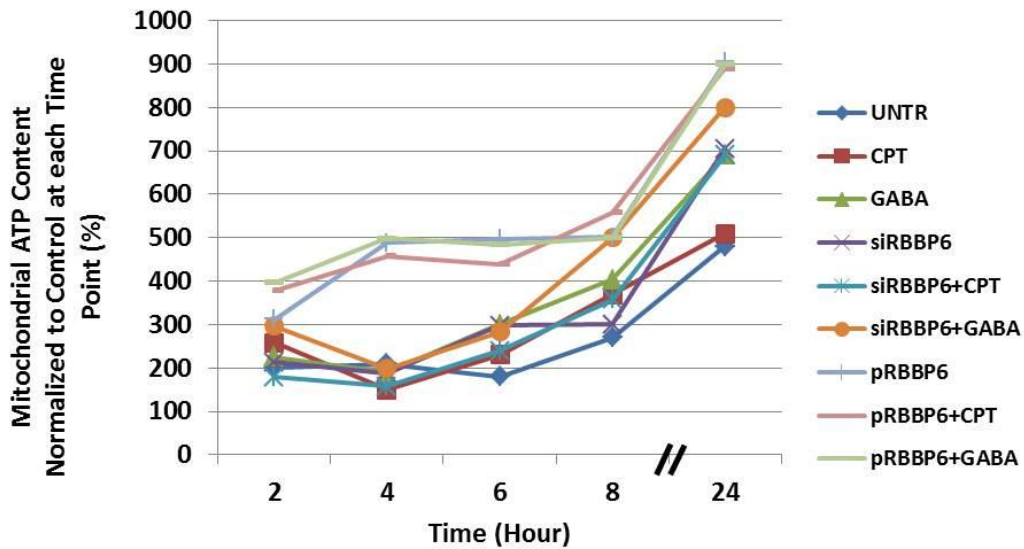
Figure 54: Mitochondrial ATP analysis in MRC-5 cells at different time intervals in hours.
The assay was conducted in triplicate and ATP levels were reported as mean % of relative light units
(RLU) normalized to control at each time point.

5.2.5. Analysis of cell cycle arrest

Cell proliferation and division are governed by proper progression of the cell cycle in which cell in the quiescence stage (G0) are allowed to enter the first gap phase (G1) before transition into the next phase (Besson *et al*., 2008) . During the G1 phase, all requirements needed for proper DNA replication in the S-phase get checked. This is followed by a second checkpoint, G2, in which ploidy or the correct number of sets of chromosomes is ensured before transition to mitosis (M phase) where the cells undergo division (Khodjakov and Rieder, 2009; Collins *et al*., 1997). In response to DNA damage, the cell becomes arrested in G0 where it undergoes DNA repair, failure of which triggers apoptosis. However, during carcinogenesis cell divide rapidly such that regulatory process become impaired thus leading to progression into either the S-phase or the M phase without going through the cell cycle checkpoints (DiPaola, 2002). The present study therefore analyses the effect of transfection and co-treatment on the cell cycle checkpoints G0/G1 and G2/M as well as the S-phase.

In breast cancer cell line (MCF-7), populations in G0/G1 arrest were increased following treatment with camptothecin at 67.45% and in GABA at 54.9% (Figure 55). Following RBBP6 silencing cells in G0/G1 arrest increased to 70.3% from initial value of 26.35 % in untreated cells; similarly a treatment with siRBBP6+camptothecin and siRBBP6+GABA showed a moderate increase of cells to 58.05% and 45.65% respectively (Figure 55). In up-regulated RBBP6, there was no change in cell populations in G0/G1 arrest following treatment with pRBBP6+camptothecin (37.35%) and pRBBP6+GABA (38.1%). For S-phase analysis, there was insignificant change in cell populations following treatment with camptothecin (10.65%), GABA (7.2%), siRBBP6 (8.65%), siRBBP6+camptothecin (6.35%) and a significant decrease in

siRBBP6+GABA at 4.7%. Transfection with pRBBP6 significantly increased the amount of cells in the S-phase to 26.9%; however a moderate decrease was observed in pRBBP6+camptothecin at 17.25% as well as in pRBBP6+GABA at 17.6% (Figure 55). Induction of G2/M arrest was insignificant at 13.9% in camptothecin and 16.8% in GABA in comparison to untreated at 25.5%. In treatment cells in G2/M was reduced in siRBBP6 (14.85%), siRBBP6+camptothecin (14.05%) and siRBBP6+GABA (9.1%). A significant increase in populations of G2/M arrest was evident in pRBBP6+GABA at 31% (Figure 55).

In MDA-MB-231 cell line induction of G0/G1 arrest increased to 59.7% after treatment with camptothecin and to 55.6% in GABA from 31.25% in untreated cell (Figure 55). Treatment with siRBBP6 significantly increased cells in G0/G1 arrest to 61.35% while co-treatment with camptothecin (58.6%) and GABA (63.35%) did not have a significant change in G0/G1 arrest from silencing only. In RBBP6 overexpression and co treatments, there were little differences in cell populations in G0/G1 arrest with pRBBP6 (48.65%), pRBBP6+camptothecin (37.25%) and pRBBP6+GABA (38.1%). The cells in the S-phase showed a little difference from untreated to treated cells with 9.1% in untreated, 5.7% in camptothecin, 7.15% in GABA, 8.45% in siRBBP6, 7.95% in siRBBP6+camptothecin and 6.45% in siRBBP6+GABA. On the other hand a significant increase was observed in pRBBP6 at 15.4% cell population whereas co-treatment with pRBBP6+camptothecin and pRBBP6+GABA slightly increased cells in the S-phase to 17.75% and 17.6%, respectively. Cells in G2/M arrest were fairly high in GABA treatment at 40.5% and in siRBBP6+GABA at 40.4%. Insignificant changes were observed in cells treated with camptothecin at 31.75%, siRBBP6 at 38.15% and siRBBP6+camptothecin at 30.55%

relative to untreated at 35.8%. Transfection with pRBBP6 led to 18.75% cell populations in

G2/M arrest with 13.15% in pRBBP6+camptothecin and 24% in pRBBP6+GABA (Figure 55).
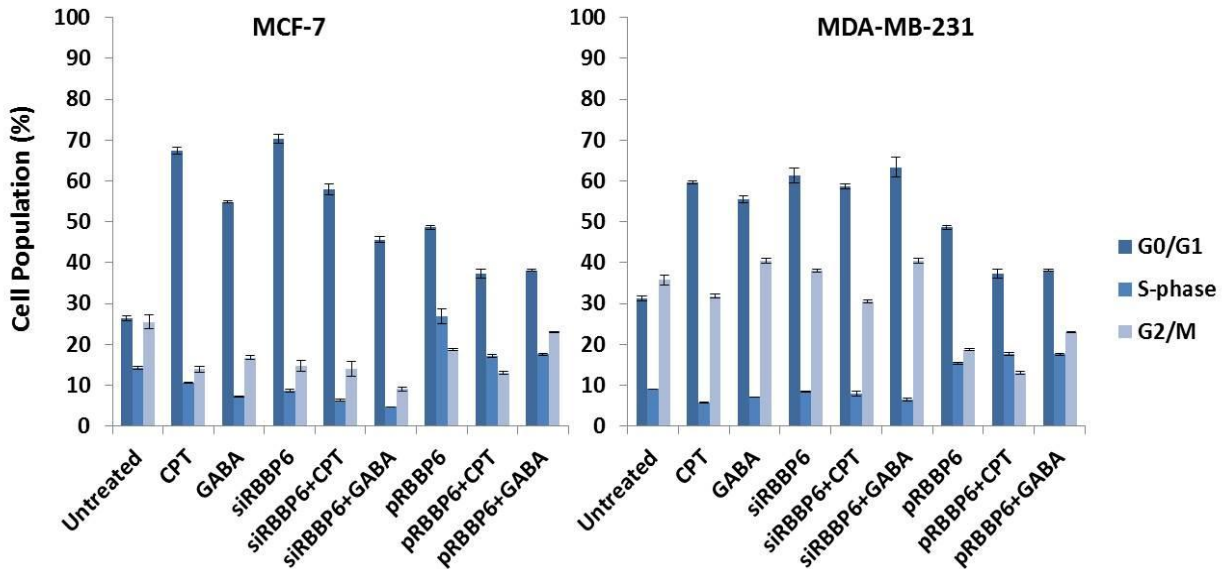
Figure 55: Cell cycle analysis in MCF-7 and MDA-MB-231 cell lines.

In normal lung fibroblasts (MRC5) induction of G0/G1 arrest was observed however there was

no significant change following different treatments (Figure 56). The cells in G0/G1 arrest were

recorded as 53.85% in untreated, 59.55% in camptothecin, 50.2% in GABA, 50.55% in

siRBBP6, 55.35% in siRBBP6+camptothecin, 50.95% in siRBBP6+GABA, 55.5% in pRBBP6,

57.75% in pRBBP6+camptothecin and 57.6% in pRBBP6+GABA. Slight changes were

observed in cell populations in the S-phase with camptothecin treatment at 19.55% in

comparison to untreated at 13.85%. There was a decrease, however, to 10.2% in GABA, 10.55%

in siRBBP6 and 10.95% in siRBBP6+GABA. The S-phase cell populations were increased to

15.4% in pRBBP6, 17.75% in pRBBP6+camptothecin and 16% in pRBBP6+GABA. Cells in G2/M arrest remained around 30% across all treatments, with 32.75% in untreated, 33.55% in camptothecin, 25.75% in GABA, 37.8% in siRBBP6, 38.75% in siRBBP6+camptothecin, 38.7% in siRBBP6+GABA, 38.65% in pRBBP6, 27.25% in pRBBP6+camptothecin and 28.1% in pRBBP6+GABA (Figure 56).
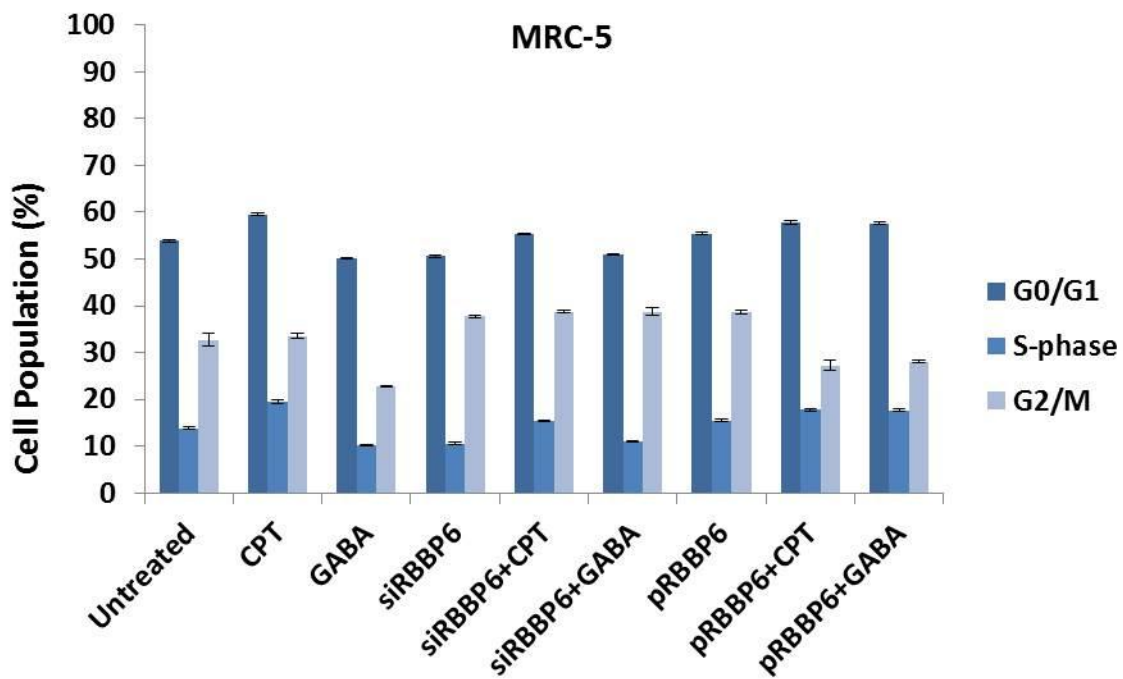
Figure 56: Cell cycle analysis in MRC-5 cell line.

5.2.6. Quantification of genes involved in apoptosis

Apoptosis is mainly regulated by the Bcl-2 family proteins and therefore understanding their expression levels in response to RBBP6 targeting and co-treatment might help determine the mode of the observed apoptosis. Quantitative PCR was therefore used to analyse the mRNA expression levels of the following pro-apoptotic genes: bax, bak1, bad, caspase-3 and caspase-8, the Bcl-2 anti-apoptotic gene as well as the p53 transcription factor. Relative gene expression was calculated using REST 2009 software (version 2.0.13) which computes expression ratios based on real time PCR efficiency and delta crossing points (ΔCP) of unknown samples versus a control.

In MCF-7 cell line, treatment with camptothecin led to no change in RBBP6, TP53 and bax gene expression (Figure 57). Bak1 and bad were up-regulated by 39.397 and 4.691, respectively, whereas caspase-3 and Bcl-2 were down-regulated by 0.600 and 0.398, respectively. No significant change was observed in the expression of Caspase-8 following treatment with camptothecin. GABA treatment had no effect on RBBP6, TP53, and bax genes; however Bak1 was down-regulated by a mean factor of 0.123. Bad gene expression was increased by a mean factor of 2.014 whilst the expression of Bcl-2 anti-apoptotic gene remained the same following GABA treatment. RBBP6 was down-regulated by 0.109 following silencing in MCF-7 and this led to TP53 up-regulation by a mean factor of 4.611. No change was observed in bad expression following RBBP6 silencing; however, Bcl-2 expression was increased by 3.797 and bax by 11. In contrast, silencing of RBBP6 led to a decrease in bak1 (0.247) and caspase-3 (0.700). RBBP6 up-regulation (5.657) had no effect on TP53, bad and bcl-2 expression; however bax and bak1 were down-regulated by 0.177 and 0.500, respectively. Furthermore, caspase-3 and caspase-8

were down-regulated in response to RBBP6 upregulation by mean factors 0.400 and 0.203, respectively.

RBBP6 silencing in combination with camptothecin led to down-regulation of RBBP6 by 0.086 whereas TP53, bax, bak1, bad and caspase-8 were up-regulated by 30.065, 9.514, 5.796, 2.969 and 9.350, respectively (Figure 58). In contrast, the anti-apoptotic Bcl-2 was down-regulated by a mean factor of 0.273 following siRBBP6+camptothecin treatment. In combination with GABA, RBBP6 silencing (0.041) in combination with GABA increased the expression of TP53 by 5.657 and caspase-8 by 2.828. siRBBP6+camptothecin led to no change in bax, caspase-3 and bad expression; however down-regulation was observed in bak1 (0.488) and Bcl-2 (0.540). In RBBP6 overexpressing cells treated with camptothecin, RBBP6 overexpression (8.282) was coupled with TP53, bax and Bcl-2 down-regulation by 0.203, 0.297 and 0.371, respectively. No significant change in expression was observed in bak1 and bad genes; however, caspase-8 was up-regulated by a 2-fold change. Combination of RBBP6 overexpression with GABA increased expression of RBBP6 and caspase-8 by 34.535 and 2.144, respectively, however no change was observed in TP53 and bax expression. Bak1, bad and Bcl-2 genes were down-regulated by 0.392; 0.196 and 0.215, respectively, following treatment with pRBBP6+GABA.
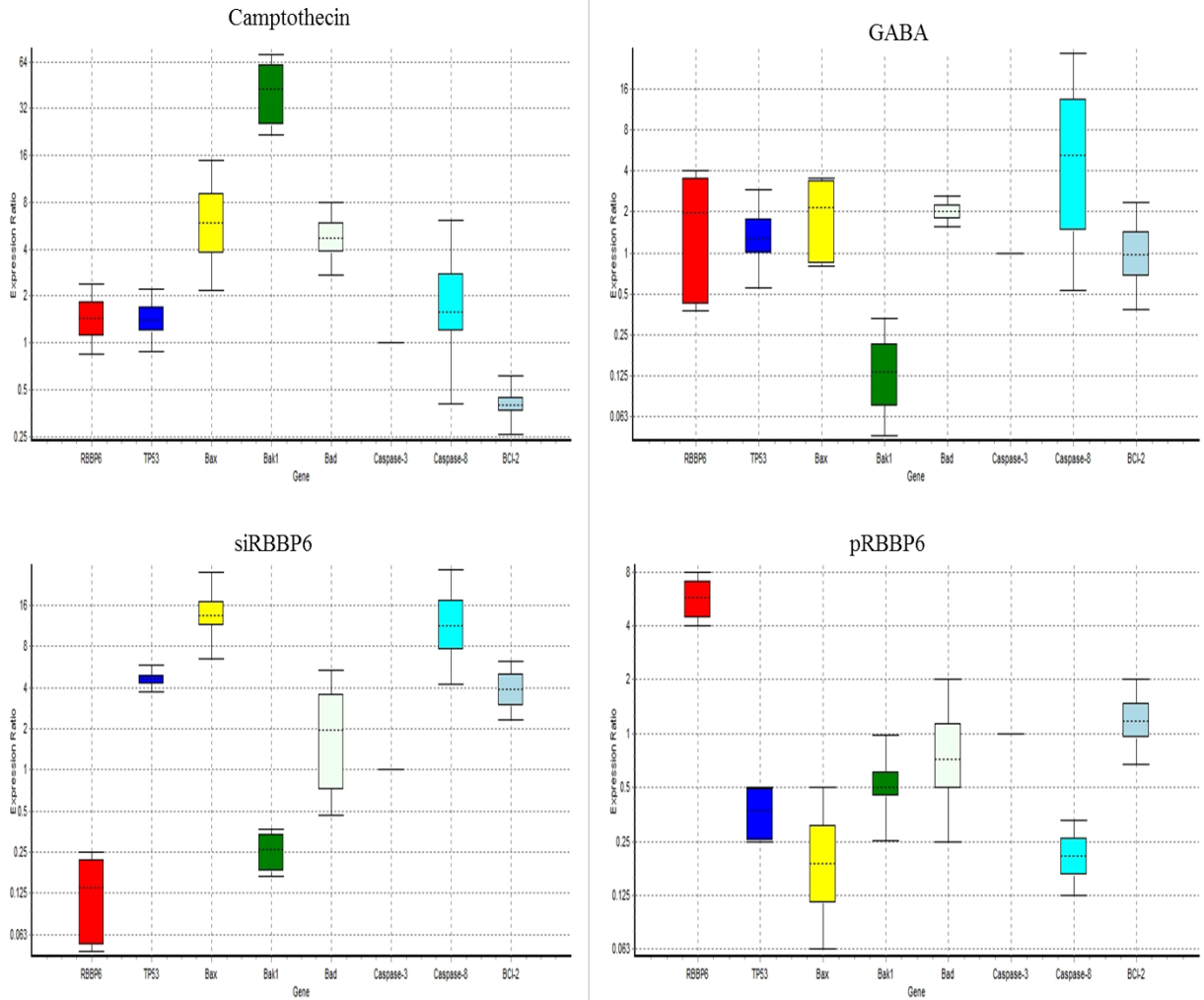
Figure 57: Relative quantification of gene expression in MCF-7 cell line performed using qPCR. Whisker box plots with expression ratio on the y-axis and gene type on the x-axis indicate quantification of mRNA isolated from cells treated with camptothecin (top left), GABA (top right), siRBBP6 (bottom left) and pRBBP6 (bottom right). Gene expression is depicted by the colour coded whisker bars in the following manner: RBBP6 (red), TP53 (blue), bax (yellow), bak1 (light green), bad (grey), caspase-3 (dark green), caspase-8 (cyan) and Bcl-2 (light blue).

Figure 58: Relative quantification of gene expression in MCF-7 cell line was performed using qPCR. Gene expression in response to treatment with siRBBP6+camptothecin (top left), siRBBP6+GABA (top right), pRBBP6+camptothecin (bottom left) and pRBBP6+GABA (bottom right).

In MDA-MB-231 cell line, RBBP6, TP53, bax, bak1 and caspase-8 expression changed slightly whereas bad was up-regulated by a mean factor of 3.317. Caspase-3 and Bcl-2 were down-regulated by a 0.5-fold and 0.281-fold changes, respectively, in camptothecin treatment (Figure 59). GABA treatment did not have any effect on the expression of RBBP6, TP53, caspase-3, Bcl-2 and caspase-8 as well; however bax and bak1 were down-regulated by mean factors 0.052

and 0.087, respectively. Bad was up-regulated by a mean factor of 1.424 in response to GABA treatment. RBBP6 silencing (0.077) had no effect on the expression of TP53, bax and bad; however bak1 and caspase-3 expression were down-regulated by 0.255. Caspase-8 and Bcl-2 were up-regulated by 5.521 and 5.370, respectively. Overexpression (5.657) had no significant effect on TP53 expression, whereas caspase-3 and Bcl-2. Bax, bak1, bad and caspase-8 were down-regulated by 0.125, 0.354, 0.500 and 0.287, respectively.

Combination of RBBP6 silencing with camptothecin led to a decrease in RBBP6 expression by a mean factor of 0.171 and had no significant effect on TP53, bax, caspase-3 and Bcl-2 gene expression (Figure 60). Bak1, bad and caspase-8 gene expression was increased by 3.215, 3.182 and 4.675, respectively, in response to siRBBP6+camptothecin co-treatment. Co-treatment with siRBBP6+GABA reduced the expression of RBBP6 by a mean factor of 0.041; however the change in expression of TP53, bax, caspase-3 and bad was insignificant. Bak1 and caspase-8 genes were up-regulated by 1.231 and 2.828, respectively, however Bcl-2 was down-regulated by 0.419. RBBP6 overexpression combined with camptothecin led to an increase in the expression of RBBP6 and caspase-8 by mean factors 3.701 and 1.741, respectively; however the change in expression of TP53, bak1 and bad was not significant. A decrease in Bcl-2 (0.320) as well as bax and caspase-3 (0.500) were observed following treatment with pRBBP6+camptothecin. Co-treatment with pRBBP6+GABA increased RBBP6 expression to 34.535 and bax expression to 2.378 whereas no significant change was observed in the expression of TP53, bak1 and caspase-8. Bad, caspase-3 and Bcl-2 were down-regulated by 0.049, 0.4972 and 0.137, respectively following co-treatment with pRBBP6+GABA.
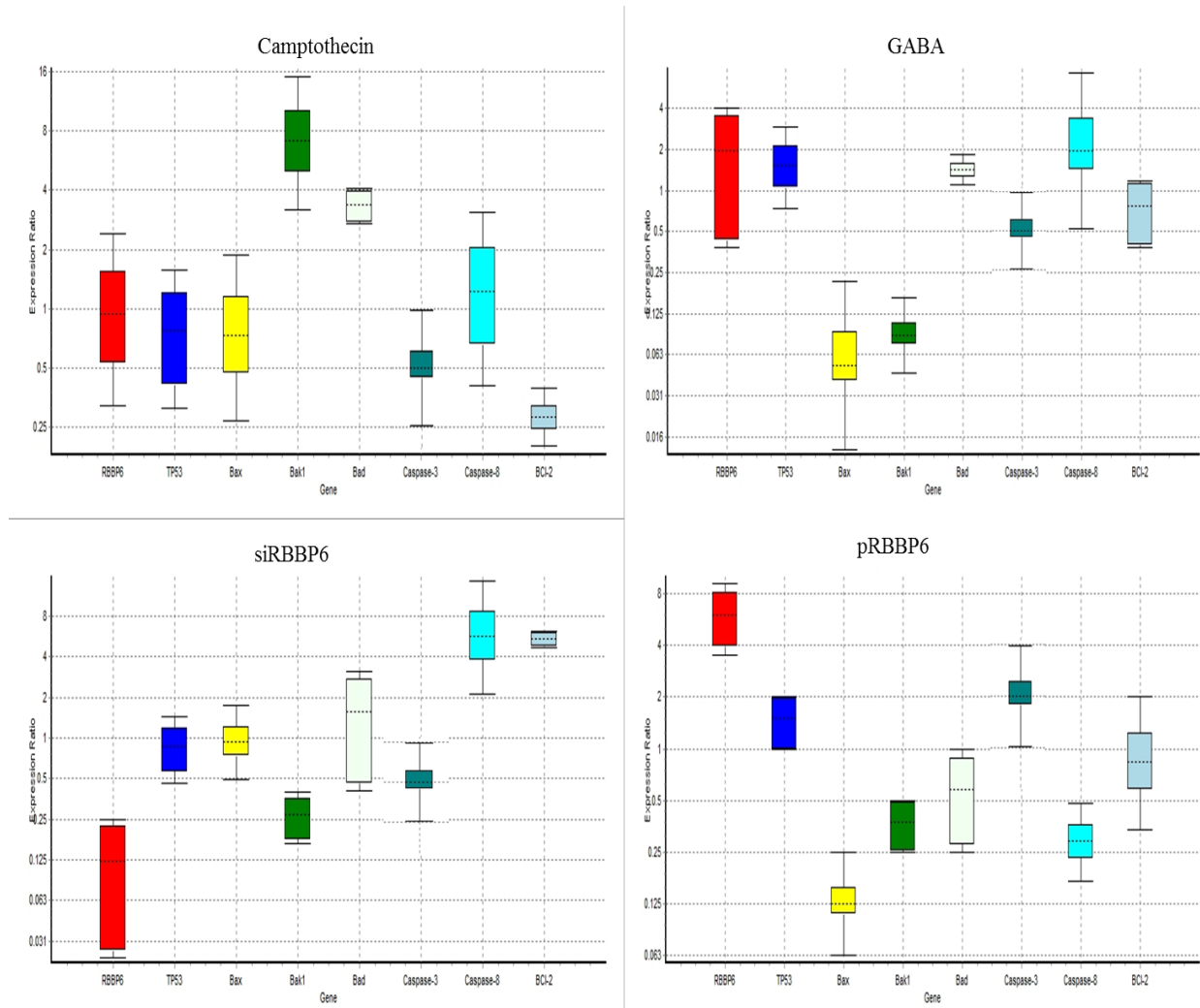
Figure 59: Relative quantification of gene expression in MDA-MB-231 cell line by qPCR.

The cells were treated with camptothecin (top left), GABA (top right), siRBBP6 (bottom left) and pRBBP6 (bottom right).
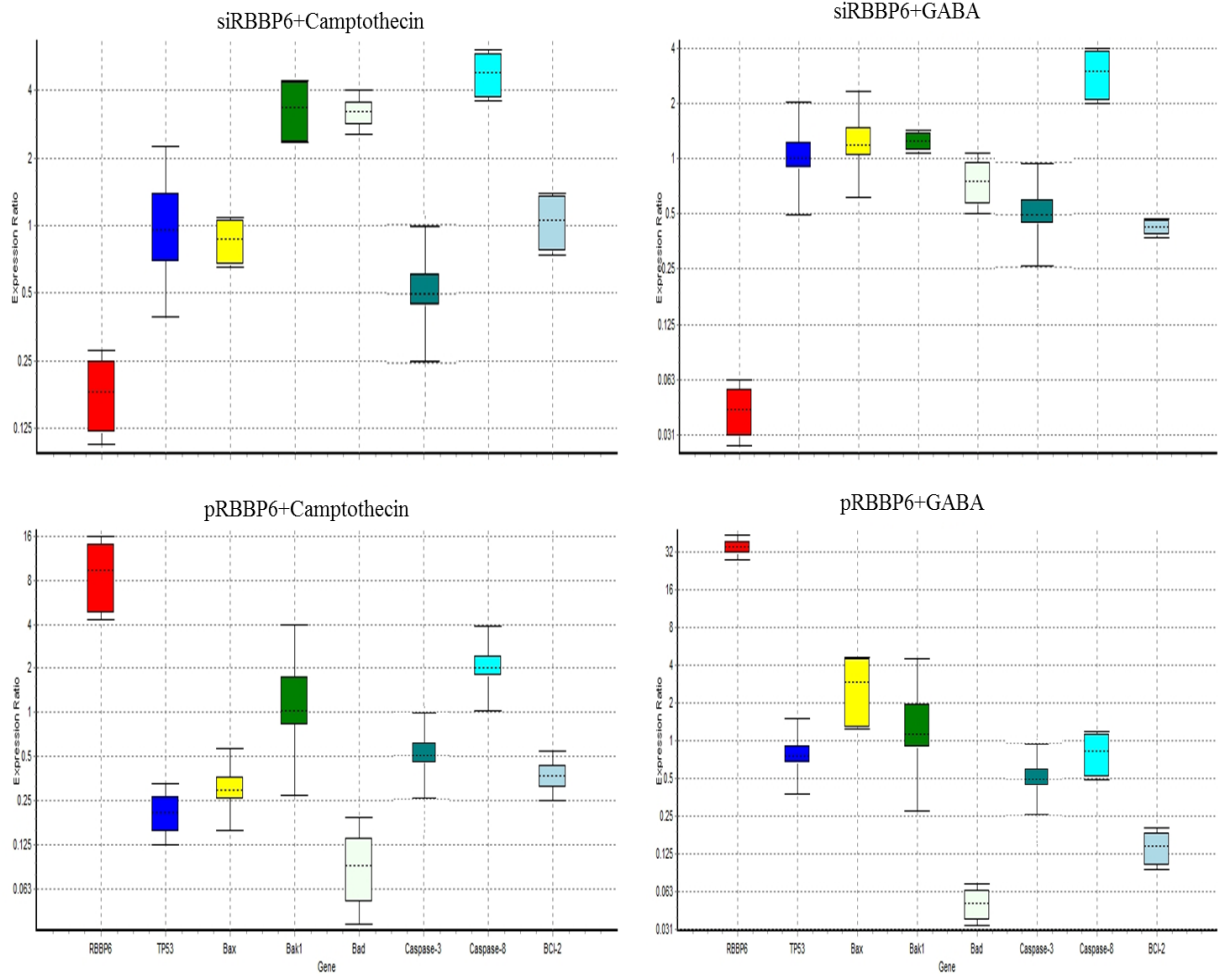
Figure 60: Relative quantification of gene expression in MDA-MB-231 cell line by qPCR.

The cells were treated with siRBBP6+camptothecin (top left), siRBBP6+GABA (top right), pRBBP6+camptothecin (bottom left) and pRBBP6+GABA (bottom right).

No significant changes were observed in gene expression following treatment with camptothecin and GABA in normal lung fibroblasts (Figure 61). RBBP6 silencing (0.518) led to an increase in caspase-8 expression by 1.414 and had no significant effect on the expression of other genes. Similarly, no significant changes were observed in the expression of all genes in response to RBBP6 up-regulation (2.462). siRBBP6+camptothecin reduced RBBP6 expression by a mean

factor of 0.154, whilst siRBBP6+GABA reduced RBBP6 and bak1 by 0.330 and 0.500, respectively. Furthermore, siRBBP6+GABA increased gene expression of bax by 1.414 (Figure 62). RBBP6 was up-regulated by a mean factor of 5.856 in response to co-treatment with pRBBP6+camptothecin, and no significant effect was observed on the expression of other genes. Co-treatment with pRBBP6+GABA increased RBBP6 expression by a mean factor of 3.053 and bax by a mean factor of 1.809.
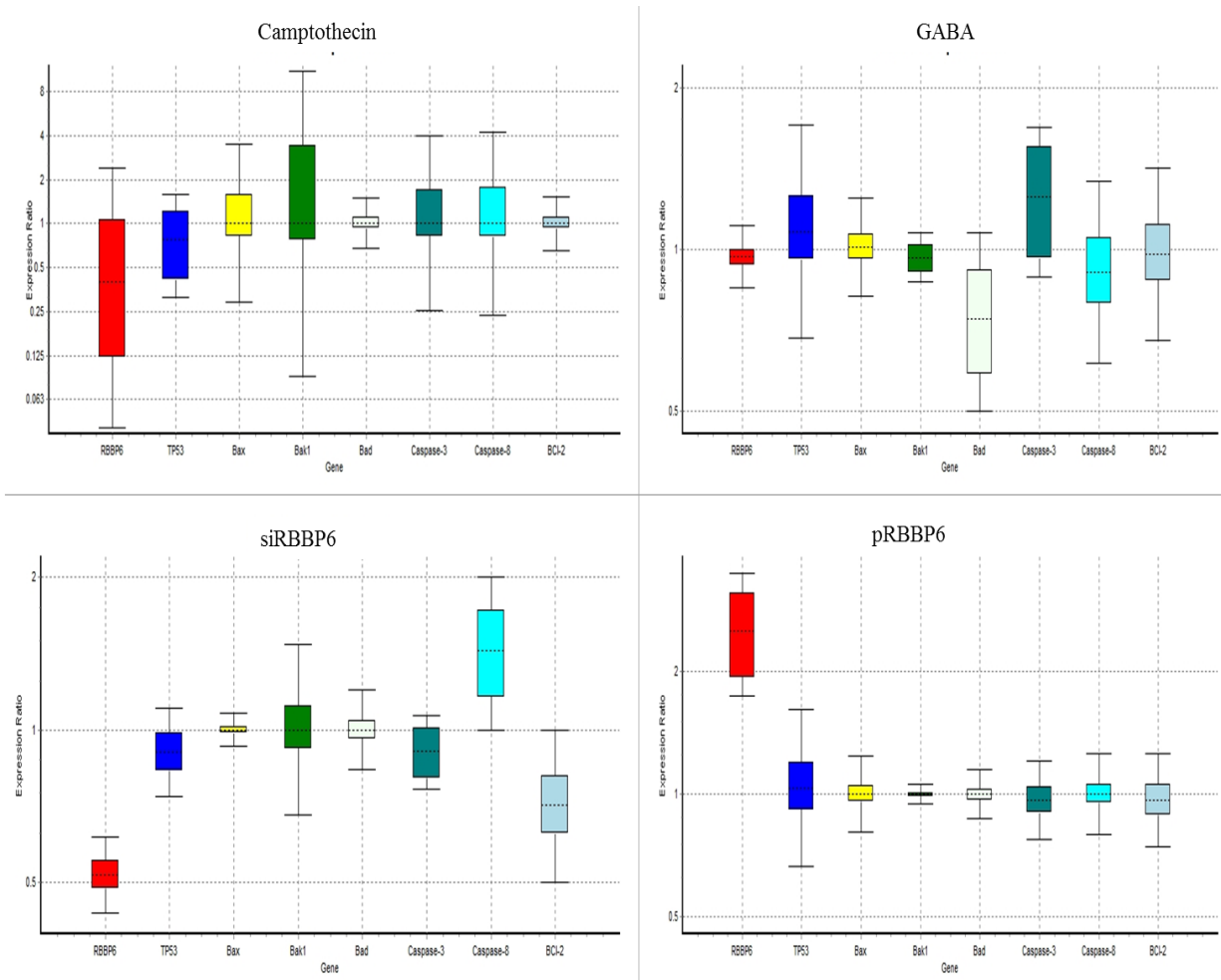
Figure 61: Relative quantification of gene expression in MRC-5 cell line was performed using qPCR. The cells were treated with camptothecin (top left), GABA (top right), siRBBP6 (bottom left) and pRBBP6 (bottom right).
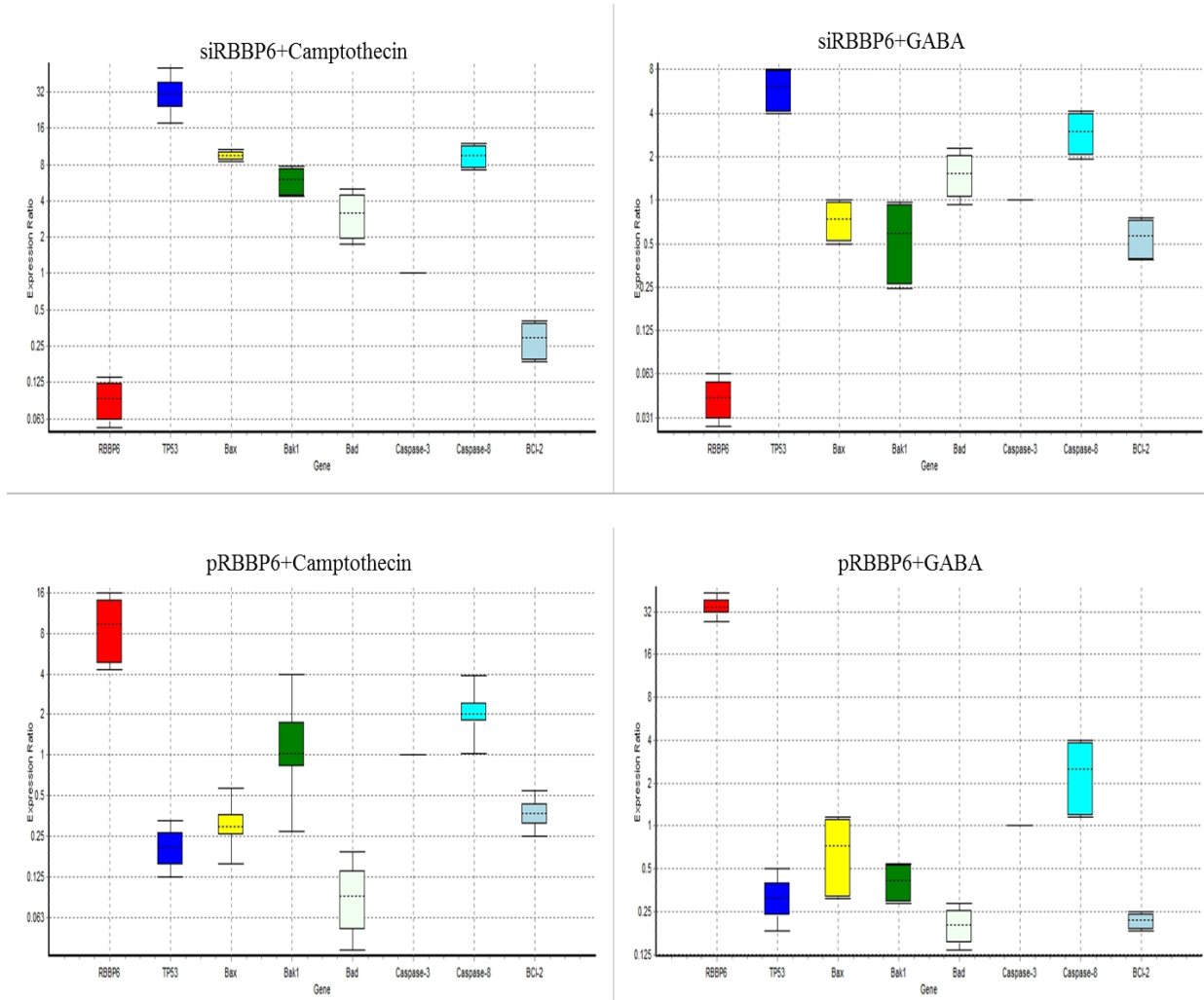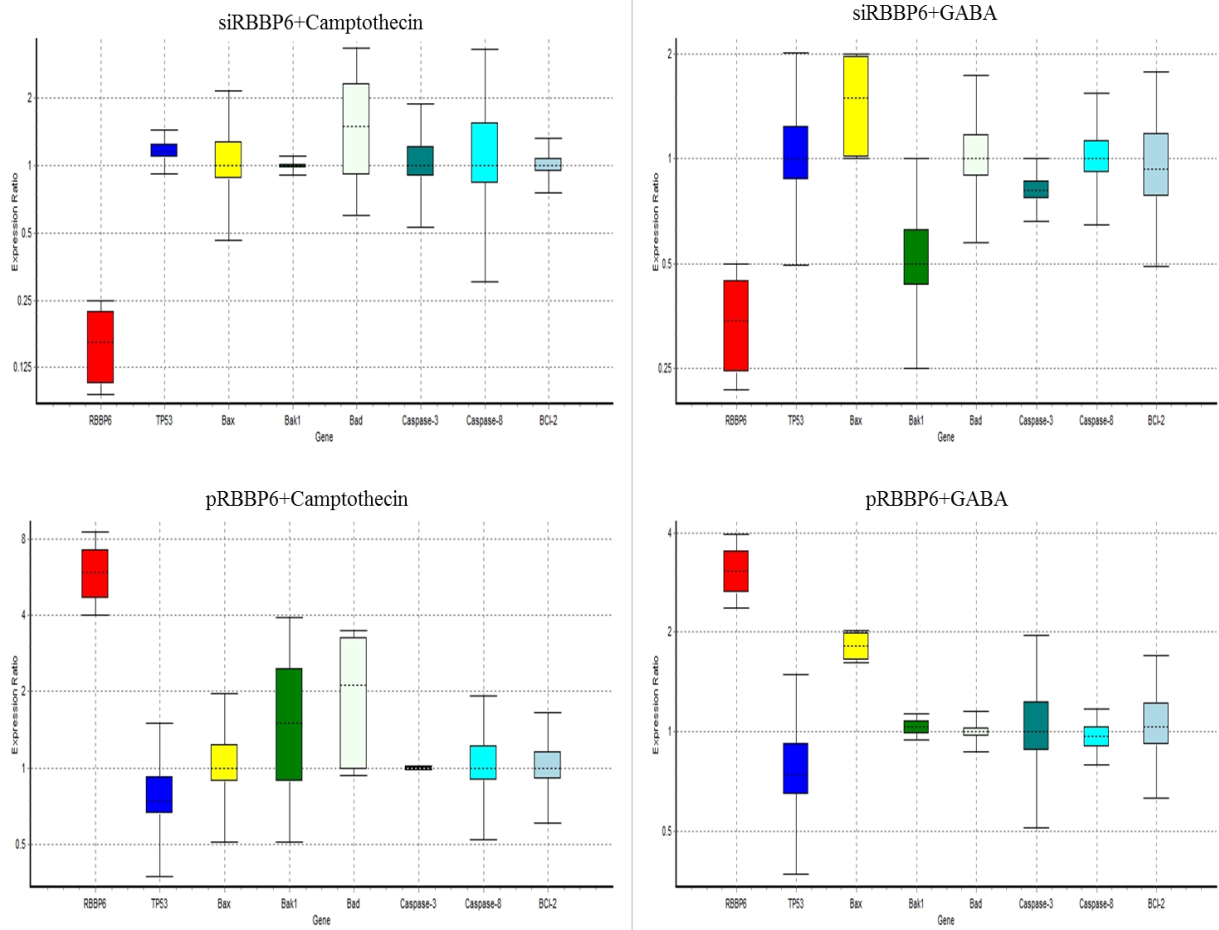
Figure 62: Relative quantification of gene expression in MRC-5 cell line was performed using qPCR. The cells were treated with siRBBP6+camptothecin (top left), siRBBP6+GABA (top right), pRBBP6+camptothecin (bottom left) and pRBBP6+GABA (bottom right).

5.3.   Discussion

The present study demonstrates the effects of RBBP6 silencing and overexpression in combination with anticancer agents on breast cancer progression. The study further provides a comparative analysis of the two genetically different breast cancer cell lines, MCF-7 and MDA-MB-231, as far as their response to RBBP6 targeting is concerned. The two cell lines display different p53 expression levels, with MCF-7 being shown to generally express wt. p53 and MDA-MB-231 expressing mt. p53 (Gartel *et al.*, 2003). It was therefore interesting to investigate the effect of RBBP6 on these cell lines since it (RBBP6) associates with p53 (Pugh *et al.*, 2006).

Silencing in both cell lines was achieved by delivery of siRNA oligonucleotides targeted at RBBP6 in which analysis using qPCR and western blotting revealed close to 90% silencing at both mRNA and protein levels (Figure 40). This successful silencing is credited to the high specificity elicited by the synthetic short interfering RNA molecules in directing homology-dependent control of gene activity using the cell's own machinery (Aagaard and Rossi, 2007; Meister and Tuschl, 2004). In terms of RBBP6 overexpression, the expression vector system was employed to transiently deliver RBBP6 cDNA clone into the cells. The cytomegalovirus (CMV) promoter present in the expression construct led to an efficient transcription of the RBBP6 transcript variant 3 sequence, which was subsequently translated into protein as confirmed by expression of the GFP reporter (Figure 34 to Figure 39).

It was interesting to see growth of both cell lines ceasing about 24 hours later into RBBP6 silencing as shown by the RTCA assay (Figure 41 and Figure 42). More so, the responses were

distinct as can be seen by the sharp decrease in MCF-7 cell proliferation as opposed to the cytostatic response in MDA-MB-231. This observation highlights early insights into the involvement of RBBP6 in cell proliferation, more especially because RBBP6 overexpressing MCF-7 cells maintain their growth at higher cellular index (Figure 41). This conforms to the high percentages of cell populations in the S-phase of RBBP6-overexpressing cells (Figure 55), suggesting that indeed RBBP6 promotes cell proliferation. It is not clearly known why or how RBBP6 affects cell proliferation; however the fact that it stabilizes chromosomal fragile sites (CFSs) during DNA replication might account for this (Miotto *et al.*, 2014). It has been reported that the instability of these sites on chromosomes perturb proper splitting of the chromatids thus leading to improper cell division (Miotto *et al.*, 2014). Based on this, RBBP6 is therefore necessary for promoting cell division and proliferation, as suggested by the observed continued cellular growth following RBBP6 overexpression.

Another possible explanation could be that RBBP6 negative regulation of wt. p53 inhibits cell cycle since functional p53 is needed for proper cell cycle progression (Li *et al.*, 2007). This might also help explain the different responses observed in the two cell lines where the mt. p53-expressing MDA-MB-231 appeared to be less responsive to RBBP6 silencing compared to MCF-7. These observations prompted us to quantify the mRNA expression levels of p53 in both cell lines. And it was interesting to notice that RBBP6 silencing leads to p53 accumulation (4-fold increase) in MCF-7 cells and no significant change in MDA-MB-231 cells, whereas overexpression did not seem to have a significant effect on p53 in both cell lines. In addition to p53, RBBP6 interaction with Rb tumor suppressor is known to release the E2F transcription factor which is responsible for promoting the cell cycle as well (Speidel, 2015; Bertheau *et al.*,

2014). Therefore this phenomenon could also be used to explain the observed changes in cell proliferation as a result of RBBP6 targeting.

To test whether or not the observed decrease in cell viability was as a result of apoptosis, flow cytometry and fluorescence microscopy were performed on cells transfected with either siRBBP6 or pRBBP6. Highly significant apoptosis induction was observed in MCF-7 cells and no significant apoptosis was detected in MDA-MB-231 (Figure 44, Figure 49 and Figure 50) following RBBP6 silencing. In terms of RBBP6 overexpression, no cell line elicited significant levels of apoptosis induction. These observations might suggest that RBBP6 silencing coupled with p53 accumulation in MCF-7 cells were responsible for apoptosis induction since the difference between these cell lines is the expression of functional p53. P53-mediated apoptosis occurs via the intrinsic pathway where p53 transactivates and promotes translocation of bax to the outer mitochondrial membrane, thereby oligomerizing with itself or bak1 in order to porate the membrane. Porous mitochondrial membrane allows the release of cytochrome c and other factors responsible for inducing apoptosis (Wen *et al*., 2012; Ouyang *et al*., 2012; Liu *et al*., 2011). Up-regulation of bax in MCF-7 cells might therefore be as a result of p53 activation thus confirming the observed apoptosis to be mitochondrial. Even though caspase 3/7 activity was not significant in MCF-7, probably due to the fact that the cell line is caspase-3 deficient, ATP depletion confirmed that indeed mitochondria were involved in the observed apoptosis induction (Gergely *et al*., 2002; Eguchi *et al*., 1997; Richter *et al*., 1996).

We then went on to combine RBBP6 targeting with apoptosis inducing anticancer agents, camptothecin and GABA to see if the observed apoptosis would be enhanced, especially in MDA-MB-231 cells since they failed to elicit significant levels of apoptosis induction. Camptothecin is a naturally occurring compound that was extracted from a Chinese medicinal plant and has since been shown to have anticancer effect (Chazin *et al*., 2014). GABA, the inhibitory neurotransmitter that's found in the central nervous system of vertebrates (McCarty, 2014; Takehara *et al*., 2007), was chosen as an anticancer agent in this study due to its ability to induce apoptosis, cell cycle arrest and blockade of tumour cell migration (McCarty, 2014; Caretta and Mucignat-Caretta, 2011). We have shown in our previous studies that camptothecin sensitizes MCF-7 cells to apoptosis and therefore similar observations were not surprising in the present study (Moela *et al.,* 2014). To our surprise, however; was the sensitization of MDA-MB-231 cells to camptothecin-induced apoptosis as well following RBBP6 silencing, although the effect was not comparable to that observed in MCF-7 cells. Similarly, apoptosis induced by GABA was not sensitized in MDA-MB-231 whereas it was in MCF-7 cells.

RBBP6 overexpression on the other hand did not induce nor sensitize cells to significant apoptosis levels in both cell lines. Besides the genotypic nature of p53 expression in these two cells lines, the mechanisms of action underlying the two anticancer agents might account for the differences observed in apoptosis levels. Camptothecin induces apoptosis by inhibiting topoisomerase I during the release of supercoils in DNA replication by binding to the single strand nicks created by the enzyme thus preventing religation. This leads to damaged DNA which then triggers the transactivation of p53 and a subsequent apoptosis induction (Chazin *et al*., 2014; Kümler *et al*., 2013; Hsiang *et al*., 1985). Therefore this might account for the

synergistic effect observed between camptothecin and RBBP6 silencing in inducing apoptosis since both strategies seem to reactivate the function of p53.

No distinctive mechanism of apoptosis induction has been described for GABA yet, however its affinity for G-protein coupled receptors has been shown to activate the enzyme adenylyl cyclase. This enzyme is responsible for activating the PKA/cAMP (protein kinase A/cyclin AMP) signaling pathway; which is responsible for phosphorylating several target proteins including those that lead to the induction of apoptosis (McCarty, 2014; Caretta and Mucignat-Caretta, 2011). This is in agreement with a few studies that have been carried out in other cancers where GABA was shown to induce apoptosis (McCarty, 2014; Caretta and Mucignat-Caretta, 2011; Khodjakov and Rieder, 2009; Oh and Oh, 2004). In the context of our study, apoptosis induction in response to GABA did occur as well; however at minimal levels in both cell lines. The reason for this is not known, however we suspect that the anticancer effects of GABA might be highly effective against cell migration as opposed to apoptosis. This is because several studies have been conducted, especially in liver cancer; where the effect of GABA on regulatory proteins associated with cell migration has been shown to be potent (Takehara *et al*., 2007; Schuller *et al*., 2008).

Indeed, MDA-MB-231 and MCF-7 cell lines show differential susceptibilities to apoptosis induced by RBBP6 silencing alone or in combination with either camptothecin or GABA. This might be as result of the distinct p53 statuses of the two cell line models. The p53 tumour suppressor carries missense mutations in most human cancers which are generally more stable

and highly expressed than wild-type p53 (Hui *et al*., 2006). This is not the case with the other two well-studied tumour suppressors Rb and APC as they have been shown to carry deletion and truncation mutations (Hui *et al*., 2006). Missense mutations are often associated with gain-of-function phenotypes hence mt. p53 in MDA-MB-231 cells have been reported to have the ability to interact with certain peptides in order to prevent apoptosis induction. One example of such peptides is called phospholipase D (PLD) and has been shown to be highly expressed in MDA-MB-231 cells where it interacts with mt. p53 (Hui *et al*., 2004) to generate survival signals that suppress DNA damage-induced apoptosis. This interaction might therefore be responsible for the restricted apoptosis induction observed in MDA-MB-231 cells in response to silencing and co-treatment, especially in camptothecin co-treatments since it induces apoptosis by damaging DNA.

5.4.   Summary

Successful silencing and overexpression of RBBP6 have enabled careful investigation of the effects RBBP6 has on growth of breast cancer cell lines. We have reported that both MCF-7 and MDA-MB-231 cell lines respond to RBBP6 silencing by ceasing to grow whereas overexpression of RBBP6 maintains their continued proliferation. In addition, MDA-MB-231 cell line seemed to respond at a slower rate to RBBP6 silencing as opposed to MCF-7s. This pattern appeared to be repetitive in apoptosis detection since MDA-MB-231 cells showed a weak response whereas MCF-7 cells showed significant amount of apoptosis induction. This apoptosis was further confirmed by ATP depletion assay as well as expression of pro-apoptotic genes. These differential characteristics elicited by the two cell lines were coined on their p53 statuses wherein wt. p53 accumulation in MCF-7 was associated with apoptosis induction especially because bax pro-apoptotic gene was up-regulated as well. On the other hand, mt. p53 in MDA-MB-231 is suspected to have had interactions with the pro-survival PLD peptide thus preventing apoptosis induction in this cell line.

Taken all together, it is evident that the p53 genotypic status of MCF-7 and MDA-MB-231 breast cancer cell lines plays a major role in their responsiveness to RBBP6 targeting and co-treatment with apoptosis-inducing agents. Furthermore, we have demonstrated that the ability of these cells to undergo apoptosis in the absence of RBBP6 is partly as a result of p53 reactivation, which provides early insights into the possible relationship between RBBP6 and p53 in breast cancer. These observations further highlight the significance of understanding the different subtypes of breast cancer as far as treatment is concerned because the heterogeneous nature of this disease requires patient-specific therapies thus making it difficult to manage breast cancer.

CHAPTER SIX - Silencing RBBP6 (Retinoblastoma Binding Protein 6) sensitises breast cancer cells MCF7 to staurosporine and camptothecin-induced cell death

In this chapter, the manuscript "**Silencing RBBP6 (Retinoblastoma Binding Protein 6) sensitises breast cancer cells MCF7 to staurosporine and camptothecin-induced cell death**" which was published in 2014, highlights the effects of RBBP6 silencing in combination with anticancer agents, staurosporine and camptothecin in breast tumorigenesis.

Silencing RBBP6 (Retinoblastoma Binding Protein 6) sensitises breast cancer cells MCF7 to *staurosporine* and *camptothecin*-induced cell death

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A B S T R A C T

Retinoblastoma Binding Protein 6 (RBBP6) is a multi-domain protein that uses its ring finger domain to interact with p53 and pRb tumour suppressor genes. The mechanism by which RBBP6 uses to degrade p53 is still unknown; nonetheless it is well known that RBBP6 promotes cell proliferation in several cancers by negatively regulating p53 via its E3 ubiquitin ligase activity. Degradation of p53 by RBBP6 may compromise p53-mediated apoptosis in breast cancer. This study is intended to investigate, the potential applications of RNA interference (RNAi) to block RBBP6 expression, as well as its subsequent effect on cell growth and apoptosis. Our studies indicate that the knockdown of RBBP6 by siRNA modulates p53 gene expression involved in cell death pathways and apoptosis, showing statistically significant gene expression differences. RBBP6 siRNA significantly reduced cell growth compared to the control samples and inhibition of cellular proliferation was observed between 24 and 48 h, as shown in the data obtained by real time cell analysis using the *xCELLigence* system. These results were further confirmed by flow cytometer which showed some apoptotic activity. About 20.7% increase in apoptosis was observed in cells co-treated with RBBP6 siRNA and *camptothecin* when compared to *camptothecin*-only whereas in siRBBP6 and *staurosporine* treated cells there was only an 8.8% increase in apoptosis. These findings suggest that silencing RBBP6 may be a novel strategy to promote *camptothecin*-induced apoptosis in breast cancer cells.

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Introduction

Screening tests performed over a decade ago revealed Retinoblastoma Binding Protein 6 (RBBP6) as a multi-domain protein that uses its ring-finger domain to interact with the p53 and pRb tumour suppressor genes (Pugh et al. 2006; Pretorius et al. 2013). Also known as RBQ-1, PACT or P2P-R, the RBBP6 mRNA occurs in the form of three major transcripts, transcript 6.1, 6.0 and 1.1 kb as a result of alternative splicing, which code for protein isoforms 1, 2 and 3, respectively (Pugh et al. 2006).

RBBP6 is highly expressed in cancer of the oesophagus as well as other cancers and this makes it a potential target in the treatment of cancers with intact p53 (Pugh et al. 2006; Ntwasa 2008). In addition to several of its biological functions including transcription, translation and ubiquitination, it is also associated with the execution of p53 degradation following inhibition of the tumour suppressor gene by MDM2, thus facilitating cell proliferation (Ntwasa 2008; Pretorius et al. 2013). However, little is known about the mechanism with which RBBP6 uses to degrade the p53 tumour suppressor protein. Taking note of the fact that the most efficient regimens for

anticancer activity including multidrug combinations still present patients with harsh side effects, it is important to continue evaluating any possible safe therapy (Aagaard and Rossi 2007; Arakawa et al. 2009). In this study, we report on the therapeutic potential of RBBP6 gene silencing in combination with *camptothecin* and *staurosporine* in human breast cancer cells.

Materials and methods

Materials

MCF-7 human breast cancer cell line was obtained from the Japan Health Resource Centre. Silencing was achieved by the use of Ambion's *Silencer*® Select Pre-designed siRNAs supplied by Life Technologies™, which target the RBBP6, MDM2 and p53 genes. *Staurosporine* and *camptothecin* were purchased from Calbiochem®.

Tissue culture and treatments

MCF-7 cell line was cultured in DMEM (Dulbecco's Modified Eagle Medium) growth medium supplemented with 1% pen/strep and 10% FBS (Foetal Bovine Serum) and incubated in a humidified atmosphere at $37\,^\circ$C and $5\%CO_2$. Once they reached ~70–80% confluency, cells were trypsinized and resuspended in an antibiotic-free media. The cell suspension was then mixed with siRNA/transfection agent (Ambion®) complexes, at 100 nM concentrations, targeting RBBP6, MDM2 and p53 genes in a 24-well plate and incubated at $37\,^\circ$C for 24 and 48 h. Post transfection cells were exposed to 0.25 μM *staurosporine* and 0.25 μM *camptothecin* for an additional 24 h. The cells were then harvested for subsequent analysis, i.e. RNA and protein were extracted 48 and 72 h post transfection, respectively.

Real time RT-PCR

RNA was extracted using Nucleospin® RNA II total RNA isolation kit according to the manufacturer's protocol and quantified using a nanodrop (NanoDrop Technologies, USA). RNA integrity was confirmed using ethidium bromide-stained agarose gel to analyse the 18s and 28s rRNA bands. Following RNA extraction, cDNA was synthesised using ImProm-II™ Reverse Transcription system from Promega®. RT-PCR was then performed in a 20 μl reaction mixture containing 2 μg/μl cDNA, SYBR Green (SIGMA®), reverse and forward primers (GADPH: Forward – 5′-GAG TCA ACG GAT TTG GTC GT-3′, Reverse – 5′-TTG ATT TTG GAG GGA TCT CG-3′; RBBP6: Forward – 5′-CAG CGA CGA CTA AAA GAA GAG TCT-3′, Reverse – 5′-GGT AAT TGC GGC TCT TGC CT-3′ and p53: Forward – 5′-GTT CCG AGA GCT GAA TGA GG-3′, Reverse – 5′-TGA GTC AGG CCC TTC TGT CT-3′) under the following conditions: 36 cycles of $94\,^\circ$C for 35 s, $59\,^\circ$C for 45 s, and $72\,^\circ$C for 45 s.

Western blot

Whole cell protein was extracted using RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 10% sodium dodecylsulfate (SDS), 3 μl/ml aprotinin and 5 μg/ml leupeptin in PBS, pH 7.4). Seventy-two hours post transfection and co-treatment with either *staurosporine* or *camptothecin*, cells were washed twice with cold PBS then resuspended in 500 μl RIPA buffer and collected by scraping. The total protein was then separated from cell debri by centrifugation at 14,000 rpm for 15 min and quantified with Pierce® BCA Protein Assay Kit. The protein was heated at $95\,^\circ$C for 5 min and 30 μg was loaded per well for electrophoretic separation in 40% acrylamide gel preparation at 100 V for 1 h. The protein was transferred onto a nitrocellulose membrane using wet electro-transfer method overnight at 30 V followed by incubation with primary

antibody after 1 h of blocking with 5% non-fat milk buffer. The weak light signal produced by HRP-linked secondary antibody was detected and enhanced using the Pierce® ECL Western Blotting Chemiluminescence Substrate and the blots were imaged by the CCD-based ChemiDoc™ MP system.

xCELLigence system

Before cells were seeded, 16-well E-plates containing antibiotic-free medium were imposed to current flow on the xCELLigence instrument placed in a $37\,^\circ$C incubator to record background readings. In each well of the E-plates, 1×10^5 cells were seeded simultaneously with 100 nM siRNA targeting RBBP6, MDM2 and p53 genes in the same antibiotic-free medium. After leaving the E-plates at room temperature for 30 min to allow for cell attachment, they were locked in the RTCA xCELLigence instrument and the experiment was allowed to run for 24 h. Twenty-four hours post transfection, cells were further treated with 0.25 μM apoptosis-inducing agents (*staurosporine* and *camptothecin*). The experiment was continued for an additional 24 h. Cell Index values were recorded at 15 min interval sweeps until the end of the experiment under the following xCELLigence parameters: [1st step: 1 sweep, 1 min interval, 00:00:39 total time; 2nd step: 100 sweeps, 15 min interval, 24:45:39 total time; 3rd step: 100 sweeps, 15 min interval, 49:30:39 total time].

Flow cytometry

Cultured cells were seeded in 24-well plates and simultaneously transfected with siRNAs targeting RBBP6, MDM2, and p53 for 24 h and treated for an additional 24 h with apoptosis-inducing agents (0.25 μM *staurosporine* and *camptothecin*). The treated cells were then trypsinized, resuspended in growth medium and transferred to 15 ml tubes, pelleted for 2 min at 1500 rpm and resuspended in 100 μl 1X binding buffer (annexin V-FITC Apoptosis Detection Kit, abcam®) at a concentration of 1×10^4 cells/ml. The cell suspensions were then transferred into 1 ml tubes and 5 μl of Annexin V FITC and 5 μl of PI were added. This was followed by gentle vortexing and incubation for 15 min at room temperature in the dark. To each tube, 400 μl of 1X binding buffer was then added and the cell solutions were analysed by flow cytometry within 1 h.

Statistical analysis

The results of each series of experiments (performed in duplicates) are expressed as the mean values ± standard deviation of the mean (SD). Levels of the statistical significance were calculated using the paired Student's *t*-test when comparing two groups, or by analysis of variance (ANOVA). *P*-values of ≤0.05 were considered significant.

Results

Gene silencing and mRNA expression analysis

Quantitative PCR analysis was used to evaluate the transcript on the silenced RBBP6, p53, MDM2, Bax and Bcl2 genes as shown in Fig. 1. As expected, a 37% decrease in RBBP6 expression was observed following silencing with 100 nM siRBBP6 while in combination with *camptothecin* the expression was further reduced by about 49%. In cells that were treated with *Staurosporine* and siRBBP6, expression was reduced by only 22%. In siMDM2, we observed a much higher silencing following co-treatment with both *staurosporine* and *camptothecin*, at 50% and 51%, respectively (Fig. 1.1A and B). The present study emphasizes the important role
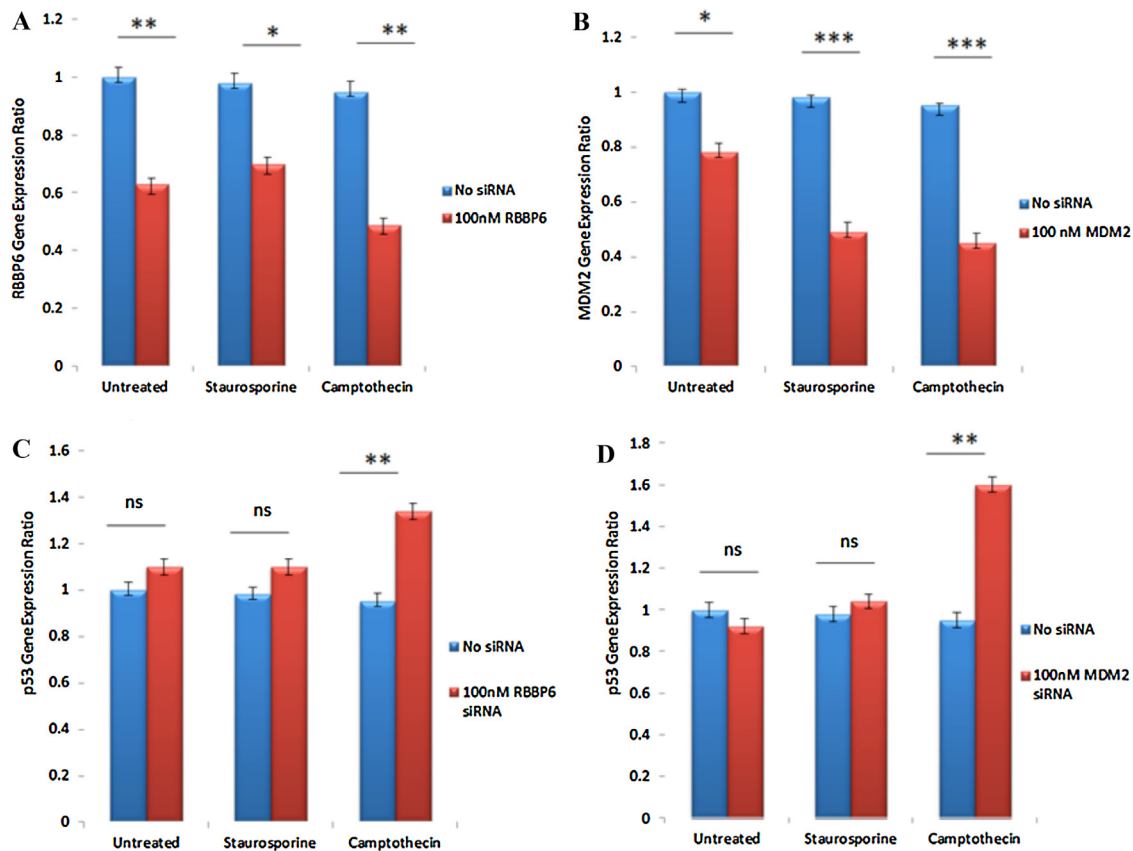
Fig. 1. (1.1) Relative quantification of gene expression in MCF-7 cells was performed using real time RT-PCR. (A) and (B), gene expression of RBBP6 and MDM2 in cells that were not transfected and either not treated, treated with *staurosporine* or *camptothecin*, co-treated with either MDM2 or RBBP6 siRNAs and treated with either *staurosporine* or *camptothecin*. (C) and (D), p53 gene expression after co-treatment of MCF-7 cells with siRNAs targeting RBBP6 and MDM2, respectively, and the apoptosis-inducing agents (*staurosporine* and *camptothecin*). The experiments were done in duplicates and the data is statistically significant, *p*-value <0.05.
(1.2) Relative quantification of gene expression in MCF-7 and MRC-5 cells was performed using real time RT-PCR. (A) and (C), gene expression of Bax and Bcl-2 in MCF-7 and MRC-5, respectively, that were not transfected and either not treated, treated with *staurosporine* or *camptothecin*, silenced with either RBBP6-, p53- and MDM2-targeting siRNAs then treated with either *staurosporine* or *camptothecin*. (B) and (D), Bax/Bcl-2 ratios after co-treatment of MCF-7 and MRC-5 cells, respectively, with siRNAs targeting RBBP6, p53 and MDM2, and the apoptosis-inducing agents (*staurosporine* and *camptothecin*).
(1.3) Relative quantification of gene expression in MRC-5 cells was performed using real time RT-PCR. The figure shows gene expression of p53 in cells that were not treated, silenced with either MDM2 or RBBP6 siRNAs and then treated with either *staurosporine* or *camptothecin*.
(1.4) Agarose gel electrophoretic analysis of real time RT-PCR products. (A) and (B), bax and bcl-2 RT-PCR products in MCF-7 cell line before and after treatments. (C) and (D), bax, bcl-2 and p53 RT-PCR products of MRC-5 cells after co-treatment.

of p53, Bcl-2 and Bax pro-apoptotic genes in activating apoptosis and reducing cell proliferation by gene silencing. The effect of RBBP6 and MDM2 silencing on p53 expression is shown in Fig. 1.1C and D. As observed, siRBBP6 in combination with *camptothecin* treatment led to a 13% increase in the expression of p53, whereas siMDM2 in combination with *camptothecin* resulted in 31% increased expression of p53 (Fig. 1.1C and D). However, co-treatment with *staurosporine* showed little to no-effect in the expression of p53 after silencing with either RBBP6 or MDM2 (Fig. 1.1C and D). One important factor in apoptosis induction is the Bax/Bcl2 ratio which determines whether cells survive or die. We have observed up to double increase in the Bax/Bcl2 ratio following MDM2 silencing (Fig. 1.2B). Bax was significantly increased following MDM2 and RBBP6 silencing that was followed by treatment with *staurosporine* (Fig. 1.2C and D). Silencing both RBBP6 and MDM2 seem to have favoured Bax/Bcl2 ratio that is for apoptosis. In MRC5 fibroblast cells that we considered normal cells, p53 was only slightly increased when RBBP6 and MDM2 were silenced even when treated with both agents (Fig. 1.3).

Protein expression by Western blot analysis

The expression of RBBP6, MDM2 and p53 proteins was significantly reduced after silencing with their respective siRNAs in comparison to protein expression in cells that were treated with *staurosporine* or *camptothecin* alone (Fig. 2). Combination of siRNAs with either *staurosporine* or *camptothecin* almost led to a complete repression of RBBP6 and MDM2 proteins, whereas reduction in p53 protein expression seemed to be constant in siRNA-only and siRNA + *staurosporine*/*camptothecin*-treated cells (Fig. 2A–C). Silencing with either RBBP6 or MDM2 led to an up-regulation of p53 protein (Fig. 2A and C) whereas p53 silencing caused MDM2 down-regulation (Fig. 2B). These findings suggest that there is indeed a relationship between RBBP6, MDM2 and p53 at both mRNA and protein level. Silencing both RBBP6 and MDM2 followed by treatment with *staurosporine* resulted in an increase in bax protein expression (Fig. 2D) whereas that of Bcl-2 seemed to be reduced or remained unchanged.

Analysis of cell growth after co-treatment

The anti-proliferative effect of gene silencing was measured using xCELLigence system. In accordance with the previous results, we verified the growth inhibitory effect of siRBBP6, siMDM2 and sip53 (100 nM) on MCF-7 breast cancer cells by measuring cell growth in real time using xCELLigence system (Roche) (Fig. 3). Growth curves were normalised to the CI at the last measured time point before compound addition for each well. MCF7 growth curve,
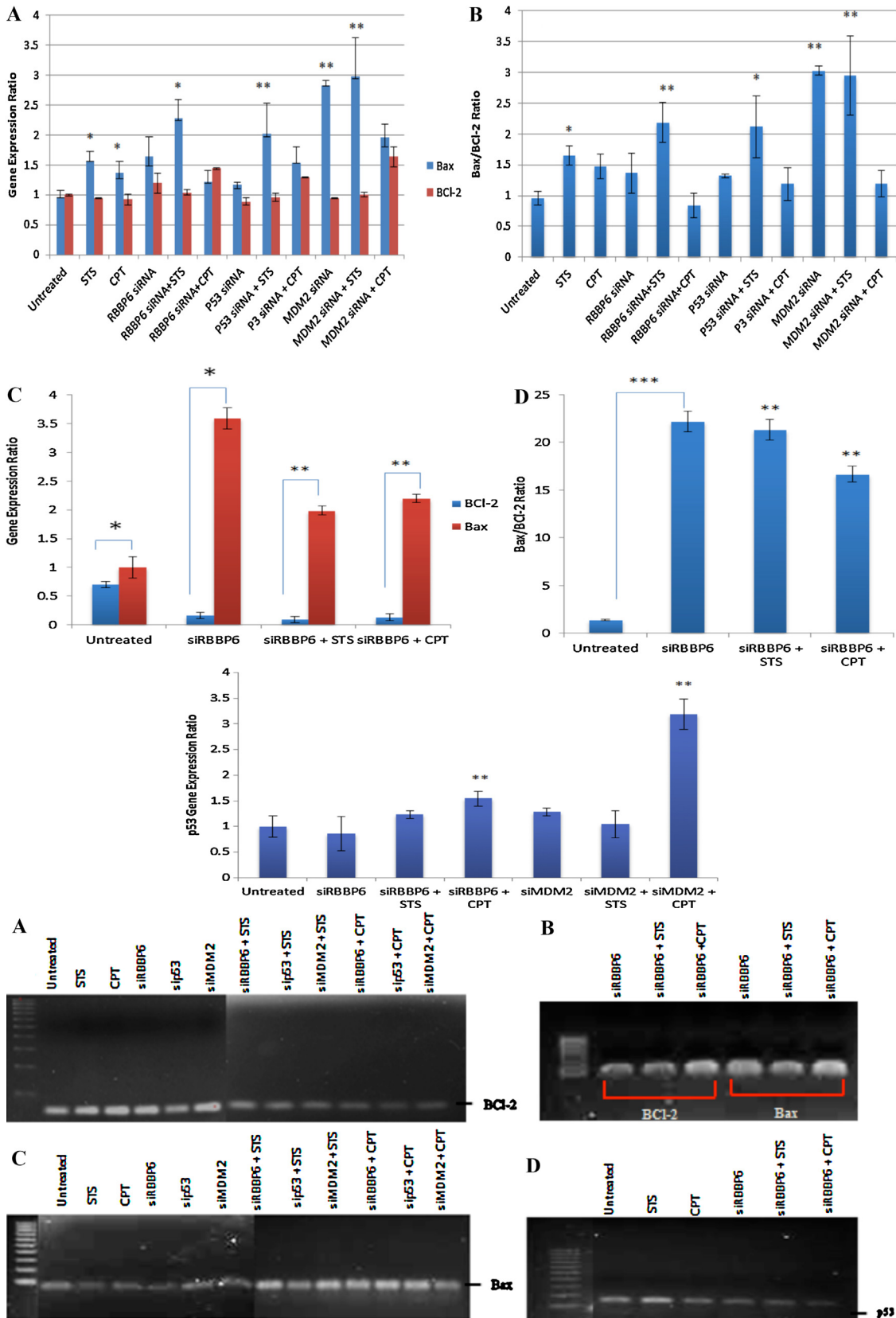
Fig. 1. (Continued)

which is presented in Fig. 3 showed a significant reduction in cellular growth after silencing siRBBP6 and siMDM2, whereas sip53 reduced cell growth at a slower rate. Combination of siRNAs and the apoptosis-inducing agents (*staurosporine* and *camptothecin*) further reduced cell growth as shown in Fig. 3B and C. *Staurosporine* reduced cell growth at a much faster rate as compared to *camptothecin* (Fig. 3B and C). These findings suggest that both co-treatments (siRNAs + *staurosporine* and siRNAs + *camptothecin*)
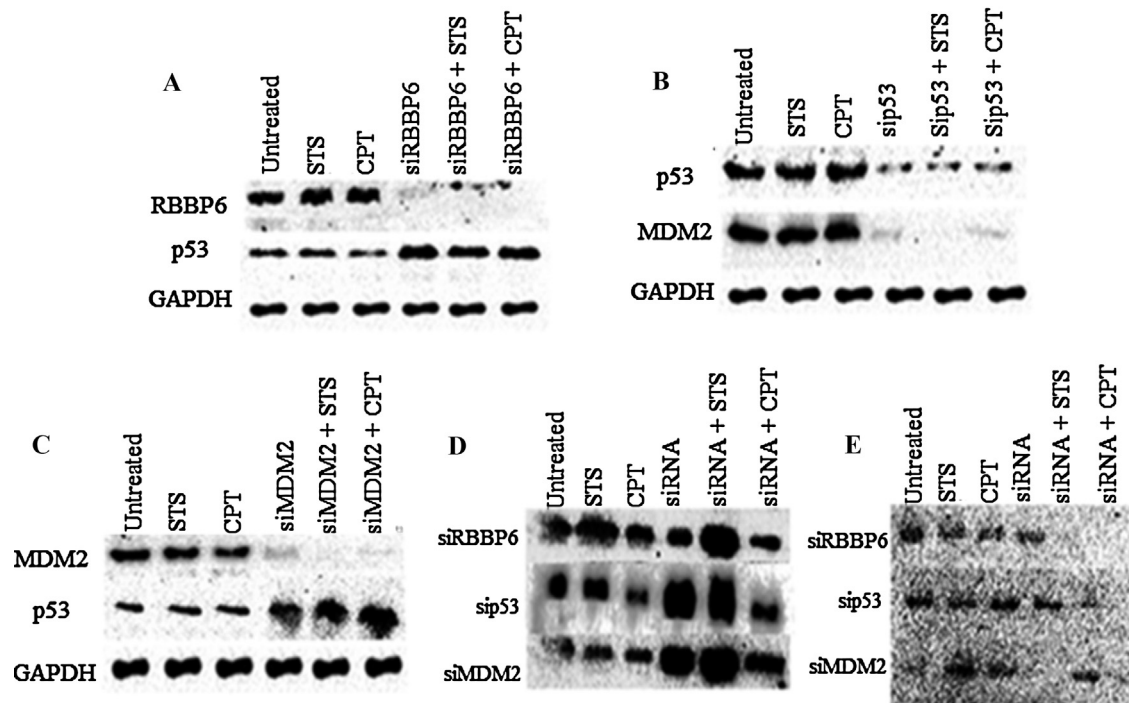
Fig. 2. Protein expression in MCF-7 cells was performed using Western blot technique. GAPDH was used to confirm that an equivalent amount of protein was loaded into each well. (A) protein expression of RBBP6 and p53 in cells that were either not treated or co-treated with RBBP6 siRNAs and *staurosporine* (STS) or *camptothecin* (CPT). (B) p53 and MDM2 protein expression after co-treatment of MCF-7 cells with siRNA targeting p53 and the apoptosis-inducing agents (*staurosporine* and *camptothecin*). (C) MDM2 and p53 protein expression after silencing with siRNAs targeting MDM2 and the apoptosis-inducing agents (*staurosporine* and *camptothecin*). (D) and (E), protein expression of Bax and Bcl-2, respectively, in cells that were either not treated or co-treated with RBBP6-, p53- and MDM2-targeting siRNAs and *staurosporine* (STS) or *camptothecin* (CPT).

induce cell death; however, the siRNAs + *staurosporine* combination might be more effective on the cells as seen by the rapid fall in cellular growth (Fig. 3C). When comparing these results to those observed in MRC5 cells, an almost similar pattern of growth inhibition was observed but it was at a minimal level (Fig. 3D–F). In this case, *staurosporine* and RBBP6 silencing did not have as much effect as in MCF-7 cells.

Apoptosis detection

Annexin V and PI staining make flow cytometry an effective technique to distinguish between early and late apoptosis in cultured cells. Analysis by flow cytometry revealed that the percentage of apoptotic cells following treatment with *camptothecin* and *staurosporine* was significantly increased to 53.2% and 55.9% respectively in MCF-7 cells ($p < 0.05$) (Table 1). siRBBP6 significantly increased apoptosis in cells treated with *camptothecin* (20.7% increment), however, only 8.8% increase in apoptosis was observed in cells treated with siRBBP6 and *staurosporine* (Table 1). siMDM2 significantly induced apoptosis in *camptothecin* by 26.6% as compared to that of *staurosporine* and this was insignificant at 0.5% (Table 1). sip53 reduced *camptothecin*-induced and *staurosporine*-induced apoptosis by 26% and 30.7%, respectively (Table 1) which was far less than those of MDM2 and RBBP6. In MRC5, silencing RBBP6 and MDM2 could only induce apoptosis to about 14 and 15% in combination with apoptosis inducing agents (Table 2 and Figs. 4 and 5).

Discussion

Several E3 enzymes are associated with cancer development and are therefore highly expressed in a number of tumours (Motadi et al. 2011). RBBP6 has been identified as an E3 ubiquitin ligase that is markedly up-regulated in tumours of the lung (Motadi

et al. 2011). Because RBBP6 possesses a p53 binding domain, the p53 tumour suppressor is prone to ubiquitination by the ring finger domain of RBBP6 thus leading to cancer progression (Vassilev et al. 2003; Malloy et al. 2012). RBBP6 therefore serves as a hallmark target in the development of anticancer therapeutics and findings in this manuscript reveal how a synergistic effect of two biological compounds could become a potential treatment against breast cancer by targeting RBBP6. In this study we demonstrated at both the mRNA and protein level that it is possible to markedly silence the expression of RBBP6 in human breast cancer cells (MCF-7) using RNA interference. We further demonstrated that combinational therapy of siRBBP6 and either *camptothecin* or *staurosporine* further reduces expression of RBBP6 and lead to reduction in cell proliferation. However, siRBBP6 in combination with *staurosporine* does not significantly reduce RBBP6 gene expression. These differences in the effects of these two combinational therapy (RBBP6 siRNA/*staurosporine* and RBBP6 siRNA/*camptothecin*) on RBBP6 expression may be attributable to the fact that the two apoptosis-inducing agents possess different mechanisms of apoptosis induction.

Camptothecin acts as a topoisomerase I inhibitor thus causing DNA strand breaks during the S-phase of the cell cycle which leads to apoptosis in cancer (Arakawa et al. 2009). The mechanism by which *staurosporine* uses to induce apoptosis is not well understood, however it is suspected that it uses its protein kinase inhibiting function to mediate translocation of bax from the cytosol to the mitochondria where it causes the release of cytochrome c from the outer mitochondrial membrane (Xue et al. 2003; Motadi et al. 2007). Silencing of RBBP6 in these cells markedly enhanced the level of apoptosis induced by *camptothecin*; however, apoptosis induced by *staurosporine* was not significantly enhanced by RBBP6 silencing. The results also strongly suggest that the enhanced apoptosis is as a result of the synergistic effect between RBBP6 siRNA and *camptothecin* as treatment with *camptothecin* alone (or siRNA
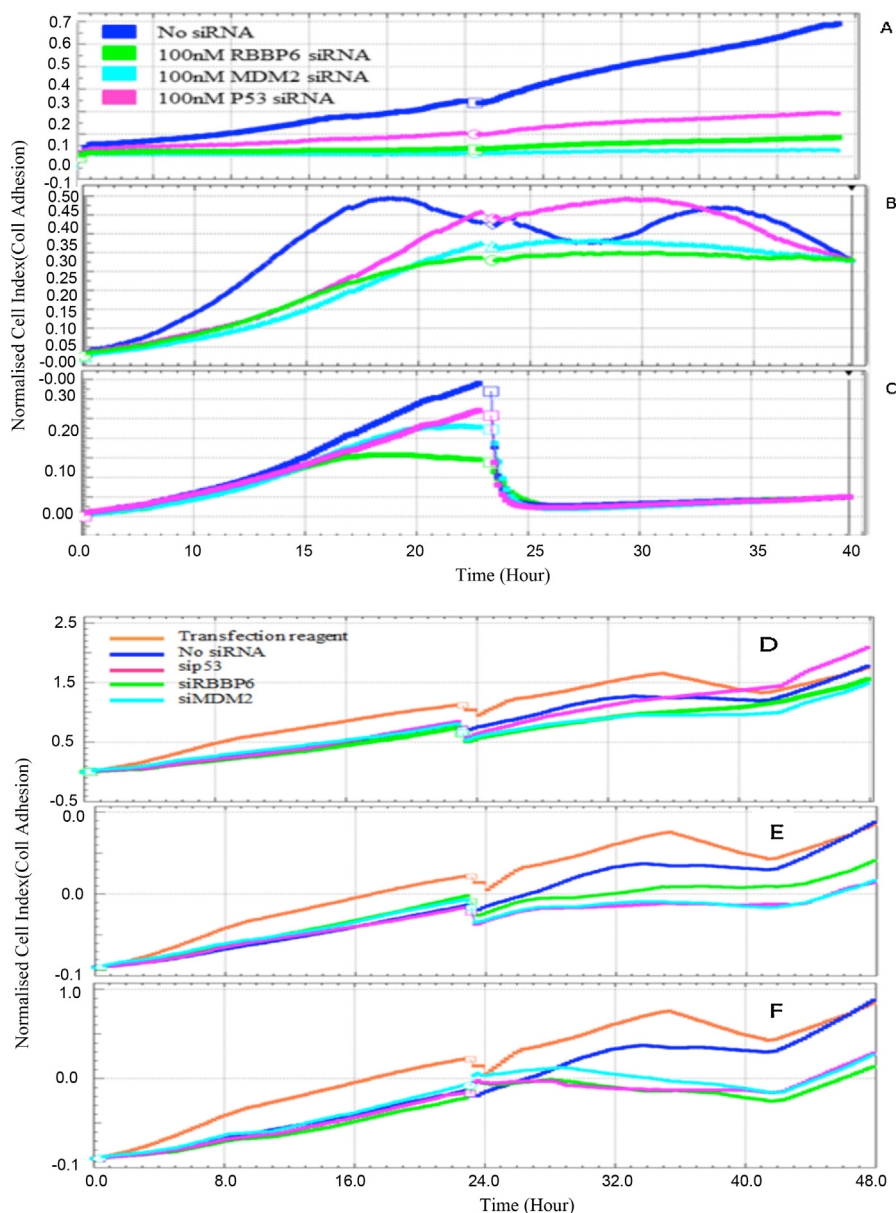
Fig. 3. Cell growth of MCF-7 (top) and MRC-5 (bottom) cells was analysed using the xCELLigence system which relies on the generation of electrical impedance as cell growth by 16-well plates coated with a gold microelectrode covering the base of each well. The *y*-axis shows normalised cell index or cell adhesion over a period of about 48 h (*x*-axis). The growth patterns in (A) and (D) show cells that are not treated (blue), those that were exposed to transfection reagent only (orange) and those transfected with siRBBP6, siMDM2 and sip53 (pink, green and light blue). (B) and (E) curves of cells co-treated with siRNAs and the *camptothecin* and (C) and (F) curves of cells co-treated with siRNAs and *staurosporine*.

alone) induces less apoptosis (Fig. 9). p53 induces apoptosis by transcriptionally activating bax and causing its translocation to the mitochondria where it porates the outer mitochondrial membrane (Shen and White 2001). p53-silenced cells were not undergoing as much apoptotic cell death as those that were RBBP6-silenced and from this it is arguable that some of the observed apoptosis might be attributed to some p53-mediated pathway. These findings suggest that, by blocking the gene expression of both MDM2 and RBBP6, the activation of other proapoptotic genes is promoted, leading to the activation and upregulation of different intracellular signalling pathways, including BCL-2 gene family, inflammatory caspases and death receptors (Morissette et al. 2007; Porichi et al. 2009). These signalling pathways promote p53 mediated cell death.

Table 1
Apoptosis of MCF-7 cells analysed using flow cytometer with annexin V and PI following co-treatment with 100 nM siRNAs (siRBBP6, sip53 and siMDM2) and 0.25 μM staurosporine or 0.25 μM camptothecin.

	Untreated	Staurosporine (STS)	Camptothecin (CPT)	siRBBP6 + STS	siRBBP6 + CPT	sip53 + STS	sip53 + CPT	siMDM2 + STS	siMDM2 + CPT
Live cells (%)	85.5	42.0	46.2	31.7	25.0	74.5	50.3	41.2	19.6
Necrotic cells (%)	13.6	2.1	0.7	3.1	0.2	0.0	0.4	2.5	0.4
Apoptotic cells (%)	0.8	55.9	53.2	65.3	74.7	25.7	49.3	56.3	79.2

Table 2
Apoptosis of MRC-5 cells analysed using flow cytometer with annexin V and PI following co-treatment with 100 nM siRNAs (siRBBP6, sip53 and siMDM2) and 0.25 μM staurosporine or 0.25 μM camptothecin.

	Untreated	Staurosporine (STS)	Camptothecin (CPT)	siRBBP6 + STS	siRBBP6 + CPT	sip53 + STS	sip53 + CPT	siMDM2 + STS	siMDM2 + CPT
Live cells (%)	94.0	79.5	79.0	85.1	86.5	85.2	84.1	85.9	83.4
Necrotic cells (%)	3.5	5.2	2.4	1.4	1.5	1.5	1.2	1.5	1.2
Apoptotic cells (%)	2.5	14.3	18.7	13.5	12.0	13.3	14.8	12.6	15.4

Under normal physiological conditions, wild type TP53 tumour suppressor has a short half-life due to its targeted degradation by MDM2 E3 ubiquitin ligase (Bai and Zhu 2006). This auto-regulatory feedback loop keeps TP53 under tight regulation to maintain normal cell growth and prevent tumorigenesis (Vassilev et al. 2003; Malloy et al. 2012). In response to stress signals such as DNA damage, activated TP53 stimulates genes such as p21 and Bax which are responsible for the induction of cell cycle arrest and apoptosis, respectively (Bai and Zhu 2006). Inhibition of RBBP6 led to over-expression of the p53 tumour suppressor at both the mRNA and protein level. This suggests that by silencing RBBP6 we were able to block the interaction between RBBP6 and p53 and thus prevented p53 degradation.

During their auto-regulatory feedback loop, p53 activates MDM2 which in turn has the ability to block the transactivation activity of p53 by tightly binding the p53 protein (Bai and Zhu 2006). MDM2 is also involved in the nuclear transport of p53 and also serves as an E3 ubiquitin ligase that promotes degradation of p53 (Vassilev et al. 2003). We demonstrated in this study that by tempering with the p53:MDM2 auto-regulatory feedback loop, i.e. silencing MDM2, also frees p53 as evidenced by the up-regulation of both p53 mRNA and p53 protein, and the fact that

p53 silencing leads to MDM2 down-regulation tells us that indeed the p53:MDM2 interaction does exist and it compromises the function of p53 during cancer development (Shangary and Wang 2008). These findings suggest that in the absence of RBBP6 and the p53:MDM2 interaction, p53 degradation by the E3 ubiquitin ligases is abrogated thus leading to p53-mediated apoptosis. Normal skin fibroblasts were less responsive to the co-treatment and this somehow suggests that silencing RBBP6 is not cytotoxic to non-cancerous cells.

We showed a good correlation between cellular growth reduction and apoptosis induction in MCF-7 cells, for example siRBBP6 combined with *camptothecin* led to a significant cellular growth rate reduction and an increase in apoptosis induction in comparison to cells that were untreated or treated with *camptothecin* only. However combination of siRBBP6 with *staurosporine* led to a much faster reduction in growth rate of MCF-7 cells. The observed difference in the potency of *camptothecin* and *staurosporine* on cell growth may be attributable to the fact that the two agents induce apoptosis using different mechanisms. We have shown that *camptothecin* induces a much higher apoptosis than *staurosporine* when combined with siRBBP6 and this may be due to the fact that *camptothecin* interacts directly with proapoptotic genes in order to
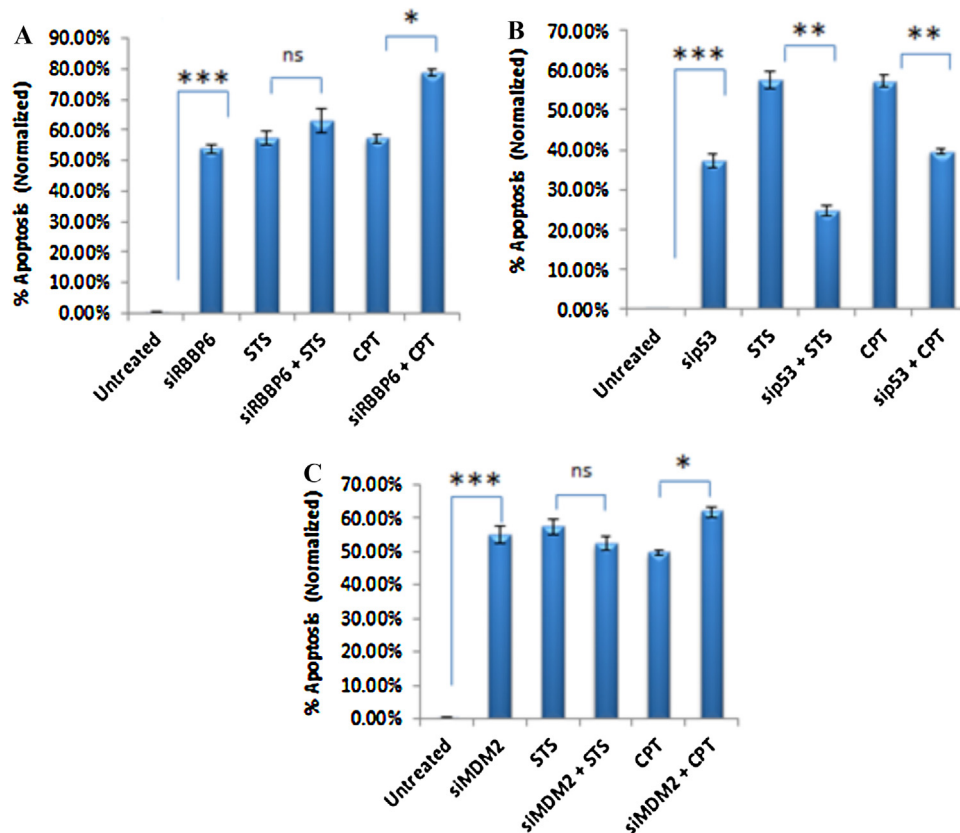
Fig. 4. Statistical analysis of flow cytometry-obtained apoptosis (%) in MCF-7 cell line. *t*-Test was used to generate *p*-values in order to compute the difference between treated and untreated scores. A, B and C, statistical analysis between untreated and silenced and between treated and silenced + treated in MCF-7 cells. $^* p \leq 0.05$, $^{**} p \leq 0.01$, $^{***} p \leq 0.001$ and $^{ns} p \geq 0.05$.
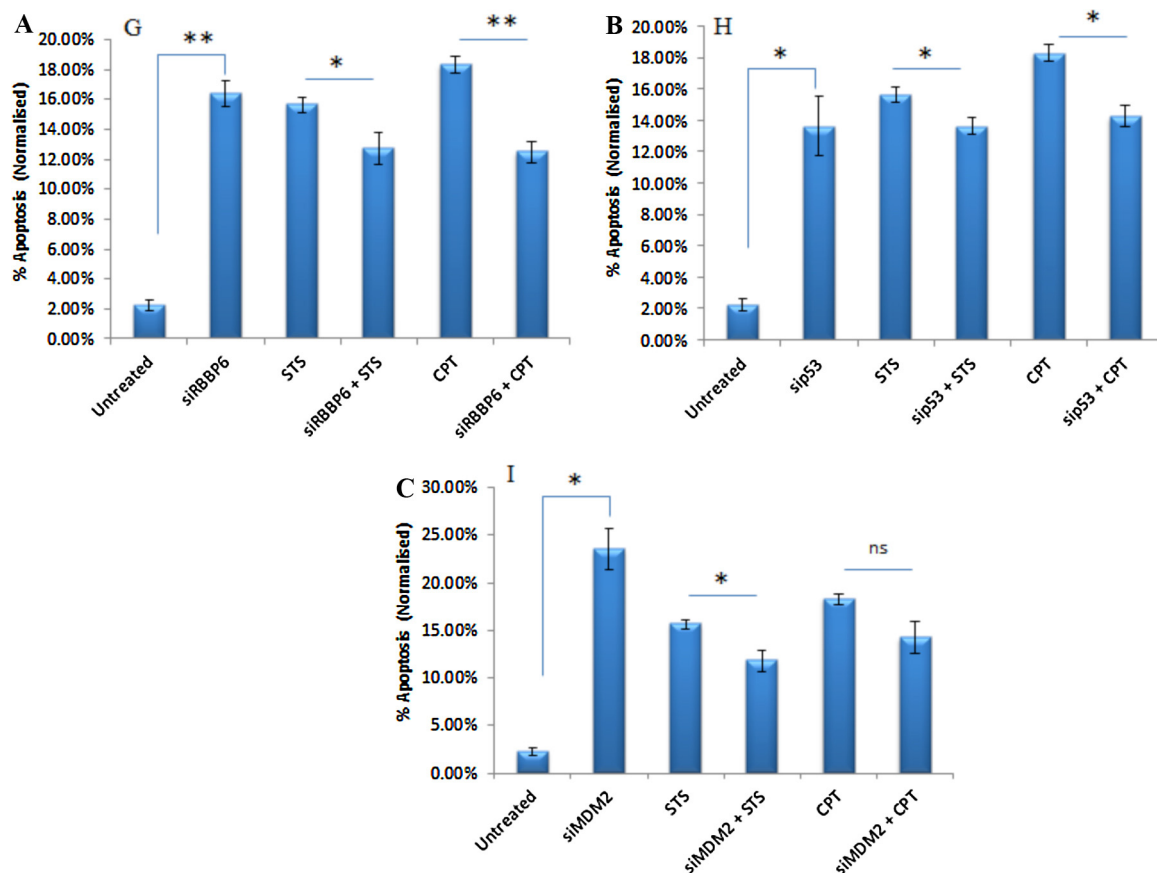
Fig. 5. Statistical analysis of flow cytometry-obtained apoptosis (%) in MRC-5 cell line. *t*-Test was used to generate p-values in order to compute the difference between treated and untreated scores. G, H and I, statistical analysis between untreated and silenced and between treated and silenced + treated in MCF-7 cells. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{ns}p \geq 0.05$.

induce apoptosis (Zeng et al. 2012). Wild type p53 has been shown to enhance *camptothecin*-induced apoptosis (Zhang et al. 2000; Li et al. 2000) and this may also explain the observed enhanced apoptosis in cells co-treated with siRBBP6 + *camptothecin*.

Interestingly, we also showed that sip53 combined with either *camptothecin* or *staurosporine* leads to a slight increase in cell growth in comparison to co-treatment with siRBBP6 or siMDM2 and either of the agents. This observation may suggest that p53 is required in the induction of cell death and to explore this further, apoptosis detection was carried out in p53-knockdown MCF-7 cells. We showed that there is minimal apoptosis induction in p53-silenced cell population in comparison to cells co-treated with either siMDM2 or siRBBP6. P53 is involved in the induction of apoptosis via the intrinsic apoptotic pathway by promoting transcription; translocation and oligomerization of bax on the outer mitochondrial membrane in order to facilitate cytochrome c release (Bai and Zhu 2006) and these findings suggest that silencing p53 reduces p53-mediated apoptosis.

Conclusion

p53 could promote the convergence of the extrinsic and intrinsic apoptotic pathways, including in MCF-7 breast cancer cells. The present study provides early insight into the mechanisms of RBBP6 pathways in mediating p53 in breast cancer cells, as the mRNA and protein expression data show. Manipulating the expression of the RBBP6 and MDM2 genes is required for the induction of apoptosis by different Bcl-2 gene family members. Therefore, these results provide a promising path to understanding further the role of RBBP6 in cancer development and treatment.

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CHAPTER SEVEN - General Discussion and Conclusions

7.1. Introduction

According to dictionary.com, a biomarker is defined as a distinct biochemical, genetic, or molecular substance that is used as an indicator of a particular biological condition, e.g. a blood test can be used to measure protein biomarkers for cancer, which indicate either the extent of disease progression or effectiveness of therapy. The question raised in this study is whether or not Retinoblastoma binding protein 6 (RBBP6) through its molecular functioning serves as a biomarker for breast or cervical cancer. We show through gene silencing, overexpression, apoptosis and cell cycle analysis that indeed RBBP6 presents molecular characteristics of a cancer biomarker.

RBBP6 is a 250 kDa multi-domain protein whose molecular functions include ubiquitin-protein transferase activity, ligase activity, protein binding, zinc ion binding as well as poly(A) RNA binding (Pugh *et al*., 2006; Miotto *et al*., 2014). RBBP6 localizes in the chromosomes, microtubules, nucleolus and cytoplasmic cellular components during utero embryonic development where it facilitates progression of the embryo from the formation of a zygote until growth into a multicellular organism (Miotto *et al*., 2014). In terms of its protein binding function, recent research has successfully showed that RBBP6 plays a role in the regulation of tumour suppressor proteins p53 and pRB via its p53-binding and pRB-binding domains (Pugh *et al*., 2006, Chen *et al*., 2013; Li *et al*., 2007). This functioning of RBBP6 in cell biology suggests an important role in cancer progression and development.

7.1.1. RBBP6 protein expression and localisation in cervical cancer tissue sections

Conventional prognostic techniques in tumorigenesis are denoted by lymph node status, FIGO stage, characteristics of primary tumour which include tumour size, depth of cervical invasion, type of histology and uterine diffusion (Gadducci *et al*., 2013). However, recent identification of tissue biomarkers such as EGFR, VEGF, Tap73, p53 and INK4 family proteins has played a major role in assessing and predicting a positive response to treatment (Gadducci *et al*., 2013). Previously, RBBP6 has been shown to be highly expressed in several cancer type with the expression higher around well-differentiated cells of carcinomas (Li *et al*., 2007; Mbita *et al*., 2012; Motadi *et al*., 2011; Chen *et al*., 2013, Moela *et al*., 2014; Harutyunyan *et al*., 2016). This findings support the hypothesis that RBBP6 is a cancer causing gene by playing a major role in cancer progression.

Through tissue sections collected from cervical cancer patients, we show an increased expression of RBBP6 in the stromal and well-differentiated non-keratinizing squamous cell carcinoma tissues. These findings are consistent with previous studies (Mbita *et al.,* 2012; Motadi et al., 2011) and reinforce the role played by RBBP6 in cancer development and progression. In keratinized and columnar cells of the glandular element of the less prevalent adenocarcinoma, there was aggressive expression of RBBP6. Keratin formation is associated with aggressiveness of the cancer and increased cell division (Motadi et al., 2011). This suggests that the presence of RBBP6 in such areas might be the driving force of cancer aggression through its perceived role in stabilizing chromosomal fragile sites thereby ensuring proper cell division (Motadi et al., 2011). By implication, this suggests that at the onset of cancer development RBBP6 might be

required to make sure that cell division occurs thereby acting as a facilitator for cancer development.

7.1.2. Overexpression and silencing of RBBP6 gene in tumorigenic cell lines

RBBP6 gene manipulation was highly important in this study so as to understand its role in breast and cervical cancer biology. New and exciting findings on the involvement of RBBP6 in tumorigenesis continue to emerge, however, this area of study still needs to be thoroughly investigated, more so because there are contradictions from one set of findings to the other. For example, Gao and Scott (2003) showed that P2P-R overexpression sensitizes MCF-7 cells to apoptosis, which is conflicting to the observations made by Motadi *et al* (2011) where RBBP6 overexpression plays an inhibitory role by promoting cell proliferation in lung cancer. On the other hand, Chen *et al*. (2013) argues that RBBP6 overexpression together with mutant TP53 is deemed predictive of poor prognosis in colon cancer. In terms of silencing, Li *et al*. (2007) showed that RBBP6 silencing was linked to early embryonic lethality coupled with p53 accumulation and a widespread apoptosis which is in agreement with our previous findings (Moela *et al*., 2014) where RBBP6 silencing led to p53 accumulation and sensitized MCF-7 cells to camptothecin-induced apoptosis. Nonetheless, taken all together these observations highlight the important role played by RBBP6 in tumorigenesis and puts cancer research a step further in exploring the potential of RBBP6 to become a novel biomarker in anticancer therapy.

To gain a better understanding of the possible role of RBBP6 in breast and cervical cancer progression, we first overexpressed and silenced the gene in various breast and cervical cancer cell lines. RBBP6 overexpression in HeLa, SiHa, MCF-7, MDA-MB-231 cell lines as well as the

normal lung fibroblasts was achieved by transient transfection using an expression vector that contained transcript variant 3 of RBBP6 tagged with a GFP reporter gene to allow for positive identification of RBBP6 overexpression. Transcript variant 3 consists of the DWNN domain which plays a central role in the functioning of the full length RBBP6 (Kappo *et al*., 2012; Pugh *et al*., 2006). The use of expression vectors to overexpress proteins is a long established method which has seen a lot of recombinant proteins being FDA-approved as anticancer drugs (Kinch, 2015). The aforementioned tumorigenic cell lines were chosen based on their p53 status because the tumour suppressor has been shown to be involved in the functioning of RBBP6 (Pugh *et al*., 2006, Li *et al*., 2007).

Overexpression studies of RBBP6 were monitored using GFP tag protein in which immunoprecipitation showed expression that was directly proportional to that of RBBP6. Furthermore, RBBP6 protein was analysed using 50kDa antibody that was able to detect all isoforms of the gene. Gene expression was also evaluated at mRNA levels to confirm the success of our expressional studies. From the results as described in chapter 4, overexpressional studies were successful in all cervical cancer cell lines, whereas HeLa showing statistically significant ($P < 0.001$) up-regulation of RBBP6. This successful transfection of HeLa cells is similar to a study that has shown it to be an easy to transfect cell line and therefore remains the most commonly used in transfection studies (Ning and Tang, 2012). The difference in overexpression between HeLa and SiHa was insignificant (11%). This was further confirmed by analysis of expression in live cells using FACS and confocal microscopy where a strong GFP fluorescence signal was detected in both cell lines. Similarly, mRNA quantification showed an average of 12-fold increase of gene expression in all cell lines further confirming the observed overexpression.

RBBP6 overexpression was successful in breast cancer as indicated by Figure 34 and Figure 35. To the best of our knowledge this is the first study to successfully overexpress RBBP6 in human cancer cell lines using transient transfection. The success of this study is similar in sense to that by Gao and Scott (2003) in which they used stable transfection (knockout) of P2P-R in MCF-7. The successful overexpression of RBBP6 will enable us to understand and identify its molecular mechanism in cell biology. This understanding of its mechanism will be able to present RBBP6 as a potential target for cancer therapy.

RBBP6 gene silencing was achieved by the use of siRNA technology, a method that is rapidly becoming important for studying gene functioning and thus holds promise for the development of therapeutic gene silencing (Aagaard and Rossi, 2007; Meister and Tuschl, 2004). The main question that exists in RBBP6 studies is whether it binds to p53 or Rb and leads to their degradation thereby leading to cancer development or whether that ubiquitin function results in the activation of spindle and speed up cell division. Depending on which theory is true, siRNA technology will assist in clarifying the functions of RBBP6 in cell biology.

Silencing studies were successful as indicated in our earlier results where we managed to knockdown RBBP6 by over 90% in all cell lines. These results are similar to those conducted by other researchers in both breast and cervical cancer (Jung *et al*., 2015; Shen *et al*., 2013; Peralta-Zaragoza *et al*., 2010; Alvarez-Salas and DiPaola, 2007). RNAi technology assisted many researchers in the identification of cancer biomarkers (Milani *et al*., 2016; Koivusalo *et al*., 2006)

and we show in this study that it was able to assist in the understanding of RBBP6 function. As described through central dogma, protein synthesis is much dependent on mRNA transcription. In many cases, degradation or blocking of mRNA results in deficiency of that particular protein. The success of RBBP6 gene silencing should thus be evident in translation. It was clear from western blot as indicated in Figure 7 and Figure 40 that silencing RBBP6 mRNA resulted in a decreased protein expression confirming the success of the transient transfection.

7.1.3. The effect of RBBP6 gene targeting on cancer cell progression

Cancer progression and development is characterised by many molecular defects. One such defect is the inactivation of the cell cycle arrest machinery which is tasked with redirecting damaged cells to a cell repair mechanism. Many researchers have identified several genes including p53 and Rb as either being mutated or inactivated during cancer development (Bai and Zhu, 2006). However, to date there is still no clear biomarker derived from our understanding of cell cycle. Perhaps the solution lies in unlocking the molecular functions of RBBP6 domains responsible for binding p53 and Rb.

Cell proliferation occurs very rapidly in embryonic cells due to increased levels of RBBP6 in order to fulfil early development, which is followed by cell differentiation to produce cell types that are more specialised and can make up a multicellular organism (Miotto *et al*., 2014). In contrast, the rate of cell proliferation decreases during differentiation and as a result most cells in a fully developed organism remain arrested in the interphase (G0) of the cell cycle (Huang *et al*., 2013). However, this is not the case with differentiated cancer cells; instead they acquire the ability to invade the cell cycle checkpoints so as to continue proliferating uncontrollably at much higher rates (Huang *et al*., 2013). Through the use of RBBP6 silencing, overexpression and

xCELLigence systems, cell proliferation was monitored. Interesting results were observed in RBBP6 silencing and overexpression in normal cell lines. In these cells when RBBP6 was silenced the cell continued at the same growth rate as untreated whereas overexpression of RBBP6 in the same cell line resulted in increased cell growth rate. MRC5 is a foetus-derived cell line (Friedman and Koropchak, 1978) and increasing RBBP6 will automatically as described in the previous sections, speed up cell division. This further places RBBP6 at the centre of cell proliferation as an important marker. For cancer cells, silencing RBBP6 as shown in Figure 7 and Figure 40 reduced cancer cell progression while overexpression resulted in moderately high cell growth rate. The reduced cell growth rate highlights the importance of RBBP6 in cancer progression and that if this gene was to be managed it might assist in combating cancer progression.

In response to DNA damage in the cell, ATM (ataxia telangiectasia mutated) protein kinase becomes active and in turn activates Chk2 kinase, both of which activate p53 by phosphorylation at distinct sites (Speidel, 2015; Bertheau *et al*., 2014). The active p53 therefore up-regulates the expression of a cyclin-dependent kinase inhibitor known as p21waf1/Cip1 which functions to induce G1 arrest by inhibiting cyclin E/CDK2-mediated phosphorylation of Rb and the release of E2F transcription factor; which is responsible for the activation of genes required for entry into the S-phase (Speidel, 2015; Bertheau *et al*., 2014). Through cell cycle analysis, RBBP6 silencing was shown to arrest cancer cells in G0/G1 which suggested that following reduction of the gene cell biology mechanisms were restored. However, it was not clear whether it was cell cycle arrest that was restored or apoptosis activated. But the fact that many of the cells were arrested at G0 stage is a suggestion that apoptosis might be induced. Interestingly, when RBBP6

was overexpressed, more cells accumulated in the S-phase which is a stage in cell cycle that involves the replication of DNA and thereby cell proliferation. This evidence suggests that RBBP6 expression promote DNA replication which by implication, promotes cell proliferation. In normal cell, similar results were observed after overexpression of RBBP6 where most cells appeared to be accumulating in the S-phase suggesting increased cell proliferation. This was also confirmed by the xCELLigence that indeed the cells were growing faster. It is now clear that RBBP6 silencing has an inhibitory effect on breast and cervical cancer cell proliferation whereas overexpression promotes the cell growth process; however, the mechanism of action responsible for this phenotype is not fully understood.

7.1.4. Implicated apoptosis induction by RBBP6 silencing

The process of cancer progression involves transformation of normal cells into malignant types as a result of genetic alterations that lead to dysregulation of cellular processes especially cell cycle and apoptosis (Dashzeveg and Yoshida, 2015). Current therapeutic strategies therefore are aimed at targeting these pathways in order to combat cancer (Dashzeveg and Yoshida, 2015). Morphologically, apoptosis is characterised by externalisation of phosphatidyl serine (PS) peptides on the outer cell membrane coupled with cell shrinkage and pyknosis which eventually lead to irregular cell shape (Kerr *et al*., 1972). Indeed, staining of live cells with the annexin V/FITC fluorescent dye that has maximum binding affinity to PS as well as the DNA-intercalating DAPI enabled visualisation of PS externalisation and DNA fragmentation.

In support of our earlier observations and arguing for apoptosis induction, cancer cells treated with the siRNA presented morphological similarities to that of apoptotic cells, namely cell

shrinkage, irregular shape and decrease in size. Whereas those stained with annexin V/PI, stained positive for both late and early apoptosis as observed through flow cytometer. In cells treated with overexpression vector, the opposite was true with apoptosis at its lowest levels of about 5% which was in line with untreated cells. The two opposing observation provide a clear evidence that RBBP6 plays a major role in cancer development that the observed cell cycle arrest at G0 might be that apoptosis was restored in proliferating cells following gene silencing. Normal cell lines treated with both siRNA and overexpression vector did not induce a significant amount of apoptosis.

7.1.5. Mechanism of cell death as induced by siRBBP6

Mitochondria are the main source of ATP generation and loss of mitochondrial membrane potential due to permeabilization of the outer mitochondrial membrane during intrinsic apoptosis induction often leads to ATP depletion (Gergely *et al*., 2002; Eguchi *et al*., 1997; Richter *et al*., 1996). However, lack of ATP restoration may be indicative of necrotic cell death because apoptosis is a highly organised and co-ordinated form of cell death that requires energy (Gergely *et al*., 2002; Eguchi *et al*., 1997; Richter *et al*., 1996). It is also worth noting that permeabilization of the outer mitochondrial membrane is the final decider of apoptosis induction because it releases apoptosis inducing factors such as cytochrome c (Gergely *et al*., 2002). This is followed by the activation of effector caspases 3 and 7 by proteins released from the mitochondrial intermembrane space, which ensures execution of apoptosis (Namura *et al*., 1998; Parton *et al*., 2001). These caspases trigger apoptosis by cleaving the inhibitor of endonucleases, ICAD, thus promoting fragmentation of DNA in the nucleus by the activated endonucleases (Namura *et al*., 1998; Parton *et al*., 2001).

In the present study, we measured mitochondrial ATP content and caspase 3/7 activity following treatment with both siRBBP6 and cDNA construct at different time points. Treatment with siRBBP6 led to ATP depletion for up to 6 hours followed by restoration in both cervical cancer cell lines. ATP depletion continued for longer (8 hours) in MCF-7 cells whereas no significant restoration was observed in MDA-MB-231. The observed mitochondrial ATP depletion suggests that mitochondrial transmembrane potential ($\Delta\Psi$m) decreased and the outer mitochondrial membrane of the cells in question had undergone permeabilization, the point of no return in apoptosis induction. Indeed, loss of $\Delta\Psi$m has been associated with ATP depletion with a subsequent necrotic cell death in the absence of ATP restoration (Gergely *et al*., 2002). Based on findings in this study, ATP generation appeared to increase exponentially in RBBP6 overexpressing cells suggesting that the cells remained intact and functional. These observations validate the observed apoptosis induction as discussed in the previous section because for apoptosis to occur ATP generation is necessary.

Caspase 3 and 7 activities were enhanced in both breast and cervical cancer cell lines following siRBBP6. A significant change in activity was observed in HeLa, SiHa and MDA-MB-231 after RBBP6 silencing. Caspase-3 and 7 mediate apoptosis upon activation via proteolytic cleavage by upstream caspases, followed by cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins, ultimately leading to morphologic manifestation of apoptosis such as DNA condensation, fragmentation and membrane blebbing (Namura *et al*., 1998). This significant change in caspase activity is in correlation with the observed apoptosis induction as detected by flow cytometry as well as the changes in cell shape detected by fluorescence microscopy. The observed statistically insignificant caspase 3/7 activity in MCF-7 cell line is not surprising since

MCF-7 cell do not express caspase-3 due to a frame shift mutation within exon 3 of the caspase-3 gene (Los *et al*., 1997). This means that the activity observed in this cell line is that of caspase-7 and not caspase-3, or the apoptosis induction observed in this cell line was caspase-independent.

7.1.6. Expression of apoptotic genes in response to RBBP6 gene targeting

Since the observed caspase activity coupled with ATP depletion seem to confirm the observed apoptosis induction in both breast and cervical cancer cell lines, understanding the effect of RBBP6 targeting on the expression of key apoptotic genes was necessary. Not only did this help understand the mechanism behind the observed apoptosis induction in this study, it also helped determine any possible link between RBBP6 and p53 gene expression. We analysed the expression levels of wild type p53 and key apoptotic genes bax, bak1, bad, caspase-3, caspase-8 and the anti-apoptotic Bcl-2 in order to understand their relationship with RBBP6 gene.

Silencing of RBBP6 led to a significant up-regulation of wild-type p53 coupled with an increased expression of bax and caspase-3 especially in HeLa and SiHa cells. Similar results were observed in MCF-7 with the exception of caspase-3 (Figure 57 and Figure 58). These observations suggest that RBBP6 gene silencing might results in the release of p53 from inactivation, allowing it to trigger intrinsic apoptotic pathway probably by facilitating the translocation of bax to the outer mitochondrial membrane. Bax oligomerises with itself or forms a complex with bak1 in order to porate the outer mitochondrial membrane for the release of cytochrome c and apoptosis inducing factors and a subsequent activation of effector caspases as affirmed by caspase 3/7 activity assay (Ouyang *et al*., 2012). It is however important to note that

bak1 expression did not change in response to RBBP6 silencing meaning the observed ATP depletion was as a result of bax dimerization.

Our observations are supported by clinical findings made by Chen *et al*. (2013) in which RBBP6 overexpression coupled with mutant TP53 was associated with poor prognosis in colon cancer tissues. The study showed that patients with RBBP6 overexpression coupled with mutants TP53 protein accumulation presented with disease recurrence followed by death. Furthermore, our observations that RBBP6 silencing promotes p53-mediated apoptosis are reminiscent of the phenotype observed in PACT knockout mice where widespread apoptosis was accompanied by wild-type p53 accumulation, and concomitant deletion of p53 rescued the mice (Li *et al*., 2007). Moreover, the insignificant apoptosis induction observed in the wild-type p53-null MDA-MB-231 cell line further implicates the importance of p53 in apoptosis detected in this study. Overall, RBBP6 silencing suggests a biomarker function in intrinsic pathway induction.

7.1.7. Combinational therapy the new success in cancer treatment

Targeted therapy is one approach that is directed at inhibiting growth and spread of tumour cells by specifically targeting molecular mechanisms responsible for tumour growth and survival without detrimental side effects (Vanneman and Dranoff, 2012; Wu *et al*., 2006). Despite high specificity and low cytotoxic effects however; limitations in targeted therapy can still arise (Dy and Adjei, 2013). For example, the target may change through mutations or the tumour may find a new pathway to achieve continued growth thus leading to drug resistance (Dy and Adjei, 2013; Vanneman and Dranoff, 2012; Wu *et al*., 2006). Targeted therapies therefore work best in

combinations that are aimed at different parts of the cell signalling, an approach well known as combinational therapy (Wu *et al.*, 2006).

In recent times, many reports have suggested highly effective and improved side effects in combinational therapy (Vanneman and Dranoff, 2012). Molecular targets have been reported to improve efficacy of chemotherapy by sensitizing cancer cells to the treatment (Vanneman and Dranoff, 2012). We find this approach much useful in this study as we have shown earlier that RBBP6 plays a critical role in cancer cell progression. The function of RBBP6 on cell cycle and proliferation has been elucidated where silencing seemed to inhibit cancer progression whilst overexpression appeared to favour it. On the other hand, the potential of camptothecin and GABA in apoptosis induction have been extensively explored (McCarty, 2014; Caretta and Mucignat-Caretta, 2011). The present study therefore shows that RBBP6 targeting enhances cellular response to these anticancer agents in cancer management.

Combination of RBBP6 silencing with camptothecin seemed to have an additive effect on the expression of p53 meaning that both treatments do trigger TP53 restoration in all cell lines except MDA-MB-231 which is known to contain mutant p53. RBBP6 silencing most likely prevents TP53 inactivation by releasing it from ubiquitination. In this case, the combination works in a way that camptothecin leads to DNA damage while RBBP6 frees and activates TP53 thus leading to either cell cycle arrest or apoptosis induction. The mechanism does not hold in GABA-siRBBP6 in which the expression of p53 remained unchanged. However, the caspase-8 activity might suggest PERP-mediated apoptosis rather than mitochondrial-dependent apoptosis.

Pretorius *et al*. (2013) on the other hand showed that RBBP6 silencing has an inhibitory effect on apoptosis in which bax/bcl-2 ratio was less than one. These findings might appear contradictory to the present study. However, it is worth noting that the cell line model (NIH3T3) used in the aforementioned study is not tumorigenic and its RBBP6 status has not been thoroughly investigated. Silencing RBBP6 stably, as opposed to transiently as is the case in the present study, might account for the observed apoptosis resistance in their study. In fact, their findings correlate well with our non-cancerous cell line model of choice, MRC-5, in which RBBP6 silencing failed to exhibit strong apoptotic characteristics when analysed by flow cytometry, even when combined with camptothecin.

From the results observed with combinational treatment of siRBBP6 and the two selected compounds, it remained clear that somehow there is a regression of cancer cells. But the question remained whether the combinational treatment resulted in apoptosis induction or cell cycle arrest. Camptothecin-siRBBP6 somehow sensitized cancer cells to apoptosis by significantly increasing cell death through the activation of p53 and bax. On the other hand, GABA-siRBBP6, apoptosis levels remained statistically unchanged with other cell lines showing a reduction in apoptosis, which suggests an antagonistic mechanism between the two treatments. Overexpression on the other hand had little impact on the apoptosis level rather than significantly reducing cell death. Cell cycle arrest was not the preferred mechanism of the apoptotic cell response to camptothecin and siRBBP6 as was seen by low cell populations in G0 phase. On the other hand overexpression and camptothecin showed increased number of cells in the S-phase of cell cycle which is a representation of dividing cells through DNA replication phase. GABA combination did not show any significant changes to perhaps allow us to come to a conclusive

argument of its involvement in cell cycle. From all these results we are able to suggest that siRBBP6 in combination with camptothecin successfully induced cell death through intrinsic pathway by restoring functional TP53.

Treatment with GABA had no significant effect on the overall expression of apoptotic genes except TP53 which appeared to be up-regulated. The observed mRNA changes in GABA+siRBBP6 co-treatment could mainly be the effect of silencing rather than GABA since low levels of apoptosis induction were detected in GABA-only treatments. MCF-7 cells are an exception though because a synergistic apoptosis induction was observed in GABA+siRBBP6 co-treatment when detected by microscopy. Such strong apoptotic characteristics could be accounted for by the observed caspase-8 up-regulation in this treatment, implying that apoptosis induction by GABA might have been due to p53-mediated PERP activation which has been associated with overexpression of caspase 8 (Davies *et al*., 2009).

7.1.8. Summary

Taken all together, we have discovered that RBBP6 is markedly up-regulated in squamous cervical carcinoma tissues, including the less prevalent adenocarcinomas. And further investigations revealed that not only does it play a role in promoting cell proliferation, it is also associated with apoptosis. The fact that RBBP6-knockdown cells become sensitized to apoptosis induced by camptothecin coupled with cell cycle arrest implicates the gene as a potential target in breast and cervical cancer management. The additive effect observed between siRBBP6 and camptothecin could be attributed to their almost-convergent mechanisms of action. That is camptothecin damages and prevents DNA replication thus resulting in failed cell division

whereas RBBP6 is said to work alongside telomerase by stabilizing chromosomal fragile sites thus promoting cell division and cell proliferation. Based on this, combination of siRBBP6 and camptothecin seems to be effective in preventing cell proliferation and inducing apoptosis in breast and cervical cancer. Accumulation of TP53 at mRNA level following RBBP6 silencing provides early insights into the possible interaction between RBBP6 and TP53; however, whether or not RBBP6 plays a role in TP53 degradation leaves room for further investigation in this area of study. Nonetheless, the accumulation of TP53 seems to be playing a central role in the induction of apoptosis observed in the present study. The inability of GABA to show effectiveness in inducing apoptosis either on its own or in combination with RBBP6 targeting could be attributed to the fact that its mechanism of action focuses more on cell migration rather than apoptosis induction.

The cell cycle was therefore analysed in this study in order to gain insights into the possible mechanism used by RBBP6 to affect cell proliferation. RBBP6 silencing led to increased cell populations in G0/G1 phase of all tumorigenic cell lines whereas overexpression induced slightly lower G0/G1 arrest compared to those in silencing. In contrast, RBBP6 overexpression resulted in cell cycle arrested of slightly higher (60%) percentage in normal lung fibroblasts at G0/G1 phase as compared to RBBP6 knockdown (52%).   Interestingly, RBBP6 targeting led to an increase in normal cell populations of up to 40% in G2/M phase whereas all tumorigenic cells remained at around 20% in this phase. Similar studies were performed to test whether RBBP6 plays a role in the cell cycle. For example, one study in mouse embryonic fibroblasts that were stably transfected with siRBBP6 oligonucleotides showed increased cell populations in G1 phase (Pretorius *et al*., 2013). Accumulation of MCF-7 cell populations in the sub-2N phase that

precedes the G1 interphase was observed following stable overexpression of P2P-R (Gao and Scott, 2003). In HEK293T cells it was suggested that the isoform 3 of RBBP6 is needed for G2 arrest since knockdown led to depletion of cell populations in G2/M checkpoint whereas knockdown of the full length isoform 1 did not have any effect in G2/M (Mbita *et al*., 2012).

These observations demonstrate RBBP6 to be an important regulator of the cell cycle and therefore cell proliferation. Increasing cell populations in G0/G1 phase as a result of RBBP6 knockdown as well as in the S-phase in response to RBBP6 overexpression suggest that RBBP6 plays a significant role in prompting cell cycle arrest. Ever since RBBP6 was proven to be involved in protein degradation where they showed that the RING finger-like domain is able to ubiquitinate YB-1, resulting in its proteasomal degradation (Chibi *et al*., 2008), more investigations emerged in which it was suspected that RBBP6 plays a role in p53 degradation as well (Motadi *et al*., 2011). Therefore the fact that RBBP6 overexpression shifted cell populations towards the S-phase might suggest p53 deregulation. To summarise, it is evident that RBBP6 silencing inhibits cell proliferation by arresting cells in the G0/G1 phase and overexpression facilitates growth by increasing cell populations in the S-phase.

RBBP6 overexpression in cervical cancer might be reminiscent of viral E6 oncoprotein since both proteins possess E3 ligase activity and have been shown to promote proteasomal degradation of TP53 protein thereof (Fernandes *et al*., 2013; Denny, 2010; Bosch *et al.*, 2002). The E6 oncoprotein has been shown to be involved in the development of cervical carcinogenesis in which it is responsible for the transfer of ubiquitin molecules from E2 ligase to

TP53 target protein, which is subsequently recognized by the proteasome for degradation (Fernandes *et al*., 2013). Besides TP53 degradation, both proteins have been shown to interact with yet another tumour suppressor, Rb, thereby releasing E2F transcription factor which is responsible for activation of the cell cycle, thus promoting tumour cell proliferation (Denny, 2010; Bosch *et al.*, 2002). Therefore it is highly likely that overexpression of RBBP6 in cervical cancer acquaints with an additive effect to the functioning of E6, thus leading to aggressive cervical tumours. This therefore makes RBBP6 an important target in the management of cervical cancer. The status of RBBP6 expression alone is however not sufficient to identify it as a prognostic marker, and therefore its involvement in cellular proliferation, apoptosis and cell cycle had to be extensively explored in this study.

7.1.9. Conclusions and future prospects

This study extensively characterized the role of RBBP6 in cell proliferation and apoptosis. The experimental approach provided novel exploration of the RBBP6 gene towards breast and cervical cancer and the study remains significant because both malignancies are still a common cause of female-related deaths in Africa. RBBP6 overexpression is indicative of poor prognosis in breast and cervical cancer atleast *in vitro* as seen by the continued cell proliferation and minimal apoptosis induction in RBBP6-overexpressing cells. The absence of RBBP6 sensitizes cells to what seems like p53-mediated apoptosis as demonstrated by TP53 accumulation, cell cycle arrest, up-regulation of pro-apoptotic genes, ATP depletion and activation of effector caspases. Furthermore, combined treatment with camptothecin showed an overall additive effect on apoptosis induction, which is very important in anticancer therapy because the goal is to improve drug efficacy while minimizing resistance. Even though GABA managed to induce cell death to a certain extent, its anticancer effects are not entirely governed by the apoptotic avenues.

In conclusion, these observations suggest that RBBP6 might be a potential biomarker for both breast and cervical cancer, and it provides an even more promising management assay when in combination with the chemotherapeutic agent, camptothecin.

Future investigations on the effects of GABA on cell migration and chemotaxis in breast and cervical cancer would be interesting. Quantification of GABA receptors in order to understand which ones (GABA$_{A/C}$ or GABA$_B$) are being stimulated by GABA treatment might be necessary in clearly understanding the mechanism of action of GABA in breast and cervical cancer. Thorough understanding of the suspected interaction between RBBP6 and p53 can be accomplished in part by performing protein-protein interaction experiments such as co-immunoprecipitation and western blotting. Furthermore, testing this promising anticancer management assay in mouse models of breast and cervical cancer would help to better understand the function of RBBP6 *in vivo*.

CHAPTER EIGHT - References

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APPENDICES

<div style="text-align: right;">**APPENDIX A**</div>

Clearance Certificate

R14/49 Ms Pontsho Moela

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140801

NAME:
(Principal Investigator)   Ms Pontsho Moela

DEPARTMENT: School of Molecular and Cell Biology
University of the Witwatersrand

PROJECT TITLE: Functional Studies of the Retinoblastoma Binding
Protein 6 Gene and its related Genes in Cervical
Cancer: A Promising Diagnostic and Management Assay
for Cervical Cancer

DATE CONSIDERED: 29/08/2014

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Dr Lesetja Motadi and Dr Letsolo Boitelo

APPROVED BY: _____
Professor P Cleaton-Jones, Co-Chairperson, HREC (Medical)

DATE OF APPROVAL: 01/09/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor,
Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned
research and I/we undertake to ensure compliance with these conditions. Should any departure be
contemplated, from the research protocol as approved, I/we undertake to resubmit the
application to the Committee. **I agree to submit a yearly progress report**.

_____   _____
Principal Investigator Signature   Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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ORIGINALITY REPORT

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PRIMARY SOURCES

1	Moela, Pontsho, Mpho M.S. Choene, and Lesetja R. Motadi. "Silencing RBBP6 (Retinoblastoma Binding Protein 6) sensitises breast cancer cells MCF7 to staurosporine and camptothecin-induced cell death", Immunobiology, 2014. Publication	3%
2	www.science.gov Internet Source	1%
3	www.biomedcentral.com Internet Source	<1%
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Statistical Analysis (Mitochondrial Activity)

HeLa	Time (Hour) p-value, t-test				
	2	4	6	8	24
CPT	0.002975	0.002975	0.048124	0.039301	0.000553
GABA	0.018208	0.018208	0.058594	0.047711	0.055623
siRBBP6	0.012141	0.012141	0.17117	0.027422	0.049165
siRBBP6+CPT	0.032	0.031	0.432	0.16	0.003
siRBBP6+GABA	0.432	0.16	0.003	0.0021	0.0023
pRBBP6	0.012141	0.012141	0.205327	0.023623	0.041377
pRBBP6+CPT	0.046873	0.046873	0.08773	0.045402	0.067357
pRBBP6+GABA	0.002975	0.002975	0.026579	0.001517	0.006782

SiHa	Time (Hour) p-value, t-test				
	2	4	6	8	24
CPT	0.05375	0.05215	0.01324	0.039301	0.00523
GABA	0.01208	0.01208	0.04594	0.047711	0.05623
siRBBP6	0.03841	0.04141	0.17117	0.05322	0.03165
siRBBP6+CPT	0.04232	0.0031	0.04724	0.03516	0.05403
siRBBP6+GABA	0.01432	0.0161	0.01032	0.0021	0.03023
pRBBP6	0.02141	0.01276	0.05327	0.02643	0.01367
pRBBP6+CPT	0.01883	0.04833	0.08773	0.04402	0.35765
pRBBP6+GABA	0.00275	0.00279	0.02579	0.01517	0.00682

MCF-7	Time (Hour) p-value, t-test				
	2	4	6	8	24
CPT	0.017430522	0.099775366	0.156338512	0.000521904	0.000152258
GABA	0.027754288	0.029047709	0.92998554	0.017504419	9.09658E-07
siRBBP6	0.033835546	0.084388542	0.139875936	0.000606661	0.070566457
siRBBP6+CPT	0.003820205	0.430733753	0.468394605	2.4312E-05	0.004397034
siRBBP6+GABA	0.008193212	0.008496109	0.467327572	0.06663708	0.000454948
pRBBP6	0.005831468	0.189158778	0.008772099	0.000356224	0.03250804
pRBBP6+CPT	0.001110167	0.020998515	0.135790788	0.066051391	0.20764419
pRBBP6+GABA	0.000518484	0.000848091	0.093573565	0.003116928	0.000751035

MDA-MB-231	Time (Hour) p-value, t-test				
	2	4	6	8	24
CPT	0.022304786	0.624983114	0.507205489	2.08683E-05	0.000118704
GABA	0.016263	0.022627	0.012021	0.015556	0.005657
siRBBP6	0.02687	0.022627	0.020506	0.005657	0.014849
siRBBP6+CPT	0.016803	0.016147	0.022727	0.044418	0.016803
siRBBP6+GABA	0.015556	0.019799	0.032527	0.03182	0.04799
pRBBP6	0.077979	0.056035	0.077318	0.134155	0.054829
pRBBP6+CPT	0.175962	0.026729	0.099584	0.209909	0.005115
pRBBP6+GABA	0.077979	0.056035	0.077318	0.134155	0.054829

MRC-5	Time (Hour) p-value, t-test				
	2	4	6	8	24
CPT	0.026729	0.099584	0.209909	0.005115	0.028799
GABA	0.028858	0.227302	0.169609	0.04057	0.034126
siRBBP6	0.043041	0.118726	0.055998	0.128971	0.024437
siRBBP6+CPT	0.061739	0.118058	0.109818	0.025767	0.263889
siRBBP6+GABA	0.201486	0.047865	0.275889	0.026771	0.005623
pRBBP6	0.022727	0.099584	0.041572	0.03841	0.01432
pRBBP6+CPT	0.032527	0.077318	0.00209	0.04232	0.02141
pRBBP6+GABA	0.077318	0.08773	0.04402	0.35765	0.01883