TARGETING RETINOBLASTOMA BINDING PROTEIN 6 (RBBP6) AS AN ANTI-OVARIAN CANCER THERAPEUTIC STRATEGY

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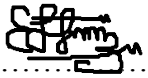
Declaration

I hereby declare that "**TARGETING RETINOBLASTOMA BINDING PROTEIN 6 (RBBP6) AS AN ANTI-OVARIAN CANCER THERAPEUTIC STRATEGY"** is my work. It has not been submitted for any degree or examination at any other University and all the sources I have used or quoted herein have been indicated and duly acknowledged by complete references.

Philemon Njende Ubanako

Signature  ……

On this 23rd day of January, 2015

"Whatever the mind can conceive and believe, it can achieve"

Napoleon Hill

Research Outputs

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Dedication

This work is dedicated to God Almighty who has been the source of my passion, inspiration, wisdom and strength. Lord, your guidance, comfort and provision have been priceless to me. Thank you for your unfailing love.

Abstract

Ovarian cancer is the most lethal gynaecological cancer. About 90% of ovarian cancers are epithelial (ovarian carcinomas), thought to arise from the ovarian surface epithelium. Diagnosed usually at clinically advanced stages, many patients show poor response to chemotherapy, with resistance and recurrent disease being prevalent. siRNA technology is currently being explored in clinical trials as a form of targeted therapeutic strategy in the disease. RBBP6 is a 250kD protein that enhances MDM2-mediated ubiquitination of p53 and also plays a role in cell cycle regulation and cell differentiation. It is upregulated in numerous cancers such as lung, oesophageal, colorectal and cervical cancer. RBBP6 suppresses p53 binding to DNA thereby inhibiting p53-dependent gene transcription. RBBP6 was knocked down using 30 nM siRNA in RMG-1 cells for 48 hours, after which the cells were treated with 50 nM paclitaxel and 0.5µM camptothecin for 24 hours. *xCELLigence* real time cell analysis was used to evaluate cell proliferation. qPCR and western blot were used to evaluate both gene expression and protein expressions respectively, of Bax, Bcl-2, MDM2, p53 and p21. Flow cytometry was used to determine the mode of cell death elicited apoptosis and also analyse changes in cell cycle progression.

qPCR and Western blot analyses showed that RBBP6 expression reduced by approximately 57%. There was a significant upregulation of p53 and a significant downregulation of Bcl-2 in siRBBP6 transfected cells ($p<0.05$). Knockdown of RBBP6 resulted in a 37±5.8% cell death. There was a significant increase in cell death in paclitaxel and siRBBP6 co-treated cells (81.6±0.79%) as compared to cells treated with paclitaxel only (76.±1.14%).

siRNA-mediated knock down of RBBP6 induces cell death in RMG-1 ovarian carcinoma cells. In addition, paclitaxel-induced cell death in RMG-1 cells is potentiated by RBBP6

siRNA transfection. A combination of chemotherapy with paclitaxel or camptothecin and RBBP6 siRNA could be a possible therapeutic strategy in combatting ovarian carcinomas.

Keywords:

Ovarian cancer	**Apoptosis**
Paclitaxel	**RBBP6**
MDM2	**Camptothecin**
siRNA	**p53**

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Table of non-standard abbreviations

AKT	Protein kinase B	NAIP	Neuronal inhibitor of apoptosis
ATP	Adenosine triphosphate	NCBI	National Centre for Biotechnology Information
Apaf-1	Apoptosis protease activating factor 1	Nm	nanometer
Bax	Bcl2-associated X protein	nM	Nanomolar
BCA	Bicinchoninic Assay	NOXA	NOXA–Long isoform protein
Bcl-2	B-cell/lymphoma 2 family	P13K	Phosphoinositide 3-kinase
BRAF	v-Raf murine sarcoma viral oncogene homolog B1	PARP	Poly (ADP-ribose) Polymerase
BRCA	Breast Cancer gene	P2P-R	Potential Related Protein
BSA	Bovine Serum Albumin	PACT	P53- Associated Cellular Protein
Bp	Base pair	p53	protein 53 (tumour protein 53)

BID	BH 3 interacting domain	PBS	Phosphate buffered saline
CD	Cluster of differentiation	PCR	Polymerase Chain Reaction
CDK	cyclin-D Dependent Kinase	PDGFR:	Platelet-derived growth factor receptor
DEPC	Diethylpyrocarbonate	pRb	Retinoblastoma protein
DMEM	Dulbecco's Modified Medium	PS	Phosphatidylserine
DMSO	Dimethyl sulfoxide	PTEN	Phosphatase and tensin homolog
DNA	Deoxyribonucleic Acid	PUMA	p53-Upregulated Modulator of Apoptosis
DR	Death Receptor	RBBP6	Retinoblastoma binding protein 6
DWNN	Domain With No Name	RING	Really Interesting New Gene
EDTA	Ethylene Diamine Tetra Acetic acid	RNA	Ribonucleic acid

EGFR	Epidermal growth factor receptor	RT-PCR	Real Time Polymerase Chain reaction
FBS	Foetal Bovine Serum	SD	Standard Deviation
FITC	Flourescein Isothiocynate	SDS	Sodium Dodecyl Sulphate
FSH	Follicle-Stimulating Hormone	siRNA	Short interfering RNA
FLIP	FLICE-like inhibitory protein	SMAC	Second Mitochondria-Derived Activator of Caspaces
G1	Gap 1	TNF	Tumour Necrosis Factor
G2	Gap 2	TRAIL	TNF-Related Apoptosis-Inducing Ligand
GADD45	Growth arrest and DNA damage gene	VEGFR:	Vasculo-endothelial growth factor receptor
GATA-4	GATA binding protein 4	XIAP	X-linked inhibitor of apoptosis
HRP	Horseradish Peroxidase		
IAP	Inhibitor of Apoptosis		

IGFR1	Insulin-like growth factor receptor 1		
Kb	Kilo base		
kDa	Kilo Dalton		
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue		
LH	Leuteinizing Hormone		
MCF-7	Michigan Cancer Foundation 7		
MDM-2	Mouse Double Minute 2		
mTOR	Mammalian target of Rapamycin		
NFκB	Nuclear factor kappa B		

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CHAPTER ONE- INTRODUCTION AND LITERATURE REVIEW

Introduction

This chapter focuses on a review of literature in ovarian cancer. It covers the epidemiology, causes and risk factors of the disease. Mechanisms of apoptosis in ovarian cancer are also reviewed. Current targeted therapeutic strategies using tyrosine kinase inhibitors and monoclonal antibodies are explored herein. RBBP6 is a 250kD protein shown to be upregulated in numerous cancers. We take a closer look at RBBP6 and its interaction with MDM2 to mediate p53 degradation. We review paclitaxel and camptothecin as current chemotherapy in ovarian cancer. Finally, we appraise a novel form of targeted therapy: siRNA technology and some advantages over other therapeutic strategies in ovarian cancer. This research applies a combination therapeutic strategy of RBBP6 siRNA transfection and paclitaxel or camptothecin in ovarian cancer; and is the first study carried out in this regard.

1.1 Ovarian cancer overview

Ovarian cancer is the leading cause of death among all gynaecological cancers in western countries. When compared to other gynaecological cancers, the mortality rate of ovarian cancer surpasses that of cervical and endometrial cancers put together (Jemal *et al*., 2008). This high death rate is due to the diagnosis at an advanced stage in most patients caused by the relative lack of specific signs and symptoms of the disease and the lack of reliable tests for early detection. Ovarian cancer is a highly metastatic disease characterized by widespread peritoneal dissemination and ascites and is the leading cause of death from gynaecologic

malignancies (Zhang *et al*., 2009; Gavalas *et al*., 2011; Kim *et al*., 2012). Despite aggressive surgery and platinum-based chemotherapy for patients presenting with clinically advanced ovarian cancer, the global five-year survival rate is only 15-20% (Parkin *et al*., 2008). Disease-associated mortality in ovarian carcinomas is most often as a result of metastasis. At the time of diagnosis, in most cases, disease has spread beyond the ovaries (Cannistra, 2004; Smith and Guidozzi, 2009). However, survival rates can be as high as 90% in stage I ovarian cancers (American Cancer Society, 2014)

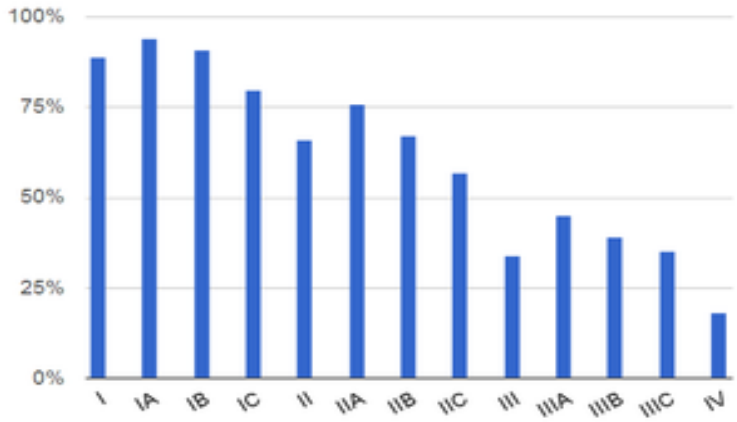


Figure 1.1: Relative 5-year survival for invasive epithelial ovarian cancer by stage.
Adapted from: "Survival rates for ovarian cancer". American Cancer Society, 2015.

There are more than 30 types and subtypes of ovarian cancer; classified into 3 major categories according to the primary cells from which they arise. They are categorized as: epithelial tumours, stromal tumours and germ cell tumours (Kaku *et al*., 2003). Epithelial ovarian tumours (ovarian carcinomas) are the most prevalent and the most aggressive; accounting for about 90% of all ovarian cancers (Sankaranarayanan, 2006; Gavalas *et al*., 2011).

1.2 Causes and Risk factors

The exact cause of ovarian cancer is not known. However, there are several factors which can increase a woman's risk of developing ovarian carcinomas. Regardless of the fact that molecular mechanisms of neoplastic transformation are inadequately understood, a handful of hypotheses have been put forward to explain the etiology of ovarian carcinomas (Zhang *et al*., 2009). The most popular of which are: the incessant ovulation theory (Fathalla, 1971) and the gonadotropin hypothesis (Ohtani *et al*., 2001). Not much is known about the causes and risk factors of stromal and germ cell ovarian tumours (American Cancer Society, 2015).

1.2.1 The gonadotropin hypothesis

This proposes that elevated serum gonadotropin levels (FSH and LH) play an important role in the development of epithelial ovarian cancers (EOCs) (Ohtani *et al*., 2001). This supports the observation that EOCs are most frequently diagnosed in postmenopausal women, with the median age of women affected being over 60 years, who usually show high serum gonadotropins levels (Cannistra, 2004). In addition, numerous studies on the risk factors and epidemiology of EOCs associate a decreasing risk of EOCs with length of lactation, increasing number of pregnancies (multiparity) and use of oral contraceptives (Schildkraut and Thompson, 1988; Gwinn *et al*., 1990); which are all typical of either lower levels of gonadotropins or decreased expression of their receptors (Daly and Obrams, 1998; Gnagy *et al*., 2000; La Vecchia, 2001).

1.2.2 The incessant ovulation theory

It proposes that frequent ovulation-induced rupture of the OSE ensued by mitotic repair, without pregnancy-induced rest periods, is a possible cause of EOCs (Fathalla, 1971). This theory proposes that repeated bouts of apoptosis and mitotic restoration of cells of the ovarian surface epithelium (OSE) predisposes them to genetic mutations and genomic instability which usually herald the onset of neoplastic transformation. Factors that protect against EOC such as oral contraceptive use, lactation and pregnancy which characterise anovuation are in agreement with this theory. Additionally, risk factors such as nulliparity and usage of ovulation stimulating drugs (fertility treatment), all characteristic of incessant ovulation also give credence to this theory (Cannistra, 2004). In 2001, Murdoch and his colleagues showed a high degree of oxidative DNA damage on OSE cells of sheep around the site of ovulation. They suggested that in case the DNA damage goes unrepaired the cell may evade apoptosis, resulting to the emergence of a transformed progenitor cell which may become propagated during mitogenic repair of the OSE post ovulation (Murdock *et al*., 2001).

1.3 Genetic risk factors

Studies have indicated that the most predominant risk factor for ovarian cancer is having a strong family history of breast cancer, ovarian cancer or both (Cannistra, 2004). The most important risk factor associated with ovarian cancer is genetic mutations in the breast cancer DNA repair genes BRCA1 and/or BRCA2 genes which are prevalent in families with a history of breast and/or ovarian cancer (Cannistra, 2004). BRCA1 mutations represent a 30-

40% chance of having the disease while BRCA2 mutations show a 10-15% lifetime risk (Antoniou *et al*., 2003). Some ethnic (Ashkenazi Jewish) groups show an increased frequency of germline BRCA1 and/or 2 mutations and show increased ovarian cancer incidence. In South Africa, germline BRCA mutations are the most common hereditary causes of ovarian cancer and affect about 1 in 500 women (Smith and Guidozzi, 2009). Lynch syndrome (also called hereditary non-polyposis colorectal cancer (HNPCC)) is a mismatch repair germline mutation that predisposes individuals to certain types of cancer including colorectal, endometrial and ovarian cancers. About 10 to 15% of hereditary cases of ovarian cancer occur in women having Lynch syndrome (Rubin *et al*., 1998).

Milder, less aggressive and genetically more stable ovarian cancers involve mutations of KRAS (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue), BRAF (v-Raf murine sarcoma viral oncogene homolog B1), PTEN (Phosphatase and tensin homolog) and B-catenin, mostly termed borderline tumours. A majority of sporadic mutations in EOC are as a result of mutations in the p53 and retinoblastoma tumour-suppressor pathways (Sherr and Mc Commick, 2002). Most high grade epithelial ovarian cancers (carcinomas) are as a result of TP53 mutations with examples such as undifferentiated tumours, high grade serous carcinomas and mixed malignant mesodermal tumours (Kurman and Shih, 2008). About 50% of human cancers have shown inactivation of p53 through mutations or loss. In most characterized tumours retaining wild type p53, disruptions have been found in the p53 pathway of tumour suppression (Harris and Hollstein, 1993). Mutations in p53 gene are frequently encountered in ovarian cancer (Kupryjanczyk *et al*., 2000). It seems reasonable to make use of therapeutic strategies that target the p53 pathway of tumour suppression.

Lifestyle factors such as cigarette smoking, alcohol consumption, poor diet and sedentary habits have been associated to increased risk of developing the disease (Anand *et al*., 2008).

1.4 Epidemiology

According to estimates from the International Agency for Research on Cancer (IARC), cancer is a leading cause of death worldwide with an estimated 7.6 million deaths in 2008 (approximately 21,000 deaths per day) (American Cancer Society, 2008). Ovarian cancer has taken its toll on health worldwide with approximately 239,000 cases diagnosed in 2012, claiming about 152, 000 lives (Cancer Research, UK, 2014). Cancer has received less focused attention in Africa partly as a result of the immense burden of infectious or communicable diseases such as HIV/AIDS and malaria because more resources are being directed to curb infectious diseases (Parkin *et al*., 2008). Survival rates of cancer are even worse in developing countries because of later diagnosis due to poorer screening facilities. Public health providers have a limited knowledge of signs and symptoms at early stages of cancer, and patients have limited access to prompt and standard treatment (WHO, 2002).

Most tellingly, the statistics of cancer in Africa, showing high rates of incidence, morbidity and mortality, emphasize the severity of the problem. Incidence rates of about 4.2 per 100,000 of the population were recorded in ovarian cancer in 2008. The corresponding mortality was as high as 3.4 women per 100,000 (GLOBOCAN, 2008).

Cancer generally results when mechanisms that control apoptosis are dysfunctional.

For us to be able to properly comprehend pathological apoptosis in the human ovary, it is imperative that we have a grasp of the underlying principles of physiological apoptotic processes in the ovary.

1.5 Apoptosis as cell division guardian

Therapies that are currently used in cancer treatment such as chemotherapy, radiotherapy and a handful of targeted therapeutic approaches exert their effects by inducing programmed cell death or apoptosis (Fulda and Debatin, 2006). It is therefore pertinent for us to understand apoptosis, the mechanisms responsible for it, its regulation and dysfunction in health and disease.

Apoptosis is a programmed mode of physiological cell death that is genetically controlled, tightly regulated and evolutionarily conserved. It functions in regulating cell populations in embryogenesis, cellular homeostasis, metamorphosis and defence (Kerr *et al*., 1972; Metzstein *et al*., 1998; Gupta, 2001; Renehan *et al*., 2001). It has been shown that both physiological and pathological stimuli can initiate or inhibit apoptosis. They include oncogenic activation, DNA damaging cytotoxic drugs, irradiation, ligation of death receptors, and lack of growth factors or survival signals (Renehan *et al*., 2001). Apoptosis is generally morphologically characterised by rapid cytoplasmic and chromatin condensation, internucleosomal DNA fragmentation and cell fragmentation. Protuberances then form on the cell membrane and are later budded-off to form membrane-bound structures. The membrane-bound bodies are then phagocytosed (Kerr *et al*., 1972; Walker *et al*., 1987). Necrosis, unlike apoptosis is pathological and traumatic form of cell death resulting in cellular inflammation and rupture Cell death by necrosis is usually as a result of external injury while apoptosis is as a result of internal or external insults that lead to programmed cell suicide (Schwartz *et al*., 1990).

1.5.1 Apoptotic pathways

Two main pathways of apoptosis have been elucidated: The death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway of apoptosis. The extrinsic pathway of apoptosis involves binding of ligands to cell surface receptors of the tumour necrosis factor-alpha (TNF-α) superfamily. The death receptor-ligand binding leads to transmission of the death signal through death domains, death effector domains and caspases recruitment domains. The caspase recruitment domains cause the activation of procaspases and adaptor proteins. Procaspases are then cleaved to form active caspases which lead to apoptosis (Hussein, 2005). The intrinsic pathway is regulated by members of the Bcl-2 family. Upon reception of a death signal such as ultra violet rays, gamma radiation oncogenic activation or chemotherapeutic agents by the cell, p53 becomes activated. Depending on the cellular context, activated p53 transcriptionally activates a combination of many pro-apoptotic genes such as Bax, Bak, Bad and BH3-only proteins (NOXA and PUMA), growth arrest gene p21, DR5, Bid, Fas and a host of others (Wu *et al*., 1997; Oda *et al*., 2000; Nakano and Vousden, 2001; Sax *et al*., 2002;). P53 has also been shown to repress the activity of anti-apoptotic genes such as BCl2 (Spierings *et al*., 2005; Vousden and Prives, 2009). Pro-apoptotic members of the Bcl-2 family are stimulated to formation of pores on the mitochondrial membrane enabling the release of cytochrome *c* out of mitochondria into the cytoplasm (Hussein *et al*., 2003a). Cytochrome *c* will then bind to Apaf 1, alongside dATP to form the apoptosome complex (Gewies, 2003; Spierings *et al*., 2005). The apoptosome complex will cleave and activate caspase 9, which in turn activates a cascade of events involving executioner caspases resulting in apoptosis (Gupta, 2001; Spierings *et al*., 2005). A p53-mediated transcriptional upregulation of DR5 (Wu *et al*., 1997) and Fas (Muller *et al*., 1998)

leads to apoptosis via the death receptor pathway. This describes a cross-talk that exists between intrinsic and extrinsic apoptotic pathways.

1.5.2 Cell cycle regulation and p21

We evaluated the expression of a cell cycle regulatory gene, p21 in this study. The p21 gene is a growth arrest gene that is transactivated by elevated p53 in response to various stress signals (Waldman *et al*., 1995; Sherr and Roberts, 1995), although it can also be activated by p53-independent mechanisms (Johnson *et al*., 1994; Kibbe *et al*., 2000). P21 protein induces G1 cell cycle arrest by inhibiting the activity of CDKs, blocking their ability to phosphorylate Rb and hence inhibiting the transcription of early S-phase genes by E2F (Chen *et al*., 1998.)

1.5.3 Regulation and failure of apoptosis

The intricacy of apoptotic regulation can be justified by an ever-expanding list of proteins and genes that are involved in the process. The pro-apoptotic and antiapoptotic BCl2 family of proteins which have been extensively studied are primarily responsible for the regulation of apoptosis. In a viable cell, the effects of pro-apoptotic members such as Bax and Bad are counterbalanced by those of pro-survival members such as BCl2 and BclX (Gewies, 2003). Another group of proteins known as inhibitors of apoptosis (IAP) inhibit apoptosis by blocking the activity of caspases. However, the effects of IAP are neutralised by Smac, a protein which is released from the mitochondria. During ovarian follicular development, X-linked IAP expression is increased. It facilitates the development of follicles by FSH and also inhibits the apoptosis of follicular cells (Hussein, 2005).

There is a fine-tuned balance between the number of cells that are produced by mitosis and those that die via apoptosis in a healthy living organism. Millions of cells are produced in a day by cell division, while a similar number dies by apoptosis. However, this balance between cell division and apoptosis becomes compromised in numerous diseased states. Excessive apoptosis contribute to neurodegenerative diseases (Parkinson's disease, Alzheimer's disease and Huntington's disease) while insufficient apoptosis may lead to autoimmunity and cancer (Reed, 2002). Dysregulation of apoptosis is usually as a result of genetic mutations or epigenetic modifications and is particularly very important in cancer. This study seeks to improve upon apoptosis by RBBP6 gene knockdown and paclitaxel and camptothecin treatments a therapeutic strategy in ovarian cancer.

1.5.4 Role of apoptosis in ovarian follicle development and maintenance

The ovary provides a paradigm for programmed cell death due to the cyclic nature of ovarian development and function (Hussein, 2005). Apoptosis is critical for ovarian function, bearing in mind that it regulates the cyclical processes in the female reproductive system. Apoptosis has been shown to be the underlying mechanism of cell death in the ovaries. It has been observed in germ cell loss (germ cell attrition), follicular atresia and corpus luteum regression (luteolysis) and ovarian surface epithelial cells prior to ovulation (Murdoch *et al*., 1995, Murdoch *et al*., 2002; Tilly, 1996; Hussein, 2005).

Follicular atresia: Follicular atresisa is the breakdown and resorption of ovarian follicles which occurs prior to ovulation (Santos *et al*., 2008). Several studies have shown that

apoptosis of granulosa cells is the main mode of cell death in follicular atresia (Tilly *et al*., 1991; Kaipia and Hsueh, 1997; Manabe *et al*., 2008).

Luteolysis: Luteolysis is the degeneration of the corpus luteum which occurs at the end of the female reproductive cycle in the absence of pregnancy (Vaskivuo and Taipanainen, 2003). The molecular effectors that have been shown to be mediators of luteolysis include Fas/Fas ligand, prostaglandin F2-α, endothelins, integrins, interferon-γ, (Moeljono *et al*., 1977; Quirk *et al*., 1995; Otani *et al*., 1996; Petroff *et al*., 2001; Wall *et al*., 2003).

Apoptosis in germ cell attrition: About seven million oocytes are produced in the ovary during the early life of the human foetus. However, there is a drastic reduction of the number of oocytes, to about one third, by apoptosis, shortly after birth (Manabe *et al*., 2008). Defects in apoptosis or sustained proliferation of germ cells may lead to germ cell tumours of the ovary.

1.6 Apoptosis in ovarian cancer and its therapeutic targets

In the last decades, basic cancer research has produced remarkable advances in our understanding of cancer biology and cancer genetics. Among the most important of these advances is the realization that apoptosis and the genes that control it have a profound effect on the malignant phenotype. For example, it is now clear that some oncogenic mutations disrupt apoptosis, leading to tumour initiation, progression or metastasis (Motadi *et al*., 2011; Chen *et al*., 2013). It is now well documented that most cytotoxic anticancer agents induce apoptosis, raising the possibility that defects in apoptotic mechanisms contribute to treatment failure (Lowe *et al*., 2000). Since apoptotic programs can be manipulated to produce massive changes in cell death, the genes and proteins controlling apoptosis are potential drug targets.

Recently, most anticancer agents now in use were developed with the sole purpose of inducing apoptosis that will selectively kill tumuor cells. So, targeting apoptosis remains the main focus and safest route towards combating cancer.

1.6.1 Targeted therapy in Ovarian Cancer

Despite optimal chemotherapeutic and surgical treatment of ovarian cancer patients presenting with advanced disease, 10 to 15% show long-term subsidence. More than 70% of chemotherapeutically treated ovarian cancer cases show resistance, and subsequent relapse to platinum-based drugs and paclitaxel (Copeland *et al*., 1994; Bartel *et al*., 2008; Gavalas *et al*., 2011). This necessitates research into alternative therapeutic strategies against ovarian cancer.

Conventional cancer chemotherapy is cytotoxic, and indiscriminately targets rapidly-dividing cells of the body. In a bid to destroy cancerous cells, some normal body cells are killed in the process, resulting in serious side effects (American Cancer Society, 2006). Damages to hair follicular cells, bone marrow cells, gastro-intestinal cells and cells lining the reproductive tract account for a great proportion of side effects experienced by chemotherapeutic use. Also, additional limitations of traditional chemotherapy include the palliative and unpredictable responses produced by patients (Arora and Scholar, 2005).

However, targeted therapy depicts a new generation of anti-cancer therapeutics that are designed to ideally interact with a specific molecule, usually a protein molecule, that is believed or shown to have a vital role in tumour progression or growth (Wu *et al*., 2006; Arora and Scholar, 2005).

Researchers (Ross *et al*., 2004) have proposed a set of criteria that qualify an ideal molecular target in cancer therapy. They include the following:

i) The molecule should be significantly expressed in vital tissues.

ii) It should be reproducibly measured in clinical samples.

iii) It should correlate with clinical outcome; yielding a clinical response in a significant amount of patients whose tumours expresses the target, and shows a trivial response in those whose tumours don not express the target. (Ross *et al*., 2004).

However, side effects have been reported in numerous clinical trials involving small molecule tyrosine kinase inhibitors some of which are as a result of unintended targets. They include fatigue, bowel perforations and severe diarrhoea, hypertension, hand-foot syndrome and proteinuria as observed in some vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) inhibitors (Friedlander *et al*., 2007; Azad *et al*., 2008; Nimeiri *et al*., 2008; Biagi *et al*., 2008).

As opposed to conventional chemotherapy, a hallmark of the action of targeted cancer therapeutics is a higher degree of selective toxicity. This aspect of an anticancer drug may be improved upon by either augmenting the concentration of the therapeutic agent that reaches the tumour tissue or decreasing the amount that reaches normal tissues (Singh *et al*., 2010). However, synergistic anticancer effects have been achieved by using targeted therapeutic approaches in combination with cytotoxic chemotherapy (Aurora and Scholar, 2005).

Various targeted therapeutic strategies have been explored in ovarian cancer management, often used in combination with chemotherapeutic agents for maximal results. Targeted cancer therapeutics can be grouped into two broad categories: kinase inhibitors and monoclonal antibodies (Wu *et al*., 2006; Aurora and Scholar, 2005).

1.6.2 Tyrosine Kinases

Tyrosine kinases are molecules that play crucial roles in signal transduction, culminating their effects by regulating gene transcription within the nucleus. They function by transferring γ-phosphate groups from adenosine triphosphate to the hydroxyl group of protein molecules responsible for signal transduction (Schlessinger, 2000). A major event that activates tumour proliferation is the phosphorylation of signal transduction molecules. The most essential cellular processes such as the cell cycle, cell division, differentiation, motility and apoptosis or cell survival are under scrupulous regulation of tyrosine kinases (Prenzel *et al*., 2001; Slichenmyer and Fry, 2001). Dysfunctions in tyrosine kinases pre-dispose signal transduction molecules to sustained phosphorylation hence abnormal cell proliferation. Tyrosine kinases have been found over-expressed or mutated in several types of tumours in humans including ovarian tumours (Levitzki and Gazit, 1995; Blume-Jensen and Hunter, 2001; Wiener *et al*., 2003) making them good targets for cancer therapy.

Small molecules have been developed to target tyrosine kinases while monoclonal antibodies target surface proteins or antigens that are differentially expressed, overexpressed or mutated in cancer cells compared to normal tissues. Monoclonal antibodies function by inducing changes in the function of the antigen or receptor in question such as invoking an immune response, or conjugating a drug to the antibody which targets a specific antigen (Scott *et al*., 2007).

1.7 Molecular therapeutic targets in ovarian cancer

1.7.1 Angiogenesis inhibitors

One of the key features leading to metastasis and invasion of normal tissues by cancerous cells is angiogenesis (the formation of new blood vessels). Poor prognosis in ovarian cancer has been confirmed to be associated with increased expression of the vascular endothelial growth factor (VEGF) which has functions such as angiogenesis, mitogenesis, improvement of vascular permeability, and endothelial cell survival (Folkman, 1997; Hartenbach *et al*., 1997). Small molecule tyrosine kinase inhibitors have been developed to target the VEGF ligand and the VEGF receptor in ovarian cancer thereby slowing angiogenesis and improving upon prognosis of disease (Friedlander *et al*., 2007; Campos *et al*., 2008; Hirte *et al*., 2008; Becker *et al*., 2013).

1.7.2 Inhibitors of the epidermal growth factor receptor (EGFR)

EGFR is a transmembrane tyrosine kinase protein receptor that is involved in cell proliferation, survival and differentiation (Herbst, 2004). About 70% of ovarian cancers show upregulated expression in EGFR, making it an attractive target for the treatment of ovarian carcinomas. Over-expression of EGFR has been shown to correlate with chemoresistance and poor prognosis. Increased cell proliferation, angiogenesis and reduced apoptosis are attributed to over-expression of EGFR (Bartlett *et al*., 1996, Fischer-Colbrie *et al*., 1997).Tyrosine kinase inhibitors such as erlonitib and gefitinib have been successfully directed against the EGFR (Sirotnak *et al*., 2000; Sirotnak, 2003). Phase 2 clinical trials with gefinitib in patients

with advanced recurrent ovarian carcinomas showed very little activity, although the drug was well tolerated (Schilder *et al*., 2005). Phase 3 trials with erlonitib, however did not show any significant improvement in activity (Vergote *et al*., 2013).

1.7.3 Aurora kinase inhibitors

Aurora A is a serine-threonine kinase that is required for many essential cellular functions such as mitosis, spindle formation and centromere separation (Bischoff *et al*., 1998; Campos and Gosh, 2010). An over-expression of Aurora A as well as amplification of its gene location have been frequently noted in human tumours, including in ovarian carcinomas (Bischoff *et al*., 1998; Zhou *et al*., 1998; Lingle *et al*., 1998; Landen *et al*., 2007). Aurora A has been shown to inhibit paclitaxel and cisplatin –mediated apoptosis in ovarian cancer cells (Yang *et al.*, 2006; Anand *et al*., 2003). Aurora kinase inhibition with a small molecule MK-0457 in combination with chemotherapy (docetaxel) has shown significant reduction in tumour growth and cell proliferation in HeyA8 and SKOV3ip1 ovarian cancer cell lines (Sun *et al*., 2007; Lin *et al*., 2008; Traynor *et al*., 2011).

1.7.4 Poly ADP Ribose Polymerase (PARP) inhibitors

BRCA1 and BRCA2 play an important role in DNA double strand break repairs and maintaining genomic stability (Lord and Ashworth, 2008). BRCA ovarian cancer patients show an impaired ability to repair damaged DNA. Mutations in these genes account for 5 to

10% of all ovarian cases. BRCA genes represent the most important risk factor in ovarian cancer with lifetime risks of between 40-50% and 10-20% for BRCA1 and BRCA2 respectively (Cannistra, 2004; Lord and Ashworth, 2008). More than 50% of high grade serous ovarian carcinomas show loss of function of BRCA genes, either by genetic or epigenetic causes (Press *et al*., 2008). Poly ADP Ribose Polymerase (PARP) is a nuclear enzyme involved in the DNA single strand break repair (Tutt *et al*., 2005). It is activated in DNA damage and its inhibition results in DNA single strand breaks which may result in double strand breaks. BRCA1 and BRCA 2 patients show high sensitivity to DNA-damaging chemotherapeutics and have also shown immense responsiveness to PARP inhibitors (Bryant *et al*., 2005; Tutt *et al*., 2005). Although the mechanism is not fully understood, Lord and Ashworth (2008) suggest that an excessive amount of DNA single strand breaks with subsequent double strand breaks leads to high irreparable genomic instability and hence, cell death. Olaparib is a small molecule PARP inhibitor that has been used and has shown efficacy in BRCA1 ovarian cancer patients and has It has passed the first phase of clinical trials (Fong *et al*., 2008).

1.7.5 Platelet-derived growth factor (PDGF) receptor inhibitors

PDGF is involved in cellular growth, survival, differentiation, vascular permeability, cellular migration and healing of wounds (Schmitt and Matei, 2008). Between 50% and 80% of ovarian cancers show activation of the PDGF receptor which is involved in neoplastic transformation (Heinrich *et al*., 2003; Apte *et al*., 2004). PDGF receptor activation is as a result of mutations, genetic amplification or chromosomal rearrangements (Carroll *et al*., 1996; Heinrich *et al*., 2003). Imatinib mesylate (STI57) is a small molecule that has been

used to target PDGF receptor. A significant induction of apoptosis and reduction in tumour weight was observed in this study when STI571 was used in combination with paclitaxel. However, STI571 alone did not cause any significant effects (Apte *et al*., 2004). Imatinib is a small molecule PDGF receptor inhibitor that has passed phase two clinical trials in ovarian cancer patients whose tumours express the PDGF receptor (Alberts *et al*., 2007).

1.7.6 MTOR inhibitors

PTEN is a lipid phosphatase that is involved in G1 cell cycle arrest and apoptosis through the AKT /PI3K /mTOR pathway and has been shown to be mutated, deleted or inactivated in gynaecologic tumours (Sansal and Sellers, 2004; Jiang and Liu, 2008; Campos and Gosh 2009). The mTOR pathway is a key regulator of cell growth, proliferation and programmed cell death (Campos and Gosh 2009). Inhibitors of mTOR such as everolimus (RAD001) have been shown to inhibit angiogenesis, tumour proliferation and ascites formation in vivo and in vitro using OVCA10 and SKOV-3 ovarian cancer cells. It also improved upon cisplatin-mediated apoptosis These suggest a promising role of mTOR inhibitors to treat ovarian tumours (Gera *et al*., 2004; Mabuchi *et al*., 2007; Okamoto *et al*., 2010).

1.7.7 Targeting Bcl-2 Family in ovarian cancer and apoptosis

Bcl-2 family of proteins is divided into two types of proteins ones that can induce apoptosis referred to as pro-apoptotic and those that inhibit apoptosis called antiapoptotic molecules.

The most common protein which is well defined is Bcl-2 which is an antiapoptotic molecule that exerts its effects by binding to Bax, blocking c-Myc-induced apoptosis, blocking mitochondrial release of cytochrome-C and also inhibiting Apaf-1 interaction with Caspase-9 (Luo *et al*., 1997). In the ovaries, Bcl-2 is expressed mainly in healthy ovarian follicles while Bax, a pro-apoptotic molecule which is a pro-apoptotic molecule, is expressed in the follicles undergoing atresia. Bcl2 and Bax expression are markedly influenced by gonadotrophin levels. Elevated gonadotrophins tend to inhibit Bax expression while increasing Bcl-2 and Bcl-xL expression, hence promoting the survival of the follicle (Tilly *et al*., 1995a; Sugino *et al*., 2000). Tilly *et al* (1995a) analyzing the expression of Bcl2 family of protein in the immature ovaries of a rat during follicular atresia found the correlation between Bax mRNA which was upregulated and that of Bcl2 and Bcl-xL down-regulated. Other pro-apoptotic molecules such as Mcl-1, Bax and Bok elicit their apoptotic effects by triggering mitochondrial cytochrome-c release. Cytochrome c binds to Apaf-1, forming the apoptosome and activating the caspase cascade which leads to apoptotic cell demise (Tilly *et al*., 1995b).

Because of the importance of these pro- and anti-apoptotic proteins in deciding if the cell undergoes apoptosis or they survive, they have become a target for many researchers who are eager to restore apoptosis in cancer cells. Ovarian cancer is mainly associated with mutations in some of the genes that might trigger apoptosis and restoring their functions might be alternative therapeutic options. In recent years several small molecules that aimed to target Bcl-2 thereby inhibiting its activity in cancer cells are identified as possible therapeutic option. One of the recently discovered molecules that act as a selective inhibitor of Bcl2 is AB-737 and its orally active product AB-263where shown to inhibit cell growth in eight different ovarian cancer cell lines, although with relatively poor potency. Further test revealed that ABT-737 increased the sensitivity of several cell lines to carboplatin when

ABT-737 was administered after carboplatin (Witham *et al.*, 2007; Jain *et al*., 2014). In addition, ABT-737 significantly enhanced the activity of carboplatin in one of three primary cultures derived directly from ascitic tumour cells in patients recently treated with chemotherapy. The increased sensitivity to carboplatin was accompanied by a decrease in time at which apoptosis was observed when assessed according to the number of attached cells, PARP cleavage, and nucleosome formation (Witham *et al*., 2007). When carboplatin was co-administered with Small interfering RNA directed to Bcl-xL or BCl2 IGROV-1 tumour xenograft growth was highly inhibited as compared to treatment with carboplatin (Witham *et al* 2007). In recent clinical trials, ABT-737 is able to prolong the survival of recipient mice transplanted with Bcl-2-transduced tumours. It was also found to be functional with co-treatment with chemotherapy.

1.7.8 Minimizing expression of Inhibitors of Apoptosis (IAP) as target for ovarian cancer

The inhibitor of apoptosis proteins (IAPs) constitutes a family of highly conserved apoptosis suppressor proteins that were originally identified in baculoviruses. Although IAP homologs have recently been demonstrated to suppress apoptosis in mammalian cells, their expression and role in human ovarian epithelial cancer and chemotherapy resistance are still unknown or not clear. To date eight IAPs have been identified, namely, NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), Apollon (BRUCE, BIRC6), Livin/ML-IAP (BIRC7) and IAP-like protein 2 (BIRC8) (Vucic and Fairbrother, 2007). IAPs are endogenous inhibitors of caspases and they can inhibit caspase activity by binding their conserved BIR domains to the active sites of caspases, by promoting

degradation of active caspases or by keeping the caspases away from their substrates (Wei *et al* 2008). Dysregulated IAP expression has been reported in many cancers. For example, Lopes *et al* demonstrated abnormal expression of the IAP family in pancreatic cancer cells and that this abnormal expression was also responsible for resistance to chemotherapy. So far, XIAP has been reported to be the most potent inhibitor of apoptosis among all the IAPs (Svane *et al*, 2004). It effectively inhibits the intrinsic as well as extrinsic pathways of apoptosis and it does so by binding and inhibiting upstream caspase-9 and the downstream caspases-3 and -7 (Svane *et al.*, 2004).

When designing novel drugs for cancers, the IAPs are attractive molecular targets. In normal ovaries, surging FSH in the ovary upregulates XIAP which leads to a suppression of granulosa cell apoptosis and promotes the growth of follicles, induced by FSH (Hussein, 2005; Xiao *et al*., 2001; Scott *et al*., 2005). It also said to effectively inhibits the intrinsic as well as extrinsic pathways of apoptosis and it does so by binding and inhibiting upstream caspase-9 and the downstream caspases-3 and -7 (Hussein, 2005; Xiao *et al*., 2001; Scott *et al*., 2005). So, if this is true even during ovarian cancer development, XIAP might provide much better molecular therapeutic target in chemo-resistant ovarian cancer. In recent years, some novel therapy targeting XIAP included antisense strategies and short interfering RNA (siRNA) molecules. In their using the antisense approach, Dai *et al* (2009) reported inhibition of XIAP resulted in an improved *in vivo* tumour control by radiotherapy. In another study by Li *et al* (2001), it was shown through the use of cisplatin-sensitive and -resistant human ovarian surface epithelial (hOSE) cancer cell lines and adenoviral antisense and sense complementary DNA expression to examine the role of IAP in the regulation of apoptosis in human ovarian cancer cells and chemoresistance that Cisplatin consistently decreased XIAP content and induced apoptosis in the cisplatin-sensitive, but not cisplatin-resistant cells.

1.7.9 Therapuetical potential of TNF family members

Tumor necrosis factor alpha (TNF-α), also known as cachectin and TNFSF2, is the prototypic ligand of the TNF superfamily. It is a pleiotropic molecule that plays a central role in inflammation, apoptosis, and immune system development. In normal ovarian development, the expression of some TNF family members such as FasL/Fas is highly influenced by gonadotrophin levels. Surging gonadotrophin levels result to a decreased expression of Fas/FasL, thereby promoting follicular survival. However, decreased gonadotropin levels result in an increased expression of Fas/FasL, leading to follicular atresia (Jiang *et al*., 2003). TNF-α is abundant in the ovarian cancer microenvironment. TNF-α modulates the expression of CD44 in normal T lymphocytes and CD44 is implicated in ovarian carcinogenesis and metastases (Muthukumaran *et al.,* 2006). TNF family members show both pro-survival and pro-apoptotic functions, depending on the type of receptors that are activated. TNF- α triggers apoptosis by activating caspases (Wang *et al*., 2008); on the other hand, TNF-α is able to aid the survival of granulosa cells by upregulating the expression of and XIAP through the NFκB system (Jiang *et al*., 2003). TRAIL is another TNF family member that has been shown to induce apoptosis in tumour cells, but not in normal cells, owing to the presence of TRAIL decoy receptors which competitively inhibit the binding of TRAIL ligands to their cognate receptors (Sheridan *et al*., 1997; Pan *et al*., 1997). TRAIL and its receptors are expressed in growing, atretic and antral ovarian follicles (Bobe and Goetz, 2001).

1.7.10 Wild-type p53: the genomic guardian target

This gene encodes a tumour suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains. The encoded protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Scientists have dubbed p53 "the guardian of the genome" (Levine, 1997) and "the cellular gate-keeper" (1992) because of its critical function in regulating responses to a wide range of cellular stress factors. P53 transcriptionally activates apoptotic and growth arrest genes and also represses pro-survival genes (C-myc repression (Ho *et al*., 2005), Bcl2 repression (Miyashita *et al*., 1994; Wu *et al*., 2001)). The response of p53 to cellular stresses is dependent on the type and duration of stress or extent of DNA damage and cellular growth conditions. P53 induces the transcription of target genes which can either lead to apoptosis, growth arrest to enable DNA repair, senescence or cellular differentiation (Hupp *et al*., 1992). P53 has a very short half-life, and becomes rapidly degraded in normal physiological conditions.

1.7.11 Regulation of p53

MDM2 is a multi-domain oncoprotein that is encoded by the MDM2 gene. It has an *N*-terminal domain that contains the binding sites of p53 and p73; a putative ZINC finger domain that binds the Rb protein; an acidic domain which contains the p14 binding site; and a p53 ubiquitination site, which is a RING finger E3 ligase domain (Momand *et al*., 2000). Under normal physiological conditions, p53 and MDM2 regulate the cellular levels of each other via their interactions in an auto-regulatory feedback loop. When p53 becomes activated,

it binds to the MDM2 P2 promoter and induces the expression of MDM2 protein. While activated p53 transcriptionally upregulates a handful of genes, including MDM2 (Picksley and Lane, 1993; Wu *et al*., 1993), MDM2 in turn represses the activity of p53 possibly via the following mechanisms:

- The activity of MDM2 as an E3 ubiquitin ligase enables it to target p53 for degradation by the proteasome.
- MDM2 inhibits p53 binding to DNA thereby preventing its functions as a transcription factor. This aspect is achieved by MDM2 binding to p53 and inducing its export out of the nucleus.
- The binding of MDM2 to the p53 transactivation domain prevents p53 from carrying out it transcription activation functions

(Chen *et al*., 1993; Oren *et al*., 1999; Tao and Levine, 1999; Honda and Yashuda, 2000)

Upon DNA damage, p53 becomes activated or stabilised by phosphorylation (Karin and Hunter, 1995; Shieh *et al*., 1997), acetylation of the C-terminus (Gu and Roeder, 1997) and other mechanisms, depending on the cellular context. Activated p53 translocates from the cytoplasm to the nucleus and acts in concert with other oncogenes and transcription factors to modulate cell proliferation by selectively activating a specific subset of proapoptotic and growth arrest genes, and repressing antiapoptotic genes. The mechanisms still remains unclear (Budhram-Mahadeo *et al*., 1999; Alarcon-Vargas and Ronai, 2002).

The effects of p53 are mainly as a result of its activity as a transcription factor. It transcriptionally activates pro-apoptotic genes such as Bax, PUMA, NOXA and the growth arrest gene p21. It represses the transcription of anti-apoptotic genes such as Bcl2 and Bcl-Xl, MDM2 (Barak *et al*., 1994; Wu *et al*., 2001; Ohtani *et al*., 2004). p53 is expressed in

apoptotic granulosa cells of atretic follicles, suggesting a possible role of p53 in follicular atresia (Kim *et al*., 2000). One of the ways by which Gonadotropins inhibit apoptosis is by repressing a host of pro-apoptotic genes including p53 gene expression (Tilly *et al.,* 1995b). APR-246 is converted to the active compound MQ that binds covalently to mutant p53, refolding it to wild type conformation2 and reactivating p53-dependent apoptosis (von Euler *et al*., 2014).

1.7.12 Molecules targeting p53

In targeting the reactivation of p53 in ovarian cancer, APR-246 was shown not only able to reactivates mutant p53, but also decreases intracellular glutathione levels in a dose-dependent manner (von Euler *et al* 2014). This unique mechanism of action, targeting both mutant p53 and glutathione, is likely to account for the strong synergistic effects of APR-246 and platinum drugs as well as resensitization of ovarian cancer cells, which were observed in the study. Ad5CMV-p53 is a recombinant adenoviral vector that encodes TP53. The co-administration of Ad5CMV-p53 intraperitoneally with chemotherapy using gemcitabine showed synergistic effects in the treatment of recurrent disease after multiple cycles of therapy (Wen *et al.,* 2003). In another study, it was shown that Ad5CMVp53 showed a substantial clinical activity when used in combination with platinum-based chemotherapy to treat recurrent ovarian cancer (Buller *et al* 2002). Another molecule, CDB3, which is a synthetic p53 binding protein-derived peptide (53BP2), is known to interact with the core domain of p53 and upregulate the transactivation activity of p53 (Samuels-Lev *et al* 2001. A fluorescently tagged CDB3 was shown to improve the proper native folding of

p53Arg273His contact and p53Arg175His mutants; induce p53-mediated transactivation of p21, MDM2 and GADD45; and induce apoptosis.

It was observed that, after treatment with CDB3 alone, apoptosis was not induced in wild-type p53-bearing cells. Notwithstanding, the cells showed enhanced sensitivity to apoptosis induced by infra-red radiation (Weill *et al*., 2000). Cp-31398, a styrylquinazoline, is a small synthetic molecule that was identified for its ability to stabilize p53 against thermal denaturation *in vitro*. Treatment with Cp-31398 blocks the ubiquitination and degradation of p53 resulting in increased DR5 cell surface exposure and activation of the intrinsic bax/mitochondrial/caspase-9 pathway, culminating in apoptosis (Vecil *et al*., 2003). Because of the critical inhibitory role of MDM2 and RBBP6 on p53, blocking the interaction of RBBP6, MDM2 and p53 has been proposed as a potential cancer therapeutic strategy. Nutlin-2 is a small-molecular-weight inhibitor that fits into the pocket where wild-type TP53 binds to MDM, a molecule required for the rapid degradation of TP53 through the ubiquitin-proteasome pathway (Wang *et al.,* 2012). Inhibition of the MDM–TP53 interaction results in the increased expression of wild-type TP53, inhibiting tumour growth and inducing apoptosis. In a study by Moela *et al.,* 2014, silencing breast cancer cell lines with RBBP6 siRNA followed by treatment with camptothecin also sensitized cancer cells to apoptosis induced cell death which serves as a promising natural cell biology pathway.

1.7.13 Other apoptotic molecular targets for ovarian cancer cells

Interferons (IFN): IFN-γ is a potent immunomodulatory, antiviral, and antiproliferative cytokine that has anticancer activity. IFN-γ directly inhibits human tumor cell growth and

induces apoptosis (Clemens, 2003). They include IFN-α, IFN-γ, IFN-β and IFN-δ. Interferons sensitize cells to apoptosis-inducing genes and proteins in apoptotic pathways (O'Connell *et al.,* 2000; Lissat *et al*., 2007). IFN- δ inhibits Fas expression in the corpus luteum, thereby inducing apoptosis in bovine luteal cells (Komatsu *et al*., 2003). This is as a result of anti-apoptotic Fas effects in the corpus luteum. IFN- γ has been shown to induce apoptosis in luteal cells and ovarian cancer cells both in vivo and in vitro (Petroff *et al*., 2001; Wall *et al*., 2003). In ovarian cancer cell linea and xenografts in nude mice IFN was found to induce apoptosis and inhibit proliferation of cancer cell (Wall *et al.,* 2003). IFN-γ might be a useful biological treatment of human epithelial ovarian cancer if sustained levels of this cytokine could be achieved within the peritoneum by improved protein or gene delivery strategies.

Integrins*:* are transmembrane proteins receptors containing α and β heterodimeric chains and a short tail in the cytoplasmic region. They possess adhesion properties that connect the cell to the cytoskeleton and are able to influence cell survival and cell death (Aoudjit and Vuori, 2012). They function in cell proliferation via signal transduction pathways by activating protein kinases (Giancotti and Ruoslahti, 1999). Integrins are expressed in primordial follicular cell surfaces, aiding their adhesion to the extracellular matrix. Integrins are weakly expressed in atretic tertiary follicles and absent in atretic primary and secondary follicles. Granulosa cells that lack the expression are the only ones that undergo apoptosis (Giebel *et al*., 1996). Volociximab, a chimeric monoclonal antibody directed against α5β1-integrin, inhibits angiogenesis and impedes tumor growth. Intetumumab (CNTO95) is a fully human anti–αv integrin monoclonal antibody that binds human αv integrin–expressing cells with high affinity (Kd, approximately 1–24 nmol/L) and has limited cross-reactivity with rat αv integrin (Kd, 220 nmol/L). Intetumumab has been reported to inhibit cell adhesion, migration, invasion, and proliferation of endothelial cells and tumor cells *in vitro* and tumor

metastasis *in vivo* in nude mice with human breast cancer xenografts by inactivating the focal adhesion kinase (FAK) and the docking protein paxillin (Chen *et al.*, 2008). Intra-peritoneal administration Etaracizumab is a a humanized monoclonal antibody that has been tested in SKOV3ip1 and HeyA8 mouse model tumours, targeting the αvβ3 integrin receptor. This study showed a decreased tumour burden by 36 and 48% respectively (Landen *et al*., 2008). However, its role in ovarian cancer patients is still to be exploited in clinical trials. Cilengitide, an αVβ3 and αVβ5 inhibitor, is a cyclic RGD containing pentapeptide. Cilengitide has been shown to cross the blood brain barrier and accumulate to appreciable levels in brain tissue in a phase 2 trial in glioblastoma. Perry *et al.* (2013) using breast cancer cell lines (MCF-7 cells and then MDA-MB-231) treated with Cilengitide has shown that it was able to induce apoptosis and arrest cell proliferation. Cilengitide target expression level may play a role in this process, and apoptosis appears to be possible mechanism for cilengitide mediated cell death in these cell lines. In another study by Lautenschlaeger *et al.* (2013), using breast cancer cell lines found that combined cilengitide and radiation therapy appears to be more efficacious than either treatment alone in breast cancer cell lines. This study together with others (Reardon *et al*., 2008) have actually supported the idea that cilengitide in combination with radiation can be useful tool against ovarian cancer associated with integrins.

Insulin-like growth factor (IGF)*:* IGFs are proteins with high sequence similarity to insulin that stimulate mitosis; regulating cell proliferation, differentiation and apoptosis. IGF binding proteins regulate the activity of IGFs by modulating IGF binding to their cognate receptors (Werner *et al*., 2008). IGF-1 functions in the ovary by potentiating the action of gonadotrophic hormones. IGF-1 stimulates and sustains signals that lead to cell proliferation

and inhibition of apoptosis by activation of the P13K/AKT pathway (Wang *et al*., 2007). A high IGF-1 expression in mice follicles suggests that it pays an important role in follicle development. The roles of IGF binding proteins may differ; while IGFBP-4 is highly expressed in atretic follicles, IGFBP-5 is up-regulated in healthy primary and secondary follicles (Besnard *et al*., 1996). IGF over-expression correlates with poor disease prognosis in some cases of ovarian carcinomas (Brokaw *et al*., 2007). Small molecule inhibitors hinder the activation of IGF-IR by binding to the pocket of the receptor that binds to ATP. The attenuation of insulin receptor signalling is a side effect that is exhibited by most tyrosine kinase inhibitors that have been developed. Nonetheless, their activity in preclinical models has made them to be evaluated in clinical trials, irrespective of their lack of specificity.

NVP-AEW541 is a potent inhibitor of IGF-1R which was demonstrated to sensitized cells to the effect of cisplatin treatment in ovarian cancer cells (Beauchamp *et al*., 2010). NVP-AEW541 induced apoptosis and decreased AKT activation (Beauchamp, *et al* 2010) in two human epithelial ovarian cancer cell lines, namely, OVCAR-3 and OVCAR-4. Another molecules exploited is, BMS-536924 which is a potent small molecule inhibitor of IGF-IR, which shows antitumor activity in multiple tumour models. It has been reported to provoke cell apoptosis in ovarian cancer cells by the activating PARP cleavage (Xu *et al* 2008). In addition, it proposes that combination therapy using BMS-536924 with a PARP inhibitor might be an effective strategy to circumvent resistance to treatment in clinical settings. In another study by Beltran *et al.* (2009) using AMG 479, a fully human monoclonal antibody against insulin-like growth factor type 1 receptor (IGF-1R) they reported that AMG479 served as second line therapy in patients with recurrent platinum-sensitive ovarian cancer by blocking the binding of IGF1 and IGF2. There is a possibility that these agents might be more

potent anticancer drugs since insulin receptor present on malignant cells may have an important role as well in carcinogenesis.

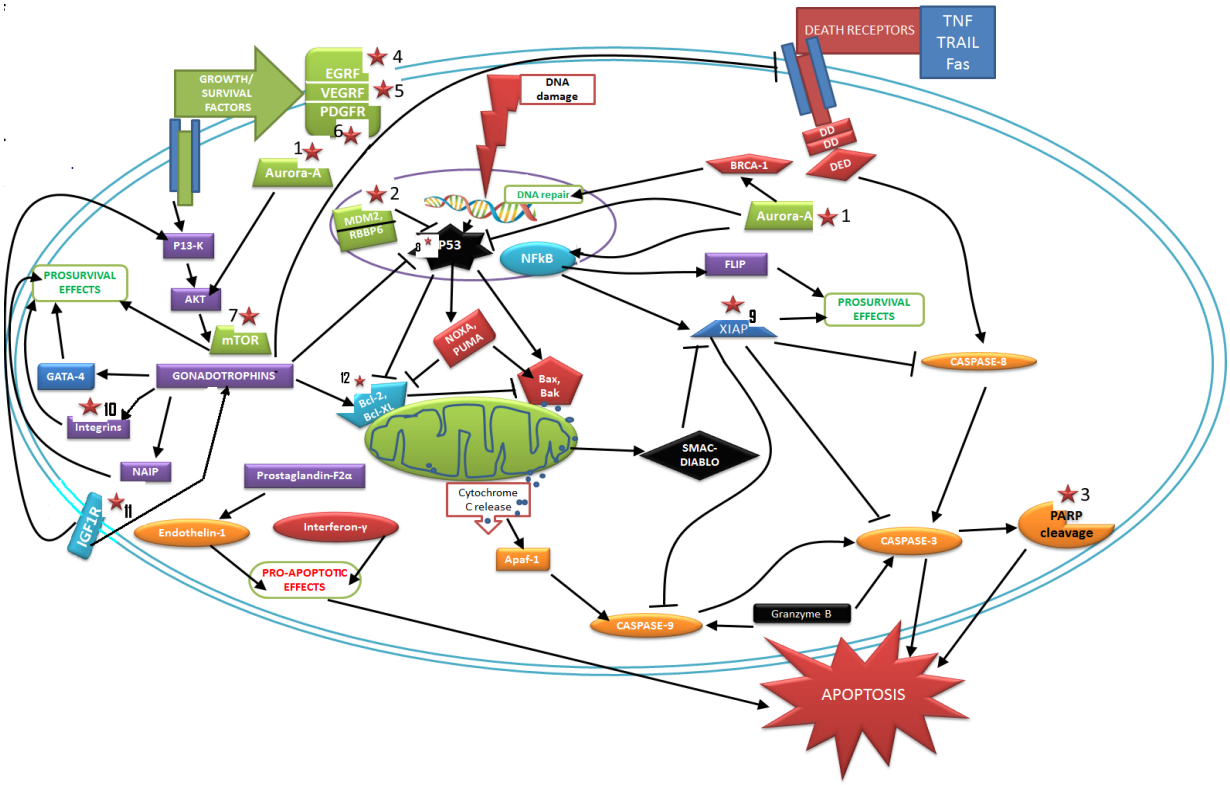


Figure 1.2: A schematic mechanism showing apoptosis in ovarian cancer and some current targeted therapeutic approaches.

Central to this mechanism is the activity of gonadotrophins and other upregulated proteins such as Aurora A, PARP, mTOR and growth factors such as EGFR, VEGFR, and PDGFR which have been exploited as molecular targets in various types of epithelial ovarian cancer and are at various phases in clinical trials. The numbered red stars (1 to 12) represent various molecular targets that have been explored in ovarian cancer (See table 1.1). 1. Aurora A is a potent prosurvival molecule that exerts its effects by activating AKT, promoting DNA repair by activating BRCA1, blocking p53 activity, and activating the transcription factor NFkB. Growth factor receptors (EGFR (4), VEGFR (5), and PDGFR (6) which exert prosurvival effects via the P13K/AKT pathway and have been targeted in ovarian cancer. XIAP, which

can be upregulated by growth signals from NFkB is the most potent known inhibitor of caspases and has also been targeted using small molecules. Intergrins such as α5β1-integrin exert prosurvival effects by activating protein kinases. IGF-1 functions in the ovary by potentiating the action of gonadotrophic hormones. IGF-1 stimulates and sustains signals that lead to cell proliferation and inhibition of apoptosis by activation of the P13K/AKT pathway. MDM2 which interacts with RBBP6 to promote p53 ubiquitination has been targeted as well with Nultin-2

Table 1.1

A table showing small molecule inhibitors/ monoclonal antibodies and their molecular targets in ovarian cancer

Small molecule drug (generic name)	Molecular Target	Clinical Trial Phase	References
1. Tozasertib (MK-0457)	Aurora kinase	Phase I	Sun *et al*., 2007, Lin *et al*., 2008, Traynor *et al*., 2011
2. Nultin-2	MDM2	Phase I	Wang *et al*., 2012
3. Olaparib	PARP (Poly-ADP ribose)	Phase I	Fong *et al*., 2008
4. Erlonitib and gefitinib	EGFR (epidermal growth factor receptor)	Phase II (gefinitib) Phase III (erlonitib)	Schilder *et al*., 2005, Vergote *et al*., 2013
5. BMS-690514	VEGFR (vasculo-endothelial growth factor receptor)	Not yet entered clinical trials for ovarian cancer	Becker *et al*., 2013
6. Imatinib mesylate (STI57)	PDGFR (Platelet-derived growth factor receptor)	Phase II	Alberts *et al*., 2007
7. Everolimus (RAD001)	mTOR	Phase I	Mabuchi *et al*., 2007, Okamoto *et al*., 2010
8. APR-246	P53	Phase II	Von Euler *et al*., 2014
9. LBW242	XIAP	Phase I	Eschenburg *et al*., 2012; Petrucci *et al*., 2012
10.	Integrin	Not entered clinical trials for ovarian cancer	Landen *et al*., 2008
11. BMS-536924 NVP-AEW541 Ganitumab (AMG-479)	IGFR1	Not entered clinical trials for ovarian cancer Phase I	Xu *et al* 2008; Beauchamp *et al*., 2010
12. ABT-737	Bcl-2	Phase I	Jain *et al*., 2014

1.8 Paclitaxel and Camptothecin derivatives as conventional chemotherapy in ovarian cancer

1.8.1 Paclitaxel

Paclitaxel is a potent natural drug that was isolated from the bark of the western yew tree *Taxus brevifolia* and shown to possess antitumour and antileukaemic activity (Wani *et al*., 1971). Paclitaxel primarily targets microtubules, which are vital for cell division, proliferation, secretion and motility (Holmes *et al*., 1991; George *et al*., 1992). Paclitaxel stimulates the assembly of microtubules, hinders microtubule depolymerisation and alters their dynamics (Rowinsky and Donehower, 1995).A disturbance of the dynamics of microtubules by mitosis-inhibiting compounds results in an inhibition of cell cycle progression with a G2/M phase growth arrest, eventually leading to apoptosis (Jordan and Wilson, 2004). Presently, paclitaxel is used for the treatment of ovarian and breast cancer however, challenges of drug resistance and undesirable side effects still abide (Copeland *et al*., 1994; Bartel *et al*., 2008). Gene expression changes in certain apoptosis-related genes and growth arrest genes such as have been observed, albeit the mechanism of apoptosis mediated by paclitaxel is not yet known (Ofir *et al*., 2001).

Drug resistance observed in several anticancer therapeutics, including microtubule inhibitors such as paclitaxel, remains a huge challenge to cancer treatment (Copeland *et al*., 1994; Bartel *et al*., 2008). With increased understanding of the mechanisms of drug resistance in cancers, synthetic derivatives of paclitaxel are being developed to curb the problem of drug resistance.

1.8.2 Camptothecin

Camptothecin induces apoptosis by inhibiting the human DNA topoisomerase I enzyme (Topo I) which is active in the S-phase of the cycle. Topo I is involved in the cleavage and re-ligation step of DNA replication in eukaryotic organisms. Topo I relaxes the supercoiling of DNA as a result of torsional stress produced during DNA replication (Wang, 1985) An abrogation of topo I function by camptothecin binding to the topoisomeras1/DNA complex results in DNA strand breaks which leads to apoptosis. (Liu *et al*., 2000)
Mutations of DNA topo I have correlated with high resistance to camptothecin in some colon cancer cells (Arakawa *et al*., 2013).

Paclitaxel and Topotecan (a derivative of camptothecin) have passed all phases of clinical trials and are currently being used for the treatment of advanced ovarian cancers (Ofir *et al*., 2001) although resistance, recurrent disease and debilitating side effects still plague many patients**.** However, a combination of chemotherapeutics with RNA interference may improve upon patient management and clinical outcomes.

1.9 RNAi as a targeted therapeutic approach

Epigenetics is the study of heritable changes in gene expression without any changes in the corresponding coding sequence of DNA. Epigenetic modifications are effected by DNA methylation, histone modification and RNA interference (RNAi) (Egger *et al*., 2004). RNAi pathway is common in many eukaryotic organisms and plays an important role in defence against viral invading viral nucleotides and transposons, developmental changes and gene

expression (Fire, 1999). Central to RNAi are three types of small RNA molecules: micro RNA, shRNA and short interfering RNA (siRNA) (Bartel 2004; Peragine *et al*., 2004). siRNA technology has received a lot of attention in the scientific community and is currently widely applied in research (Yang *et al*., 2003; Moela et al., 2014; Ghaemimanesh *et al*., 2014)

1.10 siRNA structure and Mechanism of action

With the exception of hairpin RNAs, double stranded RNA does not normally occur in eukaryotic organisms (Goodsell, 2008).Upon entry of parasitic double stranded RNA into the cell, the RNA binding domain of dicer, an RNase III endonuclease cleaves the dsRNA into approximately 22 base pairs fragments). These fragments are called siRNA. (Typically each siRNA is a 20 to 25 bp double stranded molecule characterised by a 2 base pair nucleotide overhang and a hydroxyl group at the 3' end, and a phosphate group at the 5' end of each strand) (Goodsell, 2008) Dicer-siRNA is transported from the nucleus to the cytosol and integrated into argonaute proteins and the RNA induced silencing complex (RISC) is assembled. Arganaute then uses helicase activity to unwind the siRNA and retains one of the strands (the guide strand) while the other strand (passenger strand) is degraded. The guide strand, which is now a functional part of the RISC, recruits the RISC to the mRNA complimentary to the siRNA. siRNA recognition results in the catalytic degradation of the mRNA by the RNase domain of argonaute (Patkaniowska *et al*., 2004). As a result, translational inhibition of the target mRNA is accomplished. The ability for siRNAs to specifically knock down any aberrantly expressed gene makes them attractive molecules for targeted therapy in cancer.

This study employs siRNA technology to knock down the RBBP6 mRNA in RMG-1 ovarian carcinoma cells as a targeted therapeutic approach.

1.11 The RBBP6 gene

The RBBP6 gene is located on chromosome 16p11.2 to p12, and codes for a 250 kD protein which contains numerous repeated sequences. The protein possesses a conserved *N*-terminal RING finger domain, a highly conserved DWNN, an Rb protein binding domain and a p53-binding domain (Sakai *et al*., 1995; Simmons *et al*., 1997). RBBP6 is a 250kD ubiquitin ligase protein than is known to interact with p53 and Rb proteins in humans and mice, both in vivo and in vitro (Sakai *et al*., 1995; Simons *et al*., 1997; Witte and Scott, 1997) and possibly regulating their levels. It is involved in mRNA processing and ubiquitin-like modification of proteins (Pugh *et al*., 2006). It enhances MDM2-mediated ubiquitination of p53 (Li *et al*., 1997) and also plays a role in cell cycle regulation and cell differentiation (Scott *et al*., 2003). It is upregulated in numerous cancers such as lung cancer (Motadi *et al*., 2011), oesophageal cancer (Yoshitake *et al*., 2004), colorectal cancer (Chen *et al*., 2013), and cervical cancer (Mbita *et al*., 2012). RBBP6 suppresses p53 binding to DNA (Simmons *et al*., 1997), thereby inhibiting p53-dependent gene transcription.

Figure 1.3: Structural domains of RBBP6. DWNN: Domain With No Name, ZINC, RING, NLS: Nuclear localisation signal, Rb BD: Rb binding domain, P53 BD: p53 binding domain. Adapted from Pugh *et al*., 2006.

1.12 Rationale of study

RBBP6, which has now been shown to be expressed in a handful of cancers, binds to the tumour suppressors p53 and Rb; possibly sequestering them from the cell and hence promoting cell proliferation. It has been shown that endogenous RBBP6 interacts with MDM2 and enhances MDM2-mediated ubiquitination and degradation of p53 as a result of increased p53-MDM2 affinity (Li *et al*., 2007).

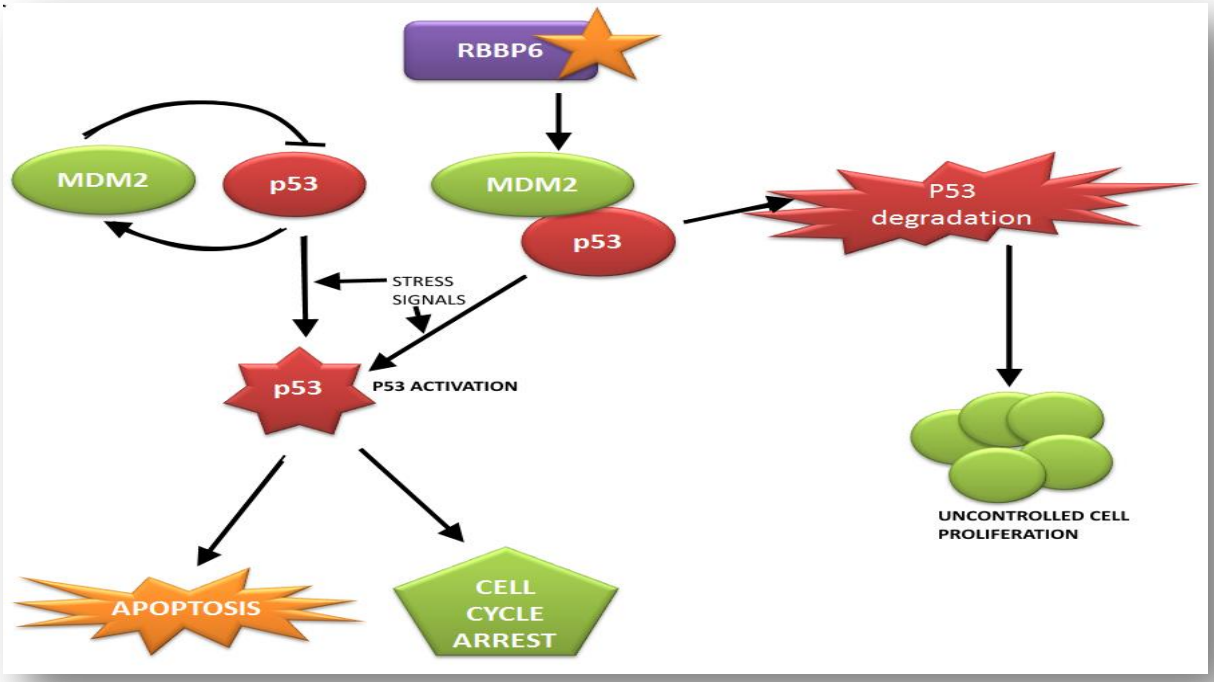


Figure 1.4 shows a proposed mechanism by which RBBP6 interacts with MDM2 to mediate cell proliferation.

Under normal physiological conditions, p53 is kept under tight regulation mainly by MDM2. Following stress an exposure of the cell to stress signals, such as DNA damage or oncogenic activation, p53 becomes activated by phosphorylation at specific residues. This

phosphorylation enables it to evade MDM2 mediated ubiquitination. Activated p53, according to the cellular context either leads to cell cycle arrest or apoptosis. However, upregulated RBBP6 may increase the MDM2-p53 affinity, thereby mediating enhanced ubiquitination and degradation of p53 by MDM2, which may lead to abnormal cell proliferation (Li *et al*., 2007).

However, the mechanism by which RBBP6 promotes cell proliferation is unclear. Knocking down RBBP6 may indirectly improve upon the availability of the tumour suppressor, p53 and hence induce apoptosis. Paclitaxel and Camptothecin derivatives are currently used for treatment of ovarian cancers, albeit problems of resistance and relapse are frequently encountered (Niimi et al., 1992; Swisher *et al*., 1997). We wish to investigate the potential role of RBBP6 knockdown in apoptosis or cell proliferation and also evaluate the chemotherapeutic efficacy of Paclitaxel and Camptothecin by knocking down RBBP6.

1.13 *Aim and objectives*

Aim

The aim of this study was to evaluate the anti-tumour activity of RBBP6 knockdown only, and in combination with paclitaxel and camptothecin-treated ovarian cancer cells

Objectives

The following are potentially achievable objectives of the research, which will enable us to achieve our primary aim. We intended to:

1. determine whether silencing RBBP6 in ovarian cancer cells causes them to induce apoptosis in ovarian cancer cells.

2. evaluate the percentages of cell death by apoptosis, necrosis, and of viable cells when RBBP6 silenced in ovarian cancer cells.

3. determine and compare gene expression levels of MDM2, p53, Bax, Bcl-2 and p21 following RBBP6 knockdown

4. evaluate cell cycle changes following siRBBP6 transfection

5. compare gene expression and cell death levels in siRBBP6 transfected cells with cells co-treated with siRBBP6 and paclitaxel or camptothecin.

In order to achieve our objectives the following experiments or methodologies will be used:

CHAPTER TWO - MATERIALS AND METHODS

2.1 Introduction

This chapter elaborates on the techniques used to acquire results in this study. As much as possible, the basic principles of each technique, the procedures and protocols followed, and the reasons thereof are outlined herein. The following techniques were used:

i) Cell culture; including siRNA transfection, paclitaxel and camptothecin treatment

ii) qPCR

iii) Western blotting

iv) *xCELLigence*® real time cell analysis

v) Flow cytometry (Apoptosis)

vi) Flow cytometry (Cell cycle analysis)

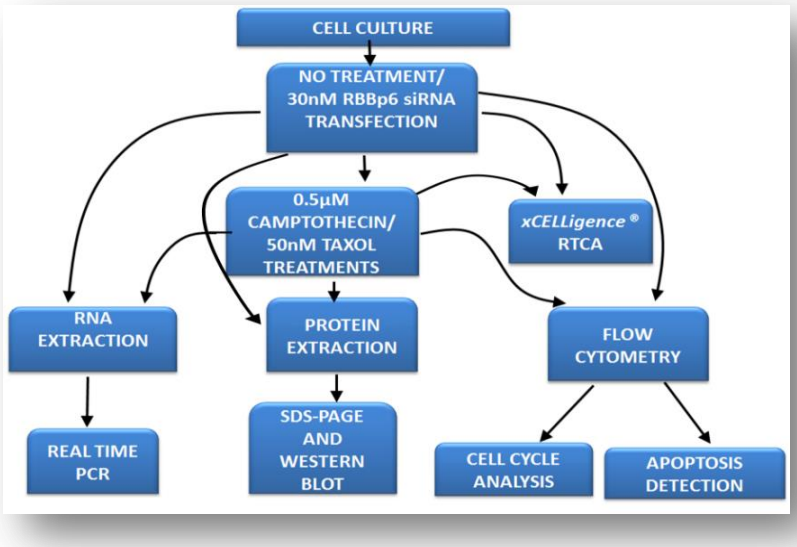


Figure 2.1 is a flow diagram illustrating the methods used to obtain results in this study.

2.2 Cell culture

The cell line used in this study were RMG-1 cells, a clear-cell adenocarcinoma of the ovary were purchased from ATCC (American Type Culture Collection), Virginia, USA.

2.2.1 Thawing and plating

The frozen cells were thawed in a 37ºC water bath for one minute. The vials were then cleaned with 70% ethanol and transferred to a 15 ml centrifuge tube, and centrifuged at low speed for 5 min to remove the cryo-protectant, DMSO. The supernatant was discarded and the cell pellet resuspended in 5 ml of pre-warmed DMEM (Hyclone, USA) supplemented with 10% Foetal bovine serum (Highveld Biological, RSA) and 1% antibiotics (Penstrep) (Sigma Aldrich, USA). Cells were cultured to 80% confluence at 37ºC in a humidified incubator maintained at 5% CO_2 concentration.

2.2.2 Sub-culturing

The spent growth medium was aspirated and cells were washed twice with 1×PBS, pH 7.5. They were then trypsinised (Trypsin-EDTA, Biowest, France) for 2 min by gently adding 1 ml of trypsin –EDTA. Resuspension of the cells was done by adding 3 ml of pre-warmed medium to stop protein degradation by trypsin. The cells were gently pipetted up and down to enable even distribution and 10 µl were pipetted out and the cells counted using a

haemocytometer. They were then transferred to other culture flasks containing 7 ml of pre-warmed culture media at their respective optimal seeding densities.

2.2.3 Freezing

Freezing medium was prepared, composed of 20% FBS, 10% DMSO and 70% DMEM. The cell freezing procedure was as follows: Growth medium was aspirated; the cells were then washed twice with PBS and trypsinized. Trypsin was neutralised by resuspension of cells in normal growth medium. The cells were gently pipetted and 1ml of cell suspension was aspirated for cell counts and viability. The cells were centrifuged as earlier described and resuspended in appropriate amounts of cold cryopreservation medium based on cell counts and viability. 1 ml of cell suspension was transferred into sterile appropriately labelled cryovials which were then stored at -70ºC overnight. The following morning, the cells were transferred to liquid nitrogen for long-term storage.

2.2.3 siRNA Transfection

siRNAs were designed to target three regions of the RBBP6 mRNA (Dr. L. R Motadi) for effective knock down of the gene. They were synthesized by Ambion Inc., Huntingdon, UK. Proper controls for siRNA were used to ensure effective gene knockdown and control for off-target effects, if any. A random double stranded oligonucleotide was used as a negative control. The transfection protocol was followed according to the manufacturers' recommendations and optimized modifications.

A 5000 cells/ml cell suspension was prepared per sample, using culture techniques described above. The 30µl of transfection reagent (siPORT, Ambion) was diluted in 600 µl of OPTIMEM in an eppendorf tube and allowed to incubate at room temperature for 10 min. To make final concentration of 30 nm of RBBP6 siRNA per well (after addition of cell suspension), 22.5 µl of 20 µM of RBBP6 siRNA was diluted in 600 µl OPTIMEM. The siRNA was then mixed with the diluted transfection reagent and pipetted up-and-down 5 times. The mixture was then allowed to incubate for 10 min at room temperature to allow for the formation of siRNA/transfection complexes. 200 µl of the siRNA/transfection complex was then dispensed per well in a 6-well plate. Finally, 2300 µl of the cell suspension was added into each of the wells. The plate was rocked back and forth a few times to enable even distribution of cells and the siRNA/transfection complex. The cells were incubated at 37ºC for 6 hours (in order for the siRNA to be transfected or delivered into the cells). 2 ml of DMEM containing 10% FBS was added per well to prevent any potential cytotoxicity, while enriching the growth medium for cell growth. Two negative controls were used in the assay: A negative control using 30nm of scrambled siRNA, and another negative control using the transfection reagent only (without siRNA) were done. This was to control for false positives, false negatives or any non-specific effects. The negative controls showed no difference in cell proliferation when compared to untransfected cells. These controls confirmed that any growth changes experienced were due to the knockdown of the RBBP6 mRNA. The cells were incubated for 24 h prior to 24 h treatments for RNA extractions, and 48 h for protein extractions and flow cytometry.

2.2.4 Camptothecin and Paclitaxel Treatments

The cells were then treated with 50 nM paclitaxel (Sigma Aldrich, USA) in DMEM and 0.5µM camptothecin (Merck, USA) in DMEM for 24 hours.

2.3 Real time PCR

2.3.1 Introduction

Numerous decisions concerning cell survival, proliferation, differentiation or death are as a result of changes in the patterns of gene expression (Zamorano *et al*., 1996). These changes in gene expression can be measured by using the polymerase chain reaction. The polymerase chain reaction is a highly sensitive molecular biology technique that is widely utilised in a vast array of applications to amplify specific template DNA sequences. mRNA levels can therefore be evaluated, which indicate important changes in gene expression following induced cellular stimuli such as drug treatments or transfections. The entire procedure involved RNA extraction, RNA purity and integrity evaluations, cDNA synthesis (reverse transcription), primer design and optimization and qPCR.

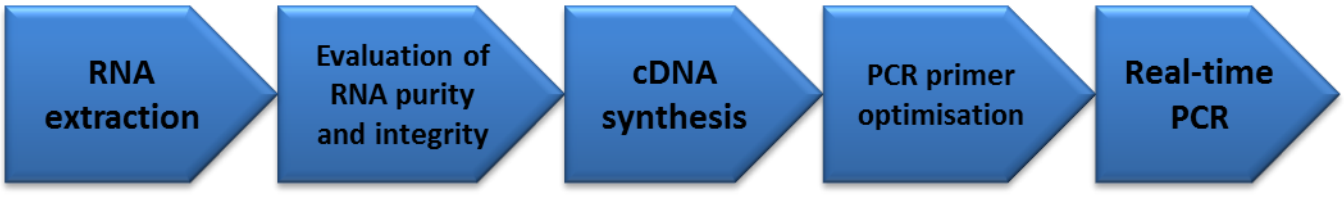


Figure 2.2: Real-time PCR workflow

2.3.2 RNA extraction

Total RNA was isolated from RMG-1 and MRC-5 using the Nucleospin RNA 2 (Promega, Wisconsin, USA) following manufacturers' instructions. Recombinant DNAse was used in the procedure in order to degrade contaminating DNA in the RNA sample before elution of the RNA in nuclease-free water.

RNA quantity, purity and integrity are very important parameters in PCR for reliable results. The RNA was quantified using the Nanodrop spectrophotometer (Thermofischer Scientific, California, USA) which also checks on RNA purity.

Concentrations of RNA from the various samples were determined using a Nanodrop spectrophotometer to measure optical densities at a wavelength of 260 nm. RNA purity assessments were established by $A_{260/280}$ of ~2.0 and $A_{260/230}$ between 1.9 and 2.2.

2.3.2.1 Denaturing agarose gel electrophoresis

To check for RNA integrity, the RNA was run on a 1% formaldehyde denaturing agarose gel stained with ethidium bromide. This was to make sure the RNA used was not degraded. In this procedure, 1 g of agarose was weighed and dissolved in 72 ml of DEPC-treated water. The agarose was melted by microwaving at high power for 1 min. 10 ml of 10X MOPS buffer was added and the mixture allowed cooling to about 60 degrees. 18ml of 37% formaldehyde was added to the mixture and mixed thoroughly in a fume hood. The gel was then poured and allowed to set.

The RNA sample was prepared by adding 5µl of DEPC-treated water and 15 µl of RNA sample buffer to 5 µl of RNA sample. The sample was heated to 65 degrees for 10 min and allowed to cool on ice for 3 min. The samples were loaded in the gel immersed in 1X MOPS buffer and run at 100V for 1.5 hours. The gel was viewed in a Gel Documentation (Biorad, CA, USA), showing the 18S and 28S, 18S and 5S rRNA subunits; therefore attesting to a satisfactory level of RNA integrity.

2.3.3 Reverse transcription (cDNA synthesis)

Reverse transcription was done using the Improm II reverse transcription kit from Promega (Promega, Wisconsin, USA) to synthesize cDNA from total RNA to be used for PCR. The reaction was carried out in a 20 µL reaction mixture, made up of a cocktail of the following: 1.4 µl (1µg) of total RNA, 1 µl of reverse transcriptase, 1 µl RNase inhibitor 2.5 µl of oligo dt primers, 2 µl of DNTPs, 3 µl of $MgCl_2$ and 7.5 µl of nuclease-free water.

Table 2.1

cDNA synthesis reaction components

Reagent	**Volume**	**Final Concentration/mass**
RNA	1.4µl	1 µg
Reverse transcriptase	1 µl	_
DNTPs	2 µl	1Mm
Oligodt primers	3 µl	75µg/ml
$MgCl_2$	3 µl	3.75mM
RNAse inhibitor	1 µl	_
Nuclease-free water	7.1µl	_
Final volume	20 µl	_

The samples were then placed in the Gene Amp thermal cycler 2700 (Applied Biosystems, USA) and set sequentially to the following thermal conditions: sample denaturation at 95ºC for 5 min, annealing of primers to mRNA at 25ºC for 10 min, cDNA synthesis at 42ºC for 60 min, enzyme (reverse transcriptase) inactivation at 95ºC for 10 min and sample cooling at 4ºC.

Table 2.2

Reverse transcription thermal cycling conditions

Step	**Temperature/ºC**	**Time**
Denaturation	95 **ºC**	5 min
Annealing	25 **ºC**	10 min
Extension/synthesis	42 **ºC**	60 min
Enzyme inactivation	95 **ºC**	10 min
Cooling	4 **ºC**	∞

2.3.4 Primer design

Specific primers for the amplification of the mRNA for GAPDH, RBBP6, MDM2, p53, Bcl2, and Bax were designed using the NCBI website. Important parameters such as melting temperature, GC content, primer length and 3'complimentarity were taken into consideration for optimal results. The sequences were then sent to Whitehead, SA for oligonucleotide synthesis.

Primers

Table 2.3

Gene	Forward primer	Reverse primer	Amplicon sizes/kbp
GAPDH	5'-TGTAGTTGAGGTCAATGAAGGG-3'	5'-ACATCGCTCAGACACCATG-3'	143
Bax	5'-AAAGATGGTCACGGTCCAAC-3'	5'-CAAACTTGGTGCTCAAGGC-3'	36
Bcl-2	5'AGCCAGGAGAAATCAAACAGAC-3'	5'-GATGACTGAGTACCTGAACCG-3'	117
MDM-2	5'-GTGCATTTCCAATAGTCAGCTAA-3'	5'-AGAAGGACAAGAACTCTCAGATG-3'	129
TP53	5'-GACGCTAGGATCTGACTGC-3'	5'-GACACGCTTCCCTGGATTG-3'	155
RBBP6	5'-ACGAAGAACCACTATAGGGAGA-3'	5'- GCAGACAGCTCATTCAAATACC -3'	122
P21	5'-AAGACCATGTGGACCTGT-3'	5'-GGTAGAAATCTGTCATGCTG-3'	133

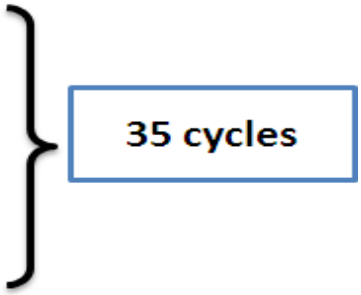
2.3.4.1 Primer optimisation

PCR was used to ascertain that our designed primers could effectively amplify our target gene products without the formation of primer dimers. Our primers were optimised by testing a range of annealing temperatures with various magnesium ion concentrations. The reaction was carried out in a 20µl mixture containing the following: 10µl Master Mix (Promega, Wisconsin, USA), 1µl forward primer, 1µl reverse primer, 1µl cDNA, 1.5µl (1.5µM) MgCl2 and 5.5µl of nuclease-free water. The samples were then run in a Gene Amp thermal cycler (Applied Biosystem , USA), with the following thermal cycling parameters:

Table 2.4

Thermal cycling parametres for PCR

Step	Temperature/°C	Time
Initial Denaturation	95 °C	5mins
Denaturation	95 °C	30 seconds
Annealing	X°C	30 seconds
Extension/synthesis	72 °C	30 seconds
Final extension	72 °C	10mins
Cooling	4 °C	∞



The PCR products were run on a 1% agarose gel to confirm the product sizes and ascertain that no primer dimmers were formed. All primers worked at 60ºC.

2.3.5 qPCR

Following optimization of primers, qPCR was run in a 96 well non-skirted PCR plate (BIOplastics BV, Netherlands). In order to monitor and quantify the amplification of cDNA from the various samples in real time, Maxima SYBR® green/ROX qPCR master mix (Thermoscientific, USA) was used. The master mix includes Maxima® Hot Start Taq DNA polymerase, dNTPs and an optimised buffer. It also contains SYBR green I dye and a ROX passive reference dye. Primers, cDNA and nuclease-free water were then added to the master mix in the right proportions per sample. To minimise variability, cocktails were prepared containing SYBR green, primers and water. 1µl of cDNA was then finally added per well and

1 µl of nuclease-free water per NTC wells. A NTC was made for each primer set, containing all reagents except cDNA. 1 µl nuclease-free water was added in the place of cDNA for all 'no template' controls.

Table 2.5

qPCR reaction reagents

Reagent	**Cocktail**	**Volume/µl per tube**	**Final Concentration**
SYBR Green	75	5	_
Forward primer	7.5	0.5	50 nM
Reverse primer	7.5	0.5	50 nM
Nuclease-free water	45	3	_
Cdna	_	1	100 ng/µl
Total volume	145	10	_

The plate was loaded into a Lightcycler qPCR machine, LC 480 (Roche, USA). Thermal cycling conditions were entered following recommendations from the SYBR green 1 protocol

Table 2.6: qPCR Thermal cycling parametres

Programme name	Temperature	Time	Cycles
Pre-incubation	95ºC	10 minutes	1
Denaturation	95ºC	15seconds	40
Annealing	60ºC	30seconds	
Extension	72ºC	30 seconds	

2.4. SDS PAGE and Western Blot

2.4.1 Introduction

Western blotting (immunoblotting) is a sensitive biochemical method that makes use of specific antibodies to identify specific proteins from a mixture of proteins that have been separated by SDS-PAGE. It makes use of three aspects: i) Separation of proteins by size, ii) transfer of proteins to a membrane and iii) using proper primary and secondary antibodies to tag the target protein and iv) using immunochemical substrates to visualise the proteins (Mahmood and Yang, 2012).

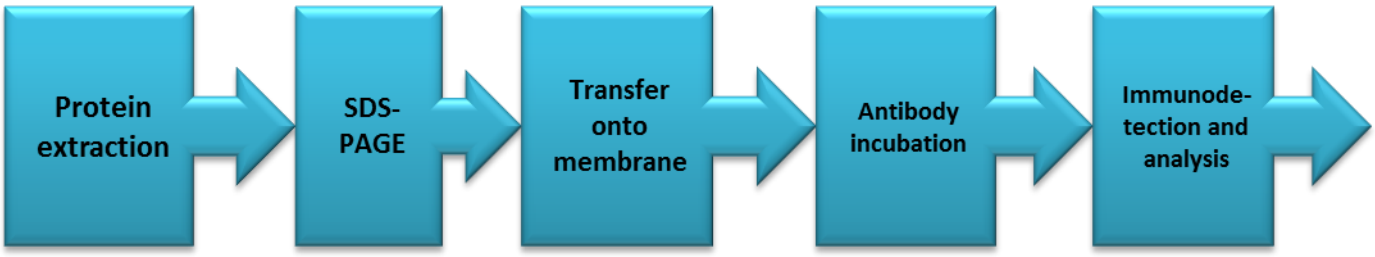


Figure 2.3: Western blot workflow

2.4.2 Protein extraction

The cells were cultured in 6-well plates, siRNA-transfected and treated with 50nm paclitaxel and 0.5uM camptothecin as previously described. Spent growth medium was aspirated and the cells were washed twice with cold 1X PBS. 0.5ml of RIPA buffer (Thermofischer Scientific, Massachusetts, USA) was added per well for cell lysis. The plate was then put on ice and gently agitated periodically to ensure even distribution. After 5 min, the wells were rapidly scraped to lyse and remove any residual cells. The cell lysates were transferred to 1.5ml eppendorf tubes. The cell lysates were clarified by centrifugation at 14000g for 15 min at 4ºC. The supernatant, containing proteins were carefully transferred to 1.5ml *eppendorf* tubes on ice. The proteins were then stored at -80ºC.

2.4.3 Coomassie blue staining and destaining

In order to confirm a successful extraction of proteins, the proteins were run on a 12% SDS-PAGE gels. Protein samples were prepared by adding 10µL of protein suspension to 10 µL of 2X Laemmli sample buffer (Biorad, California, USA). The sample was heated on a heating block at 95°C for 7 min in order to denature the proteins. The samples were loaded into the SDS PAGE gel wells and run using a 1X Tris/glycine/SDS running buffer. Power parameters were set at 100V, constant voltage and 300mA of current for 2 hours. The gel was then stained with coomassie solution overnight. The following morning, it was destained using destain solution for one hour. Protein bands were visualised, confirming a successful isolation of proteins.

2.4.4 BCA assay

In order to load known concentrations into the SDS-PAGE gels, protein quantification was done using the Bichiconinic assay (BCA) protein kit (Thermoscientific, Massachusetts, USA). The Bichiconinic assay BCA protein assay colorimetrically detects and measures total protein. It relies on the reduction of Cu^{2+} to Cu^{+} ions in alkaline conditions by protein molecules, resulting in a pale blue colour. The amount of Cu^{2+} reduction is proportional to the amount of protein present. The Cu+ ions then form complexes with BCA molecules, producing a purple product which can be quantified spectrophotometrically at 562nm. It is more sensitive and less time-consuming, compared to other protein quantification assays.

Eight BSA protein standards (25, 125, 250, 500, 750, 1000, 1500, and 2000 µg/ml) were prepared. Distilled water was used as the blank. 10ml of working reagent was prepared by adding reagent A to reagent B in a 50:1 ratio. 25µl of each of the BSA standards and unknown protein samples were pipette into different wells of a 96-well plate. 200µl of the working reagent were then added to each of the wells. The plate was then mixed thoroughly on a shaker for 1minute. It was then incubated at 37ºC for 30 min, after which the plate was cooled and the absorbance was read at 562 nm. The corrected absorbance values were gotten by subtracting the absorbance of standards and unknown samples from that of the blank. A standard curve of absorbance versus concentration was then plotted and the values of unknown samples were gotten by manual extrapolation.

2.4.5 SDS-PAGE

An 8% SDS-PAGE gel was prepared for RBBP6 (because of its large size of 250kD) and a12% gel was prepared for b actin, mdm2, Bcl-2, Bax, and p53. 30µg of total protein was

added to 10µl of 2X Laemli sample buffer (Biorad, USA). The sample was heated at 95ºC for 7 min in order to denature the proteins. The samples were loaded per well for every sample. The gels were run in 1X running buffer at 100V, constant voltage for about 2hours. Protein bands were confirmed by coomassie staining.

2.4.6 Transfer

Following SDS-PAGE, the gels were equilibrated in transfer buffer (containing Tris, glycine and 10% methanol) for 15mins on a rocking platform. Six filter papers and a 0.2µm pore nitrocellulose membrane (Biorad, California, USA) were cut, having approximately the same dimensions of the gel. The filter papers and membrane were briefly soaked in transfer buffer. The sandwich was then prepared in the following manner:

A fibre pad soaked in transfer buffer was laid on the negative side of the cassette; three filter papers were then placed on the fibre pad. The gel was carefully placed on the filter paper after which the membrane was placed on the gel. Finally, 3 filter papers were then carefully placed on the membrane. Air bubbled were avoided by rolling a 15ml tube gently on the sandwich. The cassette was then clipped tightly and fitted into the transfer tank. Transfer buffer was poured into the tank up to the blotting mark. The transfer was carried out at 25 volts at 4ºC overnight. Effective transfer was confirmed by washing the membrane in distilled water, and staining with Ponceau stain for 30mins. The membrane was then destained and blocked with 5% non-fat milk in TBS containing 1% tween (TBST) for 1hour at room temperature. The membrane was washed 5 times (5 min per wash) with 5ml TBST.

2.4.7 Antibody incubation: Antibodies, dilutions and preparations

Monoclonal antibodies for Bax, Bcl-2, p53, RBBP6, MDM2 and β-actin were purchased from Santa Cruz Biotechnology, USA. Rabbit anti-mouse Horse radish peroxidase-linked (HRP-linked) secondary antibody was purchased from Cell Signalling technology, USA. The membranes were incubated with 10ml of 1µg/ml (diluted in TBST) of primary antibody overnight at 4ºC. The following morning, the membrane was washed 5 times (5 min per wash) with 5ml TBST. 10ml of 1µg/ml of Secondary antibody diluted in TBST containing 5% non-fat milk was then added onto the membrane and incubation at room temperature was done for one hour. Final washes were then performed as above, with the membrane left in the final wash solution.

2.4.8 Immunodetection

The final wash solution was poured off the membrane and 1 ml each of chemiluminescent solutions A and B (Santa Cruz, USA) were mixed in a 15ml tube and dispensed to evenly cover the surface of the membrane. The membrane was incubated in the dark for 5min at room temperature. HRP linked to the secondary antibody catalyses the oxidation of luminol substrate present in solutions A and B, using hydrogen peroxide as an oxidizing agent, accompanied by an emission of low intensity light at 428nm (Veitch, 2004). The emission of light was visualised on the Chemidoc imaging system. (Biorad, California, USA).

2.5 *xCELLigence*® Real Time Cell Analysis

The *xCELLigence* Real Time Cell Analyser (RTCA) (ACEA Biosciences, USA) system is an automated, electrical impedance-based, label-free electronic technology that measures cellular adhesion, invasion and proliferation in real-time. In the *xCELLigence* system, adherent cells generate electrical impedance whose measurements are converted to cell indices by the RTCA software. The cell indices are a measure of the cell numbers, viability, adhesion and morphology. This novel system is currently being explored in a wide range of biomedical applications to obtain and assess distinct cytological profiles.

Cells were trypsinized and resuspended in Opti-MEM (Gibco, USA) at 5,000 cells per well. Opti-MMEM is a reduced serum medium that shows better transfection efficiency than serum-rich culture media (Young *et al*., 2004). 100 µl of cells, to be transfected were dispensed into 6 wells of an E-plate 16, containing prepared siRNA/transfection complex.

The layout page on the software was completed; indicating cell type (RMG-1), cell number per well (5000), and concentration of treatments (paclitaxel: 50nM and Camptothecin: 0.5µM).

The experiment schedule page was then completed. The experiment was scheduled to run in steps; with manual intervention involving unclamping the E-plate 16 from the RTCA instrument, performing cell culture tasks, re-clamping the plate on the RTCA instrument and clicking the "run" icon on the software were necessary to proceed to the next step. The first step measured background measurement of culture media only in each of the 16 wells of the E-plate. The second step was scheduled to run for 8 hours, to allow for siRNA transfection into the cells. 100µL of DMEM was then added to all the wells in order to inhibit any potential cytotoxic effects of the siRNA/transfection reagent complexes, and also to enrich the growth media for optimal cell growth. The third step was scheduled to run for 48hours,

monitoring post-transfection changes in cell proliferation. The growth media was then aspirated, the cells were washed and 50nM paclitaxel and 0.5µM camptothecin were added in designated wells. The fourth step was set to run for 24hours, evaluating treatment and transfection effects cell proliferation in real time.

Table 2.7

xCELLigence® RTCA experiment schedule

Step	Sweeps	Interval in min	Approximate Duration per step	Total Time
1. Background measurement	1	1	1 minute	0:01:00
2. siRNA transfection	33	15	8 hours	8:23:54
3. Adding DMEM	193	15	48 hours	55:08:36
4. Treating with 0.5µM Camptothecin and 50nM paclitaxel	100	15	24 hours	80:09:47

2.6 Flow Cytometry

2.6.1 Introduction

Flow cytometry in cell biology is a biophysical technique that measures and analyses physical properties of cells as they flow in a fluid medium bombarded by a laser beam. The

cellular characteristics measured and analysed include size, granularity, and fluorescence (Brown and Wittwer, 2000). In this study, we used flow cytometry to assess apoptosis, necrosis and cell cycle analyses. The cells are fluorescently labelled, excited using lasers such that they emit varying wavelengths of light.

Phosphatidyl serine (PS) is a cell membrane phospholipid that plays an important role in cell cycle signalling, especially in response to apoptosis. During the early phases of apoptosis, PS which is embedded within the plasma membrane of live cells becomes translocated to the external surface of the cell membrane. The externalisation of PS is used as a marker of early apoptosis. Annexin V is a calcium ion-dependent protein that has a high binding affinity for PS (Andree *et al.*, 1990). Annexin V coupled to the green fluorescent dye, FITC can be used to assess the level of apoptosis using a flow cytometer (Koopman *et al.*, 1994). Plasma membrane integrity becomes compromised during necrotic cell death. This exposes DNA. The exposure of DNA is taken advantage of by the DNA-binding dye, propidium iodide, as a marker of necrosis. Proper gating procedures to count cell populations and not debris, and fluorescence compensations were done because of similar emission profiles of both FITC and PI dyes. This was done by having some cells which were unstained, Annexin V-FITC only-stained, PI only- stained, and Annexin-V and PI-stained.

2.6.2 Method

2.6.2.1 Cell cycle analysis

Cell cycle analysis using DNA-binding dyes such as Propidium iodide (PI) make use of the fact that DNA contents change as the cell progresses from the G1, S and G2 phases of the cell cycle. Cells that are in the S-phase of the cell cycle have more DNA than those in the G1.

While G2 cells have approximately doubled the amount of DNA found in G1 cells. For cell cycle analysis, cellular DNA content is measured using the DNA-binding dye, PI. PI intercalates into the helical structure of DNA. The amount of PI binding to DNA, hence the amount of fluorescence yield is directly proportional to the amount of DNA present within the cell (Brown and Wittwer, 2000). This makes it easy to detect cells that have undergone growth arrest at any phase of the cell cycle.

2.6.3.1 Fixation of cells for cell cycle analysis

Fixation of the cells was done using 70% (v/v) ethanol. Ethanol is a dehydrating fixative agent which also permeabilizes the cells to allow for the binding of PI to DNA. The cells were transfected and treated and harvested with trypsin following the routine procedures into a 15ml tube. The cells were pelleted by centrifugation at 1500 rpm for 10min. After removing the supernatant, the cell pellet was carefully resuspended in 1ml of PBS and transferred to a 1.5ml eppendorf tube and centrifuged for 5 mins at 5000rpm. The PBS supernatant was gently removed and the pellet was carefully resuspended in 300µl of PBS. 700 µl of 100% ethanol, pre-chilled at -20ºC was added per sample and mixed by inverting the tube 5 times to ensure proper mixing and fixing. The cells were stored at -20ºC until required.

2.6.3.2 PI Staining.

The cell suspension was centrifuged at 5000g for 5 min, after which the supernatant was removed. The pellet was then washed twice with 1 ml of PBS by centrifuging at 1500rpm for

7 min per wash. The PBS supernatant was discarded and the cell pellet resuspended in 300µl of PI containing RNase and vortexed for 30seconds. The cell suspension was then incubated in the dark for 30 min, after which analysis was done at 488nm using a BD Acuri C6 flow cytometer (BD Biosciences, USA).

CHAPTER THREE-RESULTS

3.1 Introduction

In this study, our aim was to investigate the effects of silenced RBBP6 on ovarian carcinoma cells combined with standard chemotherapeutic regiments. Interesting results were observed through the use of qPCR, *xCELLigence* RTCA, Western Blotting and Flow Cytometric analysis. Both apoptosis and cell cycle progression investigations were carried out using a Flow cytometer. All siRBBP6 transfections were carried out using 30nm of RBBP6 siRNA.

3.2 The effects of RBBP6 knockdown, camptothecin and paclitaxel treatments on Bcl-2, Bax, MDM2 and p53 mRNA gene expression.

To evaluate the role played by RBBP6 knockdown on the expression of certain key proliferative and apoptotic genes, qPCR was carried out. We evaluated the afore-mentioned genes because of the role they play in cell death or proliferation

GAPDH gene was used as a reference gene to normalise gene expressions. Gene expressions were normalised to one; meaning expression levels above one showed that the gene in question was upregulated while expressions below one showed that the gene was downregulated. Bax mRNA expression was very low in this study; at protein level, Bax was not expressed. Bax expression did not change following RBBP6 knockdown. Melting curves were generated to confirm the melting points of the expected qPCR products. No template

controls were used as negative controls to ascertain that the products were not primer dimers but expected amplification of mRNA of interest. Samples were all run in duplicates in order to obtain significant statistical data. The REST (Relative Expression Software Tool) from Qiagen, Germany, was used to analyse the results at 95% confidence interval ($P<0.05$) using a two-tailed t-test.

3.2.1 mRNA expression analyses following RBBP6 siRNA transfection

We first had to ascertain the effective knockdown of the RBBP6 gene. As seen in Figure 3.1, RBBP6 was downregulated by a mean factor of 0.446 (or a 55% downregulation). Our knockdown was therefore partly confirmed by reduced RBBP6 expression following RBBP6 siRNA transfection. A down regulation of RBBP6 gene expression was expected because the gene was knocked down. Since not all mRNA changes can be translated into functional protein products, we had to further ascertain the effective knockdown of RBBP6 at protein level, using a western blot.

The following gene expression changes were observed after RBBP6 knockdown:

Bax, MDM-2 and p21 did not show any significant change in mRNA expressions. P53 showed an upregulation by a mean factor of 8.785 while Bcl-2 was downregulated by a mean factor of 0.088.

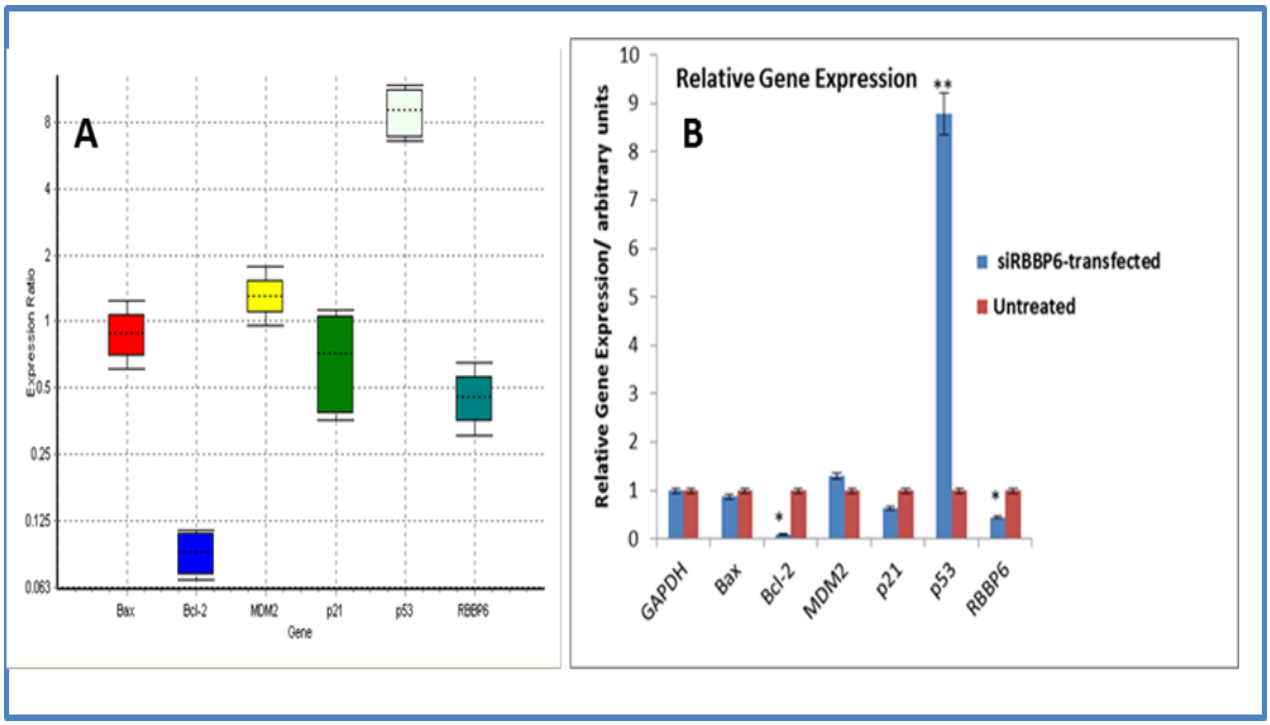


Figure 3.1: A is a box plot showing changes in gene expressions following RBBP6 siRNA transfection. The boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression while the whiskers represent the minimum and maximum observations. B is a histogram depicting a comparison between untreated and siRBBP6-transfected cells. The blue and the red bars represent relative gene expression ofsiRBBP6-transfected cells and untreated cells respectively.

3.2.2 mRNA expressions following treatment with Camptothecin and co-treatment with siRBBP6

Following treatment with camptothecin only, the following gene expression changes were observed: Bax, p21 and p53 all showed an upregulation by a mean factor of 2.173, 3.422 and 19.16 respectively. MDM2 also showed an upregulation by a mean factor of 6.797. Bcl-2,

however, showed a downregulation by a mean factor of 0.06. RBBP6 gene expression did not show any significant change following camptothecin treatment. After RBBP6 siRNA transfections followed by Camptothecin treatments, the following gene expression changes were observed: Bax and P53 gene expressions were upregulated by mean factors of 1.149 and 12.295 respectively. RBBP6 and Bcl-2 were downregulated by mean factors of 0.566 and 0.022 respectively. However, p21 and MDM2 expressions did not show any significant change but it showed that p21 was up to a mean factor of 7.1 from a mean factor of 3.8 in camptothecin treated only as compared to in co-treatment.

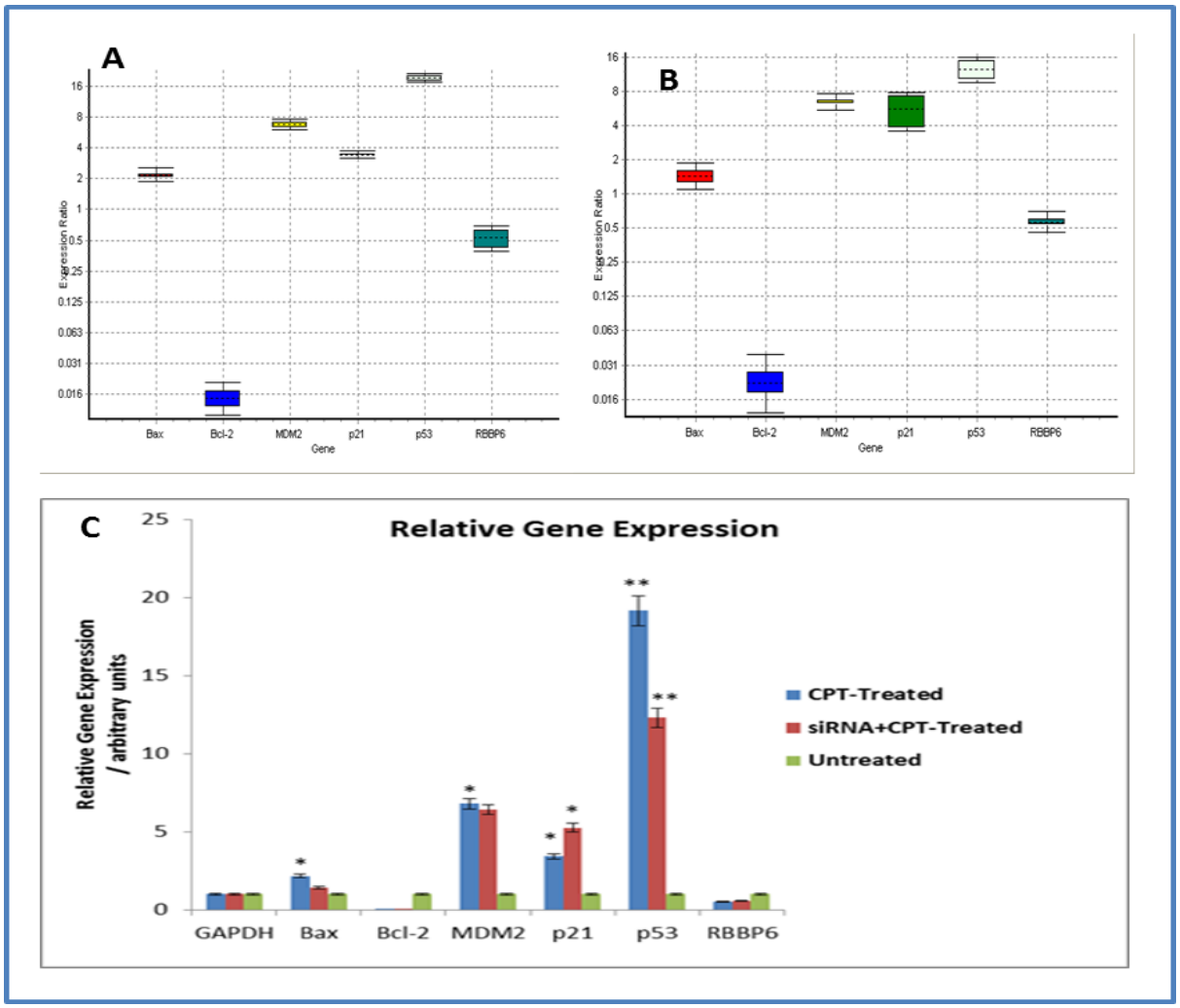


Figure 3.2: Gene expression changes following Camptothecin compared to co-treatment treatment with siRBBP6 and camptothecin. A is a Box plot analysis showing gene expressions following 0.5µM camptothecin treatment for 24 hours while B is Box plot analysis showing gene expression changes following 30nM RBBP6 siRNA transfection for 24 hours and 0.5µM Camptothecin treatment for 24 hours. C is a histogram depicting a comparison between untreated, CPT-treated and co-treatment with siRBBP6 and CPT treatment.

Bcl-2 was further downregulated in co-treated cells (0.022) compared to cells treated with CPT only (0.06).

Bcl-2 was further downregulated in co-treated cells compared to CPT-only treated cells. However, p21, p53, Bax, and MDM2 still showed a higher expression level in CPT-treated cells only as compared with co-treatments using CPT and siRBBP6.

3.2.3 mRNA expressions following Paclitaxel treatment and co-treatment with siRBBP6.

The following gene expression changes were observed following paclitaxel treatment:

Bax expression showed no significant change in gene expression. Bcl-2 and RBBP6 both showed a downregulation by mean factors of 0.053 and 0.418 respectively. MDM2, p21 and p53 genes were all upregulated by mean factors of 3.986, 2.321 and 9.126 respectively. Transfection with RBBP6 siRNA followed by Paclitaxel treatments showed the following differences in gene expression: Bax, Bcl-2 and RBBP6 genes were downregulated by mean factors of 0.459, 0.048 and 0.595 respectively. MDM-2 levels did not change significantly, while p21 and p53 were upregulated by mean factors of 3.568 and 8.028, respectively.

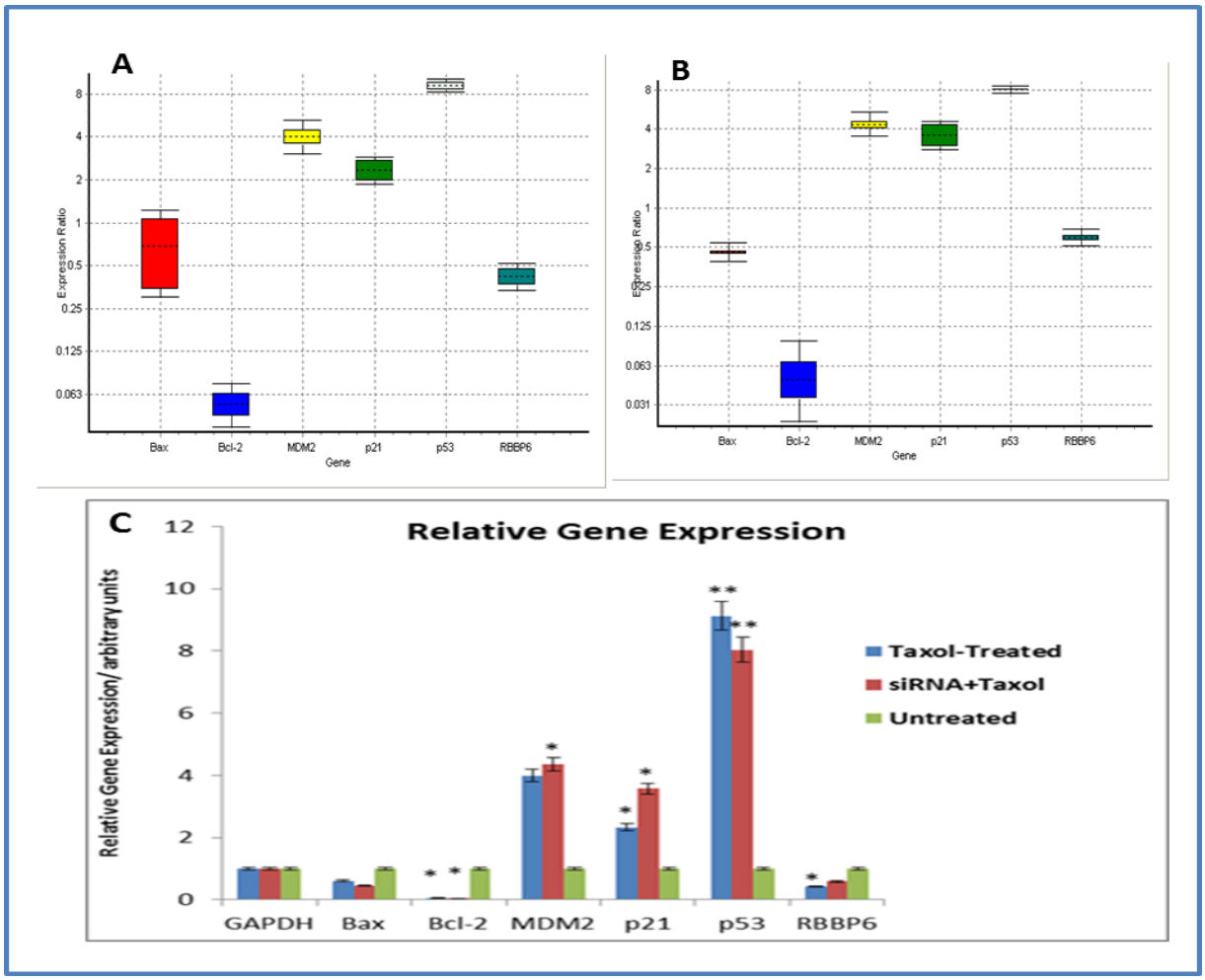


Figure 3.3: Gene expression changes following Paclitaxel treatment and co-treatment with siRBBP6 and Paclitaxel. A is a Box plot analysis showing changes in gene expressions following 50 nM paclitaxel treatment for 24 hours while B is a Box plot analysis showing gene expression changes following 30nM RBBP6 siRNA transfection for 24 hours and 50 nM paclitaxel treatment for 24 hours. C is a histogram depicting a comparison between untreated, paclitaxel-treated and co-treatment with siRBBP6 and Paclitaxel treatment.

It can be seen on Figure 3.3 that there was a higher expression of p21 cells co-treated with siRBBP6 and paclitaxel (3.568) as compared to cells that were Paclitaxel-treated only (2.321). Bcl-2 was also further downregulated in co-treated cells (0.048) as compared to cells

treated with paclitaxel only (0.053). However, p53 showed a higher expression in cells treated with taxol only (9.126) as compared with co-treated cells (8.028).

Following PCR, agarose gels were run, to ascertain that they were the expected product sizes

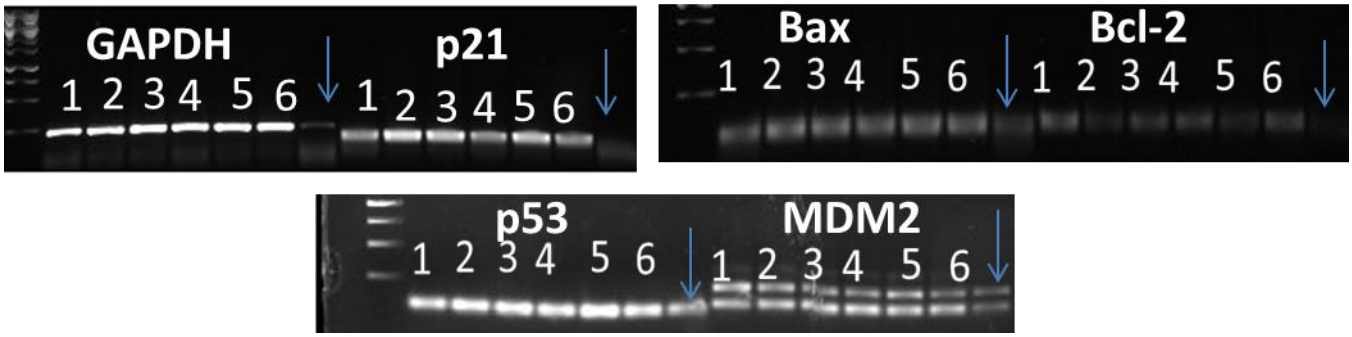


Figure 3.4: ***Post-PCR gels.*** The figure shows PCR agarose gels run for the various genes. The arrows indicate "no template controls. 1-6 represent: untreated, camptothecin-treated, paclitaxel-treated, siRBBP6-transfected, siRBBP6 plus camptothecin, and siRBBP6 plus paclitaxel respectively. The intensities of the bands indicate whether or not there were any gene expression changes following siRBBP6 transfection and treatments with CPT and paclitaxel

3.3 The effects of RBBP6 knockdown, camptothecin and paclitaxel treatments on Bcl-2, Bax, MDM2 and p53 mRNA protein expression.

In order to analyse the effects of RBBP6 knockdown, followed by camptothecin and paclitaxel treatments on Bcl-2, Bax, MDM2 and p53 protein expressions, a western blot

analysis was carried out. We first needed to confirm that RBBP6 was indeed knocked down following RBBP6 siRNA transfection.

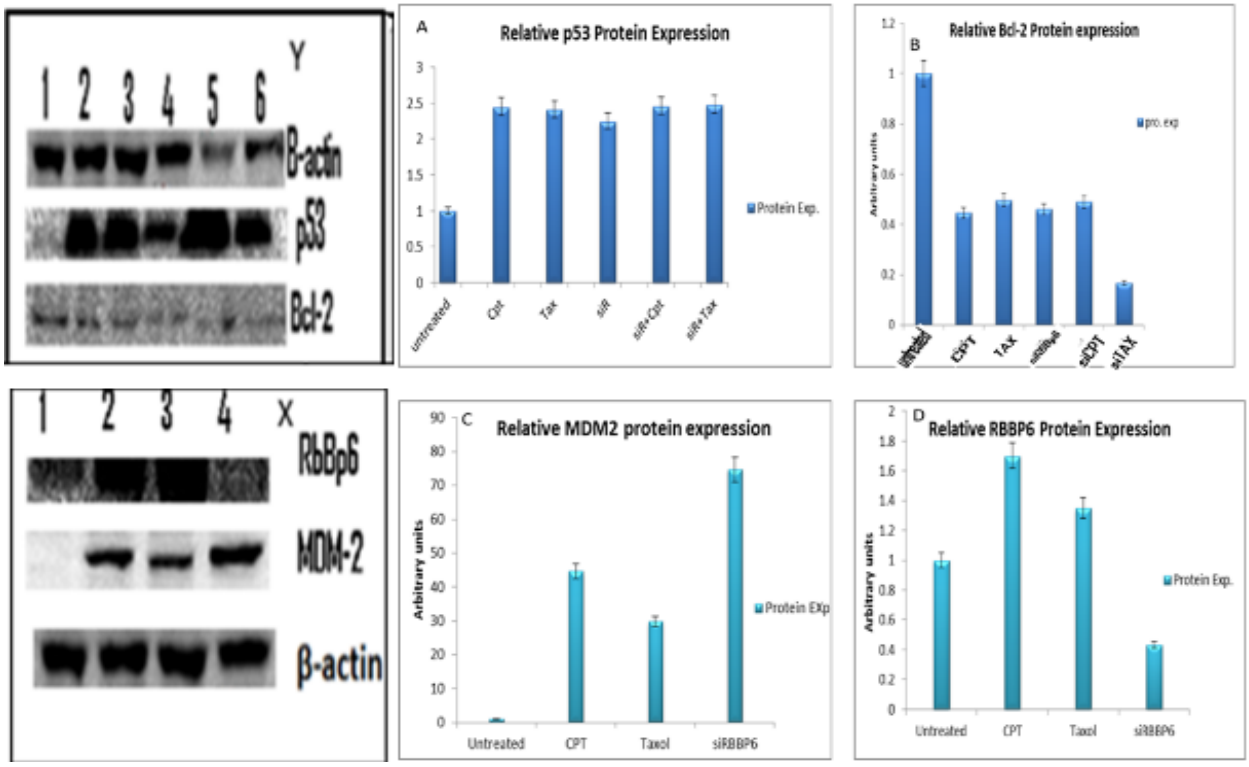


Figure 3.5 Protein expression analyses. RBBP6, MDM2, p53, Bcl-2 and Bax protein expressions following RBBP6 siRNA transfections and treatments with camptothecin and paclitaxel. X and Y represent western blot analysis of the various proteins investigated. A, B, C and D are histograms showing densitometric analysis of the various protein bands of RBBP6, MDM2, Bcl-2 and p53 respectively. The lanes numbered 1 to 6 represent the following samples: 1: untreated samples, 2: Campthothecin-treated, 3: paclitaxel-treated, 4: RBBP6 siRNA-transfected, 5. siBBP6+ Camptothecin, 6. siRBBP6+ paclitaxel.

RBBP6 showed a 57% knockdown following siRNA transfection (Lane 4, compared to Lane 1) which confirms that the protein was indeed knocked down. 30 µg of proteins were loaded per well. β-actin was used as a reference protein in this study. Bax, which is known to play a role in apoptosis, did not show any expressional changes at the protein level. Bcl-2, a pro-survival protein showed reduced protein expression following RBBP6 knockdown, camptothecin and paclitaxel treatments. MDM2 was however upregulated following treatments and RBBP6 knockdown.

3.4 xCELLigence® RTCA shows that RBBP6 knockdown, camptothecin and paclitaxel treatments reduce RMG-1 cell viability

We needed to evaluate the role played by RBBP6 knockdown, followed by camptothecin and paclitaxel treatments on RMG-1 cell proliferation. We did an *xCELLigence*® Real Time Cell Analysis. This system analyses the proliferation of cells on a real time basis. Samples were run in duplicates and the curves averaged to control for any well-to-well variations. The plot displayed changes in cell indices (y-axis) against time in hours (x-axis). Changes in cell indices are a measure of changes in cell proliferation.

3.4.1 Untreated and controls

Negative controls with the transfection reagent only and another with non-specific or nonsense siRNA did not show any growth inhibitory effects when compared to the untreated

samples. This confirmed that the observed growth changes were as a result of RBBP6 siRNA transfections which led to the knockdown of the RBBP6 gene.

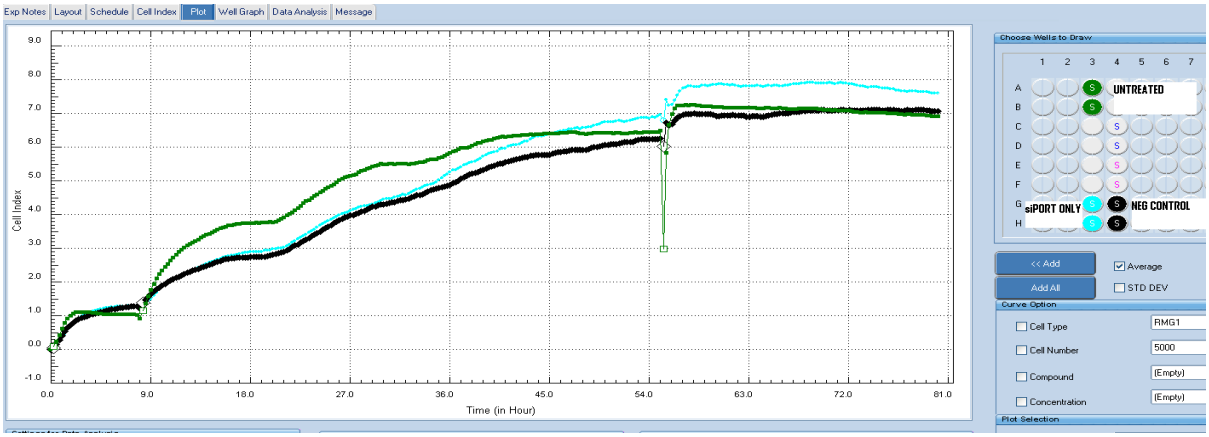


Figure 3.6: An *xCELLigence* RTCA plot showing real time cell proliferation of untreated cells and controls over a period of 80 hours. The green curve shows untreated cells, the black curve shows cells treated with 30nM of non-specific siRNA, while the turquoise curve shows cells that were treated with the transfection reagent (siPORT) only.

There was no significant difference in untreated cells compared with mock-transfected controls, enabling us to attribute any observed cell death to siRBBP6 transfection. Cell index measure as indicated by the growth curve all remained static after 54 hours suggesting that the cells were not growing anymore.

3.5 siRBBP6 transfected cells compared to untreated cells

Following RBBP6 siRNA transfection, cell proliferation reduced, as shown on the following figure:

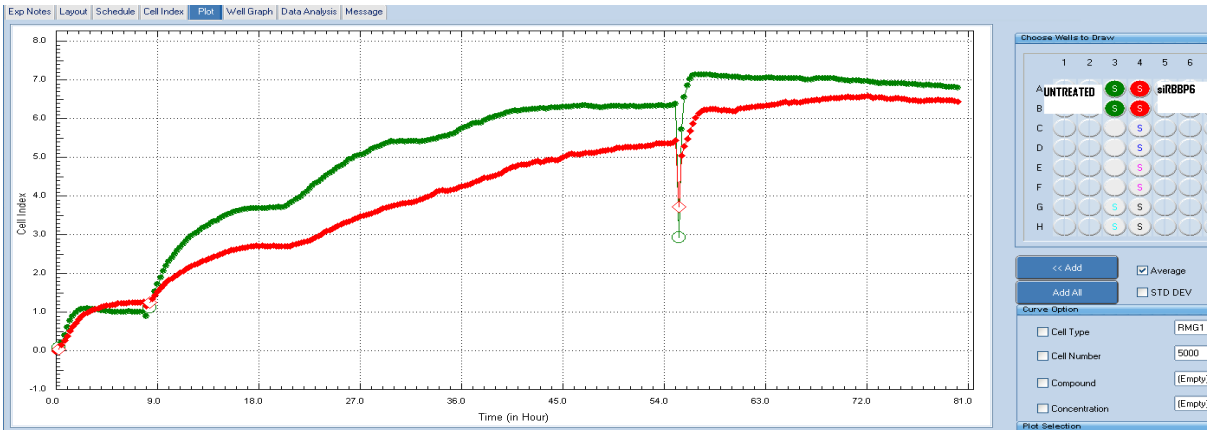


Figure 3.7.1: *xCELLigence* RTCA growth curves showing siRBBP6 transfected cells compared to untreated cells

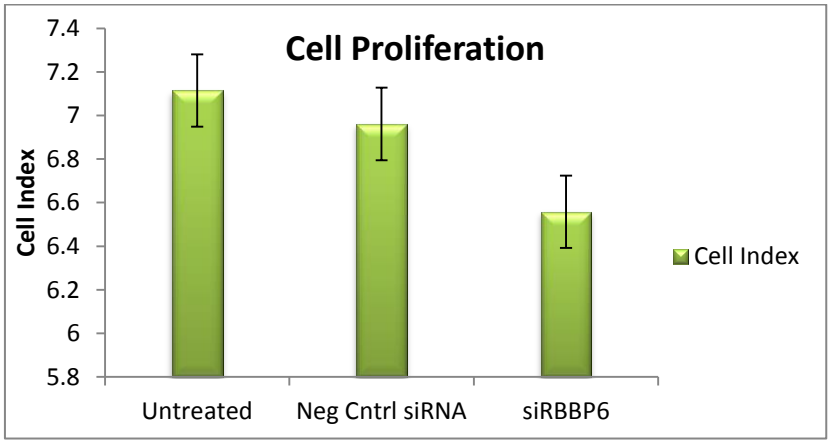


Figure 3.7.2: *xCELLigence* RTCA plot showing real time RMG-1 cell proliferation changes following 30nM RBBP6 siRNA transfection over a period of 80 hours. The green curve shows untreated cells red curve shows RBBP6 siRNA-transfected cells. ***Figure 3.7.2*** is a histogram that shows *xCELLigence* cell indices at 72 hours for untreated, negative control and siRBBP6 transfected.

It can be seen on Figure 3.7.1 that siRBBP6 transfection caused a decrease in cell proliferation. This is shown by a relative decrease in cell indices of transfected cells (red curve) compared to untreated cells (green curve) over time.

3.6 Camptothecin and Paclitaxel treatments

The following curves show real time cell proliferation following 0.5 µM camptothecin and 50 nM paclitaxel treatments over 48 h.

Figure 3.8: xCELLigence real time cell proliferation following 0.5 µM camptothecin and 50nM paclitaxel treatments over 48 hours

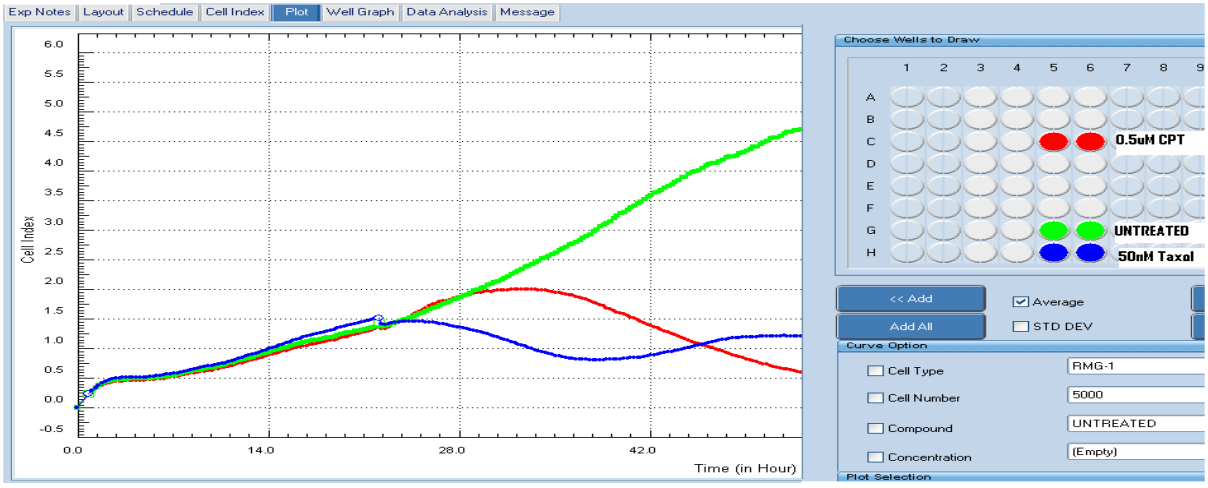


Figure 3.8: *xCELLigence* RTCA plot showing real time effects of 0.5µM camptothecin and 50nM paclitaxel on cell proliferation. The green curve shows untreated cells, the red curve shows camptothecin-treated cells while the blue curve shows paclitaxel-treated cells.

Cell indices dropped following 4 hours of paclitaxel treatment showing decreased proliferation. However, after roughly 32 hours, cell proliferation remained constant, suggesting growth arrest.

3.7 Cell proliferation after siRNA transfection, Camptothecin and Paclitaxel treatments

Following siRNA transfections and Camptothecin and Paclitaxel treatments, the following cell proliferation curves were observed:

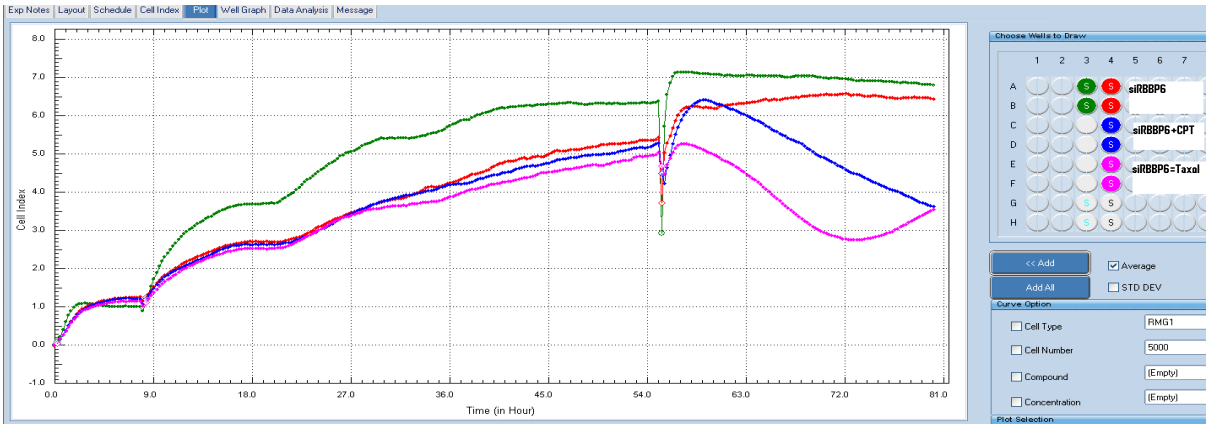


Figure 3.9: *xCELLigence* RTCA plot showing real time effects of 30nM RBBP6 siRNA transfections for 48 hours, followed by 0.5µM camptothecin and 50nM paclitaxel for 24 hours on cell proliferation.

Following about 8 hours of siRBBP6 transfection, cell indices, which is a measure of cell proliferation as shown on the purple, blue and red curves were not as high as untreated cells (the green curve). Moreover, after CPT and paclitaxel treatments at 55 hours there was a short-lived growth spurt which lasted for 4 hours. The cell indices for CPT-treated cells post

siRBBP6 transfection plummeted till the end of the experiment (at 80 hours). A rather different growth pattern was observed when cells were treated with paclitaxel following siRBBP6 transfection. There was a decrease in cell proliferation up till 72 hours, after which there was a stable growth pattern for about 2 hours. Cell proliferation then began to increase steadily, after which it finally declined.

3.8 Effects of RBBP6 siRNA transfections and paclitaxel treatments on the cell cycle

Flow Cytometry (Cell cycle analysis) (Paclitaxel induces cell cycle arrest in RMG-1 ovarian cancer cells). Paclitaxel is known to induce growth arrest and apoptosis (Vikhanskaya *et al*., 1998, Giannakakou *et al*., 2001) Cell indices dropped following 4 hours of taxol treatment showing decreased proliferation. However, after roughly 32 hours, cell proliferation remained constant, suggesting growth arrest. Also, the upregulation of p53 following RBBP6 knockdown prompted us to investigate growth arrest, given that p53 is known to upregulate p21. P21, a growth arrest gene was upregulated in our qPCR following siRBBP6 and paclitaxel treatments. The afore-mentioned points prompted us to investigate the possible effects of RBBP6 knockdown and paclitaxel treatments on the cell cycle.

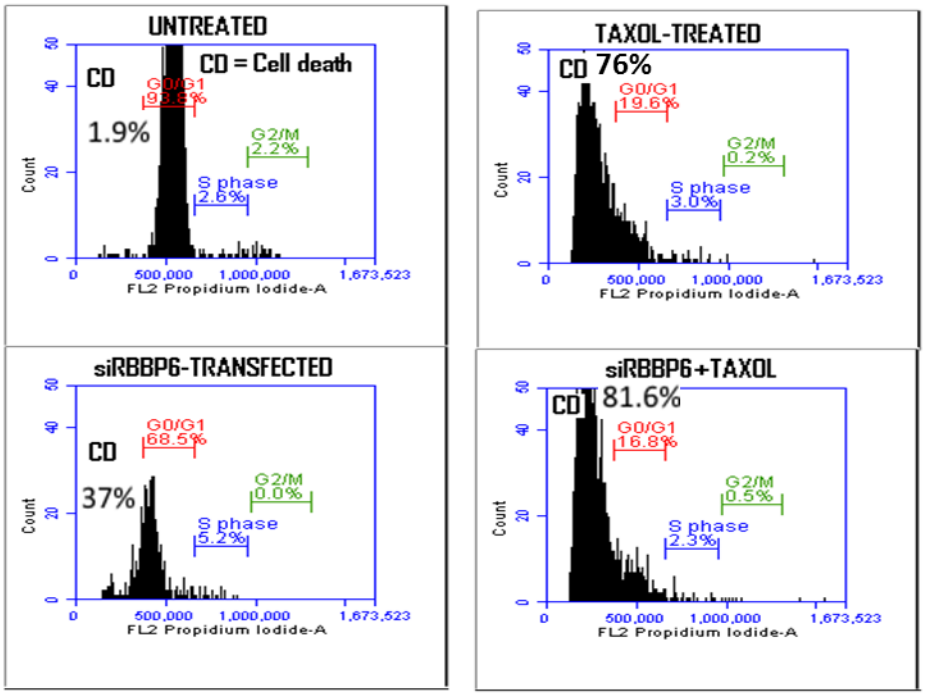


Figure 3.10: Effects of RBBP6 siRNA transfection and Paclitaxel treatment on the cell cycle of RMG-1 cells.

Untreated cells showed 93.8% at the G0/G1 stage, 2.6% at the S phase and 2.0% at the G2/M phase. Cells treated with 50nM paclitaxel showed 19.6% at the G0/G1 phase, 3.0% at the S phase and 0.2% at the G2/M phase. Cells transfected with 30nM siRBBP6 showed 68.5% at the G0/G1 phase, 5.2% at the S phase and 0.0% at the G2/M phase. Cells that were both transfected and treated showed a G0/G1 percentage of 16.8 and an S phase percentage of 2.3 and a G2/M percentage of 0.5.

3.9 An evaluation of sub-G0/G1 populations following treatments, representing cell death

Cells that showed a chromosome number of less than 2n appeared at the sub-G0/G1 cell population. This was an indication that their DNA had either been fragmented or smeared which could represent apoptosis or necrosis.

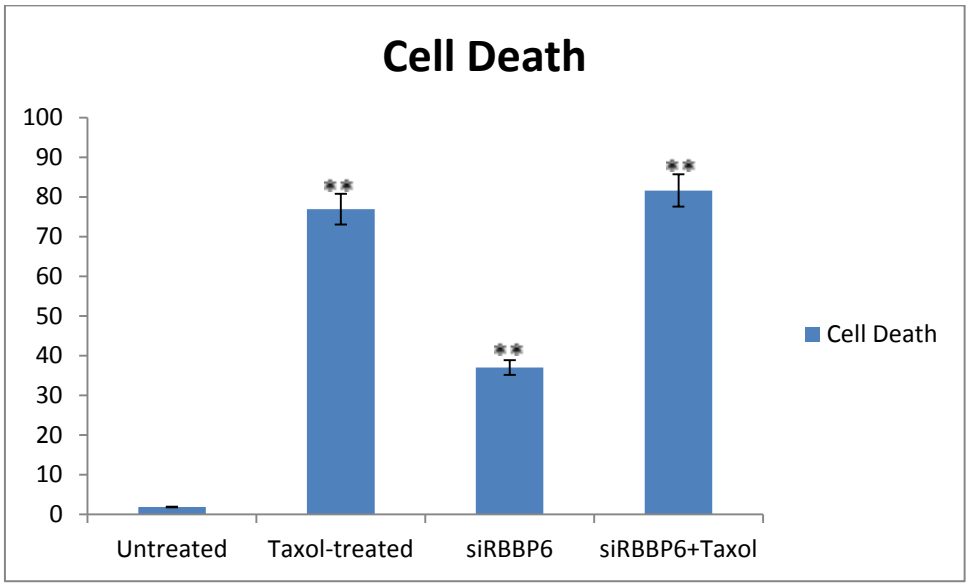


Figure 3.11: A histogram showing Sub-G0 cell populations showing cell death in untreated, paclitaxel treated (50nM), RBBP6 siRNA-transfected (30nM) and both transfected and treated.

Cell death statistical significance was calculated using a two-tailed t-test at a 95% confidence interval on the ***GraphPad QuickCalcs*** statistical program (http://www.graphpad.com/quickcalcs/ttest1.cfm). P values of less than 0.0001 were obtained when the means and SE of untreated and RBBP6 siRNA-transfected cells were used. This showed a very strong statistical significance. When means and SE of paclitaxel-treated cells were compared to those which were transfected before treatment with paclitaxel, a P-value of less than 0.0001 was also obtained; showing a strong statistical significance. The mean ± SD of cell death showed the following: As compared to untransfected cells, the RBBP6 siRNA-transfected cells showed a 37.0±5.8% while untrasfected cells showed a 1.9±0.4% cell death. Paclitaxel treated cells showed a 76±1.14% while cells that were both siRNA-transfected and paclitaxel-treated showed an 81.6±0.79% cell death.

CHAPTER FOUR- DISCUSSION

4.1 Introduction

Owing to the inadequate clinical benefits of conventional chemotherapy and surgery in ovarian cancer coupled with debilitating side effects, targeted therapeutic strategies have taken the centre stage in current research and clinical trials. In advanced stage ovarian cancers, standard treatment includes surgery, followed by chemotherapy with paclitaxel and a platinum based chemotherapy such as carboplatin or cislpatin (Markman *et al*., 2001). Moreover, the aqueous insolubility of camptothecin combined with severe and unpredictable side effects have impaired its therapeutic applications in cancer (Rothenburg, 1997). However, resistance and recurrent disease will occur in 80-85% of those diagnosed with advanced cases of ovarian cancer (Parkin *et al*., 2008).

Targeted therapeutics are dependent on an understanding of normal and oncogenic signalling pathways in the ovaries, which are usually not easy to elucidate. Aberrantly expressed oncogenes and proteins are key molecular targets in cancer. Small molecule inhibitors and monoclonal antibodies have attracted a lot of attention in the treatment of ovarian cancer (Gera *et al*., 2004; Apte *et al*., 2004; Fong *et al.,* 2008; Traynor *et al*., 2011).

The RNAi pathway, which includes shRNA, miRNA and siRNA activity, is currently being exploited by biologists to investigate gene function (Moela et al., 2014; Ghaemimanesh *et al*., 2014). Herein, we focused of a new mode of targeted therapy, in which an abnormally expressed gene, RBBP6, is knocked down by siRNA.

A very logical question in this study would be: "Why did we develop interest in RBBP6 in the first place?" RBBP6, because of its possession of a RING finger domain, has been identified as an E-3 ubiquitin ligase (Pugh *et al*., 2006). It is not surprising that RBBP6 is deregulated in several cancers (Yoshitake *et al*., 2004; Motadi *et al*., 2011, Mbita *et al*., 2012; Chen *et al*., 2013) because, a deregulation in ubiquitin pathways has been reported in numerous cancers (Hoeller *et al*., 2006). The functions of RBBP6 which include cell cycle progression, its interaction with p53, Rb, which are all implicated in tumourigenesis, (Simons et al., 1997; Sherr and McCommick, 2002; Scott *et al*., 2003) chromosomal stability and preventing DNA damage (Miotto *et al*., 2013) coupled with its overexpression in a handful of cancers suggest that RBBP6 plays a pro-survival role in the cell. Two important questions needed to be answered in this study: Firstly, could siRNA-mediated knockdown of RBBP6 in RMG-1 cells lead to a cell death or a decrease in cell proliferation? Secondly, could there be synergistic therapeutic effects of co-treatment of RMG-1 cells with Paclitaxel and siRBB6? In other words, could siRBBP6 transfection potentiate Paclitaxel or Camptothecin-induced cell death?

4.2 qPCR and Western blot analysis of gene and protein expressions.

4.2.1 qPCR

So far, qPCR is the most sensitive technique that can be used for the quantification of gene expression. We used the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines which helped us to ensure the quality, integrity, and transparency of our qPCR experiments (Bustin et al., 2009). We evaluated the levels of Bax, Bcl-2, MDM2, p21, and RBBP6 because of the role they play in cell death or proliferation and the direct or indirect relationship they may have with RBBP6. RBBP6 which is overexpressed in a handful of cancers has recently been shown to be one of the members of

the negative regulators of p53. RBBP6 has been shown to interact with p53, mediating its ubiquitination by MDM2 (Li *et al*., 2007) and MDM2 is well known to regulate p53 levels (Barak et al., 1993). The rigorous regulation of the action of p53 is critical for cell survival, death, or proliferation because of its ability to transactivate and repress several genes responsible for apoptosis and proliferation. Bcl-2 is known to aid cell survival and promote proliferation. Bax is a p53-inducible gene that plays a role in increasing mitochondrial permeability, causing the release of cytochrome *C*. This leads to apoptosis via the intrinsic pathway (Spierings, *et al*., 2005; Elmore, 2007).

The first role of qPCR was to ascertain the effective knockdown of RBBP6, following it siRNA transfection in the cells. The knockdown was confirmed, showing a 55% drop in expression. The expression of Bax, Bcl-2, MDM2, p21 and p53 were evaluated. Bax, p53 and p21 and MDM2 showed an upregulation following camptothecin treatment. Camptothecin is known to inhibit DNA topoisomerase I activity. An abrogation of topoisomeras I function by camptothecin binding to the topoisomeras1/DNA complex results in DNA strand breaks which leads to apoptosis (Liu *et al*., 2000). It is most likely that the induced apoptosis is as a result of p53-dependent pathways. This is because p53 has been shown to be upregulated following DNA damage, although its upregulation is usually as a result of post-translational modifications rather than by increases in its mRNA levels (Kanstan *et al*., 1991). MDM2, Bax and p21 can be transcriptionally upregulated by p53. In order to control the excessive tumour suppressor functions of p53, MDM2 is known to negatively regulate p53 by p53 ubiquitination (Chene, 2003)

There was a slight increase in p53 protein expression following co-treatments in comparison to either paclitaxel only or camptothecin treatments only. Also, there was a slight downregulation of the pro-survival gene, Bcl-2 expression in co-treatments compared to

treatments only. Bax/Bcl-2 ratios have been employed successfully to predict the fate of a cell (Raisova *et al*., 2001). A ratio of greater than one means that the cell will most likely die, while a ratio of less than one (showing that Bcl-2 expression is higher), indicated that the cell will most likely survive. Given that Bax expression was not seen at the protein level, we could not use mRNA expressions alone to justify the fate of our cells. However, densitometric analysis of Bcl-2 western blot showed that, Bcl-2 expressions dropped more in co-treated cells than in paclitaxel-treated cells.

4.2.2 Protein evaluations by Western Blots

An upregulation of mRNA transcripts in a cell is no guarantee that functional proteins are going to be synthesized. A lot of changes can happen to an mRNA following transcription, which explains why there may not always be correlation of mRNA expressions with actual protein expressions. Only approximately 40% of mRNA changes can show correlation with actual protein expressions (Maier *et al*., 2009; Abreu *et al*., 2009). The expression of proteins can be controlled at post-transcriptional, translational and regulation of degradation (Vogel *et al*., 2012). Following expressions at mRNA levels, we decided to investigate which genes were effectively translated into proteins, which could most likely be functional. Although Bax was upregulated following camptothecin, siRBBP6, and co-treatments, we could not find any Bax expression on western blots. This could possibly mean that the observed Bax mRNA transcripts were degraded and hence any observed cell death was Bax-independent.

Although RBBP6 mRNA expression did not show any change following camptothecin and paclitaxel treatment, there was a significant increase in RBBP6 protein levels as seen in our

western blots. This suggests that RBBP6 protein was stabilized by post-translational mechanisms, rather than from de novo synthesis of its protein products. Nonetheless, the mechanism by which camptothecin or paclitaxel may upregulate RBBP6 protein expression was in this study is still unclear. P2-PR is a 250kD an alternatively spliced form of the full length RBBP6 protein, lacking an exon that codes for a 34 amino acid peptide (Saijo et al., 1995). Paclitaxel and camptothecin are known to induce DNA damage in cells (Shao et al., 1999; Branham et al., 2004). Considering functions of P2-PR as cell cycle regulation (Gao et al., 2002; Scott *et al*., 2003), maintaining chromosomal stability and preventing DNA damage (Miotto et al., 2013), it is plausible to suggest that P2-PR could be upregulated as a protective measure, in order to induce cell cycle arrest in cells whose DNA had been damaged following camptothecin and paclitaxel treatment. MDM2 protein expressions increased to 45, 30 and 75 folds in CPT-treated, paclitaxel-treated and siRBBP6-transfected, respectively. MDM2 is known to be one of the most potent negative regulators of p53 so far (Haupt *et al*., 1997; Mommand *et al*., 2000); thereby explaining why several therapeutic strategies have been developed to target the MDM2-p53 interaction. P53 and MDM2 use an auto regulatory feedback loop to regulate each other's levels in which activated p53 transcriptionally upregulated MDM2 and MDM2 in turn targets p53 for ubiquitination and proteosomal degradation (Picksley and Lane, 1993). Moreover, it is important to note that there was a significant upregulation of p53 proteins following treatments and siRBBP6 transfection. Taken together, an upregulation of p53 as seen in this study induced an upregulation of MDM2 protein. The above observation is consistent with other studies (Barak et al., 1993; Picksley and Lane, 1993). Consequently, it is logical to suggest that MDM2 levels were upregulated in response to increased p53 expressions following CPT, paclitaxel and siRBBP6 treatments.

Multiple pathways and genes are involved in regulating cell survival, proliferation or death; however, only a handful of genes were analysed in this study. The expression of pro-apoptotic and growth arrest proteins as seen in this study should not be a conclusive indication of apoptosis or cell cycle arrest. Notwithstanding, evaluating the effects on cell proliferation thus became imperative. *xCELLigence* RTCA measured proliferation on a real time basis while an analysis of the cell cycle changes showed sub-G1 populations which were indicative of cell death.

4.3 xCELLigence RTCA measurements of cell adhesion and proliferation

Most cell death or viability/proliferation assays rely on end-point measurements for assessments following treatments. However, we used the *xCELLigence* RTCA system as our method of choice because of its superior ability to continuously monitor cell adhesion, attachment and proliferation on a real-time basis. From *xCELLigence* show that there was no significant difference between untreated and negative controls. When we analysed mock transfected to siRBBP6 transfected, we realized that there was a significant difference between cell growths or proliferation. From real-time analysis, we observed that camptothecin-treated at 24 hours after plating, cells showed a reduction in viability as shown by cell indices, approximately 7 hours after treatment. This reduction in cell proliferation continued progressively till the end of the experiment at 48 hours. This showed that the cells were highly sensitive to camptothecin. Cells that were treated with paclitaxel showed a rather different growth pattern. Cell growth seemed to be immediately arrested when the cells were treated. Cell indices dropped following 4 hours of paclitaxel treatment showing decreased

proliferation. However, after roughly 32 hours, cell proliferation remained constant, suggesting some form of resistance to paclitaxel or growth arrest.

xCELLigence RTCA showed growth arrest following paclitaxel+siRNA and paclitaxel only which were also consistent with p21 expression. Paclitaxel is known to induce G2/M growth arrest (Vikhanskaya *et al*., 1998), or even G1 growth arrest at small concentrations (Giannakakou *et al*., 2001). However, cell cycle could not show much arrest because the cells were harvested for analysis when they had already past arrest to apoptosis. Cells that have been arrested at G1 or G2/M check points are most likely to commit apoptotic cell death in case their DNA is not cannot be repaired.

4.4 Cell Cycle analysis following treatments and co-treatments

The PI used in this study contained RNAse to enable the quantification of DNA only, given that PI also bind to RNA. PI binding to DNA is proportional to the content of DNA a cell contains. Cells at G0/G1 are expected to be diploid (2n), and will therefore bind the least amount of PI, as compared to S-phase cells which contain more than 2n, and G2/M phase cells which contain 4n nuclei. This enables the cells to form different peaks based on the phase of the cell cycle in which they are found. The characteristic growth pattern displayed by *xCELLigence* RTCA suggestive of some form of growth arrest or drug resistance to paclitaxel, and existing literature confirming G2/M or G1 growth arrest (at low paclitaxel concentrations) properties of paclitaxel prompted us to investigate changes in the cell cycle following treatments and co-treatments. Also, the upregulation of p21 following paclitaxel and siRBBP6+paclitaxel treatments suggested that a G1 cell cycle arrest could be involved.

P21 is a growth arrest protein that initiates its effects by inhibiting the activity of kinases. Its upregulation, in our study could most likely be as a result of p53-dependent transactivation, given that p53 was also highly upregulated following paclitaxel treatments and co-treatments. Nevertheles, p21 has also been shown to show upregulation independent of p53 transactivation (Park *et al.,* 2000). In our study, we noticed only a slight decrease of G2/M cells following The reason cell cycle arrest was probably translated to apoptosis was based on the timing the cells were harvested.

The amount of cells that were at the G2/M reduced from 2.2 in the untreated, to 0.2 in paclitaxel-treated cells, 0.0 in siRBBP6 treated cells and to 0.5% in cells co-treated with siRBBP6 and paclitaxel. We deduced that, following treatments, cells did not progress beyond the S-phase and were therefore arrested at both G1 and S phases.

4.5 Evaluation of Sub-G1 Populations: An indication of Cell death

We noticed a significant increase in sub G1 cell populations of: 1.9% in untreated, 37% in siRNA transfected, 76.9% and 81.6% in paclitaxel and co-treated (siRBBP6 and paclitaxel-treated) cells respectively. Cells at sub G1 represent those having less than a diploid number of chromosomes in their nucleus (< 2n). These could either represent a population of cells with either fragmented or smeared DNA, characteristic of apoptosis or necrosis respectively.

4.6 Possible mechanisms of cell death

Although numerous studies indicate mutations in p53 in more than 50% of cancers (Chan *et al*., 2000) and many high grade carcinomas of the ovary are due to TP53 mutations, our study shows that p53 may most likely be in its wild type state. It may be important to sequence the protein to confirm that functional p53. Treatments showed upregulated p53 which correlated with apoptosis. The p53 pathway may be fine. P53 acts in concert with other oncogenes to modulate cell proliferation. Bax expression was not increased although other p53 downstream genes such as PUMA, NOXA, Bad, Bak which were not investigated in this study may have been upregulated. However, p53 also shows transcription-independent apoptosis in which it translocated to the mitochondria and promotes mitochondrial membrane permeability by forming complexes with pro-survival Bcl-2 and/or Bcl-Xl (Mihara *et al*., 2003) or directly activating Bax (Moll *et al*., 2005) which may also be the case. Several proteins which have not been investigated in this study may be upregulated or downregulated as a result of our transfections and treatments; and are possibly involved other mechanisms of apoptosis in our ovarian cancer cells. Knockdown of RBBP6 showed a marked increase in p53 protein expression as seen also in other studies (Li *et al*., 2007).

Apoptosis, which is a programmed and controlled mode of cell death, yielding no inflammatory response (Kerr *et al*., 1972), is the ideal mode of cell death expected in cancer therapy. Even though an apoptosis-based assay was not performed, it is plausible to infer that the observed cell death was most likely as a result of p53-mediated apoptosis. This is because of the following reasons:

1. siRBBP6 transfection led to an upregulation of p53 at both mRNA and protein levels. p53 null mutations rescued cell death in RBBP6 knock down embryos (Li *et al*., 2007). Showing that the observed cell death was p53-dependent.
2. Paclitaxel is well known to induce apoptosis. This is as a result of its mechanism of action. Paclitaxel causes the stability of microtubules, thereby inhibiting microtubule polymerization. A disturbance of the dynamics of microtubules by mitosis-inhibiting compounds results in an inhibition of cell cycle progression with a G2/M phase growth arrest, eventually leading to apoptosis (Jordan and Wilson, 2004).
3. Cells that lack RBBP6 experience chromosomal instability and spontaneous DNA damage (Miotto et al., 2013) which are hallmark trig/gers for apoptosis, suggesting one of the ways by which knockdown of RBBP6 may induce apoptosis.
4. It was noted that MDM2, which is known to be transactivated by wild type p53 levels (Barak et al., 1993) was upregulated following transfections and treatments. Mutant p53, which is not potent enough to induce apoptosis, cannot be transactivated by p53 (Midgley and Lane, 1997). As a result we suggest that the upregulated levels of MDM2 were an indication of the presence of wild type p53. Taken together, it is plausible to infer that most of the cell death observed by co-treatment regimens of paclitaxel and siRBBP6 cell death was due to p53-dependent apoptosis.

However, apoptosis-based assays are needed to confirm the assertion, given that the mechanism by which the action of siRBBP6 and paclitaxel could synergistically boost cell death is not yet understood.

4.7 Conclusions and Clinical Implications

Our findings show that siRNA-mediated knockdown of RBBP6 induces cell death. siRBBP6 alone induced approximately 37±% cell in RMG-1 cells. In addition, paclitaxel-mediated cell death in RMG-1 ovarian cancer cells is potentiated by siRBBP6. Taken together, it is most likely that RBBP6 plays an important role in inducing or sustaining cell proliferation.

To sum-up, we have shown that siRBBP6 transfection may act in a synergistic manner with paclitaxel to reduce tumour growth in ovarian RMG-1 ovarian cancer cells. On account of the upregulated levels of RBBP6 in numerous cancers, and resistance observed in chemotherapy, a combination of chemotherapy with paclitaxel or camptothecin and RBBP6 siRNA could be a possible therapeutic strategy in combatting ovarian carcinomas.

Furthermore, small molecules have been designed that interfere with the MDM2-p53 interaction. Bearing in mind that RBBP6 enhances the ubiquitination and degradation of p53, it is plausible that, combining siRBBP6 activity with MDM2-p53 inhibitors may also serve as a therapeutic strategy in tumours overexpressing RBBP6.

It is clear that RBBP6 acts in concert with several proteins to modulate cell survival; and it has been shown to be overexpressed in numerous cancers such as lung cancer (Motadi *et al*., 2011), oesophageal cancer (Yoshitake *et al*., 2004), colorectal cancer (Chen *et al*., 2013), and cervical cancer (Mbita *et al*., 2012).

The question is: What is the precise mechanism by which RBBP6 interacts with MDM2 and other proteins to cause cell proliferation? Additional techniques, including study of protein-protein interactions using techniques such as affinity chromatography, co-

immunoprecipitation and mass spectrometry may be the way forward to elucidate the precise mechanisms involved.

A lot of interesting findings involving proposed mechanisms of cancer development, drug resistance mechanisms and alternative therapeutic strategies in cancer treatment have been published.

Several studies have shown that siRNA alone or co-treatment with siRNA and some form of conventional chemotherapy actually sensitizes cells to apoptosis and cell cycle arrest. Yang *et al*., 2003, showed that retrovirus-delivered siRNA mediated knockdown of the H-ras gene reduced tumour growth in a model ovarian cancer. siRNA-mediated knockdown of RBBP6 in MCF-7 breast cancer cells followed by camptothecin treatment showed improved apoptosis as compared to camptothecin treatment alone (Moela *et al*., 2014). Nishimura and colleagues in 2013, have shown that therapeutic synergy could be realized when ovarian cancer cells were treated with siRNA and miRNA targeting EphA2 proteins. Moreover, a decreased cell survival and increased apoptosis was shown in ovarian carcinoma cells following the knockdown of the sortilin gene with siRNA (Ghaemimanesh *et al*., 2014). However, several pathways have been shown to be deregulated in ovarian cancer. The knockdown of a single gene may not provide optimal therapeutic benefits in patients. Microarray analysis may provide aberrant expression patterns which could lead to the development of multiple siRNA combinations, targeting over-expressed genes.

However, it is easy to jump into inaccurate conclusions whenever the validity of *in vitro* experimental results is not proven using *in vivo* conditions**.** The fact that numerous clinical trials have not been able to move through recommended phases before potential drugs become approved therapeutic use, gives credence to the previous assertion. Our lack of

understanding of the complex molecular mechanisms that precede neoplastic transformation necessitates further research in this intricate and fascinating field of science.

4.8 Future directions

- Evaluate co-treatment of siRBBP6 and paclitaxel on normal cells. This would enable us ascertain whether any serious cytotoxic effects could result following co-treatments.
- Also, and evaluation of co-treatment effects on a less aggressive ovarian cancer cell line would be necessary.
- Confirm the mode of cell death elicited by co-treatments using apoptosis-based assays such as the Annexin V-FITC and PI apoptosis assay
- Evaluate apoptosis elicited by camptothecin, compared with co-treatment with camptothecin and siRBBp6.
- An elucidation of the molecular mechanisms by which RBBP6 interacts with MDM2 and p53 to mediate cell proliferation using other techniques such as co-immunoprecipitation, affinity chromatography and mass spectrometry would be necessary.

REFERENCES

Alberts, D. S., Liu, P. Y., Wilczynski, S. P., Jang, A., Moon, J., Ward, J. H., Beck, J. T., Clouser, M., & Markman, M. (2007). Phase II trial of imatinib mesylate in recurrent, biomarker positive, ovarian cancer (Southwest Oncology Group Protocol S0211). *International Journal of Gynecological Cancer: Official Journal of the International Gynecological Cancer Society*, *17*(4), 784–788

Alarcon-Vargas, D., & Ronai, Z. (2002). p53–Mdm2—the affair that never ends. *Carcinogenesis*, *23*(4), 541–547.

American Cancer Society. (2015). Do we know what causes ovarian cancer? Retrieved from http://www.cancer.org/cancer/ovariancancer/detailedguide/ovarian-cancer-what-causes

American Cancer Society. (2015). Survival rates for ovarian cancer, by stage. Retrieved from http://www.cancer.org/cancer/ovariancancer/detailedguide/ovarian-cancer-survival-rates

American Cancer Society. (2015). Global Cancer Facts and Figures. Retrieved from http://www.cancer.org/research/cancerfactsstatistics/global

Amsterdam, A., Sasson, R., Keren-Tal, I., Aharoni, D., Dantes, A., Rimon, E., Lan, A., Cohen, T., Dor, Y., & Hirsh, L. (2003). Alternative pathways of ovarian apoptosis: death for life. *Biochemical Pharmacology*, *66*(8), 1355–1362.

Anand, S., Penrhyn-Lowe, S., & Venkitaraman, A. R. (2003). AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Paclitaxel. *Cancer Cell*, *3*(1), 51–62.

Anand, P., Kunnumakara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S., … Aggarwal, B. B. (2008). Cancer is a Preventable Disease that Requires Major Lifestyle Changes. *Pharmaceutical Research*, *25*(9), 2097–2116.

Andree, H. A., C. P. Reutelingsperger, R. Hauptmann, H. C. Hemker, W. T. Hermens, and G. M. Willems. "Binding of Vascular Anticoagulant Alpha (VAC Alpha) to Planar Phospholipid Bilayers." *The Journal of Biological Chemistry* 265, no. 9 (March 25, 1990): 4923–28.

Antoniou, A., Pharoah, P. D. P., Narod, S., Risch, H. A., Eyfjord, J. E., Hopper, J. L., … Easton, D. F. (2003). Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. *American Journal of Human Genetics*, *72*(5), 1117–1130.

Aoudjit, F., & Vuori, K. (2012). Integrin Signaling in Cancer Cell Survival and Chemoresistance. *Chemotherapy Research and Practice*, *2012*.

Apte, S. M., Fan, D., Killion, J. J., & Fidler, I. J. (2004). Targeting the platelet-derived growth factor receptor in antivascular therapy for human ovarian carcinoma. *Clinical Cancer Research*, *10*(3), 897–908.

ARAKAWA, Y., OZAKI, K., OKAWA, Y., & YAMADA, H. (2013). Three missense mutations of DNA topoisomerase I in highly camptothecin-resistant colon cancer cell sublines. *Oncology Reports*, *30*(3), 1053–1058.

Arora, A., & Scholar, E. M. (2005). Role of tyrosine kinase inhibitors in cancer therapy. *Journal of Pharmacology and Experimental Therapeutics*, *315*(3), 971–979.

Azad, N. S., Posadas, E. M., Kwitkowski, V. E., Steinberg, S. M., Jain, L., Annunziata, C. M., Minasian, L., Sarosy, G., Kotz, L.M., Premkumar, A., Cao, L., McNally, D., Chow, C., Chen, H. X., Wright, J. J., Figg, W. D., & Kohn, E. C. (2008). Combination targeted therapy with sorafenib and bevacizumab results in enhanced toxicity and antitumor activity. *Journal of Clinical Oncology*, *26*(22), 3709–3714.

Barak, Y., Juven, T., Haffner, R., & Oren, M. (1993). mdm2 expression is induced by wild type p53 activity. *The EMBO journal*, *12*(2), 461.

Barak, Y., Gottlieb, E., Juven-Gershon, T., & Oren, M. (1994). Regulation of mdm2 expression

by p53: alternative promoters produce transcripts with nonidentical translation potential. *Genes & Development*, *8*(15), 1739–1749.

Bartel, F., Jung, J., Böhnke, A., Gradhand, E., Zeng, K., Thomssen, C., & Hauptmann, S. (2008). Both Germ Line and Somatic Genetics of the p53 Pathway Affect Ovarian Cancer Incidence and Survival. *Clinical Cancer Research*, *14*(1), 89–96.

Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281−297.

Bartlett, J., Langdon, S., Simpson, B., Stewart, M., Katsaros, D., Sismondi, P., Love, S., Scott, W. N., Williams, A. R., Lessells, A. M., Macleod, K. G., Smyth, J. F., and Miller, W. R. (1996). The prognostic value of epidermal growth factor receptor mRNA expression in primary ovarian cancer. *British Journal of Cancer*, *73*(3), 301.

Becker, M. A., Farzan, T., Harrington, S. C., Krempski, J. W., Weroha, S. J., Hou, X., Kalli, K. R., Wong, T., W., and Haluska, P. (2013). Dual HER/VEGF Receptor Targeting Inhibits In Vivo Ovarian Cancer Tumor Growth. *Molecular Cancer Therapeutics*, *12*(12), 2909–2916.

Beltran, P. J., Mitchell, P., Chung, Y. A., Cajulis, E., Lu, J., Belmontes, B., ... & Calzone, F. J. (2009). AMG 479, a fully human anti–insulin-like growth factor receptor type I monoclonal antibody, inhibits the growth and survival of pancreatic carcinoma cells. *Molecular cancer therapeutics*, *8*(5), 1095-1105.

Besnard, N., Pisselet, C., Monniaux, D., Locatelli, A., Benne, F., Gasser, F., Hatey, F., Monget, P. (1996). Expression of messenger ribonucleic acids of insulin-like growth factor binding protein-2, -4, and -5 in the ovine ovary: localization and changes during growth and atresia of antral follicles. *Biology of Reproduction*, *55*(6), 1356–1367.

Biagi, J., Oza, A., Grimshaw, R., Ellard, S., Lee, U., Sederias, J., Ivy, S. P., & Eisenhauer, E. (2008). A phase II study of sunitinib (SU11248) in patients (pts) with recurrent epithelial

ovarian, fallopian tube or primary peritoneal carcinoma–NCIC CTG IND 185. *J Clin Oncol*, *26*, 5522.

Bischoff, J. R., Anderson, L., Zhu, Y., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., & Ginther, C. (1998). A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. *The EMBO Journal*, *17*(11), 3052–3065.

Blume-Jensen, P., & Hunter, T. (2001). Oncogenic kinase signalling. *Nature*, *411*(6835), 355–365.

Bobe, J., & Goetz, F. W. (2001). Molecular cloning and expression of a TNF receptor and two TNF ligands in the fish ovary. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *129*(2), 475–481.

Branham, M. T., Nadin, S. B., Vargas-Roig, L. M., & Ciocca, D. R. (2004). DNA damage induced by paclitaxel and DNA repair capability of peripheral blood lymphocytes as evaluated by the alkaline comet assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *560*(1), 11-17.

Brokaw, J., Katsaros, D., Wiley, A., Lu, L., Su, D., Sochirca, O., Rigault de la Longrais, I.A., Mayne, S., Risch, S., & Yu, H. (2007). IGF-I in epithelial ovarian cancer and its role in disease progression. *Growth Factors*, *25*(5), 346–354.

Brown, M., & Wittwer, C. (2000). Flow Cytometry: Principles and Clinical Applications in Hematology. *Clinical Chemistry*, *46*(8), 1221–1229.

Bryant, H. E., Schultz, N., Thomas, H. D., Parker, K. M., Flower, D., Lopez, E., … Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly (ADP-ribose) polymerase. *Nature*, *434*(7035), 913–917.

Budhram-Mahadeo, V., Morris, P. J., Smith, M. D., Midgley, C. A., Boxer, L. M., & Latchman, D. S. (1999). p53 suppresses the activation of the Bcl-2 promoter by the Brn-3a POU family transcription factor. *Journal of Biological Chemistry*, *274*(21), 15237–15244.

Buller RE, Shahin MS, Horowitz JA, *et al*. Long term follow up of patients with recurrent ovarian cancer after Ad p53 gene replacement with SCH 58500. Cancer Gene Therapy 2002; 9:567–72.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., ... & Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry*, *55*(4), 611-622.

Campos, S., Penson, R., & Matulonis, U. (2008). STAC: a randomized phase II trial of avastin or avastin+ erlotinib as first line consolidation chemotherapy after standard therapy. Presented at the British Gynecological Society Meeting.

Campos, S. M., & Ghosh, S. (2010). A current review of targeted therapeutics for ovarian cancer. *Journal of Oncology*, *2010*.

Cannistra, S. A. (2004). Cancer of the Ovary. *New England Journal of Medicine*, *351*(24), 2519–2529. Clemens, M. J. (2003). Interferons and apoptosis. *Journal of Interferon & Cytokine Research: The Official Journal of the International Society for Interferon and Cytokine Research*, *23*(6), 277–292.

Copeland, L. J., Vaccarello, L., & Lewandowski, G. S. (1994). Second-look laparotomy in epithelial ovarian cancer. *Obstetrics and gynecology clinics of North America*, *21*(1), 155–166.

Carroll, M., Tomasson, M. H., Barker, G. F., Golub, T. R., & Gilliland, D. G. (1996). The TEL/platelet-derived growth factor β receptor (PDGFβR) fusion in chronic myelomonocytic leukemia is a transforming protein that self-associates and activates

PDGFβR kinase-dependent signaling pathways. *Proceedings of the National Academy of Sciences*, *93*(25), 14845–14850.

Chen, J. C., Bartholomew, J., Campisi, M., Acosta, J. D., Reagan, & Ames, B. N

Molecular Analysis of H2O2-Induced Senescent-like Growth Arrest in Normal Human Fibroblasts: p53 and Rb Control G1 Arrest but Not Cell Replication. *Biochemical Journal* 332.Pt 1 (1998): 43–50.

Chène, P. (2003). Inhibiting the p53–MDM2 interaction: An important target for cancer therapy. *Nature Reviews Cancer*, *3*(2), 102.

Chen, J., Tang, H., Wu, Z., Zhou, C., Jiang, T., Xue, Y., ... & Peng, Z. (2013). Overexpression of RBBP6, Alone or Combined with Mutant TP53, Is Predictive of Poor Prognosis in Colon Cancer. *PloS one*, *8*(6), e66524.

Dai, Y., Lawrence, T. S., & Xu, L. (2009). Overcoming cancer therapy resistance by targeting inhibitors of apoptosis proteins and nuclear factor-kappa B. *American Journal of Translational Research*, *1*(1), 1–15.

Daly, M., & Obrams, G. I. (1998). Epidemiology and risk assessment for ovarian cancer. (Vol. 25, pp. 255–264). Presented at the Seminars in oncology.

Egger, G., Liang, G., Aparicio, A., & Jones, P. A. (2004). Epigenetics in human disease and prospects for epigenetic therapy. *Nature*, *429*(6990), 457–463.

Elmore, Susan. "Apoptosis: A Review of Programmed Cell Death." *Toxicologic Pathology* 35, no. 4 (2007): 495–516.

Eschenburg, G., Eggert, A., Schramm, A., Lode, H. N., & Hundsdoerfer, P. (2012). Smac mimetic LBW242 sensitizes XIAP-overexpressing neuroblastoma cells for TNF-α-independent apoptosis. *Cancer Research*, *72*(10), 2645–2656.

Fathalla, M., Davis, B., Johnson, D., Greenblatt, R., Mangat, H., Chaudhury, R., Devi , P.K., Kistner, R.W., Reid, B.L., & Bernard, R. (1971). Incessant ovulation--a factor in ovarian

neoplasia?[letter]. *Lancet*, *2*(7716), 163.

Fischer-Colbrie, J., Witt, A., Heinzl, H., Speiser, P., Czerwenka, K., Sevelda, P., & Zeillinger, R. (1997). EGFR and steroid receptors in ovarian carcinoma: comparison with prognostic parameters and outcome of patients. *Anticancer research*, *17*(1B), 613–619.

Folkman, J. (1997). Angiogenesis and angiogenesis inhibition: an overview. In *Regulation of angiogenesis* (pp. 1–8). Springer.

Fong, P., Boss, D., Carden, C., Roelvink, M., De Greve, J., Gourley, C., … Kaye, S. (2008). AZD2281 (KU-0059436), a PARP (poly ADP-ribose polymerase) inhibitor with single agent anticancer activity in patients with BRCA deficient ovarian cancer: results from a phase I study. *Journal of Clinical Oncology 26*(15S), Abstract–5510.

Friedlander, M., Hancock, K., Benigno, B., Rischin, D., Messing, M., Stringer, A., Tay, E. H., Kathman, S., Matthys, G., & Lager, J. (2007a). Pazopanib (GW786034) is active in women with advanced epithelial ovarian, fallopian tube and peritoneal cancers: initial results of a phase II study. *Journal of Clinical Oncol*ogy, *25*, 5561.

Fulda, S., & Debatin, K.-M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*, *25*(34), 4798–4811.

Gavalas, N. G., Karadimou, A., Dimopoulos, M. A., & Bamias, A. (2011). Immune response in ovarian cancer: how is the immune system involved in prognosis and therapy: potential for treatment utilization. *Clinical and Developmental Immunology*, *2010*.

Gao, S., & Scott, R. E. (2002). P2P-R protein overexpression restricts mitotic progression at prometaphase and promotes mitotic apoptosis. *Journal of cellular physiology*, *193*(2), 199-207

Gera, J. F., Mellinghoff, I. K., Shi, Y., Rettig, M. B., Tran, C., Hsu, J., Sawyers, C. L., & Lichtenstein, A. K. (2004). AKT activity determines sensitivity to mammalian target of rapamycin (mTOR) inhibitors by regulating cyclin D1 and c-myc expression. *Journal of*

Biological Chemistry, *279*(4), 2737–2746.

Gewies, A. (2003). Introduction to apoptosis. *Aporeview*, *1*, 1–26.

Ghaemimanesh, F., Ahmadian, G., Talebi, S., Zarnani, A. H., Behmanesh, M., Hemmati, S., ... & Rabbani, H. (2014). The Effect of Sortilin Silencing on Ovarian Carcinoma Cells. *Avicenna journal of medical biotechnology*, *6*(3), 169.

Giannakakou, P., Robey, R., Fojo, T., & Blagosklonny, M. V. (2001). Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. *Oncogene*, *20*(29), 3806-3813.

Giancotti, F. G., & Ruoslahti, E. (1999). Integrin signaling. *Science*, *285*(5430), 1028–1033.

Giebel, J., de Souza, P., & Rune, G. M. (1996). Expression of integrins in marmoset (Callithrix jacchus) ovary during folliculogenesis. *Tissue & Cell*, *28*(4), 379–385.

GLOBOCAN (2008). Global Cancer Facts and Figures, 2nd Edition. Retrieved from http://www.cancer.org/acs/groups/content/@epidemiologysurveilance/documents/document/acspc-027766.pdf

Gnagy, S., Ming, E. E., Devesa, S. S., Hartge, P., & Whittemore, A. S. (2000). Declining ovarian cancer rates in US women in relation to parity and oral contraceptive use. *Epidemiology*, *11*(2), 102–105.

Goodsell, D. S. (2008). Small Interfering RNA (siRNA). *RCSB Protein Data Bank*.

Gupta, S. (2001). Molecular steps of death receptor and mitochondrial pathways of apoptosis. *Life Sciences*, *69*(25–26), 2957-2964.

Gwinn, M. L., Lee, N. C., Rhodes, P. H., Layde, P. M., & Rubin, G. L. (1990). Pregnancy, breast feeding, and oral contraceptives and the risk of epithelial ovarian cancer. *Journal of clinical epidemiology*, *43*(6), 559–568.

Haupt, Y., Maya, R., Kazaz, A., & Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature*, *387*(6630), 296-299

Harris, C. C., & Hollstein, M. (1993). Clinical implications of the p53 tumor-suppressor gene. *The New England Journal of Medicine*, *329*(18), 1318–1327.

Hartenbach, E., Olson, T., Goswitz, J., Mohanraj, D., Twiggs, L., Carson, L., & Ramakrishnan, S. (1997). Vascular endothelial growth factor (VEGF) expression and survival in human epithelial ovarian carcinomas. *Cancer letters*, *121*(2), 169–175.

Heinrich, M. C., Corless, C. L., Duensing, A., McGreevey, L., Chen, C.-J., Joseph, N., … Town, A. (2003). PDGFRA activating mutations in gastrointestinal stromal tumors. *Science*, *299*(5607), 708–710.

Herbst, R. S. (2004). Review of epidermal growth factor receptor biology. *International Journal of Radiation Oncology*Biology*Physics*, *59*(2, Supplement), S21–S26.

Hirte, H., Vidal, L., Fleming, G., Sugimoto, A., Morgan, R., Biagi, J., Wang, L., McGill, S., Ivy, S., & Oza, A. (2008). A phase II study of cediranib (AZD2171) in recurrent or persistent ovarian, peritoneal or fallopian tube cancer: final results of a PMH, Chicago and California consortia trial. *Journal of Clinical Oncology*, *26*(15S), 5521.

Hoeller, D., Hecker, CM., Dikic, I. (2006) Ubiquitin and ubiquitin-like proteins in cancer pathogenesis. Nat Rev Cancer 6: 776–788.

Hussein, M. R. (2005). Apoptosis in the ovary: molecular mechanisms. *Human reproduction update*, *11*(2), 162–178.

Hussein, M. R., Haemel, A. K., & Wood, G. S. (2003). Apoptosis and melanoma: molecular mechanisms. *The Journal of pathology*, *199*(3), 275–288.

Jain, H. V., Richardson, A., Meyer-Hermann, M., & Byrne, H. M. (2014). Exploiting the Synergy between Carboplatin and ABT-737 in the Treatment of Ovarian Carcinomas. *PLoS ONE*, *9*(1), e81582.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T., & Thun, M. J. (2008). Cancer statistics, 2008. *CA: a cancer journal for clinicians*, *58*(2), 71-96.

Jiang, J.-Y., Cheung, C. K. M., Wang, Y., & Tsang, B. K. (2003). Regulation of cell death and cell survival gene expression during ovarian follicular development and atresia. *Frontiers in Bioscience: A Journal and Virtual Library*, *8*, d222–237.

Jiang, B.-H., & Liu, L.-Z. (2008). PI3K/PTEN signaling in tumorigenesis and angiogenesis. *Biochimica et Biophysica Acta (BBA)-Proteins & Proteomics*, *1784*(1), 150–158.

Jiang, M.-R., Li, Y.-C., Yang, Y., & Wu, J.-R. (2003). c-Myc degradation induced by DNA damage results in apoptosis of CHO cells. *Oncogene*, *22*(21), 3252–3259.

Johnson, M., Dimitrov, D., Vojta, P. J., Barrett, J. C., Noda, A., Pereira-Smith, O. M., & Smith, J. R. (1994). Evidence for a p53-independent pathway for upregulation of SDI1/CIP1/WAF1/P21 RNA in human cells. *Molecular Carcinogenesis*, *11*(2), 59–64.

Jordan, M. A., & Wilson, L. (2004). Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, *4*(4), 253–265.

Kaipia, A., Chun, S. Y., Eisenhauer, K., & Hsueh, A. J. (1996). Tumor necrosis factor-alpha and its second messenger, ceramide, stimulate apoptosis in cultured ovarian follicles. *Endocrinology*, *137*(11), 4864–4870.

Kaku, T., Ogawa, S., Kawano, Y., Ohishi, Y., Kobayashi, H., Hirakawa, T., & Nakano, H. (2003). Histological classification of ovarian cancer. *Medical Electron Microscopy*, *36*(1), 9–17.

Karin, M., & Hunter, T. (1995). Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Current Biology*, *5*(7), 747–757.

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., & Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage.*Cancer research*, *51*(23 Part 1), 6304-6311.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer*, *26*(4).

Kibbe, M. R., Li, J., Nie, S., Watkins, S. C., Lizonova, A., Kovesdi, I., … Tzeng, E. (2000). Inducible nitric oxide synthase (iNOS) expression upregulates p21 and inhibits vascular smooth muscle cell proliferation through p42/44 mitogen-activated protein kinase activation and independent of p53 and cyclic guanosine monophosphate. *Journal of Vascular Surgery*, *31*(6), 1214–1228.

Kim, K., Fisher, M. J., Xu, S.-Q., & El-Deiry, W. S. (2000). Molecular Determinants of Response to TRAIL in Killing of Normal and Cancer Cells. *Clinical Cancer Research*, *6*(2), 335–346.

Kim, A., Ueda, Y.,Naka, T. and Enomoto, T. (2012) Therapeutic strategies in epithelial ovarian cancer.. *Journal of Experimental & Clinical Cancer Research (17569966)*, *31*(1), 14-21.

Komatsu, K., Manabe, N., Kiso, M., Shimabe, M., & Miyamoto, H. (2003). Soluble Fas (FasB) regulates luteal cell apoptosis during luteolysis in murine ovaries. *Molecular Reproduction and Development*, *65*(4), 345–352.

Koopman, G., C. P. Reutelingsperger, G. A. Kuijten, R. M. Keehnen, S. T. Pals, and M. H. van Oers. "Annexin V for Flow Cytometric Detection of Phosphatidylserine Expression on B Cells Undergoing Apoptosis." *Blood* 84, no. 5 (September 1, 1994): 1415–20.

Kupryjanczyk, J., Thor, A. D., Beauchamp, R., Merritt, V., Edgerton, S. M., Bell, D. A., & Yandell, D. W. (1993). p53 gene mutations and protein accumulation in human ovarian cancer. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(11), 4961–4965.

Kurman, R. J., & Shih, I.-M. (2008). Pathogenesis of ovarian cancer. Lessons from morphology and molecular biology and their clinical implications. *International journal of gynecological pathology: official journal of the International Society of Gynecological Pathologists*, *27*(2).

La Vecchia, C. (2001). Epidemiology of ovarian cancer: a summary review. *European Journal of*

Cancer Prevention, *10*(2), 125–129.

Landen, C. N., Lin, Y. G., Immaneni, A., Deavers, M. T., Merritt, W. M., Spannuth, W. A., … Sood, A. K. (2007). Overexpression of the Centrosomal Protein Aurora-A Kinase is Associated with Poor Prognosis in Epithelial Ovarian Cancer Patients. *Clinical Cancer Research*, *13*(14), 4098–4104.

Levitzki, A., & Gazit, A. (1995). Tyrosine kinase inhibition: an approach to drug development. *Science*, *267*(5205), 1782–1788.

Lin, Y. G., Immaneni, A., Merritt, W. M., Mangala, L. S., Kim, S. W., Shahzad, M. M., Tsang, Y. T. M., Armaiz-Pena, G. N., Lu, C., Kamat, A. A., Han, L. Y., Spannuth, W. A., Nick, A. M., Landen, C. N., Wong, K. K., Gray, M. J., Coleman, R. L., Bodurka, D. C., Brinkley, W. R., & Kamat, A. A. (2008). Targeting aurora kinase with MK-0457 inhibits ovarian cancer growth. *Clinical Cancer Research*, *14*(17), 5437–5446.Lord, C. J., & Ashworth, A. (2008). Targeted therapy for cancer using PARP inhibitors. *Current opinion in pharmacology*, *8*(4), 363–369.

Lingle, W. L., Lutz, W. H., Ingle, J. N., Maihle, N. J., & Salisbury, J. L. (1998). Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. *Proceedings of the National Academy of Sciences*, *95*(6), 2950–2955.

Lissat, A., Vraetz, T., Tsokos, M., Klein, R., Braun, M., Koutelia, N., Fisch, P., Romero, M.,Long, L., Noellke, P., Mackall, C., Niemeyer, C., & Kontny, U. (2007). Interferon-gamma sensitizes resistant Ewing's sarcoma cells to tumor necrosis factor apoptosis-inducing ligand-induced apoptosis by up-regulation of caspase-8 without altering chemosensitivity. *The American Journal of Pathology*, *170*(6), 1917–1930.

Lord, C. J., & Ashworth, A. (2008). Targeted therapy for cancer using PARP inhibitors. *Current Opinion in Pharmacology*, *8*(4), 363–369.

Lowe, Scott W., and Athena W. Lin. "Apoptosis in cancer." *Carcinogenesis*21.3 (2000): 485-

495.

Luo, J., Xiao, J., Tao, Z., & Li, X. (1997). Detection of c-myc gene expression in nasopharyngeal carcinoma by nonradioactive in situ hybridization and immunohistochemistry. *Chinese Medical Journal*, *110*(3), 229–232.

Mabuchi, S., Altomare, D. A., Cheung, M., Zhang, L., Poulikakos, P. I., Hensley, H. H. Sclider, R.J., Ozols, R. F., &, Testa, J. R. (2007). RAD001 Inhibits Human Ovarian Cancer Cell Proliferation, Enhances Cisplatin-Induced Apoptosis, and Prolongs Survival in an Ovarian Cancer Model. *Clinical Cancer Research*, *13*(14), 4261–4270.

Mahmood, T., & Yang, P.-C. (2012). Western Blot: Technique, Theory, and Trouble Shooting. *North American Journal of Medical Sciences*, *4*(9), 429–434.

Maier, T., Güell, M., & Serrano, L. (2009). Correlation of mRNA and protein in complex biological samples. *FEBS letters*, *583*(24), 3966-3973

Manabe, N., Matsuda-Minehata, F., Goto, Y., Maeda, A., Cheng, Y., Nakagawa, S., … Li, J. (2008). Role of Cell Death Ligand and Receptor System on Regulation of Follicular Atresia in Pig Ovaries. *Reproduction in Domestic Animals*, *43*, 268–272.

Markman, M., Bundy, B. N., Alberts, D. S., Fowler, J. M., Clark-Pearson, D. L., Carson, L. F., ... & Sickel, J. (2001). Phase III trial of standard-dose intravenous cisplatin plus paclitaxel versus moderately high-dose carboplatin followed by intravenous paclitaxel and intraperitoneal cisplatin in small-volume stage III ovarian carcinoma: an intergroup study of the Gynecologic Oncology Group, Southwestern Oncology Group, and Eastern Cooperative Oncology Group. *Journal of Clinical Oncology*, *19*(4), 1001-1007.

Matsumoto, K., Nakayama, T., Sakai, H., Tanemura, K., Osuga, H., Sato, E., & Ikeda, J. (1999). Neuronal apoptosis inhibitory protein (NAIP) may enhance the survival of granulosa cells thus indirectly affecting oocyte survival. *Molecular Reproduction and Development*, *54*(2), 103–111.

Mbita, Z. (2012). *Molecular analysis of the domain with no name (DWNN)/RBBP6 in human cancers* (Doctoral dissertation, University of the Witwatersrand).

McCracken, J. A., Custer, E. E., & Lamsa, J. C. (1999). Luteolysis: a neuroendocrine-mediated event. *Physiological reviews*, *79*(2), 263–323.

Metzstein, M. M., Stanfield, G. M., & Horvitz, H. R. (1998). Genetics of programmed cell death in C. elegans: past, present and future. *Trends in Genetics*, *14*(10), 410–416.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., & Moll, U. M. (2003). p53 has a direct apoptogenic role at the mitochondria.*Molecular cell*, *11*(3), 577-590.

Miotto, B., Chibi, M., Xie, P., Koundrioukoff, S., Moolman-Smook, H., Pugh, D., ... & Defossez, P. A. (2014). The RBBP6/ZBTB38/MCM10 Axis Regulates DNA Replication and Common Fragile Site Stability. *Cell reports*, *7*(2), 575-587.

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* 219, no. 8 (August 2014): 593–601.

Moeljono, M., Thatcher, W., Bazer, F. W., Frank, M., Owens, L. J., & Wilcox, C. (1977). A study of prostaglandin F2alpha as the luteolysin in swine: II Characterization and comparison of prostaglandin F, estrogens and progestin concentrations in utero-ovarian vein plasma of nonpregnant and pregnant gilts. *Prostaglandins*, *14*(3), 543–555.

Moll, U. M., Wolff, S., Speidel, D., & Deppert, W. (2005). Transcription-independent pro-apoptotic functions of p53. *Current opinion in cell biology*,*17*(6), 631-636.

Momand, J., Wu, H. H., & Dasgupta, G. (2000). MDM2—master regulator of the p53 tumor suppressor protein. *Gene*, *242*(1), 15-29

Monniaux, D. (2002). Oocyte apoptosis and evolution of ovarian reserve. *Gynécologie, obstétrique & fertilité*, *30*(10), 822–826.

Motadi, L. R., Bhoola, K. D., & Dlamini, Z. (2011). Expression and function of retinoblastoma binding protein 6 (RBBP6) in human lung cancer. *Immunobiology, 216*(10), 1065-1073.

Murdoch, W. J. (1995). Programmed cell death in preovulatory ovine follicles. *Biology of reproduction, 53*(1), 8–12.

Murdoch, W. J., & McDonnel, A. C. (2002). Roles of the ovarian surface epithelium in ovulation and carcinogenesis. *Reproduction, 123*(6), 743–750.

Murdoch, W. J., Townsend, R. S., & McDonnel, A. C. (2001). Ovulation-induced DNA damage in ovarian surface epithelial cells of ewes: prospective regulatory mechanisms of repair/survival and apoptosis. *Biology of reproduction, 65*(5), 1417–1424.

Muthukumaran, N., Miletti-González, K. E., Ravindranath, A. K., & Rodríguez-Rodríguez, L. (2006). Tumor necrosis factor-alpha differentially modulates CD44 expression in ovarian cancer cells. *Molecular Cancer Research: MCR*, *4*(8), 511–520.

Niimi, S., Nakagawa, K., Sugimoto, Y., Nishio, K., Fujiwara, Y., Yokoyama, S., ... & Saijo, N. (1992). Mechanism of cross-resistance to a camptothecin analogue (CPT-11) in a human ovarian cancer cell line selected by cisplatin.*Cancer research, 52*(2), 328-333.

Nimeiri, H. S., Oza, A. M., Morgan, R. J., Friberg, G., Kasza, K., Faoro, L., Salgia, R., Stadler, W. M., Vokes, E. E., Fleming, G. F. (2008). Efficacy and safety of bevacizumab plus erlotinib for patients with recurrent ovarian, primary peritoneal, and fallopian tube cancer: a trial of the Chicago, PMH, and California Phase II Consortia. *Gynecologic oncology*, *110*(1), 49–55.

Nishimura, M., Jung, E. J., Shah, M. Y., Lu, C., Spizzo, R., Shimizu, M., ... & Calin, G. A. (2013). Therapeutic synergy between microRNA and siRNA in ovarian cancer treatment. *Cancer discovery*, *3*(11), 1302-1315.

O'Connell, J., Bennett, M. W., Nally, K., O'Sullivan, G. C., Collins, J. K., & Shanahan, F. (2000). Interferon-gamma sensitizes colonic epithelial cell lines to physiological and

therapeutic inducers of colonocyte apoptosis. *Journal of Cellular Physiology*, *185*(3), 331–338.

Ohtani, K., Sakamoto, H., Kikuchi, A., Nakayama, Y., Idei, T., Igarashi, N., Matukawa, T., & Satoh, K. (2001a). Follicle-stimulating hormone promotes the growth of human epithelial ovarian cancer cells through the protein kinase C-mediated system. *Cancer letters*, *166*(2), 207–213.

Okamoto, I., Doi, T., Ohtsu, A., Miyazaki, M., Tsuya, A., Kurei, K., Kobayashi, K., & Nakagawa, K. (2010). Phase I Clinical and Pharmacokinetic Study of RAD001 (Everolimus) Administered Daily to Japanese Patients with Advanced Solid Tumors. *Japanese Journal of Clinical Oncology*, *40*(1), 17–23.

Otani, H., Yamoto, M., Fujinaga, H., & Nakano, R. (1996). Presence and localization of endothelin receptor in the rat ovary and its regulation by pituitary gonadotropins. *European Journal of Endocrinology*, *135*(4), 449–454.

Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J., & Dixit, V. M. (1997). The receptor for the cytotoxic ligand TRAIL. *Science*, *276*(5309), 111–113.

Park, U. S., Park, S. K., Lee, Y. I., & Park, J. G. (2000). Hepatitis B virus-X protein upregulates the expression of p21waf1/cip1 and prolongs G1--> S transition via a p53-independent pathway in human hepatoma cells. *Oncogene*,*19*(30), 3384-3394.

Parkin, D. M., Sitas, F., Chirenje, M., Stein, L., Abratt, R., & Wabinga, H. (2008). Part I: Cancer in Indigenous Africans—burden, distribution, and trends. *Lancet Oncology*, *9*(7), 683–692.

Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H. L., and Poethig, R. S. (2004) SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of transacting siRNAs in Arabidopsis. Genes Dev. 18, 2368−2379.

Perez, G. I., Robles, R., Knudson, C. M., Flaws, J. A., Korsmeyer, S. J., & Tilly, J. L. (1999). Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency.

Nature Genetics, *21*(2), 200–203.

Petroff, M. G., Petroff, B. K., & Pate, J. L. (2001). Mechanisms of cytokine-induced death of cultured bovine luteal cells. *Reproduction*, *121*(5), 753–760.

Petrucci, E., Pasquini, L., Bernabei, M., Saulle, E., Biffoni, M., Accarpio, F., Simone, S., Angelo, D., Volante, D., Assunta, C., Pierliugi, B., & Testa, U. (2012). A Small Molecule SMAC Mimic LBW242 Potentiates TRAIL- and Anticancer Drug-Mediated Cell Death of Ovarian Cancer Cells. *PLoS ONE*, *7*(4), e35073.

Picksley, S. M., & Lane, D. P. (1993). What the papers say: The p53-mdm2 autoregulatory feedback loop: A paradigm for the regulation of growth control by p53? *BioEssays*, *15*(10), 689-690.

Prenzel, N., Fischer, O., Streit, S., Hart, S., & Ullrich, A. (2001). The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocrine-related cancer*, *8*(1), 11–31.

Pugh, D. J., Ab, E., Faro, A., Lutya, P. T., Hoffmann, E., Rees, D. J., 2006. DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. *BMC Struct. Biol*. 6, 1.

Quirk, S. M., Cowan, R. G., Joshi, S. G., & Henrikson, K. P. (1995). Fas antigen-mediated apoptosis in human granulosa/luteal cells. *Biology of Reproduction*, *52*(2), 279–287.

Raisova, M., Hossini, A. M., Eberle, J., Riebeling, C., Wieder, T., Sturm, I., ... & Geilen, C. C. (2001). The Bax/Bcl-2 ratio determines the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis. *Journal of investigative dermatology*, *117*(2), 333-340.

Reardon, David A., et al. "Randomized phase II study of cilengitide, an integrin-targeting arginine-glycine-aspartic acid peptide, in recurrent glioblastoma multiforme." *Journal of Clinical Oncology* 26.34 (2008): 5610-5617.

Reed, J. C. (2000). Mechanisms of Apoptosis. *The American Journal of Pathology*, *157*(5),

1415–1430.

Reed, J. C. (2002). Apoptosis-based therapies. *Nature reviews Drug discovery*, *1*(2), 111–121.

Renehan, A. G., Booth, C., & Potten. (2001). What is apoptosis, and why is it important? *BMJ: British Medical Journal (International Edition)*, *322*(7301), 1536–1538.

Ross, J. S., Schenkein, D. P., Pietrusko, R., Rolfe, M., Linette, G. P., Stec, J., Stagliano, N. E., Ginsburg, G. S., Symmans, W. F., Pusztai, L., & Hortobagyi, G. N. (2004). Targeted Therapies for Cancer 2004. *American Journal of Clinical Pathology*, *122*(4), 598–609.

Rothenberg, M. L. (1997). Topoisomerase I inhibitors: review and update.*Annals of Oncology*, *8*(9), 837-855.

Rowinsky, E. K., & Donehower, R. C. (1995). Paclitaxel (Paclitaxel). *New England Journal of Medicine*, *332*(15), 1004–1014.

Rubin, S. C., Blackwood, M. A., Bandera, C., Behbakht, K., Benjamin, I., Rebbeck, T. R., & Boyd, J. (1998). BRCA1, BRCA2, and hereditary nonpolyposis colorectal cancer gene mutations in an unselected ovarian cancer population: relationship to family history and implications for genetic testing. *American Journal of Obstetrics and Gynecology*, *178*(4), 670–677.

Saijo M., Sakai Y., Kishino T., Niikawa N., Matsuura Y., Morino K., Tamai K., Taya Y. (1995) Molecular cloning of a human protein which binds to the retinoblastoma protein and chromosomal mapping. Genomics 27, 511-519

Sakai, Y., Saijo, M., Coelho, K., Kishino, T., Niikawa, N., & Taya, Y. (1995). cDNA sequence and chromosomal localization of a novel human protein, RBQ-1 (RBBP6), that binds to the retinoblastoma gene product. *Genomics*, *30*(1), 98–101. doi:10.1006/geno.1995.0017

Sankaranarayanan, R., & Ferlay, J. (2006). Worldwide burden of gynaecological cancer: the size of the problem. *Best Practice & Research Clinical Obstetrics & Gynaecology*, *20*(2), 207–225.

Sansal, I., & Sellers, W. R. (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. *Journal of Clinical Oncology*, *22*(14), 2954–2963.

Scott, R. E., Giannakouros, T., Gao, S., & Peidis, P. (2003). Functional potential of P2P-R: A role in the cell cycle and cell differentiation related to its interactions with proteins that bind to matrix associated regions of DNA?.*Journal of cellular biochemistry*, *90*(1), 6-12.

Scott, F. L., Denault, J.-B., Riedl, S. J., Shin, H., Renatus, M., & Salvesen, G. S. (2005). XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. *The EMBO Journal*, *24*(3), 645–655.

Santos, H.B., Sato, Moro, L.Y. Bazzoli, N. and Rizzo, E. (2008). Relationship among follicular apoptosis, integrin beta1 and collagen type IV during early ovarian regression in the teleost *Prochilodus argenteus* after induced spawning. *Cell and Tissue Research*, 332(1): 159-170.

Schildkraut, J. M., & Thompson, W. D. (1988). Familial Ovarian Cancer: A Population-based Case-Control Study. *American Journal of Epidemiology*, *128*(3), 456–466.

Schilder, R. J., Sill, M. W., Chen, X., Darcy, K. M., Decesare, S. L., Lewandowski, G., Lee, R. B., Arciero, C. A., Wu, H., & Godwin, A. K. (2005). Phase II Study of Gefitinib in Patients with Relapsed or Persistent Ovarian or Primary Peritoneal Carcinoma and Evaluation of Epidermal Growth Factor Receptor Mutations and Immunohistochemical Expression: A Gynecologic Oncology Group Study. *Clinical Cancer Research*, *11*(15), 5539–5548.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. *Cell*, *103*(2), 211–225.

Schmitt, J., & Matei, D. (2008). Platelet-derived growth factor pathway inhibitors in ovarian cancer. *Clinical Ovarian Cancer*, *1*(2), 120–126.

Schwartz, L. M., Kosz, L., & Kay, B. K. (1990). Gene activation is required for developmentally programmed cell death. *Proceedings of the National Academy of Sciences*, *87*(17), 6594–6598.

Shao, R. G., Cao, C. X., Zhang, H., Kohn, K. W., Wold, M. S., & Pommier, Y. (1999). Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA: DNA-PK complexes. *The EMBO journal*, *18*(5), 1397-1406.

Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C.L., Baker, K., & Wood, W. I. (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science*, *277*(5327), 818–821.

Sherr, C. J., & Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & Development*, *13*(12), 1501–1512.

Sherr, C. J., & McCormick, F. (2002). The RB and p53 pathways in cancer. *Cancer Cell*, *2*(2), 103.

Shieh, S.-Y., Ikeda, M., Taya, Y., & Prives, C. (1997). DNA Damage-Induced Phosphorylation of p53 Alleviates Inhibition by MDM2. *Cell*, *91*(3), 325–334.

Simons, A., Melamed-Bessudo, C., Wolkowicz, R., Sperling, J., Sperling, R., Eisenbach, L., & Rotter, V. (1997). PACT: cloning and characterization of a cellular p53 binding protein that interacts with Rb. *Oncogene*, *14*(2), 145.

Singh, R. S. D., Tiwari, S., & Mohapatra, T. (n.d.). Targeted Therapies for Cancer treatment. *Journal of Pharmacy Research*, *4*.

Sirotnak, F. M., Zakowski, M. F., Miller, V. A., Scher, H. I., & Kris, M. G. (2000). Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clinical*

Cancer Research, *6*(12), 4885–4892.

Sirotnak, F. M. (2003). Studies with ZD1839 in preclinical models (Vol. 30, pp. 12–20). Presented at the Seminars in oncology, Elsevier.

Slichenmyer, W. J., & Fry, D. W. (2001). Anticancer therapy targeting the erbB family of receptor tyrosine kinases (Vol. 28, pp. 67–79). Presented at the Seminars in oncology, Elsevier.

Smith, T., & Guidozzi, F. (2009). Epithelial ovarian cancer in Southern Africa. *Southern African Journal of Gynaecological Oncology*, *1*(1), 23–27.

Spierings, D., McStay, G., Saleh, M., Bender, C., Chipuk, J., Maurer, U., & Green, D. R. (2005). Connected to death: The (unexpurgated) mitochondrial pathway of apoptosis. *Science*, *310*(5745), 66-67.

Sugino, N., Suzuki, T., Kashida, S., Karube, A., Takiguchi, S., & Kato, H. (2000). Expression of Bcl-2 and Bax in the human corpus luteum during the menstrual cycle and in early pregnancy: regulation by human chorionic gonadotropin. *The Journal of Clinical Endocrinology and Metabolism*, *85*(11), 4379–4386.

Sun, C., Chan, F., Briassouli, P., & Linardopoulos, S. (2007). Aurora kinase inhibition downregulates NF-κB and sensitises tumour cells to chemotherapeutic agents. *Biochemical and Biophysical Research Communications*, *352*(1), 220–225.

Survival rates for ovarian cancer, by stage. (n.d.). Retrieved April 29, 2014, from http://www.cancer.org/cancer/ovariancancer/detailedguide/ovarian-cancer-survival-rates

Svane, I. M., Pedersen, A. E., Johnsen, H. E., Nielsen, D., Kamby, C., Gaarsdal, E., Nikolajsen , K., Buus, S., & Claesson, M. H. (2004). Vaccination with p53-peptide-pulsed dendritic cells, of patients with advanced breast cancer: report from a phase I study. *Cancer Immunology, Immunotherapy: CII*, *53*(7), 633–641.

Swisher, E. M., Mutch, D. G., Rader, J. S., Elbendary, A., & Herzog, T. J. (1997). Topotecan in platinum-and paclitaxel-resistant ovarian cancer.*Gynecologic oncology*, *66*(3), 480-486.

Thatte, U., & Dahanukar, S. (1997). Apoptosis. *Drugs*, *54*(4), 511–532.

Tilly, J. L., Tilly, K. I., Kenton, M. L., & Johnson, A. L. (1995). Expression of members of the bcl-2 gene family in the immature rat ovary: equine chorionic gonadotropin-mediated inhibition of granulosa cell apoptosis is associated with decreased bax and constitutive bcl-2 and bcl-xlong messenger ribonucleic acid levels. *Endocrinology*, *136*(1), 232–241.

Tilly, K. I., Banerjee, S., Banerjee, P. P., & Tilly, J. L. (1995). Expression of the p53 and Wilms' tumor suppressor genes in the rat ovary: gonadotropin repression in vivo and immunohistochemical localization of nuclear p53 protein to apoptotic granulosa cells of atretic follicles. *Endocrinology*, *136*(4), 1394–1402.

Tilly, J. L. (1996). Apoptosis and ovarian function. *Reviews of reproduction*, *1*(3), 162–172.

Tilly, J. L. (2001). Commuting the death sentence: how oocytes strive to survive. *Nature Reviews Molecular Cell Biology*, *2*(11), 838–848.

Traynor, A. M., Hewitt, M., Liu, G., Flaherty, K. T., Clark, J., Freedman, S. J., Scott, B. B., Leighton, A. M., Watson, P. A., Zao, B., O'Dwyer, P. A., & Wilding, G. (2011). Phase I dose escalation study of MK-0457, a novel Aurora kinase inhibitor, in adult patients with advanced solid tumors. *Cancer Chemotherapy and Pharmacology*, *67*(2), 305–314.

Tutt, A., Lord, C., McCabe, N., Farmer, H., Turner, N., Martin, N., Jackson, S. P., Smith, J. C., & Ashworth, A. (2005). Exploiting the DNA repair defect in BRCA mutant cells in the design of new therapeutic strategies for cancer. (Vol. 70, p. 139). Presented at the Cold Spring Harbor Symposia on Quantitative Biology.

Vaskivuo, T. E., Anttonen, M., Herva, R., Billig, H., Dorland, M., te Velde, E. R., … Tapanainen, J. S. (2001). Survival of Human Ovarian Follicles from Fetal to Adult Life: Apoptosis, Apoptosis-Related Proteins, and Transcription Factor GATA-4 1. *Journal of*

Clinical Endocrinology & Metabolism, *86*(7), 3421–3429.

Vaskivuo, T. E., Ottander, U., Oduwole, O., Isomaa, V., Vihko, P., Olofsson, J. I., & Tapanainen, J. S. (2002). Role of apoptosis, apoptosis-related factors and 17β-hydroxysteroid dehydrogenases in human corpus luteum regression. *Molecular and cellular endocrinology*, *194*(1), 191–200.

Vaskivuo, T. E., & Tapanainen, J. S. (2003). Apoptosis in the human ovary. *Reproductive biomedicine online*, *6*(1), 24–35.

Veitch, N. C. (2004). Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry*, *65*(3), 249–259.

Vergote, I. B., Jimeno, A., Joly, F., Katsaros, D., Coens, C., Despierre, E., Marth, C., Hall, M., Steer, C. B., Colombo, N., Lesoin, A., Casado, A., Reinthaller, A., Green, J., Buck, M., Ray-Coquard, I., Ferrero, A., Favier, L., Reed, N. S., Curé, H., & Pujade-Lauraine, E. (2013). Randomized Phase III Study of Erlotinib Versus Observation in Patients With No Evidence of Disease Progression After First-Line Platin-Based Chemotherapy for Ovarian Carcinoma: A European Organisation for Research and Treatment of Cancer-Gynaecological Cancer Group, and Gynecologic Cancer Intergroup Study. *Journal of Clinical Oncology*, JCO.2013.50.5669.

Vikhanskaya, F., Vignati, S., Beccaglia, P., Ottoboni, C., Russo, P., D'Incalci, M., & Broggini, M. (1998). Inactivation of p53 in a human ovarian cancer cell line increases the sensitivity to paclitaxel by inducing G2/M arrest and apoptosis. *Experimental cell research*, *241*(1), 96-101.

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews Genetics*, *13*(4), 227-232.

Vucic, D., & Fairbrother, W. J. (2007). The inhibitor of apoptosis proteins as therapeutic targets in cancer. *Clinical Cancer Research: An Official Journal of the American Association for*

Cancer Research, *13*(20), 5995–6000.Wall, L., Burke, F., Barton, C., Smyth, J., & Balkwill, F. (2003). IFN-γ Induces Apoptosis in Ovarian Cancer Cells in Vivo and in Vitro. *Clinical Cancer Research*, *9*(7), 2487–2496.

Walker, N., Harmon, B., Gobe, G., & Kerr, J. (1987). Patterns of cell death. *Methods and Achievements in Experimental Pathology*, *13*, 18–54.

Waldman, T., Kinzler, K. W., & Vogelstein, B. (1995). p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Research*, *55*(22), 5187–5190.

Wang, J. C. (1985). DNA topoisomerases. *Annual Review of Biochemistry*, *54*(1), 665–697.Wang, L., Du, F., & Wang, X. (2008). TNF-α Induces Two Distinct Caspase-8 Activation Pathways. *Cell*, *133*(4), 693–703.

Wei, Y., Fan, T., & Yu, M. (2008). Inhibitor of apoptosis proteins and apoptosis. *Acta Biochimica Et Biophysica Sinica*, *40*(4), 278–288.

Wen, S. F., Mahavni, V., Quijano, E., Shinoda, J., Grace, M., Musco-Hobkinson, M. L., … Buller, R. (2003). Assessment of p53 gene transfer and biological activities in a clinical study of adenovirus-p53 gene therapy for recurrent ovarian cancer. *Cancer Gene Therapy*, *10*(3), 224–238.Werner, H., Weinstein, D., & Bentov, I. (2008). Similarities and differences between insulin and IGF-I: Structures, receptors, and signaling pathways. *Archives of Physiology & Biochemistry*, *114*(1), 17–22.

World Health Organisation (WHO). (2002). National Cancer Control Programmes. Policies and Managerial Guidelines, 2nd Edition.

Retrieved from http://www.who.int/cancer/media/en/408.pdf

Wiener, J. R., Windham, T. C., Estrella, V. C., Parikh, N. U., Thall, P. F., Deavers, M. T., … Gallick, G. E. (2003). Activated Src Protein Tyrosine Kinase Is Overexpressed in Late-Stage Human Ovarian Cancers. *Gynecologic Oncology*, *88*(1), 73–79.

Witham, J., Valenti, M. R., De-Haven-Brandon, A. K., Vidot, S., Eccles, S. A., Kaye, S. B., & Richardson, A. (2007). The Bcl-2/Bcl-XL family inhibitor ABT-737 sensitizes ovarian cancer cells to carboplatin. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *13*(23), 7191–7198.

Wu, G. S., Burns, T. F., McDonald, E. R., Jiang, W., Meng, R., Krantz, I. D., Kao, G., Gan, D.D., Zhou, J.Y., Muschel, R., Hamilton, S.R., Spinner, N.B., Markowitz, S., Wu, G., & El-Deiry, W. S. (1997). KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nature Genetics*, *17*(2), 141–143.

Wu, Y., Mehew, J. W., Heckman, C. A., Arcinas, M., & Boxer, L. M. (2001). Negative regulation of bcl-2 expression by p53 in hematopoietic cells. *Oncogene*, *20*(2), 240–251.

Wu, H.-C., Chang, D.-K., & Huang, C.-T. (2006). Targeted-therapy for cancer. *J Cancer Mol*, *2*(2), 57–66.

Xiao, C.W., Ash, K., Tsang, B.K., 2001. Nuclear factor-B-mediated X-linked inhibitor of apoptosis protein expression prevents rat granulosa cells from tumor necrosis factor-induced apoptosis. Endocrinology 142, 557–563.

Yang, G., Thompson, J. A., Fang, B., & Liu, J. (2003). Silencing of H-ras gene expression by retrovirus-mediated siRNA decreases transformation efficiency and tumorgrowth in a model of human ovarian cancer. *Oncogene*, *22*(36), 5694-5701.

Yang, H., He, L., Kruk, P., Nicosia, S. V., & Cheng, J. Q. (2006). Aurora-A induces cell survival and chemoresistance by activation of Akt through a p53-dependent manner in ovarian cancer cells. *International Journal of Cancer*, *119*(10), 2304–2312.

Yoshitake, Y., Nakatsura, T., Monji, M., Senju, S., Matsuyoshi, H., Tsukamoto, H., . . . Nishimura, Y. (2004). Proliferation potential-related protein, an ideal esophageal cancer antigen for immunotherapy, identified using complementary DNA microarray analysis. *Clinical Cancer Research*, *10*(19), 6437-6448.

Zamorano, P., Mahesh, V., & Brann, D. (1996). Quantitative RT-PCR for neuroendocrine studies. *Neuroendocrinology*, *63*(5), 397–407.

Zhang, Z., Jia, L., Feng, Y., & Zheng, W. (2009). Overexpression of follicle-stimulating hormone receptor facilitates the development of ovarian epithelial cancer. *Cancer letters*, *278*(1), 56–64.

Zhang, Z., Liao, H., Chen, X., Zheng, Y., Liu, Y., Tao, X., & Yang, Y. (2011). Luteinizing hormone upregulates survivin and inhibits apoptosis in ovarian epithelial tumors. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *155*(1), 69–74.

Zhou, H., Kuang, J., Zhong, L., Kuo, W., Gray, J., Sahin, A., Brinkley, B., & Sen, S. (1998). Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. *Nature Genetics*, *20*(2), 189–193.