The Role and Regulation of p53-associated, Parkin-like Cytoplasmic Protein (PARC)

in p53 Subcellular Trafficking and Chemosensitivity in Human Ovarian Cancer Cells

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A thesis submitted to the Faculty of Graduate and
Postdoctoral Studies, University of Ottawa,
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Resistance to cisplatin (CDDP)-based therapy is a major hurdle to the successful treatment of human ovarian cancer (OVCA) and the chemoresistant phenotype in OVCA cells is associated with Akt-attenuated, p53-mediated apoptosis. Pro-apoptotic functions of p53 involve both transcription-dependent and -independent signaling pathways and dysfunctional localization and/or inactivation of p53 contribute to the development of chemoresistance. PARC is a cytoplasmic protein regulating p53 subcellular localization and subsequent function. Little is known about the molecular mechanisms regulating PARC. Although PARC contains putative caspase-3 cleavage sites, and CDDP is known to induce the activation of caspases and calpains and induce proteasomal degradation of anti-apoptotic proteins, if and how PARC is regulated by CDDP in OVCA is unknown. Here we present evidence that CDDP promotes calpain-mediated PARC down-regulation, mitochondrial and nuclear p53 accumulation and apoptosis in chemosensitive but not resistant OVCA cells. Inhibition of Akt is required to sensitize chemoresistant cells to CDDP in a p53-dependent manner, an effect enhanced by PARC down-regulation. CDDP-induced PARC down-regulation is reversible by inhibitor of calpain but not of caspase-3 or the 26S proteasome. Furthermore, *in vitro* experiments confirm the ability of calpain in mediating Ca^{2+}-dependent PARC down-regulation. The role of Ca^{2+} in PARC down-regulation was further confirmed as ionomycin induced PARC down-regulation in both chemosensitive and chemoresistant ovarian cancer cells. The data presented here implicates the regulation of p53 subcellular localization and apoptosis by PARC as a contributing factor in CDDP resistance in OVCA cells and Ca^{2+}/calpain in PARC post-translational processing and chemosensitivity.

THESIS FORMAT

The current thesis is written in the "Classical thesis" format as outlined in the guidelines provided by the Faculty of Graduate and Postdoctoral Studies and the Department of Cellular and Molecular Medicine, University of Ottawa. The main body is divided as follows:

Chapter 1 (Introduction) provides a critical review of the literature and knowledge gaps in the field, providing the rationale for work performed in this thesis. Main topics examined are: 1) ovarian cancer and chemoresistance, 2) apoptosis and p53, 3) PI3K/Akt in chemoresistance and 4) role and regulation of PARC in chemosensitivity; and how these topics are related.

Chapter 2 (Objectives and Hypotheses) provides the overall and specific objectives for the experiments conducted in this thesis to investigate the validity of our hypothetical model.

Chapter 3 (Materials and Methods) describes the reagents and experimental procedures used to investigate the proposed hypotheses and to achieve the objectives of this thesis.

Chapter 4 (Results) presents the findings of the current thesis. The results demonstrate that cisplatin promotes calpain-mediated PARC down-regulation, mitochondrial and nuclear p53 accumulation and apoptosis in chemosensitive but not resistant ovarian cancer cells. Inhibition of Akt is required to sensitize chemoresistant cells to CDDP in a p53-dependent manner, an effect enhanced by PARC down-regulation.

Chapter 5 (Discussion) consists of in depth analysis of the results presented in

the current thesis, emphasizing the contributions of the findings to the field of study. This chapter also discusses experimental limitations and shortcomings of results and possible questions that may be addressed in future experiments.

Chapter 6 (References) provides source information of ideas and results cited in the present thesis.

Chapter 7 (Appendices) contains additional results that are not fundamental to addressing the hypotheses proposed in the current thesis, but were required to address related questions.

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Table 1. Supplemental information on experimental cell lines examined, including the characterization of tumour of origin, endogenous p53 status and description of tumour formation from intraperitoneal xenograft models.

LIST OF ABBREVIATIONS

AIF	Apoptosis Inducing Factor
ANOVA	Analysis of Variance
APAF-1	Apoptosis Protease Activating Factor 1
API-2	Akt Pathway Inhibitor-2
ATM	Ataxia Telangiectasia-Mutated
ATP	Adenosine Triphosphate
ATR	ATM and Rad3-Related
Bad	Bcl-2-Antagonist of Cell Death
Bax	Bcl-2-Associated X Protein
Bak	Bcl-2 homologous Antagonist/Killer
Bid	BH3 interacting-domain
BSA	Boivine Serum Albumin
BCL-2	B-Cell Lymphoma-2
BCL-XL	B-Cell Lymphoma-Extra Large
BH	Bcl-2 Homology
BID	BH3-Interacting Domain Death Agonist
Bp	Base Pair
BRCA1/2	Breast Cancer Type1/2 Susceptibility protein
CDDP	Cis-diamminedichloroplatinum (II)
Ca^{2+}	Calcium Ion
cAMP	3'-5'-cyclic Adenosine Monophosphate
CCH	C-terminal Cullin Homology
CHK 1/2	Checkpoint Kinase 1, 2
Cl^{-}	Chloride Ion
CTR1	Copper Transporter 1
CUL	Cullin
DAPI	4',6-Diamidino-2-Phenylindole
DBD	DNA-Binding Domain
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
DTT	Dithiothreitol
DMEM	Dulbeco's Modified Eagle Medium
DMSO	Dimethysulfoxide

DNA	Deoxyribonucleic Acid
DN-Akt	Dominant-Negative Akt
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-tetraacetic acid
EGF	Epidermal Growth Factor
ERK	Extracellular Signal-Related Kinase
FasL	Fas Ligand
FIGO	International Federation of Gynecology and Obstetrics
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GSH	Glutathione
GSK-3β	Glycogen Synthase Kinase-3 Beta
GSTP1	Gene encoding the Glutathione S-transferase P enzyme
HA	Hemagglutinin
HGSOC	High-Grade Serous Ovarian Carcinoma
HRP	Horseradish Peroxidase
Hsp	Heat-Shock Protein
IBR	In-Between RING
ICAD	Inhibitor of Caspase-Activated DNase
IgG	Immunoglobulin G
ILK	Integrin-Linked Kinase
IOSE	Immortalized Ovarian Surface Epithelium
IF	Immunofluorescence
IP	Immunoprecipitation
KDa	Kilo-Dalton
Lys	Lysine
MDM2	Murine Double-Minute-2
MEF	Mouse Embryonic Fibroblast
MOI	Multiplicity of Infection
MMP	Mitochondrial Membrane Potential
MOMP	Mitochondrial Outer Membrane Permeabilization
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NO	Nitric Oxide
ORF	Open Reading Frame
OS	Overall Survival

OVCA	Ovarian Cancer
PAGE	Polyacrylamide Gel Electrophoresis
PARC	p53-Associated, Parkin-like Cytoplasmic Protein
PARP	Poly(Adenosine Disphosphate) Polymerase
PBS	Phosphate-Buffered Saline
PBS-T	PBS-Tween
PDK-1	Phosphotidylinositol-Dependent Protein Kinase-1
PDL	Poly-D-Lysine
PFS	Progression-Free Survival
PH	Pleckstrin Homology
PI3K	Phosphoinositol 3-OH Kinase
PI3KCA	Gene that codes for PI3K
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PIPES	Piperazinediethanesulfonic Acid
PKB	Protein Kinase B
PMSF	Phenylmethylsulfonyl Fluoride
PTEN	Phosphatase and Tensin Homolog
PUMA	p53-Upregulated Mediator of Apoptosis
RING	Really Interesting New Gene
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCF	Skp-Cullin-Fbox
SCOTROC1	Scottish Randomized Trial in Ovarian Cancer 1
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
Ser	Serine
siRNA	Small Interfering RNA
SMAC	Second Mitochondria-Derived Activator of Caspases
TAE	Tris-Acetate-EDTA
TBS	Tris-Buffered Saline
TBS-T	TBS-Tween
Thr	Threonine

TNFR	Tumour Necrosis Factor Receptor
TOM-20	Translocase of the Outer Mitochondrial Membrane 20
TRADD	TNFR-1-Associated Death Domain
UPP	Ubiquitin Proteasome Pathway
WB	Western Blot
WCL	Whole Cell Lysate
XIAP	X-Linked Inhibitor of Apoptosis Protein

DEDICATION

This thesis is dedicated to my wonderful parents, Jim and Kitty Woo.

AKNOWLEDGEMENTS

I would first like to thank Dr. Benjamin Tsang for his friendship and mentorship throughout my Ph.D. training. Through his shared wisdom, I have learned much about science and research. He has helped me develop the skills to ask the right questions and has provided me with the tools to find the answers. For that I will always be grateful.

I would also like to thank the past and present members of the Tsang Laboratory for their support and encouragement. The friendships developed during this time have made everything easier, both inside and outside of the lab.

I would like to thank the members of my Ph.D. Advisory Committee, Drs. Michael Schlossmacher, Ajoy Basak, and Jim Dimitroulakos, for their encouragement and guidance throughout my Ph.D. program.

I would like to thank my parents Jim and Kitty, and my sister Michelle, for all their love and support. They have taught me the values of honesty and hard work, values that are essential in science, research and life.

Finally, I would like to thank Michelle Brown, who is an inspiration to me, and whose love and encouragement gives me the strength to persevere.

CHAPTER 1: INTRODUCTION

1.1 HUMAN OVARIAN CANCER

1.1.1 Epidemiology

Ovarian cancer is the most lethal of all gynecologic malignancies in the developed world, and in Canada alone, there will be an estimated 2,600 new cases and 1,750 deaths resulting from this disease in 2011 (Canadian Cancer Society, 2012).

Ovarian cancer of epithelial origin is the most common type, accounting for 90% of ovarian tumours. The most common histological subtype, accounting for >50% of ovarian epithelial malignancies, is serous ovarian carcinoma. Due to poor screening methods and non-specific symptoms early in the disease, the majority of serous ovarian carcinomas are diagnosed at advanced stage with extra-pelvic metastasis [International Federation of Gynecology and Obstetrics (FIGO) stage III/IV]. In addition to late diagnosis, the development of drug resistance in tumours has contributed to a high mortality rate [5-year overall survival rate of only 15%-25% (Jemal et al, 2011)] that has remained relatively unchanged since the 1970s.

1.1.2 Risk factors

The two greatest risk factors for developing ovarian cancer are age and family history. Risk increases sharply in post-menopausal women with almost 85% of all diagnosed cases occurring in woman over the age of 50. Women younger than 30 have a remote risk, even in those with a family history (Amos & Struewing, 1993).

The evidence that suggests a possible genetic basis for this disease comes in the form of increased incidence of ovarian cancer in women with a family history. Based on a large meta-analysis study, the risk of ovarian cancer associated with at least one first-degree relative with ovarian cancer provided an odds ratio of 3.1 (Stratton et al, 1998). In addition, families with multiple affected members show a pattern compatible to an autosomal dominant inheritance disorder. Linkage analyses have identified several highly penetrant genes that are associated with the development of ovarian cancer in many families. Some of the most common hereditary cancer syndromes associated with ovarian cancer risk include: hereditary breast and ovarian cancer (HBOC) syndrome, Lynch syndrome, Peutz-Jeghers syndrome (PJS) and nevoid basal cell carcinoma syndrome (NBCCS; Gorlin syndrome) (American Society of Clinical Oncology, 2011). Of note, sporadic mutations in genes associated with these disorders are rare and account for no more than 5%-10% of ovarian cancer cases.

Other factors reported to increase risk include obesity, use of fertility drugs, androgens, and estrogen therapy and hormone therapy after menopause. Contrary to this, risk is decreased with increasing number of pregnancies, use of oral contraceptives, and a balanced diet low in fat, red meat and processed meats (American Cancer Society, 2012).

1.1.2 Surgical staging and treatment of ovarian cancer

According FIGO, prognosis of ovarian cancer is dependent on the initial stage at diagnosis. The surgical staging system of ovarian cancer is as follows:

Stage I – The earliest form, the tumour is confined to the ovary/ovaries.

Stage II – Tumour involves one or both ovaries with spread to other pelvic organs

or surfaces.

Stage III – The tumour involves one or both ovaries, with tumour spread outside the pelvis to the abdominal area, including metastases to the liver surface.

Stage IV-Metastases or spread to the liver or outside the peritoneal cavity to areas such as the chest or brain.

The standard treatment in advanced stage III/IV ovarian cancer consists of staging laparotomy and combined surgical debulking to minimize the disease followed by combination intravenous platinum/taxane based chemotherapy. The extent of cytoreductive surgery and the amount of residual disease are the most important factors impacting the survival of women with advanced ovarian cancer (Bristow & Chi, 2006; Griffiths, 1975).

Since ovarian tumours often remain localized to the peritoneal cavity during most of their development, the benefits of intraperitoneal chemotherapy treatment has been investigated for many years and a number of phase III prospective clinical trials have reported significant increases in progression-free and overall survival time with decreased drug-induced systemic toxicity for intraperitoneal chemotherapy over intravenous chemotherapy in cases of small volume residual disease (Rothenberg et al, 2003). However, despite these advantages, there is a reluctance to accept intraperitoneal chemotherapy as a standard modality in an outpatient setting. Placement of the intraperitoneal catheter requires expertise and this process is associated with complications such as infection and pain, bowel perforation and bleeding. Fibrin sheath formation may also develop and cause obstruction of fluid distribution and outflow (Willemse & de Vries, 2003).

In advanced ovarian cancer, where often there is upper abdominal disease and where patients may present with co-morbidities, the concept of interval cytoreduction and neoadjuvant therapy prior to surgical debulking has been investigated with the aim of reducing perioperative morbidity and mortality. Compared with the conventional treatment strategy of primary surgery followed by chemotherapy, interval cytoreduction showed no survival advantages (Vergote et al, 2010). Use of neoadjuvant chemotherapy did reduce postoperative mortality, sepsis and hemorrhage, but did not impact overall survival (OS) and progression-free survival (PFS) in women with advance stage disease (Vergote et al, 2010). It is possible that in a neoadjuvant setting, increased exposure to chemotherapy could result in residual tumour cells acquiring platinum resistance, leading to the lack of benefits to PFS and OS. This observation further emphasizes the importance of understanding the cellular and molecular mechanisms associated with the development of chemoresistance in ovarian cancer in order to develop new and more efficacious treatment strategies.

1.1.3 Histological subtypes

Ovarian carcinoma is a heterogeneous disease consisting of tumours with different histological subtypes that are associated with different genetic risk factors and molecular events during oncogenesis (Narod & Boyd, 2002). In addition, these subtypes differ in frequency, as early stage carcinomas consist mainly of non-serous carcinomas, while advanced stage carcinomas consist predominantly of the serous subtype.

Response to chemotherapy is also subtype specific. Clear cell carcinomas respond poorly to chemotherapy compared to high-grade serous, resulting in a lower 5-

year survival rate in patients with advanced stage tumours (du Bois et al, 2003; Takano et al, 2006). It is important not to treat ovarian cancer as a single entity, but to recognize that major differences exist between subtypes and this should be reflected in research designs and ultimately in the management of the disease (Kobel et al, 2008).

There are three main types of ovarian cancer. Each type is defined by where the cancer cells originated in the ovary.

1) Epithelial cell tumours are thought to start in the cells that cover the outer surface of the ovary.

2) Germ cell tumours start in the germ cells within the ovary and generally occur in younger women. Germ cell cancer can even develop in children.

3) Stromal tumours start in the connective tissue cells that hold the ovary together.

Approximately 90% of ovarian cancers are of epithelial origin, and are further subdivided into histological subtypes: serous, mucinous, endometroid, clear cell, transitional cell, mixed epithelial and undifferentiated. Morphologic heterogeneity is likely a reflection of the genetic heterogeneity of these tumours which could represent transformation or progression from other tumour types. Of these subtypes, serous tumours comprise 80%-85% of epithelial ovarian carcinomas in Western countries and represent 95% of patients with FIGO stage III-IV disease. Most serous ovarian carcinomas are classified as "high-grade" based on their degree of nuclear atypia and high mitotic index. High-grade serous ovarian carcinoma (HGSOC) stands out from other subtypes both for its aggressive nature and because it contains unique genetic alterations in *TP53* and the DNA-damage repair genes *BRCA1* and *BRCA2* (gene products: breast cancer type 1/2 susceptibility protein) (Karst et al, 2011).

In 2004, Kurman and Shih proposed a classification system in which ovarian cancers are either designated as Type I or Type II based on morphologic, molecular and histogenic characteristics (Shih Ie & Kurman, 2004; Shih Ie & Kurman, 2005). Type I ovarian tumours are slow growing, often confined to the ovary or peritoneum upon diagnosis, and develop from well-established precursor lesions or "borderline" tumours. These tumours include endometrioid, clear cell, mucinous, and low-grade serous carcinomas. They are genetically stable and are characterized by gene mutations that include *KRAS* (gene product: GTPase KRas protein), *BRAF* (gene product: Ser/Thr protein kinase BRaf), *ERBB2* (gene product: human epidermal growth factor receptor 2; HER2/neu) , *PTEN* (gene product: phosphatase and tensin homologue), *PIK3CA* (gene product: p110α catalytic subunit of PI3K), and *CTNNB1* (gene product: β-catenin) (Chien et al, 2007; Kurman & Shih Ie, 2008; Wei et al, 2011).

Type II tumours are often considered to have originated from a single layer of ovarian surface epithelial cells or inclusion cyst cells in the ovary. These include high-grade serous carcinoma, malignant mixed Mullerian tumours, and undifferentiated carcinomas. This group of tumours has a high level of genetic instability and is characterized by mutation of *TP53* (gene product: tumour suppressor p53) and the DNA-damage repair genes *BRCA1* and *BRCA2* (Landen et al, 2008; Wei et al, 2011). Although originally thought to arise from ovarian surface epithelial cells, recent studies suggest that a large proportion of HGSOC may originate from the fallopian tube epithelium (Karst et al, 2011). It was discovered that women predisposed to HGSOC, because of inherited BRACA1/2 mutations, frequently displayed serous carcinoma precursor lesions in the fimbriated ends of their fallopian tubes, immediately next to the ovary. These precursor

lesions, comprised of fallopian tube secretory epithelial cells, often share identical somatic *TP53* mutations with coexisting serous tubal intraepithelial carcinomas and HGSOCs, suggesting for a common origin of the tumours (Lee et al, 2007). Although compelling, the evidence is based largely on descriptive pathological evidence which has not been experimentally verified (Karst & Drapkin, 2010). There remains a lack of evidence to demonstrate that fallopian tube secretory epithelial cells can be transformed into HGSOCs. Efforts to demonstrate this and the molecular mechanisms involved are currently under intensive study.

1.2 CISPLATIN AND ITS DERIVATIVES AND CHEMORESISTANCE

1.2.1 Compound overview

Cisplatin (cis-Pt(II)$(NH_3)_2Cl_2$; cis-diamminedichloroplatinum(II); CDDP) and its derivatives (carboplatin and oxaliplatin) are platinum complexes widely used to treat various tumour types including testicular, lung and ovarian cancers and other solid tumours. They are DNA-damaging agents that exert their toxic effects by forming inter- and intra-strand platinum-DNA crosslinks (Perez, 1998). Although effective against many cancers, the therapeutic outcome of platinum-based chemotherapy in treating ovarian cancer is impaired by intrinsic or acquired resistance and toxicities associated with treatment. Although the dose-limiting nephrotoxicity of cisplatin has been managed to some extent by procedures such as forced diuresis and pharmacological interventions with sulfhydrl agents, the cumulative and irreversible toxicities associated with cisplatin such as nephrotoxicity, ototoxicity and peripheral neuropathy, has led to the intense investigation of analogue development. The first commercially available cisplatin

analogue was carboplatin. Modification of the cisplatin compound by replacing the chloride leaving groups with a cyclo-butanedicarboxylic-leaving group, decreased the toxicity profile; however, it also reduced drug potency and did little to change the resistance pattern (Gosland et al, 1996). Cisplatin and carboplatin are commonly used in the treatment of ovarian cancer in combination with taxanes.

1.2.2 Cisplatin structure and pharmacology

The molecular structure of CDDP consists of a central platinum ion bound by two amino groups and two chloride ions in the *cis* configuration. Of note, the *trans* isomer of cisplatin exhibits similar binding kinetics towards nucleobases but shows no biological effects. This is thought to be the result of the ability of the drug to cause large DNA distortions that are more easily recognized and removed by DNA repair enzymes.

In its uncharged native state, CDDP crosses the cell membrane via passive diffusion and by facilitated diffusion through a gated channel (Gately & Howell, 1993). Recent evidence now includes facilitated and active transport mechanisms involving the copper transporter CTR1 and the organic cation transporters OCT1-3 (Hall et al, 2008; Larson et al, 2009). Information regarding the factors influencing CTR1 and OCT1-3 mediated CDDP influx is limited.

Intracellular cisplatin becomes activated as the chloride ions are released creating a positive charged platinum core. In the body, the extracellular Cl^- concentration is relatively high (100 mM), preventing hydrolysis, but inside the cell, the Cl^- concentration is about 4 mM favoring the hydrolysis reaction to occur (Reedijk, 2003). The positively charged complex, in principle can bind to all sites with a lone pair of electrons, although

DNA binding and the formation of inter- and intra-strand crosslinks are thought to be primarily responsible for the drug's cytotoxic effect. The majority of these adducts are at the 1,2-intra-strand ApG and GpG crosslinks (Kelland, 1993), resulting in adducts that distort the DNA helix, resulting in cellular DNA damage responses and apoptosis.

1.2.3 Mechanisms of resistance to CDDP

Understanding the mechanisms that mediate tumour cell cytotoxicity could provide novel strategies to enhance the efficiency of platinum-based chemotherapy. Cellular mechanisms of resistance to platinum-based chemotherapy are multifactorial. They include molecular events that inhibit drug-DNA interaction including inactivation by thiol groups of proteins and peptides (Glutathione; GSH) which show high affinity for platinum (Pompella et al, 2006), and reduced intracellular drug accumulation resulting from increased efflux, reduced influx or both. Other important mechanisms acting downstream of the initial reaction of CDDP with DNA include an increase in adduct repair and a decrease in the induction of apoptosis. Although DNA binding is the primary role of CDDP activity, other cytotoxic effects may exist.

Reduced drug accumulation has been frequently observed in cisplatin resistant cell lines and decreased influx mechanisms may be the primarily contributing factor. As mentioned above, influx of platinum complexes are mediated by passive diffusion and by facilitated and active transport through gated channels. It is thought that passive diffusion dominates in the early influx phase, with transport proteins becoming involved in the late phase as the concentration gradient is reduced. Indeed, in the cisplatin sensitive and resistant ovarian carcinoma cell line pair A2780s/A2780cp, there was lower

intracellular platinum accumulation and a significantly lower degree of DNA platination in the resistant variant, with no differences in efflux rate (Zisowsky et al, 2007). Resistant cells expressed lower levels of CTR1 which correlated to decreased platinum accumulation, DNA platination and cytotoxicity. However, cellular accumulation of platinum complexes does not always translate to increased cytotoxicity. To overcome accumulation defects, oxaliplatin derivatives with enhanced lipophilicity were tested but failed to increase DNA platination and cytotoxicity even with higher cellular accumulation in A2780cp OVCA cells (Buss et al, 2011). These results further stress the importance of the DNA repair mechanisms and apoptotic response as factors involved with the development of cisplatin resistance.

Cellular responses to platinum agents are regulated by genes involved in DNA detoxification [*GST-π* (glutathione-S-transferase π), *MPO* (myeloperoxidase)] and repair [*ERCC1* & *ERCC2* (excision repair cross-complementing 1/2), *XRCC1* (gene product: X-ray repair cross-complementing protein 1), and *BRCA1*]. As such, dysregulation of these gene products are seen in cisplatin resistant cell lines and in tumour tissue obtained from ovarian cancer patients unresponsive to platinum-based therapy (Dabholkar et al, 1992). As well, several reports have correlated gene variants to trends in cisplatin resistance in ovarian cancer, and to progression-free survival and overall survival of OVCA patients (Marsh, 2009). Contrary to this, the largest ovarian cancer pharmacogenomics study (SCOTROC1 trial), could not confirm the previous results and found no significant association with variants in *ERCC1, GST-π,* or *XRCCI* with platinum toxicity outcome (Marsh, 2009).

Other sources of gene expression variability include changes in DNA methylation

and gene amplification. Methylation of the *FANCF* (Fanconi anemia complementation group F) gene and demethylation of the *MLH1* (mutL homolog 1) gene confer cisplatin sensitivity to platinum agents in ovarian cancer cells.

Reduced apoptotic response is an important factor contributing to cisplatin resistance in ovarian cancer. Disruption of pro-apoptotic genes or of the factors that modulate the apoptotic pathway may contribute to drug resistance. The development of cisplatin resistance in ovarian cancer has been characterized by overexpression of Xiap (X-linked inhibitor of apoptosis protein) (Sasaki et al, 2000), survivin (Wang et al, 2005), and Bcl-2 (B-cell lymphoma-2) (Raspollini et al, 2004) or Bcl-xL (B-cell Lymphoma-extra large) (Williams et al, 2005). Although many resistant cells display increased p53 content, often p53 function is inhibited by functional mutations and the regulatory effects of elevated Akt activity (Buttitta et al, 1997; Fraser et al, 2008; Shaw & Vanderhyden, 2007; Yang et al, 2006).

CDDP resistance in OVCA may in part be the result of differences in mitochondrial biology. Chemoresistant ovarian cancer cells have demonstrated increased plasma membrane and mitochondrial membrane potential compared to the sensitive parental line (Andrews & Albright, 1992). Differences also exist in mitochondrial organization, structure and morphology. The mitochondria of chemosensitive OVCA cells are perinuclear with normal tubular structure. In contrast, mitochondrial in resistant OVCA cells are dispersed and disorganized, with thicker and more irregular cristae (Andrews & Albright, 1992). How these differences are associated with CDDP resistance requires further study. Although it would be easiest to believe that these differences are merely a byproduct of the resistant phenotype, the crucial role of the

mitochondria death pathway and the complex regulation of the mitochondrial membrane potential (MMP) would suggest otherwise. Indeed MMP is a key regulator of mitochondrial Ca^{2+}, as a decrease in MMP is associated with Ca^{2+} efflux (Bardsley & Brand, 1982; Brookes et al, 2004). Intracellular Ca^{2+} signaling is involved in apoptosis and CDDP sensitivity (Al-Bahlani et al, 2011). In addition, there is evidence to suggest that dysregulated p53-dependent apoptosis may be involved in the pathophysiology of chemoresistance. Indeed, cells from p53 (-/-) mice can resist certain stimui that are known to induce apoptosis by mechanisms involving the collapse of the mitochondrial inner membrane potential and cytochrome c release. This implies a failure of p53-deficient cells to engage in apoptosis at the mitochondrial level (Castedo et al, 1995). Furthermore, cisplatin resistance in ovarian cancer cells is associated with decreased p53 accumulation at the mitochondria (Yang et al, 2006). *In vitro* experiments targeting p53 to isolated mitochondria from chemoresistant and chemosensitive ovarian cancer cells resulted in comparable release of death proteins (Yang et al, 2006). This is consistent with the idea that attenuation of the mitochondrial death program in which p53 is an important regulator contributes to chemoresistance.

1.3 APOPTOSIS

1.3.1 Overview

Classically there are 2 types of cell death in biological systems: 1) necrosis (accidental cell death) and 2) apoptosis (programmed cell death). Apoptosis is a normal physiological process involving many genes and requires the active use of energy in the form of ATP to safely dispose of cells once they have fulfilled their intended biological

role. This process is initiated by a wide variety of stimuli and is characterized by a number of stereotypical morphological features where the cell first shrinks and deforms. After becoming detached from neighbouring cells, it undergoes chromatin condensation and internucleosomal cleavage of the DNA before fragmentation into compact membrane-enclosed structures named "apoptotic bodies". These apoptotic bodies are then engulfed by macrophages before elimination from the tissue with no significant inflammatory damage to surrounding cells. Although the classic apoptotic pathways are caspase-dependent, details regarding caspase-independent apoptotic pathways are being discovered (Cregan et al, 2002; Liu et al, 2008).

The ordered steps characteristic of apoptosis are in contrast to the process of necrosis, where a traumatic injury causes a cell to lose membrane integrity and rupture. This uncontrolled released of cytoplasmic content into the cell's environment results in further damage to surrounding cells from the ensuing inflammatory response in the tissue. The process of apoptosis and necrosis represent only the extreme ends of a wide range of possible morphological and biochemical deaths.

1.3.2 Intrinsic pathway

The intrinsic apoptotic pathway is initiated by intracellular signals or intrinsic stresses such as DNA damage, hypoxia, oncogene activation and survival factor deprivation. p53 is a crucial sensor of cellular stress and an activator of the intrinsic pathway. The DNA checkpoint proteins ataxia telangiectasia-mutated (ATM) and checkpoint kinase 2 (Chk2) phosphorylates and stablizes p53 and inhibits murine double-minute-2 (MDM2)-mediated ubiquitination and degradation of p53. MDM2 binds to p53

and promotes nuclear export and prevents p53 transcriptional activity. p53 initiates apoptosis by activating the transcription of proapoptotic Bcl-2 family members such as Bax (Bcl-2-associated X protein), Bad (Bcl-2-antagonist of cell death), Bak (Bcl-2 homologous antagonist/killer) and Bid (BH3 interacting-domain) and repressing antiapoptotic Bcl-2 proteins and cIAPs. This results in mitochondrial membrane permeabilization resulting in the release of apoptotic activators into the cytosol (Smac, HtrA2/Omni, and cytochrome c). Cytochrome c binds to APAF1 (apoptotic protease activating factor-1) and recruits pro-caspase-9, forming a complex known as the apoptosome. This complex cleaves and activates pro-caspase-9, which leads to caspase-9 dependent pro-caspase-3 cleavage and activation. The substrate for caspase-3 is the DNA fragmentation factor ICAD (Inhibitor of Caspase-Activated DNase) which binds to and inhibits CAD (Caspase-Activated DNase). Cleaved ICAD dissociates from CAD promoting CAD oligomerization and activation of DNase activity. The active CAD oligomer causes internucleosomal DNA fragmentation which is a hallmark of chromatin condensation and apoptosis (Pirnia et al, 2002).

1.3.3 Extrinsic pathway

As the name suggests, the extrinsic pathway begins outside the cell. Based on the external stimulus, apoptosis is mediated by binding of a death ligand to a death receptor (DR). DR activation quickly activates death caspases that effect apoptosis. Death receptors belong to the TNFR (Tumour necrosis factor receptor) superfamily, which are characterized by a Cys-rich extracellular domain and a homologous intracellular death domain. Adaptor molecules like FADD (Fas-associated via death domain), TRADD

(Tumour necrosis factor receptor-1-associated death domain) or Daxx contain death domains so that they can interact with the DRs and transmit and amplify the apoptotic signal. The best studied death receptors are Fas and TNFR1 (Tumour necrosis factor receptor-1). Other DRs include Apo2 and Apo3 (Varfolomeev et al, 2005).

The binding of Fas Ligand to the Fas receptor induces receptor oligomerization and clustering of death domains and the binding of cofactor FADD. Through its DED (Death effector domain) FADD recruits pro-caspase-8 and -10. The Fas, FADD and pro-caspase-8 and -10 complex is known as the death inducing signaling complex (DISC). Once the DISC is formed, these caspases are activated by oligomerization and proteolytic self-processing (Varfolomeev et al, 2005) which in turn, activates downstream effector caspases (caspase-3, -6, and -7) that mediate the execution phase of apoptosis.

Amplification of the death signal can also be initiated by caspase-8-mediated BID (Bcl-2 interacting protein) cleavage. This is followed by subsequent cytochrome c release and mitochondrial outer membrane permeabilization (MOMP), thus representing cross-talk between both extrinsic and intrinsic apoptosis pathway.

1.4 TP53 TUMOUR SUPPRESSOR

1.4.1 Overview

As one of the most commonly mutated tumour suppressor genes associated with cancer development, and often referred to as the "guardian of the genome", p53 remains a hot topic in cancer research. p53 is a sequence specific transcription factor, activated by diverse stress signals. The transcriptional function of p53 is dependent on both nuclear import and export, processes that are tightly regulated and are enabled by three nuclear

localization signals (NLS) and two nuclear export signals (NES) respectively (**Figure 1**). Upon DNA damage, p53 is imported to the nucleus and undergoes tetramerization and binds and activates DNA damage-response genes. There are several proteins that influence p53 nuclear import and export, the most important being MDM2.

MDM2 is a ubiquitin E3 ligase that promotes p53 degradation and ultimately inhibits p53 transcriptional activity. MDM2 itself is a transcriptional target of p53, creating a negative feedback loop where p53 activation leads to expression of its own negative regulator. The importance of this regulatory loop is reflected in the embryonic lethality of MDM2 knock-out mice (de Rozieres et al, 2000), and in tumours with hyperactive MDM2 display a "p53-null" phenotype resulting from rapid p53 degradation (Cuny et al, 2000).

Both MDM2 and p53 move between the cytoplasm and nucleus. Nuclear translocation is required for transactivation of target genes; however, re-entry to the cytoplasm is required for degradation of both MDM2 and p53. Subcellular localization of MDM2 is important to p53 function, and is regulated by factors such as p14ARF and the PI3K/Akt pathway. P14ARF binds to MDM2 and can sequester the entire p53-MDM2 complex in the nucleolus, thereby inhibiting nuclear export (Xirodimas et al, 2001). Akt-mediated MDM2 phosphorylation promotes nuclear MDM2 translocation and p53 degradation (Mayo & Donner, 2001).

1.4.1 Transcription-dependent role of p53 in apoptosis

The importance of p53 in cancer progression is evident by the fact that nearly half of all human cancers exhibit inactivating mutations of p53, and in those without, p53

activity is often decreased as a result of increased inhibitors, decreased activators or inactivation of downstream targets. To complicate matters, evidence now suggests that inactivating mutations of p53 can result in gain of functions contributing to tumour growth and metastasis (Muller et al, 2009).

Classically known as a transcription factor, p53 promotes apoptosis by up-regulating pro-apoptotic genes such as *BAX* (Miyashita et al, 1994), *APAF1* (Fortin et al, 2001), *BBC3* (BCL2 binding component 3) (Nakano & Vousden, 2001), and *PTEN* (Stambolic et al, 2001), and down-regulating anti-apoptotic genes such as *BCL2* (Haldar et al, 1994), and *BIRC5* (baculoviral IAP repeat containing 5) (Hoffman et al, 2002). Bax was one of the earliest gene products shown to be regulated by p53 (Miyashita et al, 1994), and is required for apoptosis induced by numerous stimuli (Deckwerth et al, 1996; Knudson et al, 1995). The protein product of BBC3 is p53-upregulated mediator of apoptosis (PUMA), is a BH3-only member of the Bcl-2 family and is upregulated in a p53-dependent manner (Nakano & Vousden, 2001). PUMA is a critical regulator of p53-dependent apoptosis, as PUMA knockout confers resistance to DNA damage-induced apoptosis (Villunger et al, 2003), while over-expression of PUMA induces apoptosis in neuronal cells (Cregan et al, 2004).

1.4.2 Transcription-independent role of p53 in apoptosis

In addition to its nuclear functions, p53 induces apoptosis in a transcription-independent fashion. Studies have shown that transactivation-incompetent p53 mutants can induce apoptosis in human cells even when the DNA binding domain is lacking (Haupt et al, 1995). Furthermore, p53 has been shown to induce apoptosis in conditions

where transcriptional or translational blockade is complete (Caelles et al, 1994; Chipuk et al, 2003).

It is now widely accepted that p53 has an additional transcription-independent role in the regulation of pro-apoptotic signaling at the mitochondria (Erster et al, 2004; Marchenko et al, 2000; Sansome et al, 2001; Yang et al, 2006). In response to multiple stress signals, including DNA-damage and hypoxia, a fraction of stress-induced p53 rapidly translocates to the mitochondria. Once there, p53 directly interacts with and inhibits anti-apoptotic $BclX_L$ and Bcl2 proteins located at the outer membrane, and induces the oligomerization of the BH1, 2, 3 effectors of membrane permeabilization such as Bak (Mihara et al, 2003). p53 has also been shown to directly activate Bax to promote mitochondrial outer-membrane permeabilization (MOMP). This in turn leads to the release of proapoptogenic factors from the mitochondria such as cytochrome c, with subsequent activation of effector caspases, rapidly leading to apoptosis. Binding of p53 to the anti-apoptotic Bcl2 proteins occur through the DNA-binding domain of p53. Therefore, oncogenic mutations of p53 represents a "double hit" attenuating its tumour suppressive function by reducing its transactivation potential and its ability to induce MOMP. Studies have also shown that the proline-rich region is involved with Bax activation and interaction with Bcl2 proteins. Binding affinities of p53 to these molecules are distinct and may reflect sequential steps involved with MOMP. Based on the higher affinity of p53 to $BclX_L$ than for Bax, binding to Bcl2 proteins may act as a sensitizer to apoptosis, followed by direct activation of apoptosis resulting from p53-Bak interaction.

The pro-apoptotic effects of mitochondrial p53 accumulation are not dependent

on gene transcription. However, the transcription-dependent and -independent roles of p53 both contribute to the final outcome of apoptosis. The initiation of stress-induced apoptosis promotes the translocation of p53 to the mitochondria, an early event in p53-dependent apoptosis, and results in caspase activation and MOMP. These events precede and promote p53 target gene activation, required to amplify and "finish off" the apoptotic response. Indeed, in gamma-irradiated thymocytes, these events are represented as a rapid first wave of apoptosis that is transcription-independent, followed by a second slower wave that is transcription-dependent (Erster et al, 2004). This biphasic response may reflect a cell's ability to monitor degrees of stress and allow for repair mechanisms during the lag phase if required. Whether or not this represents a point of no return is not known. It is possible that the transcription-independent role of p53 once activated, serves to mark cells for apoptosis, and the lag phase is to allow time for the expression and translation of death related proteins.

1.4.3 Regulation of apoptosis by p53 phosphorylation

TP53 mutations occur in >50% of human ovarian cancers and dysregulation of p53 function is a critical determinant of tumourigenesis. However, wild-type *TP53* is not a direct predictor of successful chemotherapeutic response, suggesting that additional mechanisms unrelated to genotype is important in regulating CDDP sensitivity, and may include direct modification of p53 including phosphorylation [for a detailed review on p53 stabilization and activation, refer to (Lavin & Gueven, 2006)].

CDDP resistance in OVCA is associated with increased Akt activation, which inhibits p53 phosphorylation and nuclear function (Fraser et al, 2008). Evidence suggests

that the key p53 phosphorylation sites required for CDDP-induced apoptosis is at ser15 and ser20 as they are phosphorylated in chemosensitive but not resistant OVCA cells, and mutations at these sites attenuates CDDP sensitivity (Fraser et al, 2008). Further study is required to examine if this process requires phosphorylation of other p53 residues.

The mechanism by which Akt regulates p53 phosphorylation is unclear. Phosphorylation of p53 is dependent on numerous protein kinases that are activated in a stimulus-specific manner. A primary cellular response to CDDP-induced DNA damage is the activation of the pathway involving members of the ataxia telangiectasia-mutated (ATM) family, including ATM and ATR (ATM and Rad3 Related) (Appella & Anderson, 2001; Shieh et al, 2000). In response to DNA damage, ATR/ATM is known to mediate phosphorylation at multiple sites on p53, including ser15, ser20, ser33 and ser46 (Lavin & Gueven, 2006). However, it appears that phosphorylation of Ser33 and Ser46 does not play a major role in CDDP-induced apoptosis as there is no change or no detection of these species in chemosensitive OVCA cells compared to their resistant counterparts (Fraser et al, 2008). This is not surprising as, depending on the stress signal, there is considerable overlap in phosphorylation sites of p53 (Lavin & Gueven, 2006). Interestingly, ATR contains a putative Akt phosphorylation site, which offers the possibility that Akt activation may phosphorylate and inhibit ATR activation and subsequent p53 phosphorylation. Further studies are required to explore this hypothesis.

The molecular basis for chemosensitivity and resistance in ovarian cancer is complex, although the p53 tumour suppressor protein appears to play a major role in mediating the cellular response to platinum-based chemotherapies. The impact of p53 gene status and protein modification will vary between tumour types as the result of

differences in: a) cellular transduction pathways; b) the specific type of drug-induced cellular toxicity; c) the inherent cell-specific responses by the cell.

Figure 1. A schematic representation of p53.

The human p53 protein consists of several domains. The amino transactivation domain is responsible for the activation of target genes and contains the site of MDM2 binding as well as several key phosphorylation sites (including ser15, thr18, and ser20), implicated in inhibition of MDM2-p53 binding. This is followed by a proline-rich domain that is essential for the p53 apoptotic response to DNA damage. A central DNA-binding domain is required for the recognition of the p53 consensus sequence [two copies of the 10-bp motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', separated by 0-13bp (el-Deiry et al, 1992)] within the promoters of p53-responsive genes. This core domain is the target of the majority of p53 mutations found in human cancers, where a single mutation within this domain can cause major conformational changes. The C-terminus oligomerization domain consists of a β-strand–turn-α-helix motif which is involved in p53 dimer and tetramer formation. This domain is involved in p53/PARC interaction (Kaustov et al, 2007; Nikolaev et al, 2003). The p53 C-terminal contains three nuclear localization signals (NLS). Furthermore, two nuclear export signals (NES) have been identified. It is suggested that oligomerization of p53 can mask the NES, promoting p53 nuclear retention.

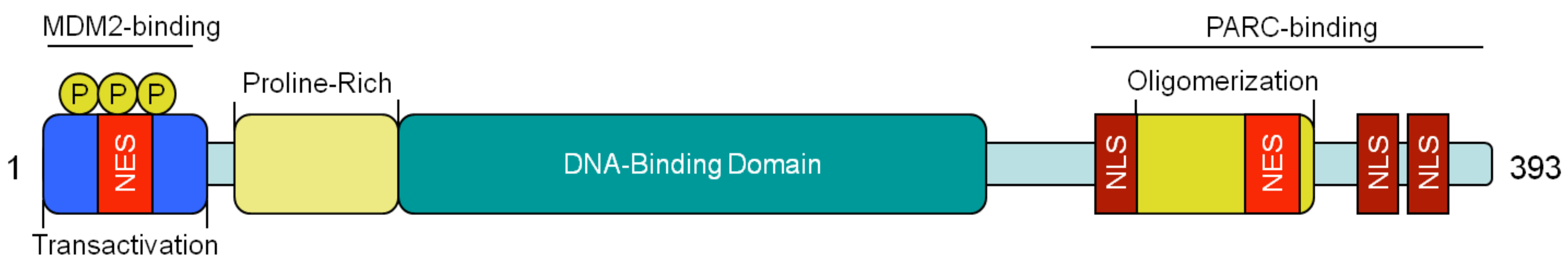


Figure 1

1.5 p53-ASSOCIATED, PARKIN-LIKE CYTOPLASMIC PROTEIN (PARC)

1.5.1 PARC structure

The human *PARC* gene (also known as *CUL9*) is located on chromosome 6p21.1 and encodes a 2517 amino acid protein measuring ~270 kDa in size (**Figure 2**). It is ubiquitously expressed in various tissues, including the ovary (Nikolaev et al, 2003). PARC is a cytoplasmic protein that forms a multi-protein complex of approximately 1 million kDa in size. The N-terminus of PARC contains a CPH motif [domain that is conserved in Cul7, PARC, and HERC2 proteins; (Kaustov et al, 2007)] that binds to the C-terminus of p53 and acts as a cytoplasmic anchor for p53 in unstressed cells, inhibiting p53 mitochondrial and nuclear accumulation and function (Dowell et al, 2007; Mulhall et al; Nikolaev et al, 2003; Vitali et al, 2008). As expected, PARC expression is increased in cells where cytoplasmic p53 accumulation is high and studies that reduce endogenous levels of PARC show increased p53 nuclear accumulation and transcriptional activity (Mulhall et al; Nikolaev et al, 2003).

Sequence analysis of PARC reveals the presence a Really Interesting New Gene (RING)-In between RING (IBR)-RING domain and CCH signature motifs that are associated with proteins having E3 ubiquitin ligase activity. The RING-IBR-RING domain was first identified in the Parkin protein, where loss of function as a result of gene mutations is associated with an autosomal recessive form of Parkinson's disease. This domain is responsible for the intrinsic ubiquitin ligase activity of Parkin, a function that is crucial to prevent the accumulation of critical substrates resulting in toxicity to dopaminergic neurons.

PARC also contains a C-terminal Cullin Homology (CCH) domain that is unique

to the Cullins family of proteins. The Cullins are critical components of several ubiquitin ligase megacomplexes, such as the anaphase-promoting complex (APC/C) and the Skp-Cullin-Fbox (SCF) (Nikolaev et al, 2003). Recently, PARC was classified as a genuine cullins protein (renamed CUL9) based on its binding to RBX1 and its covalent modification by NEDD8 (Skaar et al, 2007). Among the cullins, PARC is highly homologous to CUL7, sharing up to 60% identity in several domains and containing a "spacer domain" of unknown function and the C-terminal RING-IBR-RING domain (absent in CUL7). As in CUL7, PARC contains several discrete domains present in other proteins found in E3 ligase complexes, such as HERC2, APC10/DOC1 and Parkin. The presence of both of these domains suggests that PARC regulates protein stability through ubiquitination, although PARC fails to promote p53 ubiquitination *in vivo* and *in vitro* and, in contrast to over-expression of Mdm2, PARC over-expression has no effect on cellular p53 content (Mulhall et al; Nikolaev et al, 2003). This suggests that the primary role of PARC is not to mediate p53 degradation but instead, to regulate p53 subcellular localization. The endogenous substrates of PARC have yet to be determined (Nikolaev et al, 2003).

Interestingly, the N-terminal, p53-binding domain of PARC is not sufficient to induce cytoplasmic retention of p53, but requires additional sequences such as the CCH and the RING-IBR-RING domain (Nikolaev et al, 2003). It is possible that these domains are required to link PARC to stable cytoplasmic complexes or structures such as vimentin (Klotzsche et al, 1998), actin (Metcalfe et al, 1999), and microtubules (Giannakakou et al, 2000; Maxwell et al, 1991). The possible interaction of PARC and microtubules in the regulation of p53 localization would be especially interesting as

Paclitaxel, a microtubule-stabilizing agent, is commonly used in combination with cisplatin and its derivatives for the treatment of ovarian cancer. This interaction requires further investigation, although it is known that the Parkin protein binds to and regulates degradation of tubulins via its E3 liagase activity (Ren et al, 2003).

Figure 2. A schematic representation of PARC, Cul7 and Parkin.

The human PARC protein consists of several domains that are highly homologous to motifs found in the Parkin and Cul7 protein. PARC shares 60% sequence identity to Cul7, both having the CPH (Cul7, PARC, and HERC2), DOC (from Doc1/Apc10), and C-terminal Cullin homology (CCH) domains (Kaustov et al, 2007). The CPH domain of PARC and Cul7 can interact directly with the oligomerization domain of p53. PARC is distinct from Cul7 by having a spacer domain and a Really Interesting New Gene (RING)-In Between RING (IBR)-RING domain at the C-terminus (Nikolaev et al, 2003), a signature motif first identified in the C-terminus of Parkin that is responsible for its intrinsic ubiquitin ligase activity (Shimura et al, 2000).

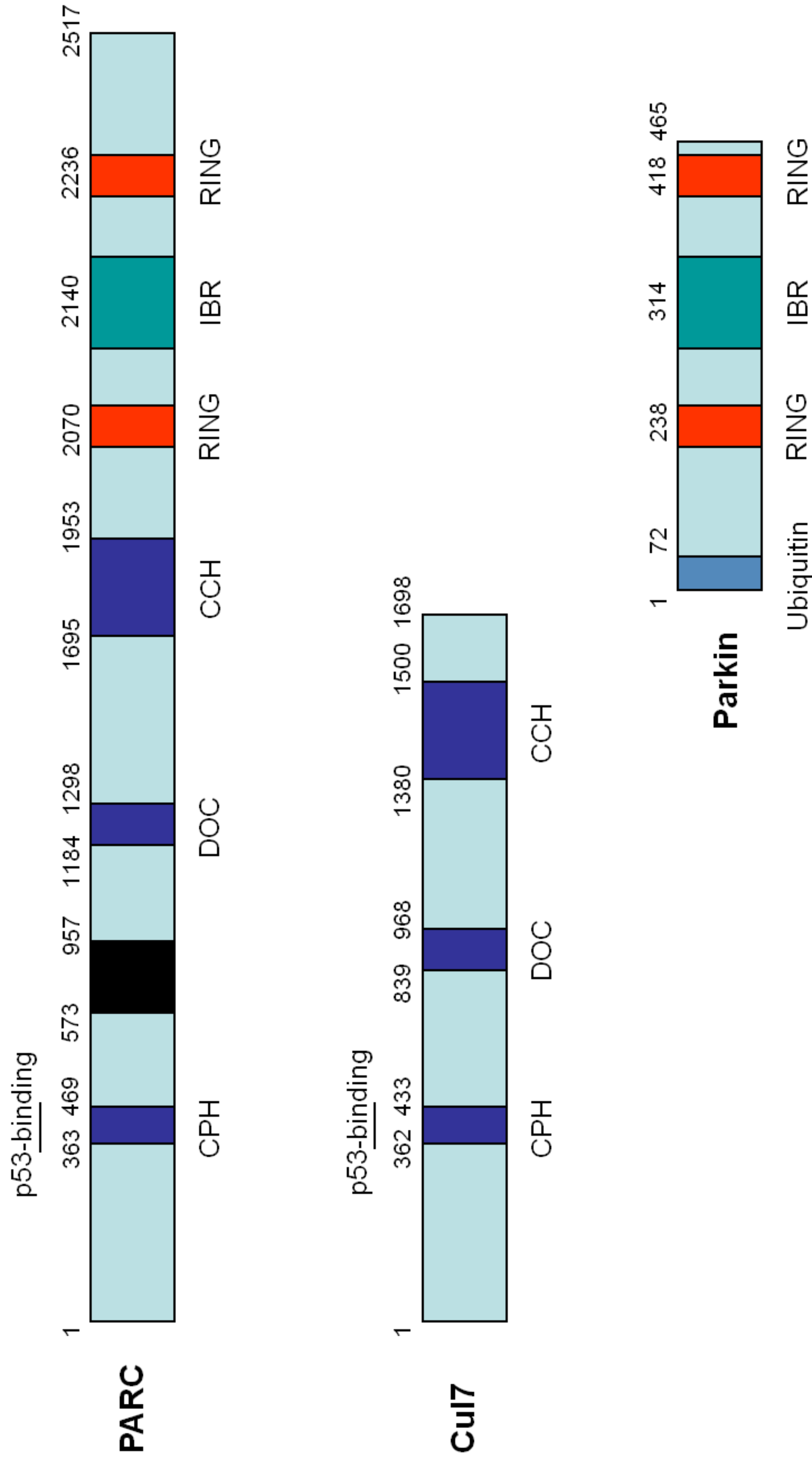


Figure 2

1.5.3 PARC-mediated regulation of p53 localization and apoptosis

p53 induces apoptosis by mechanisms that are both transcription-dependent and independent, and require navigation from the cytoplasm to the nucleus and mitochondria. Tumour cells that respond poorly to chemotherapy or radiotherapy often show high cytoplasmic p53 content (Brown et al, 1993; Mulhall et al; Nikolaev et al, 2003; Vitali et al, 2008), and it is in these cells that mitochondrial and nuclear p53 accumulation and subsequent function is attenuated.

PARC promotes p53 cytoplasmic sequestration. Immunodepletion assays suggest that the majority of cytoplasmic p53 is associated with PARC (Nikolaev et al, 2003). In cells exhibiting high basal p53 content where the p53 pathway may be dysregulated, PARC down-regulation induces nuclear p53 localization and p53-dependent apoptosis (Mulhall et al; Nikolaev et al, 2003). However, the effects of PARC on mitochondrial p53 localization have not been examined. Since cisplatin-resistant ovarian cell lines are often characterized by high basal p53 content (Brown et al, 1993), and dysregulation of p53-mediated apoptosis (Yang et al, 2006), PARC down-regulation may represent a novel strategy to reverse resistance to cisplatin-based chemotherapy in ovarian cancer. Indeed, p53 reactivation can occur in tumours demonstrating cytoplasmic p53 using a p53 C-terminal peptide containing the PARC-interacting region (Vitali et al, 2008). By disrupting the interaction of wt-p53 and PARC, peptide treatment increased nuclear p53 accumulation, transcriptional activation and enhanced genotoxic stress-induced apoptosis (Vitali et al, 2008). However, it has been also been suggested that PARC is not important in the regulation of p53-mediated apoptosis and paradoxically functions as a tumour suppressor to promote p53-dependent apoptosis (Pei et al, 2011; Skaar et al, 2005).

These studies are flawed in two major ways: 1) in assuming that exogenous manipulations of PARC can regulate p53-function in cells that exhibit normal p53 signaling, where basal p53 content is low, and 2) by not considering the mechanisms by which genotoxic agents may regulate PARC/p53 interaction and apoptosis. However, these studies do highlight the idea that dysregulation of subcellular p53 localization and its regulation by PARC is cell type-specific. Specifically, PARC down-regulation promotes apoptosis primarily in models where basal p53 content is high and there is dysregulation of p53-dependent apoptosis. As such, PARC manipulation may offer a treatment strategy that is very relevant in reversing chemoresistance in ovarian cancer.

1.6 PI3K/Akt PATHWAY

1.6.1 PI3K/Akt pathway and regulation/dysregulation

The serine/threonine protein kinase, protein kinase B or more commonly known as Akt (PKB/Akt) regulates a wide variety of cellular processes including apoptosis, proliferation, differentiation and metabolism (Dan et al, 2004; Fraser et al, 2008; Gottlieb et al, 2002; Song et al, 2005; Yamaguchi et al, 2001; Yang et al, 2006). As such, dysregulation of PKB/Akt signaling has been implicated in the development of several cancers. Akt is the mammalian homologue of the viral oncogene v-Akt. There are 3 isoforms encoded by separate genes termed PKBa/Akt1, PKBb/Akt2 and PKBg/Akt3 located on chromosomes 14q32, 19q13 and 1q44, respectively (Song et al, 2005). The isoforms are similar, having an amino terminal pleckstrin homology (PH) domain, a central kinase domain and a carboxyl-terminal regulatory domain that contains the hydrophobic motif, a characteristic of the cAMP-dependent protein kinase A/protein

kinase G/protein kinase C (ACG) super family of protein kinases.

All Akt isoforms (except an Akt3 splice variant) contain two regulatory phosphorylation sites: Thr308 in the activation loop within the kinase domain and Ser473 in the C-terminal regulatory domain. Activation of Akt1 is dependent on a PI3-Kinase (PI3K)- and PH domain-dependent membrane translocation mechanism, followed by phosphorylation at the two sites. Thr308 phosphorylation results in partial activation, while phosphorylation at both sites is required for full activation. Phosphorylation at Ser473 alone does not significantly affect Akt activation.

Akt is the downstream effector of PI3K (Class 1A and 1B), that in turn, are activated by tyrosine kinases and G-protein-coupled receptors, respectively. PI3K is a heterodimer composed of a p85 regulatory and a p110 catalytic subunit. Once recruited to these receptors at the plasma membrane, PI3K is activated and converts PtdIns(4,5)P_2 (PIP2) on the 3-OH group to generate the second messenger PtdIns(3,4,5)P_3 (PIP3). PIP3 does not activate Akt directly, but instead recruits Akt to the plasma membrane where its conformation is changed to facilitate subsequent phosphorylation by the phospho-inositide- dependent kinases-1 (PDK1) at Thr308. The kinase(s) responsible for Ser473 phosphorylation is not completely understood, and may be the result of autophosphorylation, the involvement of serine kinases including the integrin-linked kinase (ILK). Phosphorylation of tyrosine residues Tyr315, Tyr326 and Tyr474 may also play an important part in regulating Akt activity although further studies are required to confirm this notion. It has been suggested that Akt can be activated in a PI3K-independent manner, as agents that increases intracellular cAMP can induce PKA-dependent Akt activation (Cullen et al, 2004). This process does not require the PH

domain of Akt nor Ser473 phosphorylation although it requires phosphorylation of Thr308. How PKA activates Akt remains unclear.

As a critical regulator of cell survival and proliferation, Akt activity is regulated by direct interactions with various proteins. Several proteins have been shown to bind to and inhibit Akt phosphorylation and activation. Carboxyl-terminal modulator protein (CTMP) reduces Ser473 phosphorylation and attenuates tumour growth in mice (Ono et al, 2007). Trb3, a mammalian homologue of tribbles, a cdc25 binding protein in *Drosophila*, binds to the kinase domain of Akt and inhibits activation (Du et al, 2003). Keratin K10, a cytoskeletal component, binds to Akt and sequesters it to the cytoskeletal structure, preventing intracellular translocation and activation of Akt (Santos et al, 2002). Conversely, positive regulators of Akt activity include proteins such as heat shock protein (Hsp)90, Hsp27, growth factor receptor-bound protein 10(Grb10), and Ft1 (Song et al, 2005).

1.6.2 PI3K/Akt in cancer and chemoresistance

The *PIK3CA* gene, located on chromosome 3q26, encodes the p110α catalytic subunit of PI3K. *PIK3C*A is frequently amplified in ovarian cancer (Shayesteh et al, 1999) with increased expression of RNA and protein content (Shayesteh et al, 1999). Approximately 40% of ovarian cancers show increased copy numbers at 3q26 (Iwabuchi et al, 1995), and inhibition of PI3K can attenuate ovarian tumour growth in vivo (Hu et al, 2000).

PI3K activation is mainly the result of ligand-dependent activation of tyrosine kinase receptors and G-protein-coupled receptors, although receptor-independent

activation of PI3K can occur, primarily in cells expressing constitutively active Ras proteins. As cell surface receptors are commonly over-expressed or constitutively active in many cancers, downstream signaling pathways are as a result also active. The most extensively studied example is the ErbB2 tyrosine kinase receptor, which is over-expressed as a result of gene amplification in many cancer types including 25-30% of breast and ovarian cancers. Over-expression of ErbB2 correlates with poor prognosis and decreased survival, and has been shown to confer cisplatin resistance to human ovarian cancer cells (Smith et al, 2002). Reports indicate that ErbB2 acts to preserve the phosphorylation and prolong Akt activation by inhibiting the phosphatase activity of PP1, a serine/threonine phosphatase associated with Akt regulation. Therefore, ErbB2 promotes PI3K/Akt signaling by promoting its activation while inhibiting its inactivation (Xu et al, 2003).

Another phosphatase involved in the regulation of the PI3K/Akt signaling pathway is PTEN (phosphatases and tensin homologue deleted on chromosome 10). PTEN primarily regulates PI3K signaling by dephosphorylating membrane PtdIns(3,4,5)P_3. In addition to its lipid phosphatase ability, PTEN also expresses protein phosphatase activity against a number of substrates and has been shown to dephosphorylate ILK, a regulator of Akt activation (Persad et al, 2000). Interestingly, evidence suggests that p53 may promote apoptosis through binding of the PTEN promoter, in which case p53 inactivation can promote PI3K-mediated cell survival (Stambolic et al, 2001). Additional aberrations in the PI3K pathway reported in ovarian cancer include mutational activation of the p85 regulatory subunit of PI3K, and the overexpression and amplification of *Akt2* (Astanehe et al, 2008).

Akt activation promotes growth factor-mediated cell survival and inhibits apoptosis. Among Akt family members, Akt2 is the predominant isoform involved in human malignancies, including ovarian cancer. No modified or mutated *Akt* genes have been reported in mammals, although there are many reports of Akt gene amplifications in human cancers. *Akt2* amplification is associated with high-grade aggressive ovarian tumours and appears to occur as part of the frequent amplification of the 19q13.1-q13.2 chromosomal region (Bellacosa et al, 1995; Thompson et al, 1996). In addition, *Akt2* amplification has been demonstrated in numerous ovarian cancer cell lines, corresponding with elevated protein content and high kinase levels in half of primary ovarian carcinoma examined (Yuan et al, 2000).

1.7 CALPAIN FAMILY OF Ca^{2+}-DEPENDENT PROTEASES

1.7.1 Calpain structure and function

Calpains constitute a family of intracellular Ca^{2+}-dependent neutral cysteine proteases, mediating regulatory cleavage of specific substrates involved in processes of cellular differentiation, cell migration and apoptosis (Goll et al, 2003). Sixteen gene products of the family are known in mammals, among which two isoenzymes μ-calpain (calpain I) and *m*-calpain (calpain II) are ubiquitously expressed in human tissue, are the best characterized, and are considered as the 'conventional' members of the calpain family (**Figure 3**). These calpains are heterodimers consisting of a catalytic "large" subunit (80 kDa) and a common regulatory "small" subunit (28 kDa). The large subunit has four domains; the second and fourth domain comprise the cysteine protease (domain

II) and the Ca^{2+}-binding domain (domain IV), respectively. The function of domain I and III is unclear. The protease activity is therefore attributed to the large subunit. The small subunit consists of two domains, an N-terminal glycine-clustering hydrophobic region (domain V) and a C-terminal Ca^{2+}-binding domain (domain VI) similar to that found in the large domain. The C-terminal domains of both the large and small subunits are Ca^{2+}-binding domains, each containing five EF-hand structures. These structures are important in calcium binding and is crucial for heterodimer subunit formation (Sorimachi et al, 1997).

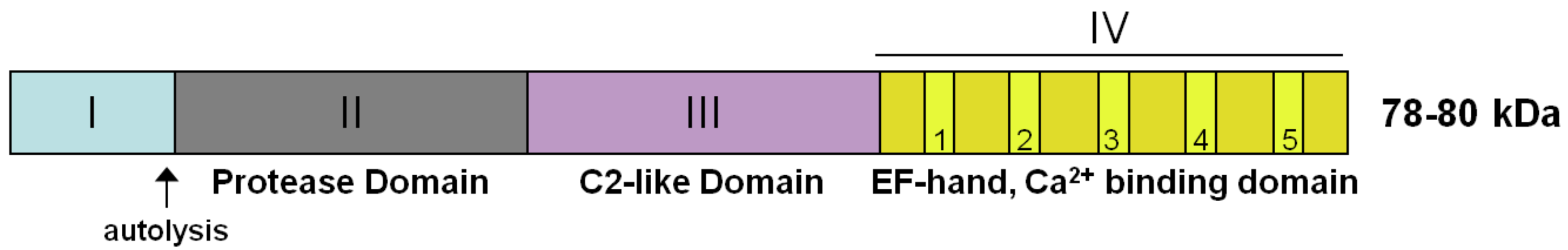
Other 'tissue-specific' calpains, such as p94 (skeletal muscle-specific) and nCL-2 (stomach-specific) are probably related to the specific functions of the organs in which they are predominantly expressed. These 'atypical' homologues differ from the 'conventional' calpains in possessing unique large subunit domains (Sorimachi et al, 1997).

Figure 3. A schematic representation of calpain structure.

The structural motifs of the "conventional" μ or m-calpain and their common 28 kDa regulatory subunit. Both μ and *m*-calpain are heterodimers comprised of a large catalytic (80 kDa) subunit and a small regulatory (28 kDa) subunit, consisting of six domains with distinct functions. Domain I is a short domain that is cleaved by autolysis when calpains are activated by calcium (Storr et al, 2011). Domain II is similar between calpains as it contains the conserved protease domain. It is divided into two parts (IIa, IIb) which forms a single domain containing the catalytic centre upon calcium binding (Glading et al, 2002). Domain III, a C2-like domain, is involved in membrane targeting and contains sites for phosphorylation (Glading et al, 2002). Domains IV and VI of the catalytic and regulatory subunit respectively, contains five EF- hand Ca^{2+}-binding domains and are also involved in the dimerization of the subunits through interaction of the fifth EF-hand motifs (Storr et al, 2011). Domain V of the regulatory subunit, contains multiple glycine residues that may be involved with plasma membrane interaction and are autolysed upon calpain activation (Storr et al, 2011).

Calpain I/II

Large Catalytic Subunit



Small Regulatory Subunit

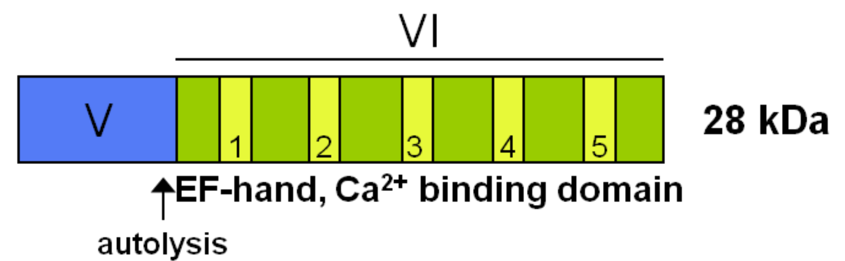


Figure 3

1.7.2 Ca2+-dependent regulation of calpain activity

The activities of conventional calpains are regulated in mammals by the specific endogenous inhibitor protein calpastatin. Subdomains of the reactive site of calpastatin bind to the Ca^{2+}-binding domains of both the large (domain IV) and small (domain VI) subunits. There are also numerous synthetic calpain inhibitors, however, their use *in vivo* is limited as they also frequently inhibit the proteasome and other cysteine proteinases. As such, the study of calpain *in vivo* is difficult, requiring combination treatments to specifically target these proteinases (Sorimachi et al, 1997).

Calpains are the only known proteolytic enzymes that depend on direct calcium binding (Goll et al, 2003). Following activation, calpains undergo autocatalytic processing, yielding calcium-independent isoforms. Calpain activation has been the subject of intense investigation to try and reconcile the nanomolar Ca^{2+} concentration in the cytosol with the high Ca^{2+} requirements for activation *in vitro*. In addition to Ca^{2+}, several mechanisms have been proposed to facilitate calpain activation in physiological conditions. These include association to specific membrane phospholipids, interaction with activating proteins, caspase-mediated degradation of endogenous inhibitor calpastatin, and extracellular signal-related kinase (ERK)-mediated phosphorylation (Glading et al, 2004). It is also possible that calpains are exposed to high Ca^{2+} transients in local microenvironments such as the case in neuronal synapses.

1.7.3 Calpain and apoptosis

Calpains are non-lysosomal Ca^{2+}-regulated cysteine proteases known to cleave specific substrates involved with many cellular processes, including cell death (Demarchi

& Schneider, 2007). Their importance in apoptosis signaling is reflected in the growing list of substrates including p53 (Kubbutat & Vousden, 1997), PARP , Bax, Bid (Liu et al, 2008), AIF and several cytoskeletal proteins (Schafer et al, 2009). However, observations concerning the role of calpains in apoptosis differ depending on the cell line and stimulus studied, and it is not yet established what fundamental mechanisms of apoptosis are regulated by calpains. For instance treatment of thymocytes with the calpain inhibitors PD150606, E-64d, MDL28170 and calpain inhibitor I prevented dexamethasone-induced apoptosis but had no effect on valinomycin- and heat-shock-induced apoptosis (Squier & Cohen, 1997). Furthermore, in this system, calpain inhibitor I could not prevent Ca^{2+} induced DNA fragmentation, suggesting that calpain function is independent of caspases (Squier & Cohen, 1997). Calpain also induces apoptosis in murine neuronal cells by p53 activation (Chua et al, 2000), while calpain inhibition attenuated p53 upregulation in melanoma cells (Del Bello et al, 2007). This latter observation is contrary to reports that treatment of calpain inhibitors I and II increases endogenous p53 content in breast cancer (MCF-7) and colon cancer (RKO) cells (Kubbutat & Vousden, 1997). It has been noted that the specificity of inhibitors used for experiments is crucial in the interpretation of results, as the effects of proteasome, lysozomes and caspases must be eliminated to appreciate the mechanisms of calpain-mediated apoptosis.

CHAPTER 2: OBJECTIVES AND HYPOTHESES

Resistance to cisplatin (CDDP)-based chemotherapy is a major hurdle to the successful treatment of human ovarian cancer (OVCA) and the chemoresistant phenotype in OVCA cells is associated with Akt-attenuated p53-mediated apoptosis. Pro-apoptotic functions of p53 involve both transcription-dependent and independent signaling pathways and dysfunctional localization and/or inactivation of p53 contribute to the development of chemoresistance. PARC is a cytoplasmic protein regulating p53 subcellular localization and subsequent function. Due to the pleiotropic role of p53 in apoptosis, and the emerging discoveries of p53 gain-of-function mutations in cancer progression, it is possible that cytoplasmic sequestration of p53 by PARC represents the most basic, yet important, form of regulation (**Figure 4**). Although PARC is ubiquitously expressed in various tissue and cell types, and is over-expressed in cancer cells that have high cytoplasmic p53 content that respond poorly to chemo- and radiotherapy, little is known about the molecular mechanisms regulating PARC. Determining the cellular and molecular mechanisms by which PARC regulates CDDP-induced p53-dependent apoptosis is crucial in solving the underlying mechanisms of CDDP resistance. Therapeutic strategies that focus on PARC down-regulation could prove useful in reversing the molecular brakes that contribute to the development of chemoresistance. In addition, enhanced response to chemotherapy as a result of PARC down-regulation could reduce current dosing regimen leading to less adverse effects and patient morbidity.

2.1 OVERALL OBJECTIVE

The overall objective is to improve our understanding of the cellular and

molecular mechanisms of chemoresistance in human OVCA; specifically to examine the role of PARC in the contribution of aberrant p53 subcellular localization in the development of chemoresistance.

2.2 OVERALL HYPOTHESIS

PARC contributes to CDDP resistance in human OVCA cells through its ability to regulate p53 subcellular localization and function.

2.3 SPECIFIC HYPOTHESES

1. CDDP induces p53 mitochondrial and nuclear accumulation in chemosensitive but not chemoresistant OVCA cells, an effect attenuated by PARC.
2. Inhibition of Akt is required to sensitize chemoresistant OVCA cells to CDDP in a p53-dependent manner, an effect enhanced by PARC down-regulation.
3. PARC-p53 interaction is more stable in CDDP-treated chemoresistant compared to chemosensitive OVCA cells.
4. CDDP decreases PARC content and Akt activation while increasing total p53, phospho-p53 (ser15) content and apoptosis in chemosensitive but not chemoresistant OVCA cells.
5. CDDP-induced PARC processing is mediated by calpain.
6. Calpain-mediated PARC processing in OVCA cells is dependent on intracellular calcium concentrations.
7. Increased intracellular calcium content can induce calpain activation and PARC processing and apoptosis in chemoresistant OVCA cells.

2.4 SPECIFIC OBJECTIVES

1. To examine the effect of CDDP on PARC content in chemosensitive and resistant OVCA cells.
2. To investigate the role of PARC and its modulation by Akt in the regulation of CDDP-induced, p53-dependent apoptosis in human ovarian cancer cells.
3. To investigate if CDDP sensitivity is dependent on PARC-p53 interaction and subsequent effects on cytoplasmic, mitochondrial and nuclear p53 accumulation in OVCA cells.
4. To investigate the cellular and molecular mechanisms by which CDDP induces PARC down-regulation and chemosensitivity in OVCA cells, including effects on mRNA abundance, and the activation of calpains, caspases and the ubiquitin proteasome pathway.

Figure 4: Hypothetical model illustrating the role and regulation of PARC in CDDP-induced apoptosis in chemosensitive and chemoresistant OVCA cells.

In chemosensitive OVCA cells, CDDP treatment induces p53 phosphorylation and PARC/p53 dissociation. As a result, activated p53 is able to accumulate in the mitochondria and nucleus to induce apoptosis via transcription-independent and -dependent mechanisms. CDDP also increases intracellular calcium concentration ($[Ca^{2+}]_i$) that activates calpain-mediated PARC processing. In chemoresistant OVCA cells, high Akt activity (as shown by increased Akt phosphorylation) inhibits CDDP-induced p53 phosphorylation and PARC/p53 dissociation. As a result, mitochondrial and nuclear p53 accumulation and apoptosis is attenuated. Furthermore, CDDP treatment fails to change $[Ca^{2+}]_i$ in chemoresistant cells, preventing calpain mediated PARC processing and further contributing to cytoplasmic retention of p53 [activation (→); inhibition (⫞); increase (solid lines); decrease (dotted lines); increase change (↑Δ); no change (ØΔ)].

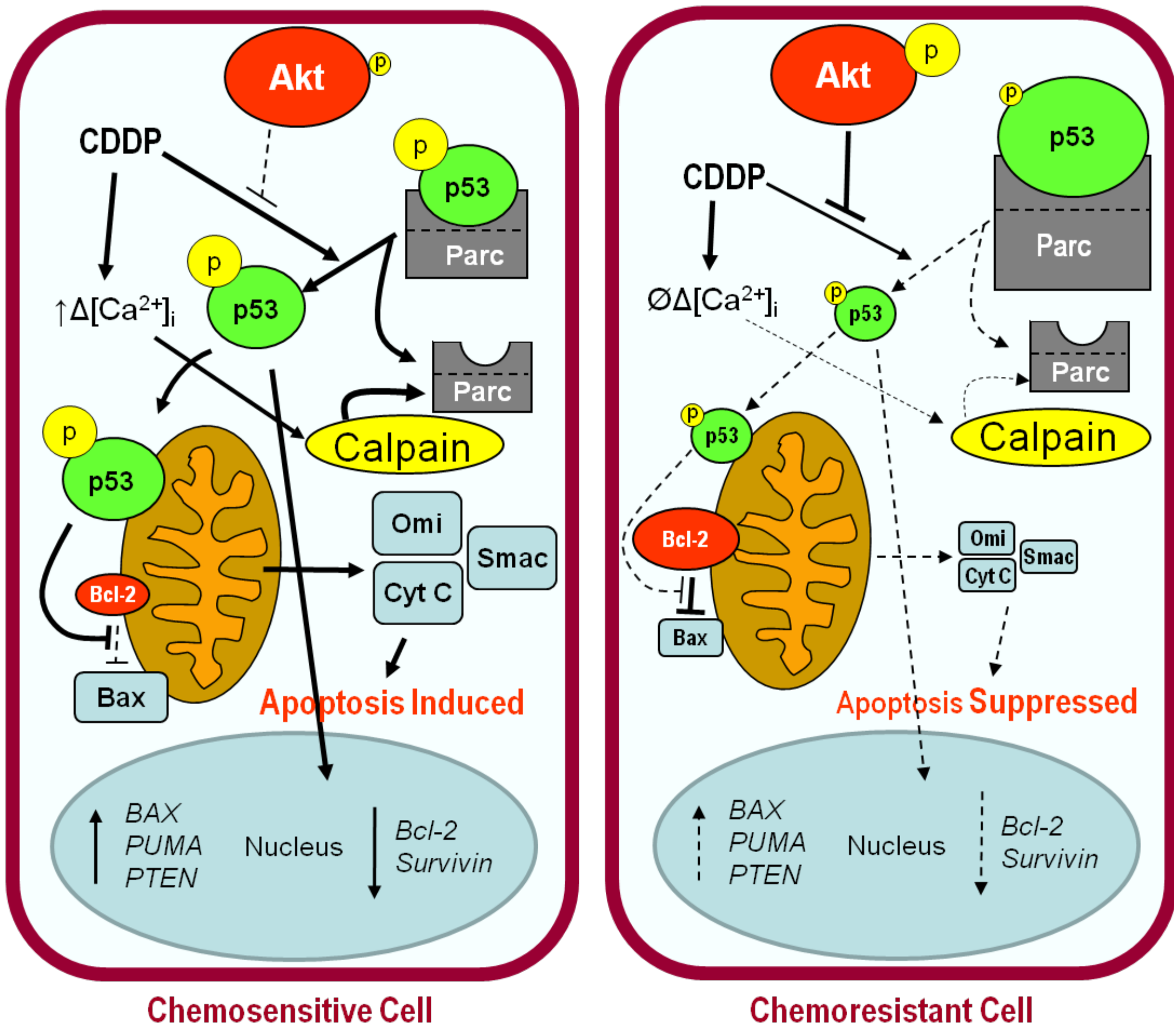


Figure 4

CHAPTER 3: MATERIALS AND METHODS

3.1 Reagents

Cis-diaminedichloroplatinum (Cisplatin; CDDP), dimethyl sulfoxide (DMSO), Hoechst 33258, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), aprotinin, Ethylene glycol-bis (2-aminoethylether)-N,N,N`,N`-tetraacetic acid (EGTA), calcium chloride dehydrate ($CaCl_2$-$2H_2O$), Lactacystin and Calpain 1 and ionomycin calcium salt were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Rabbit polyclonal PARC/H7-AP1 antibody was purchased from Bethyl Laboratories, Inc. (Montgomery, TX). Rabbit polyclonal α-Fodrin and PARP antibodies were from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal GAPDH and active- and pro-Caspase-3 were from Abcam (Cambridge, MA). Goat polyclonal Calpain 1 antibody and peroxidase-conjugated donkey anti-goat immunoglobulin were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Peroxidase-conjugated goat anti-mouse and goat anti-rabbit immunoglobulin were purchased from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, ON). Calpeptin and Epoxomicin were obtained from EMD Chemicals (Gibbstown, NJ). API-2, Z-DEVD-FMK and Z-VAD-FMK were from Tocris Bioscience (Ellisville, MO).

3.2 Cell lines and cell culture

CDDP-sensitive [OV2008 (DiSaia et al, 1972) and A2780s (Molthoff et al, 1991; Shaw et al, 2004)], and CDDP-resistant [C13* (Andrews & Albright, 1992), OVCA420 (Havrilesky et al, 1995), OVCA433 (Bast et al, 1974a; Bast et al, 1974b), and HEY

(Buick et al, 1985)] human ovarian cancer cell lines were derived from serous cystadenocarcinomas of the ovary. The C13* cell line is the isogenic resistant counterpart to OV2008, selected by chronic exposure to increasing concentrations of CDDP. IOSE397 (Xu et al, 2006) is a human ovarian surface epithelial cell line immortalized by transfection of the SV40 large T antigen. All cell lines are p53 wild-type as previously reported (Astanehe et al, 2008; Havrilesky et al, 1995; Wolf et al, 1999; Yan et al, 2006; Yang et al, 2006).

Cells were maintained in RPMI 1640 [chemosensitive (OV2008); chemoresistant (C13* and HEY)] and DMEM/F12 [Chemosensitive (A2780s); chemoresistant (OVCA420 and OVCA433)], and immortalized ovarian surface epithelial cell line (IOSE397) were cultured in MCDB/M199. RPMI 1640 and DMEM/F12 media contained heat-inactivated FBS (10%), streptomycin (50,000 μg/L), penicillin (50,000 U/L) and Fungizone (625 μg/L; Invitrogen Canada Inc., Burlington, ON) (Fraser et al, 2003). MCDB/M199 media (1:1) contained heat-inactivated FBS (15%). Cells were plated (60 mm culture dishes) at 40% confluency unless stated otherwise and cultured at 37^oC with 5% CO_2/95% O_2. Refer to Appendix 7.1 (Table 1) for supplemental information on cell lines used.

3.3 Assessment of apoptosis

Apoptotic cells were identified morphologically using Hoechst 33258 nuclear stain (Asselin et al, 2001b; Grusch et al, 2002; Sasaki et al, 2000). At least 300 cells per treatment group were assessed in randomly selected fields with the counter blinded to the sample group to avoid experimental bias. These results were further confirmed with the

detection of PARP cleavage by Western blot. Specific proteolytic cleavage of PARP is a reliable marker of apoptosis in many cell lines (Mullen, 2004).

3.4 Protein extraction and Western blot detection

Protein extraction and Western analysis were performed as previously reported (Fraser et al, 2003). Primary antibody dilutions: [PARC (1:10,000); α-fodrin (1:2,500); PARP (1:2,000); active- and pro-Caspase-3 (1:1,000); Calpain 1 (1:1,100); GAPDH (1:20,000)] and subsequently with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1,000; RT, 1 h; 1:10,000 for GAPDH). Signal intensity [enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Arlington Heights, IL)] was assessed densitometrically (Scion Image software, version 4.02; Scion Corporation, Frederick, MD).

3.5 Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (PCR) was carried out as previously described (Fraser et al, 2008). PCR primers (Invitrogen) were: PARC (sense: 5'-TGTACCCTTTGCCGTACCTC-3'; PARC antisense: 5'-AGACGAGCTGCTTGGTTCAT-3'); GAPDH (sense 5'-ACAGTCAGCCGCATCTTCTT-3'; GAPDH antisense 5'-GACAAGCTTCCCGTTCTCAG-3'). PCR was performed using HotStarTaq Polymerase (Qiagen) after activation (15 min; 95°C) as follows: denaturation (30 sec; 94°C), annealing (30 sec; 60°C) and, extension (1 min; 72°C), 34 and 21 cycles for PARC

and GAPDH, respectively. Prior to experiments, PCR cycle numbers were optimized to be in the linear range of the assay.

3.6 Determination of p53 subcellular localization

Cells were plated on Poly-D-Lysine [0.05% w/v; Sigma; **Appendix 7.2** (**Figure A1**)] coated 8-well glass culture slides (Becton, Dickinson and Company) and cultured in growth media (48 h) prior to CDDP-treatment. For immunostaining, slides were fixed in paraformaldehyde (4%, 1 h, RT), washed in PBS and blocked with 1% BSA and 1% goat serum. p53 was detected using an anti-p53 mouse monoclonal antibody (1:50; Santa Cruz) and Alexa Fluor 488 goat anti-mouse secondary antibody (1:500; Invitrogen). TOM20 was detected with an anti-TOM20 rabbit polyclonal antibody (1:200; Santa Cruz) and Texas Red goat anti-rabbit secondary antibody (1:500; Invitrogen). Confocal images were obtained (100X objective NA1.4) on an Olympus IX81 inverted microscope with appropriate argon lasers (DAPI, 405 nm; Alexa Fluor 488; 488 nm; Texas Red, 543 nm).

Cells on culture slides were incubated with anti-p53, TOM20 (mitochondria marker) and DAPI (nuclear stain). Subcellular localization of p53 is reported as the percentage of cells showing co-localization with the subcellular compartments examined (Sengupta et al, 2000). The categorization of cells showing p53 accumulation in the mitochondria, nucleus or both mitochondria and nucleus was determined based on co-localization of p53 signal with TOM20 and/or DAPI. The absence of p53 co-localization with either TOM20 or DAPI was taken as cytoplasmic accumulation. At least 400 cells were analyzed per treatment group.

3.7 Immunoprecipitation

The cell lysate was incubated (4°C, 1 h) in Pierce IP Cell Lysis buffer (Thermo Fisher Scientific Inc., Rockford, IL) supplemented with 1X Protease Inhibitor Cocktail I (Sigma) and 1X PhoSTOP phosphatase inhibitor (Roche) and centrifuged (14,000 g, 10 min, 4°C). One mg protein of the supernatant [200 µl; or lysis buffer control(LB)] was incubated with Protein A Dynabeads (Invitrogen) coated with rabbit polyclonal anti-PARC antibody (2 µg/200 µl; 1 h, RT; IgG control) and immunoprecipitated (4°C, 2 h). The beads were pelleted, re-suspended in Laemmli sample buffer (2X; 40 µl; Bio-Rad Laboratories Ltd., Mississauga, ON), boiled (10 min) and loaded onto 9% SDS-PAGE. Following protein transfer to nitrocellulose, PARC and p53 were detected by Western blotting. Refer to Appendix 7.3 (**Figure A2**) for IP protocol optimization results.

3.8 RNA interference

According to manufacturer's instructions, cells were transfected (48 h) with PARC (Santa Cruz) and p53 siRNA (Accell SMARTpool; Dharmacon) and scrambled sequence control (Dharmacon), using Lipofectamine 2000 (Invitrogen). Refer to Appendix 7.4 (**Figure A3**) for PARC siRNA optimization results.

3.9 Adenovirus infections

Cells were infected with an HA-tagged "triple-A" (K179A, T308A, S473A) dominant-negative Akt (DN-Akt) or LacZ cDNA control (Yang et al, 2006). Infection efficiency at multiplicities of infection (MOI) of 40, as determined by X-gal staining, was

>90%. DN-Akt expression was confirmed by Western blot against the HA epitope.

3.10 Construction of PARC over-expression plasmid

The plasmid pcDNA3-HA2-PARC and primers for PCR were from Addgene (Cambridge, MA) and Invitrogen (Carlsbad, CA), respectively. Primers for cloning were: PARC-forward (F): 5'-AAGGTACCATGGTGGGGAAC-3'; PARC-reverse (R): 5'-GGTCTAGACCGTCATAGGCCTCA-3'. The human coding sequence of PARC was amplified from the pcDNA3-HA2-PARC plasmid using the primers above. The PARC-V5-His fusion was constructed by cloning the human coding sequence of PARC into a pEF6/V5-His-TOPO/LacZ expression vector (Invitrogen) though KpnI and XbaI sites. Refer to Appendix 7.5 (**Figure A4**) for additional details regarding the construction, and optimization of use, of the PARC over-expression plasmid.

3.11 Transient transfection

Transfection of cells with the pEF6-derived full-length PARC over-expression vector, and the empty vector control (0-1 μg; 1 ml serum-free medium) used Lipofectamine and PLUS reagent (Invitrogen). Media was removed (24 h post-transfection) and cells were harvested or treated as required.

3.12 In vitro degradation of PARC protein by purified calpain

Purified calpain (μ-calpain; human plasma, 1 unit/6.25 μg protein) was incubated with whole cell lysate (30 μg protein; 1 h, 30°C) as described (Jang et al, 1999). Calpain

was inactivated by boiling (10 min), and/or addition of EGTA (10 mM) and $CaCl_2$ (0-1 mM). The samples were boiled (10 min), centrifuged, and supernatant proteins were resolved by 9% SDS-PAGE.

3.13 In vitro treatment with caspase-3

Whole cell lysates (50 μg) were incubated (30°C, 2 h) in Pipes assay buffer [1,4-piperazinediethanesulfonic acid (Pipes, 20 mM), NaCl (100 mM), dithiothreitol (DDT, 10 mM), EDTA (1 mM), 3-[(3-cholamindopropyl)-dimethylammoniol]-1-propanesulfonic acid (Chaps, 0.1%, w/v), sucrose (10%, w/v), pH 7.2] containing recombinant active caspase-3 (0-20 μg/ml). The samples were boiled in SDS (10 min), centrifuged, and supernatant proteins resolved by 9% SDS-PAGE.

3.14 Statistical Analysis

Results are presented as mean ± standard error of the mean (SEM) of at least three independent experiments. Data were analyzed by one-, two- and three-way ANOVA and subsequently by Bonferroni post-hoc test (PRISM software version 3.0, GraphPad, San Diego, CA). Statistical significance was inferred at $p < 0.05$.

CHAPTER 4: RESULTS

4.1 The effect of CDDP on PARC, p-p53 (ser15) and Akt content in chemosensitive and resistant OVCA cells.

Chemoresistance in OVCA is associated with high Akt expression and activity, and decreased CDDP-induced p53 phosphorylation and function (Fraser et al, 2008; Yang et al, 2006). Since PARC regulates p53 localization and nuclear function (Nikolaev et al, 2003; Vitali et al, 2008), we compared the influence of CDDP on PARC, p53 and Akt in chemosensitive and chemoresistant OVCA cells. Chemosensitive (OV2008) OVCA cells and its chemoresistant isogenic counterpart (C13*) were incubated with CDDP (0, 5, 10 μM; 24 h). DMSO was used as a control and maintained at a concentration of 0.01% (v/v) in all groups. Here, we demonstrate for the first time a regulatory effect of CDDP on PARC expression. CDDP decreased PARC content, increased PARP and α-fodrin cleavage as determined by Western blotting (WB), and induced apoptosis in OV2008 but not C13* cells. Furthermore, basal PARC content was elevated in the C13* cells compared to OV2008 cells. CDDP also decreased Akt activation in OV2008 but not C13* cells, as determined by p-Akt (ser473) content and the subsequent phosphorylation of the downstream Akt target, p-GSK-3β (ser9). Although basal p53 content was higher in the chemoresistant cells, CDDP increased phospho-p53 (ser15) content in OV2008 but not C13* cells, providing further evidence that CDDP sensitivity may be dependent on p53 activation and not just total p53 content (**Figure 5A**). Two-way ANOVA indicates that there was a significant effect of CDDP treatment ($p<0.001$) and cell line ($p<0.001$)

and interaction between both factors ($p<0.001$).

To investigate the association of PARC expression with chemoresistance, the effect of CDDP on PARC content was assessed in a panel of chemosensitive and resistant OVCA cells. CDDP treatment decreased PARC content and induced apoptosis in the chemosensitive (IOSE397 and OV2008) but not the chemoresistant (C13*, HEY, OV420 and OV433) cells. In addition, basal PARC content was higher in most of the chemoresistant OVCA cells compared to the chemosensitive cells [**Figure 5B**; 2-Way ANOVA: Cell line ($p<0.001$), CDDP ($p<0.001$) and cell line x CDDP interaction ($p<0.001$)].

OV2008 cells were treated with and without CDDP (0, 10 μM) for various durations (0, 3, 6, 10, 24 h) and changes in mRNA abundance were assessed by reverse transcriptase (RT)-PCR using PARC specific primers and GAPDH as control. The CDDP-induced PARC down-regulation in chemosensitive cells was not associated with changes in PARC mRNA content [**Figure 5C**; 2-Way ANOVA: CDDP, time effect and CDDP x time ($p>0.05$ for all)] but rather with caspase and calpain activation (as determined by PARP and α-fodrin cleavage respectively), and apoptosis [**Figure 5D**; 2-Way ANOVA: time ($p<0.001$), CDDP ($p<0.001$) and time x CDDP interaction ($p<0.001$)].

Figure 5. CDDP down-regulates PARC and Akt activity and increases p-p53 (ser15) content and apoptosis in chemosensitive but not chemoresistant OVCA cells.

A) CDDP treatment (24 h) increased total p53 content in both chemosensitive and resistant cells, with increased p-p53 (ser15) content caspase and calpain activation (represented by PARP and α-fodrin cleavage, respectively) in the sensitive cells only. CDDP decreased PARC content and Akt activity [represented by p-Akt (ser473) and p-GSK-3β (ser9)] in the sensitive cells only. A dose-dependent increase in apoptosis was seen only with CDDP-treated OV2008 cells [***p<0.001 (versus no CDDP)]. **B)** CDDP induced PARC down-regulation and apoptosis in chemosensitive (IOSE397, OV2008) but not chemoresistant (C13*, HEY, OVCA420, OVCA433) OVCA cells. Cells were treated with CDDP (10 μM; 24 h; DMSO control) and PARC content and apoptosis was assessed. Basal PARC content was lower in the chemosensitive cells compared to the resistant cells, except in the OVCA420 cell, where both PARC and GAPDH (loading control) content were consistently low. ***p<0.001. **C)** CDDP does not affect PARC mRNA content in chemosensitive (OV2008) OVCA cells. OV2008 cells were treated with CDDP (10 μM; DMSO control) for various durations and PARC mRNA content was assessed by RT-PCR using PARC specific primers and GAPDH as the loading control. **D)** The temporal effects of CDDP treatment (10 μM) on PARC content, calpain and caspase-3 activity (represented by α-fodrin and PARP cleavage) and apoptosis in OV2008 cells were examined. CDDP decreased PARC content and increased calpain and caspase-3 activity and apoptosis in a time-dependent manner starting at 12h. [**p<0.01, ***p<0.001 (versus no CDDP); $^{++}$p<0.01, $^{+++}$p<0.001 (versus time = 0)]. Results are expressed as mean ± SEM (n = 3 - 4 replicate experiments).

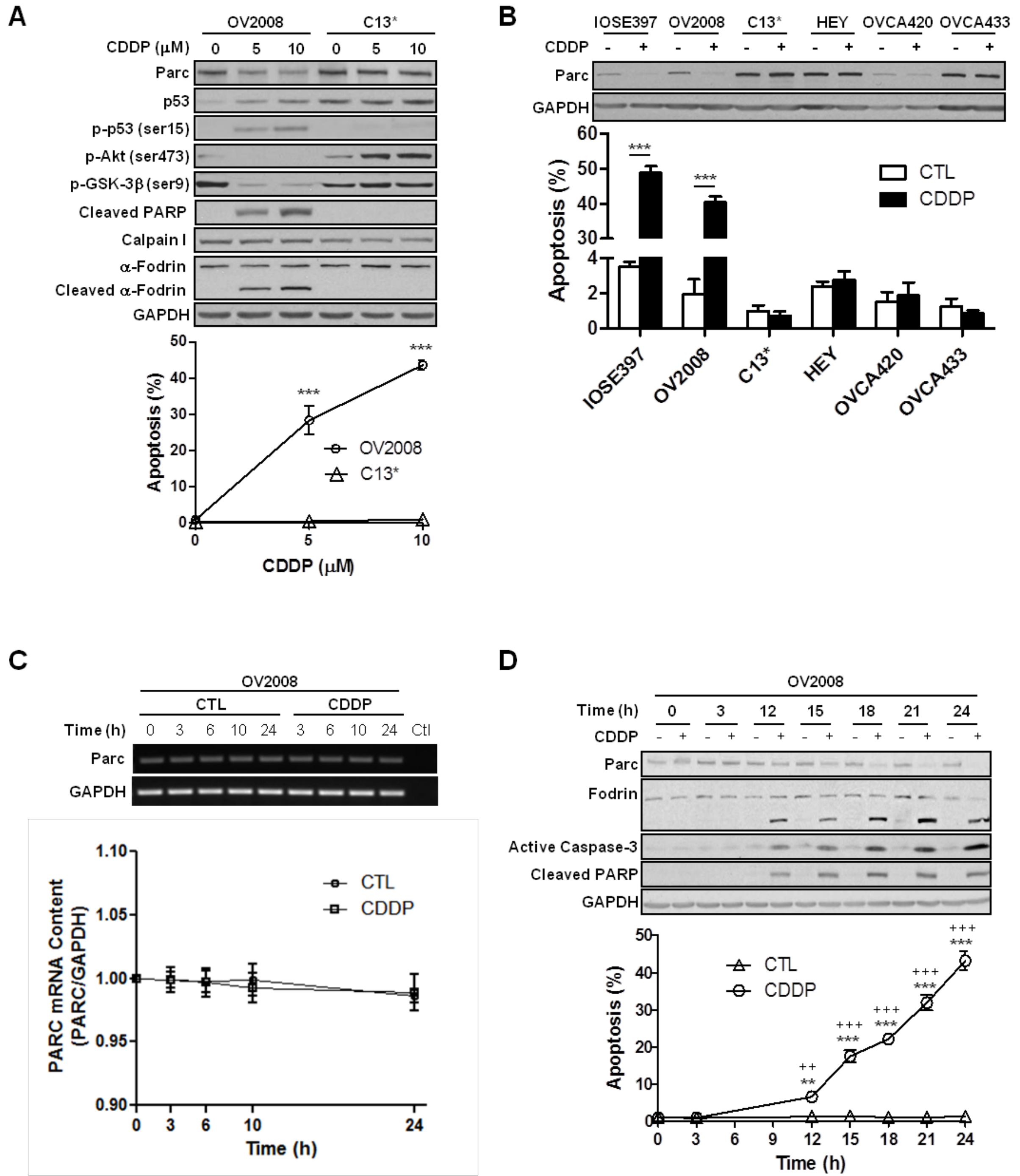


Figure 5

4.2 CDDP-induces p53 mitochondrial and nuclear accumulation in chemosensitive but not chemoresistant OVCA cells.

The mechanism of p53-mediated apoptosis requires both transcriptionally-dependent and independent pathways. At the nucleus, p53 induces cell death by activating target genes such as Bax, Fas/APO-1 and KILLER/DR5 and evidence is accumulating of the pro-apoptotic role of p53 at the mitochondria. Reports indicate that p53 translocation to the mitochondria is rapid (Marchenko et al, 2000) and occurs only during p53-dependent apoptosis but not p53-independent apoptosis or p53-mediated cell cycle arrest (Marchenko et al, 2000). Since CDDP-induced apoptosis in OVCA cells is p53-dependent and the development of chemoresistance is partly a result of p53-loss-of-function at the nucleus and mitochondria (Fraser et al, 2008; Yang et al, 2006), the effects of CDDP on p53 subcellular localization in chemosensitive and resistant OVCA cells was examined.

Chemosensitive (OV2008) and chemoresistant (C13*) OVCA cells were treated with CDDP (10 μM) for various durations (0, 6 and 24 h) and p53 subcellular localization was assessed by immunofluorescence/confocal microscopy. TOM20 was used as a mitochondrial specific marker and the nucleus was stained by DAPI. As shown in **Figure 6A**, we demonstrate by immunofluorescence that CDDP induces changes in p53 subcellular accumulation in OV2008 cells from cytoplasmic → mitochondrial → nuclear over time. In contrast, chemoresistant cells displayed only cytoplasmic p53 at all time points (**Figure 6B**). Cells that were categorized as "Cyto", only displayed cytoplasmic p53 accumulation and did not show p53 at the mitochondria or the nucleus. Cells categorized as "Mito", displayed mitochondrial p53 accumulation, did not have p53

in the nucleus, but could still have cytoplasmic p53. Cells categorized as "Mito/Nuc" displayed p53 co-localization at both the mitochondria and nucleus, but could still have p53 in the cytoplasm. Similarly, the category "Nuc" contained cells that displayed nuclear p53 accumulation, did not have mitochondrial p53 co-localization, but could still have p53 in the cytoplasm. 1-Way ANOVA indicates that there was a significant time effect in all subcellular fractions examined in OV2008 cells ($p<0.01$) but not C13* cells. These results are consistent with published work from our laboratory indicating that CDDP induces p53-dependent mitochondrial death protein release in chemosensitive but not resistant cells, and that this occurs prior to the pro-apoptotic nuclear activities of p53 (Yang et al, 2006).

Figure 6. CDDP induces p53 mitochondrial and nuclear accumulation in chemosensitive but not chemoresistant OVCA cells.

A) Chemosensitive (OV2008) and **B)** chemoresistant (C13*) OVCA cells were cultured with CDDP (10 μM; DMSO as vehicle control). Accumulation of p53 in the mitochondria (TOM20) and nucleus (DAPI) was assessed by immuno-fluorescence/confocal microscopy. Arrows indicate exact locations across fields and serve to highlight the presence or absence of p53 co-localization with TOM20 and DAPI. Shown are representative examples of cells categorized as displaying cytoplasmic p53 localization [OV2008 (0 h) and C13* (0, 6, 24 h)]. Arrows (white) in these fields show an absence of p53 co-localization with the mitochondria (TOM20) with no p53 signal in the nucleus (DAPI). Also shown are representative examples of cells categorized as displaying only mitochondrial p53 accumulation [OV2008 (CDDP; 6 & 24 h)], where the arrows (yellow) highlight the overlap of p53 with the mitochondria (TOM20) with no punctate p53 signal in the nucleus (DAPI). Quantitation of the confocal images shows the population of cells exhibiting p53 accumulation in the cytoplasm, mitochondria, both mitochondria and nucleus, and only the nucleus. At least 200 cells were counted for each time point in each replicate. Results are expressed as mean ± SEM (n = 3 replicate experiments); *** $p<0.001$.

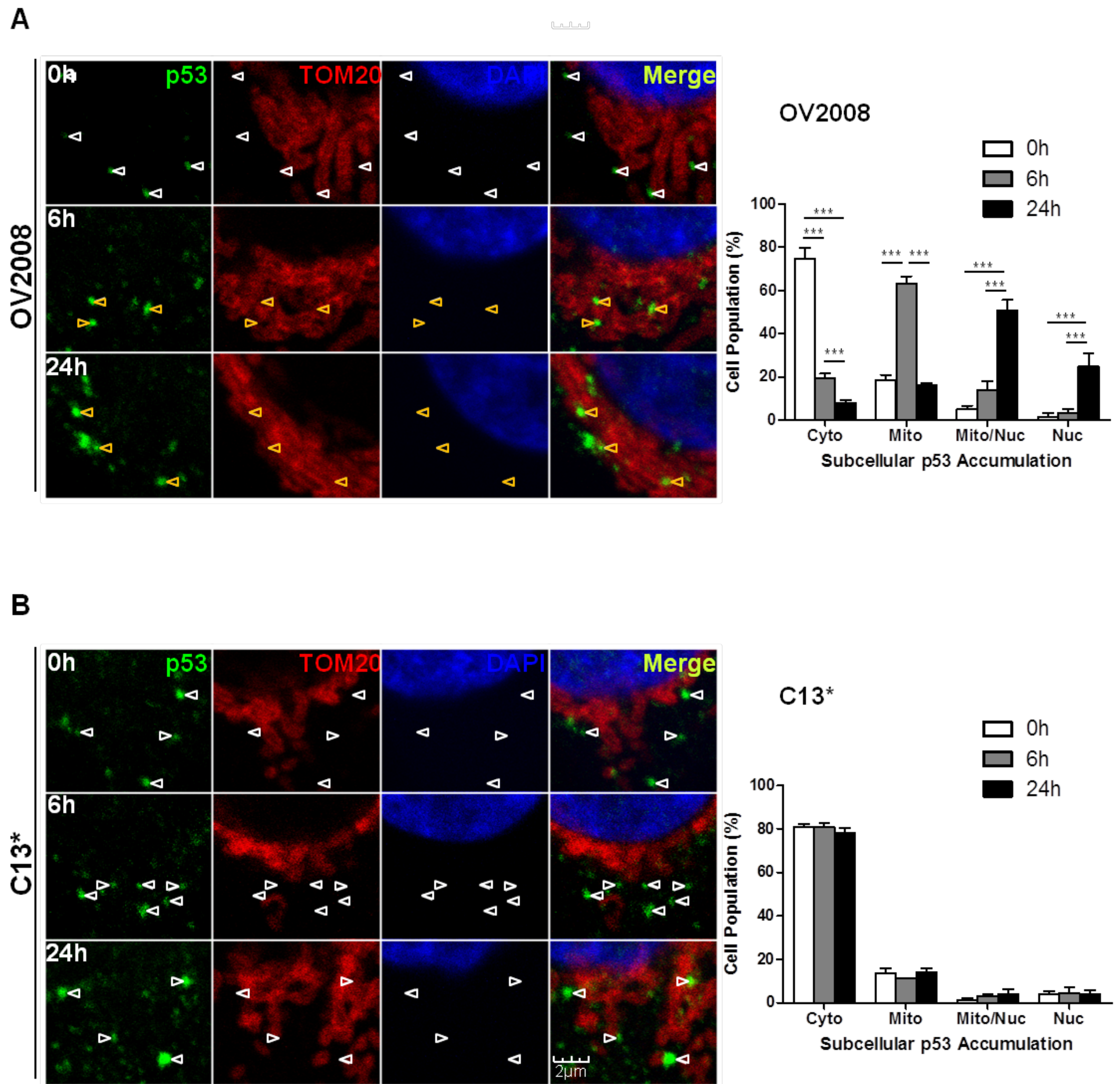


Figure 6

4.3 PARC attenuates CDDP-induced p53 mitochondrial accumulation and apoptosis in chemosensitive OVCA cells.

PARC down-regulation increases p53 nuclear localization and transcriptional activity in cells expressing high cytoplasmic p53 content (Mulhall et al; Nikolaev et al, 2003). In **Figure 6** we demonstrate that CDDP induced changes in p53 subcellular localization as early as 6 hours in chemosensitive but not resistant OVCA cells. To examine the physiological role of PARC in the regulation of p53 subcellular localization and CDDP sensitivity, we constructed a recombinant PARC plasmid to express a human PARC-V5-His fusion protein in OVCA cells [**Appendix 7.5** (**Figure A4**)].

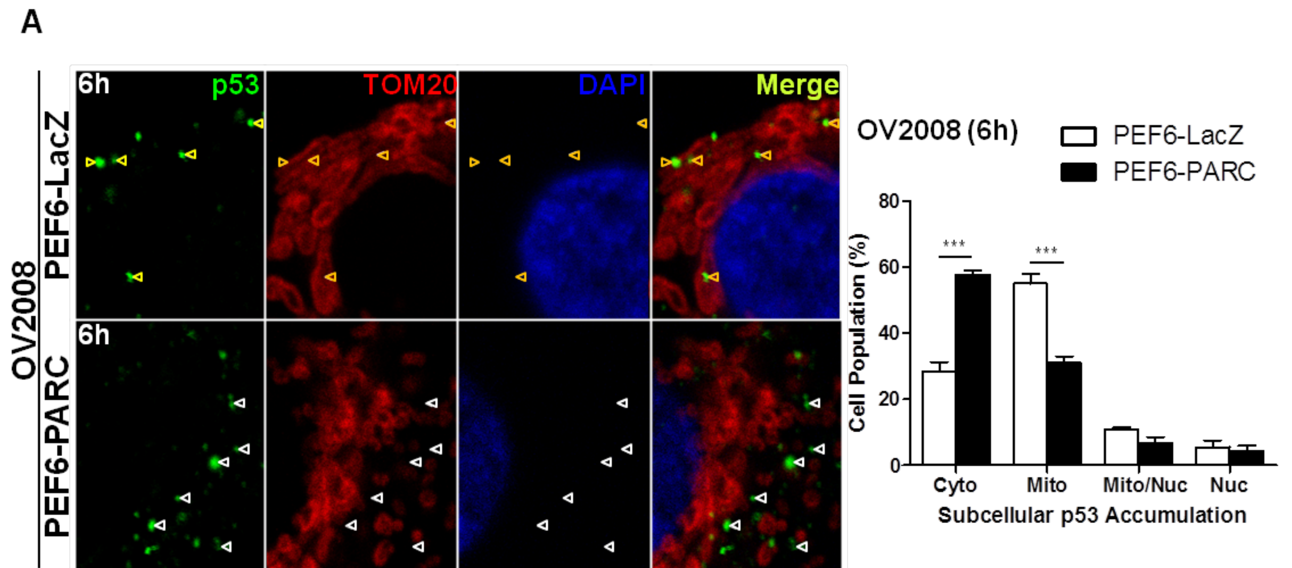
To determine the role of PARC in p53 subcellular trafficking and apoptosis, PARC was over-expressed (pEF6-PARC-V5-His, 1 μg, 24 h; pEF6-V5-LacZ as empty vector control) in OV2008 cells treated with CDDP (10 μM, 6 h; DMSO as control) and p53 subcellular localization was assessed by immunofluorescence (IF)/confocal microscopy as above. Here we report that PARC over-expression attenuated CDDP-induced mitochondrial p53 accumulation and increased cytoplasmic p53 retention at 6 hours [**Figure 7A**; 2-Way ANOVA, pEF6-PARC ($p<0.001$), fraction ($p<0.001$) and interaction between the two factors ($p<0.001$)], suggesting that PARC is involved in the regulation of p53 subcellular localization. Furthermore, PARC over-expression in PARC-silenced, chemosensitive A2780s cells [**Appendix 7.7** (**Figure A6**)] decreased CDDP-induced apoptosis without affecting p53 content (**Figure 7B**). 2-Way ANOVA indicates a significant effect of PARC expression ($p<0.001$) and CDDP ($p<0.001$) and interaction between these 2 factors ($p<0.01$). The fusion PARC protein produced from this expression vector interacted with p53 as determined by PARC immunoprecipitation

(IP) and co-IP of p53 (**Figure 7C**).

Figure 7. PARC attenuates CDDP-induced p53 mitochondrial accumulation and apoptosis in chemosensitive OVCA cells.

A) PARC was over-expressed in OV2008 cells (PEF6-PARC; PEF6-LacZ as control; 0.5μg, 24 h) and treated with CDDP (10 μM, 6 h). Subcellular p53 localization was assessed by IF. Arrows indicate exact locations across fields and serve to highlight the presence or absence of p53 co-localization with TOM20 and DAPI. A representative example of a cell categorized as displaying mitochondrial p53 localization is shown [OV2008 (PEF6-LacZ; 6 h)]. Arrows (yellow) across these fields show the overlap of p53 with the mitochondria (TOM20). In contrast, a representative example of a cell categorized as displaying cytoplasmic p53 localization is shown [OV2008 (PEF6-PARC; 6 h)]. Arrows (white) across these fields show the absence of p53 co-localization with the mitochondria (TOM20) while no p53 signal is detected in the nucleus (DAPI). Results are expressed as mean ± SEM (n = 3 replicate experiments); *** $p<0.001$. **B)** PARC was over-expressed (PEF6-PARC; PEF6-LacZ as control; 1 μg; 24 h) in PARC-silenced chemosensitive (A2780s) OVCA cells and treated with CDDP (24 h). PARC over-expression attenuated CDDP-induced PARP cleavage and apoptosis. [**$p<0.01$, ***$p<0.001$] with no effect on p53 content (WB). Results are expressed as mean ± SEM (n = 3 replicate experiments). **C)** Interaction of the recombinant PARC protein with p53 was confirmed by PARC immunoprecipitation and co-IP/Western blot of p53 using whole cell lysates (WCL) of A2780s PARC-silenced OVCA cells transfected with the pEF6-PARC expression vector as above (n = 1).

A



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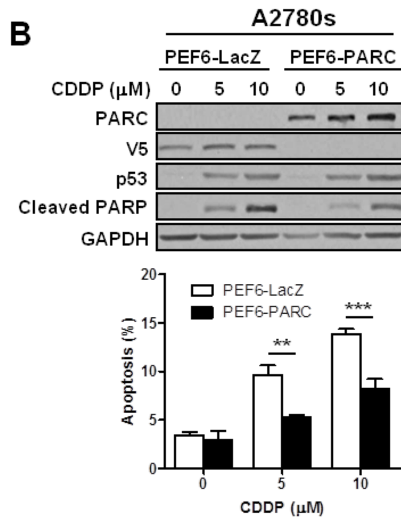

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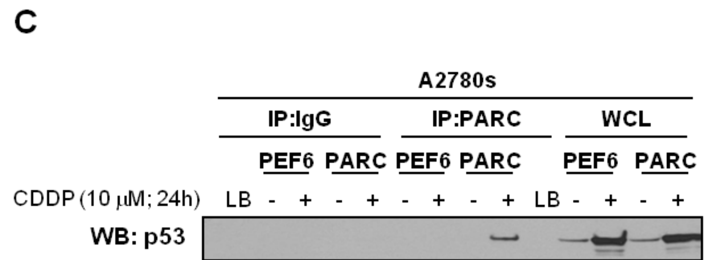


Figure 7

4.4 PARC down-regulation enhances CDDP-induced apoptosis in chemosensitive but not chemoresistant OVCA cells.

To determine the physiological role of PARC in the phenomenon of CDDP-induced apoptosis and its impact in mediating chemosensitivity in OVCA cells, PARC content was down-regulated using RNA interference followed by CDDP treatment. Chemosensitive (OV2008) and chemoresistant (C13*) OVCA cells were cultured in the presence or absence of PARC siRNA (0, 100 nM; 48 h) followed by various treatment concentrations of CDDP (0, 2.5, 5, 10 μM; 24 h) or DMSO control. As expected, CDDP alone induced apoptosis in a dose-dependent manner in OV2008 but not the C13* cells. PARC down-regulation significantly enhanced CDDP-induced apoptosis in chemosensitive OVCA cells (10 μM; 24 h) but not in CDDP-treated or untreated C13* cells [**Figure 8**; 2-Way ANOVA: OV2008: PARC siRNA ($p<0.001$), CDDP ($p<0.001$) and PARC siRNA x CDDP ($p<0.01$); C13*: PARC siRNA, CDDP effect and PARC siRNA x CDDP ($p>0.05$ for all)].

Figure 8. PARC down-regulation enhances CDDP-induced apoptosis in chemosensitive but not chemoresistant OVCA cells.

PARC silencing by RNAi [scrambled sequence (S. Seq.) as control; 100 nM; 48 h] enhanced CDDP-induced apoptosis (24 h) in chemosensitive (OV2008) but not in chemoresistant (C13*) OVCA cells [**$p<0.01$, ***$p<0.001$ (versus S. Seq.)].

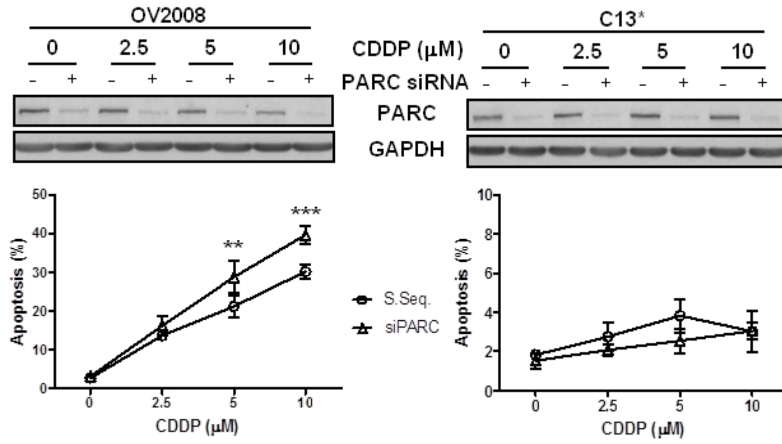


Figure 8

4.5 The role of PARC in the regulation of CDDP-induced apoptosis; regulation by Akt.

Elevated Akt activity is a determinant of chemoresistance preventing the activation and phosphorylation of p53 required for CDDP-induced apoptosis (Fraser et al, 2008). The unresponsiveness to CDDP resulting from PARC knock-down in the chemoresistant C13* cells (as shown above) may indicate that the liberation of sequestered p53 may not be sufficient to induce apoptosis and may require p53 activation. As such, we then determined if p53 function and CDDP-sensitivity in OVCA cells is dependent on both PARC and Akt-mediated regulation.

PARC down-regulation was achieved as above, and down-regulation of Akt activity was accomplished using an adenoviral dominant-negative Akt (0, 40, 80 MOI; 48 h). Akt down-regulation had no effect on CDDP-induced apoptosis in OV2008 cells, irrespective of PARC silencing. In contrast, down-regulation of Akt activity significantly induced apoptosis in C13* cells, an effect enhanced by PARC down-regulation [**Figure 9**; 3-Way ANOVA - OV2008: PARC siRNA ($p<0.01$), DN-Akt ($p<0.01$), CDDP ($p<0.001$), with no interaction between these factors; C13*: PARC siRNA ($p<0.001$), DN-Akt ($p<0.001$), CDDP ($p<0.01$), with interaction between PARC siRNA and DN-Akt ($p<0.05$)]. PARC down-regulation and adenoviral infection was confirmed by WB for PARC and an HA-epitope respectively. These results suggest that Akt is a determinant of CDDP resistance in OVCA cells, and that PARC down-regulation can enhance CDDP sensitivity once Akt activity is reduced.

Figure 9. The role of PARC in CDDP-induced apoptosis; regulation by Akt.

Down-regulation of Akt activity (DN-Akt; LacZ as control; 48 h) induced apoptosis in chemoresistant (C13*) but not chemosensitive cells following CDDP treatment (0-10 μM; 24 h). Apoptosis was significantly enhanced by PARC down-regulation (PARC siRNA; scrambled sequence control; 100 nM; 48 h) in the absence and presence of CDDP (10 μM; 24 h). [$^{***}p<0.001$ (versus S. Seq.); $^{++}p<0.01$, $^{+++}p<0.001$ [versus respective DN-Akt (MOI: 0)]; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ (versus respective CDDP CTL)].

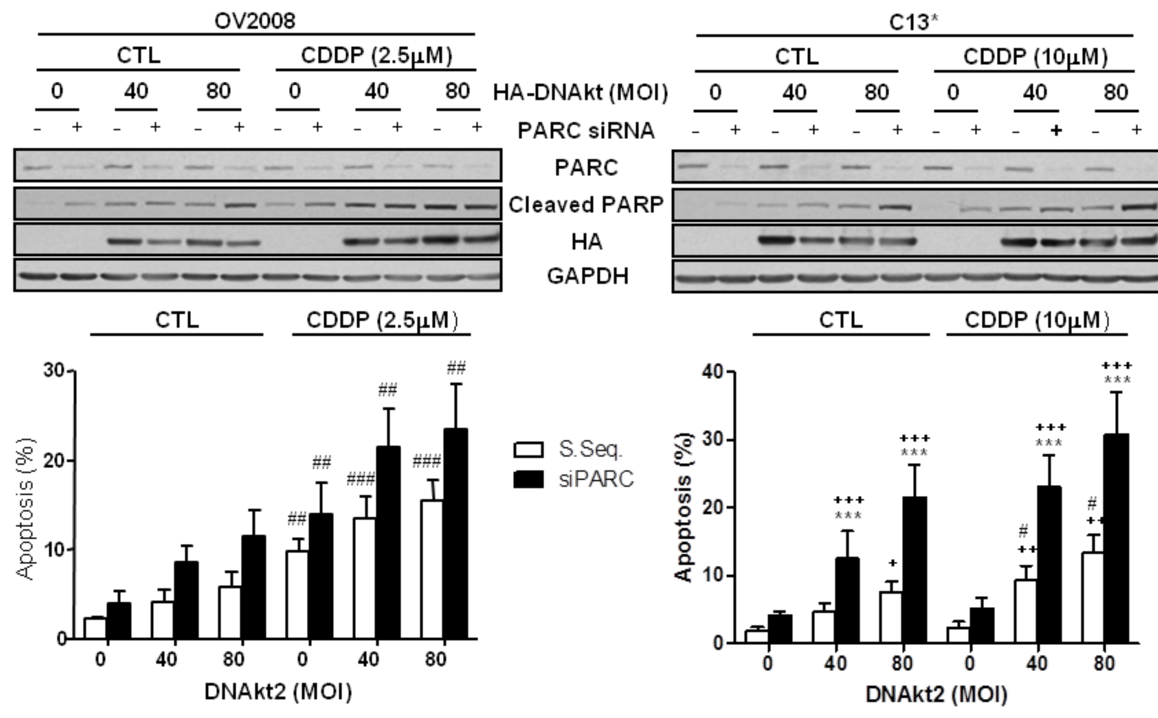


Figure 9

4.6 PARC and Akt-mediated regulation of CDDP-sensitivity in OVCA cells is p53-dependent.

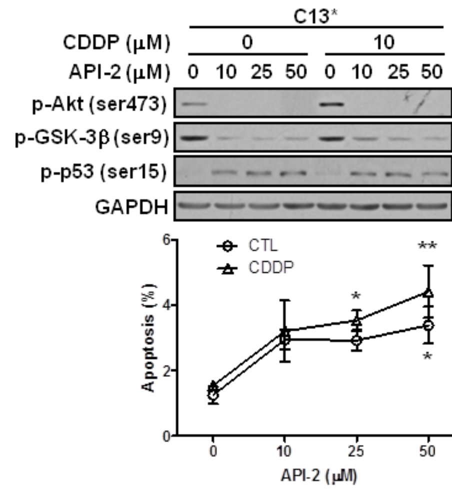
API-2 is a highly selective small molecule inhibitor of Akt and has been shown to inhibit tumour growth and induce apoptosis in human cancer cells with high Akt expression (Yang et al, 2004). Pretreatment of C13* with API-2 (0-50 μM; 1 h) inhibited the activation and phosphorylation of Akt and the downstream target GSK-3β and induced apoptosis in the presence and absence of CDDP (0-10 μM; 24 h) [**Figure 10A**; 2-Way ANOVA - API-2 dose response: API-2 ($p<0.01$)].

We then examined the role of PARC in Akt-mediated CDDP-resistance in OVCA cells and if the enhancement of CDDP sensitivity as a result of PARC down-regulation is p53-dependent. Down-regulation of Akt activity with API-2 (25 μM; 1 h) facilitated CDDP-induced apoptosis in chemoresistant cells (C13*), a response further enhanced by PARC down-regulation (100 nM; 48 h). These responses were p53-dependent and were attenuated by p53 silencing (0-100 nM; 24 h) [**Figure 10B**: 3-Way ANOVA - API-2 (25 μM): CDDP ($p<0.01$), PARC siRNA ($p<0.001$), p53 siRNA ($p<0.001$), PARC siRNA x p53 siRA interaction ($p<0.05$)]. Taken together, these findings suggest that PARC down-regulation can enhance p53-dependent CDDP sensitivity once Akt activity is inhibited. Consistent with the above experiment, PARC down-regulation enhanced the pro-apoptotic effect of decreasing Akt activity, in the presence and absence of CDDP. Apoptosis resulting from these treatments were p53-dependent as treatment with p53 siRNA attenuated these effects. PARC and p53 down-regulation was confirmed by WB. The effect of API-2 in decreasing Akt activity is confirmed by the decrease in phosphor-Akt (ser473) content (WB).

Figure 10. PARC and Akt-mediated regulation of CDDP-sensitivity in OVCA cells is p53-dependent.

A) API-2 (1 h pretreatment) decreased Akt activity [as determined by p-Akt (ser473) and p-GSK-3β (ser9) content] and increased p-p53 (ser15) content and induced apoptosis in chemoresistant C13* OVCA cells in the presence and absence of CDDP (24 h) [*p<0.05, **p<0.01 (versus no API-2)]. Results are expressed as mean ± SEM (n = 3 replicate experiments). **B)** PARC down-regulation (as above) significantly enhanced API-2-induced (25 μM; 1 h pre-treatment) apoptosis in C13* cells treated with CDDP (24 h), and this effect was p53-dependent as p53 siRNA treatment (100 nM; 48 h) inhibited the induction of apoptosis. Results are expressed as mean ± SEM [n = 5 replicate experiments; **p<0.01, ***p<0.001 (versus no PARC siRNA and no p53 siRNA); ++p<0.01, +++p<0.001 (versus PARC siRNA), ##p<0.01, ###p<0.001 (versus respective CDDP CTL)].

A



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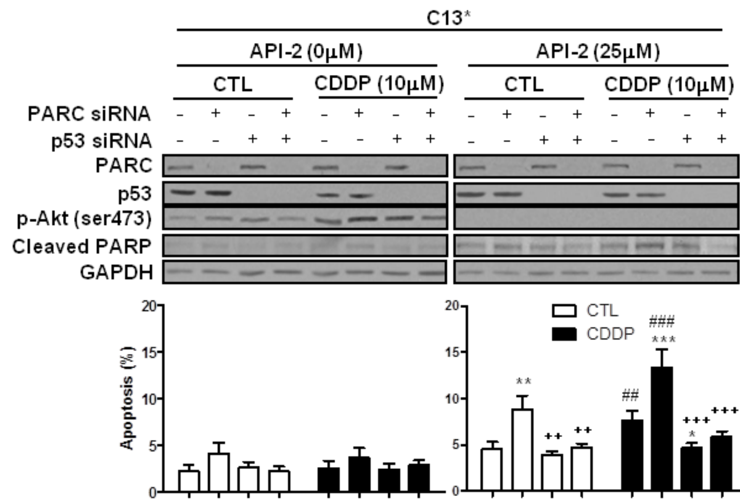


Figure 10

4.7 CDDP has no effect on PARC and p53 interaction in chemoresistant OVCA cells.

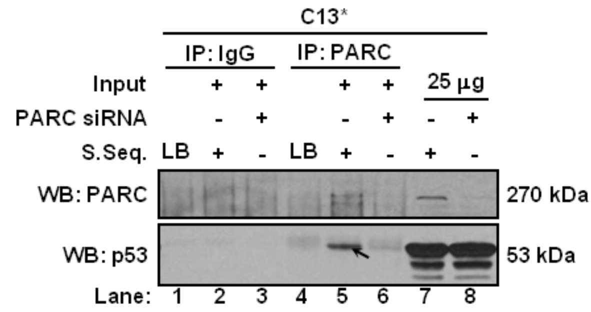
PARC and p53 have been shown to interact and this interaction is more stable in cancer cells where cytoplasmic p53 content is high (Nikolaev et al, 2003). We have demonstrated that in chemoresistant OVCA cells, p53 content is high regardless of CDDP treatment, and p53 accumulates in the cytoplasm. To determine if CDDP decreases PARC and p53 interaction in chemoresistant cells, C13 cells were treated with CDDP (10 μM) for various durations and PARC/p53 interaction was examined by immunoprecipitation and Western blot.

Basal PARC and p53 contents are abundant in C13* cells and could be co-immunoprecipitated (**Figure 11A**, **Lane 5**), a phenomenon sensitive to PARC down-regulation (**Figure 11A, Lane 6**) but unaffected by CDDP treatment (**Figure 11B**, **Lanes 7-10**). CDDP had no effect on PARC and p53 content and did not induce apoptosis, as expected (**Figure 11B**, **bottom).** The effect of CDDP on PARC/p53 interaction in chemosensitive OV2008 cells could not be determined by IP/co-IP as CDDP induced changes in both PARC and p53 content at 24 h. The effect of CDDP on PARC/p53 interaction at the earlier time points (1.5 - 6 h) were also difficult to examine as p53 content is maintained at relatively low levels [**Appendix 7.6** (**Figure A5**)].

Figure 11. CDDP has no effect on PARC and p53 interaction in chemoresistant OVCA cells.

A) Chemoresistant (C13*) OVCA cells were treated with PARC siRNA or scrambled sequence control (100 nM; 48 h). Whole cell lysates were subjected to PARC immunoprecipitation (IP). IP of normal rabbit IgG (Lanes 1-3) and input of lysis buffer (Lanes 1 & 4) are included as controls. Whole cell lysates were also included to demonstrate the efficacy of PARC down-regulation by siRNA (Lanes 7 & 8). The heavy-chain IgG of the PARC antibody migrates slightly slower than p53 (Lanes 4-6; arrow) and is used as a reference to indicate the relative abundance of p53 that is co-immunoprecipitated with PARC (n = 1).

B) PARC-p53 interaction is unaffected by CDDP in chemoresistant (C13*) OVCA cells. Protein A Dynabeads were incubated with PARC antibody (Bethyl, A300-096A; 2 ug/mg lysate; 1 h) and subsequently incubated for 1 hour with C13* whole cell lysate [1000 μg; lysis buffer control (LB)] obtained from CDDP treated (0-10 μM) cells for various durations. PARC pull-down and p53 content resulting from co-IP is unaffected by time or CDDP treatment (Lanes 3-10). Also shown are controls for potential non-specific bands involving antibody IgG (Lanes 1 & 2) and from the lysis buffer (Lanes 1 & 11). To confirm the effectiveness of the CDDP treatment, PARC, p53, GAPDH content and apoptosis counts of the above samples were examined. Results are expressed as mean ± SEM (n = 3 replicate experiments)



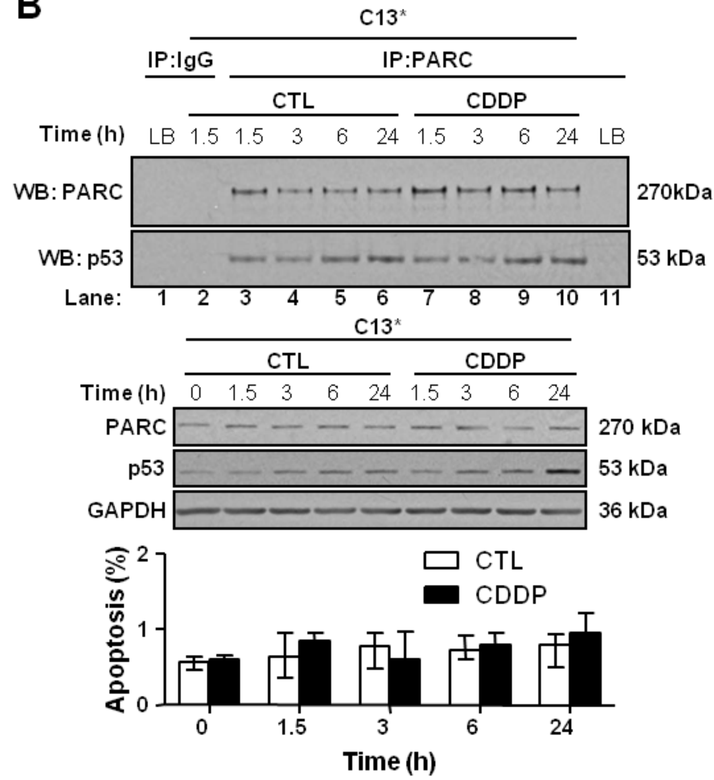


Figure 11

4.8 Calpain mediates CDDP-induced PARC processing and chemosensitivity in OVCA cells.

Since calpain is involved in CDDP-induced apoptosis (Liu et al, 2009; Liu et al, 2008), we then examined whether PARC is a substrate for calpain. *In vitro* experiments confirm that PARC is a calpain substrate (**Figure 12A**) and that calpain-mediated PARC down-regulation is Ca^{2+}-dependent (**Figure 12B**) The simultaneous incubation of OV2008 whole cell lysate with calpain and the calcium chelator EGTA prevented the degradation of PARC and α-fodrin (a known calpain substrate; (Sato et al, 2004; Siman et al, 1984; Takamure et al, 2005). Calpain inactivation (by boiling) also prevented the calpain-induced loss of PARC and α-fodrin (**Figure 12A**). Furthermore, the concurrent degradation of both PARC and α-fodrin by calpain in a Ca^{2+}-dependent manner, was independent of caspase activity as PARP content was unchanged (**Figure 12B**). These results suggest that calpain is the primary mediator of PARC processing.

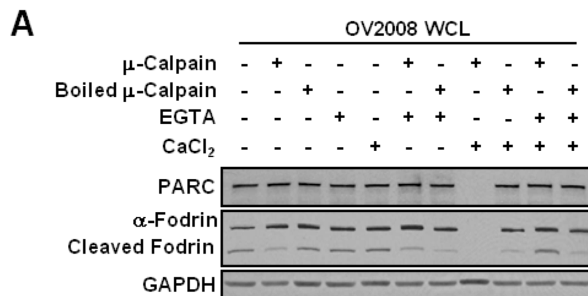
To determine the effects of the calpain system on CDDP-induced PARC processing, OV2008 cells were pre-treated with the cell-permeable calpain inhibitor Calpeptin followed by CDDP. Pretreatment with Calpeptin prevented CDDP-induced PARC down-regulation, α-fodrin cleavage and apoptosis. Maximal effects were seen at 25 μM Calpeptin, with cytotoxic effects becoming apparent at higher concentrations **(Figure 12C)**. 2-Way ANOVA indicates that there was a significant effect of Calpeptin ($p<0.001$) and CDDP ($p<0.001$) and Calpeptin x CDDP interaction ($p<0.001$).

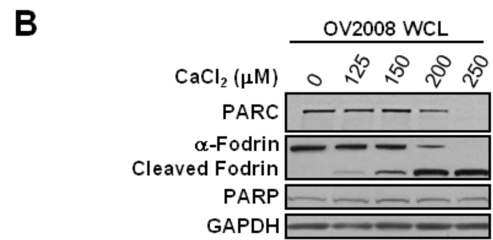
Calpain activation is Ca^{2+}-dependent and CDDP increases intracellular Ca^{2+} levels in chemosensitive OV2008 cells but not in its resistant variant (C13*) (Al-Bahlani et al, 2011). Since calpain content is the same in both chemosensitive and resistant OVCA

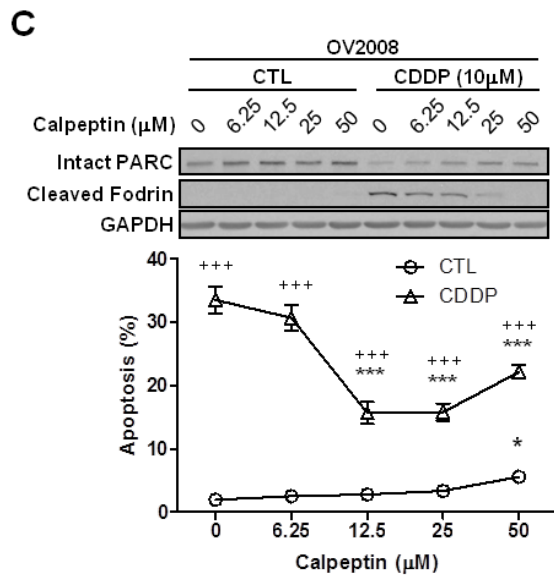
cells (**Figure 5A**), we examined if Ionomycin-induced Ca^{2+} influx in chemosensitive and resistant OVCA cells would result in PARC degradation and apoptosis. As demonstrated in **Figure 12D**, ionomycin decreased PARC content in both chemosensitive (OV2008) and chemoresistant (C13*, HEY and OVCA420) OVCA cells and induced apoptosis in all cell types examined. 2-Way ANOVA indicates that there was a significant effect of Ionomycin ($p<0.001$) and cell line ($p<0.001$) and interaction between both factors ($p<0.001$). Ionomycin did not induce PARC down-regulation in the chemosensitive IOSE397 and chemoresistant OVCA433 OVCA cells, which may reflect alternative mechanisms of Ca^{2+} regulation in these cells. These differences may include cell specific Ca^{2+} response, expression and activiation of calpain and endogenous inhibitors. These data implicate Ca^{2+}/calpain in CDDP-induced PARC processing and chemosensitivity.

Figure 12. CDDP-induced PARC processing and chemosensitivity is regulated in part by calpain activation.

A) OV2008 cell lysates (30 μg) were incubated for 1 hour at 30°C with purified calpain I from human plasma. Calpain activity was inhibited by boiling, EGTA treatment and absence of $CaCl_2$ during the incubation process. PARC processing occurs when native calpain is activated by $CaCl_2$ (lane 8). α-fodrin cleavage is indicative of calpain activity (n = 3 replicate experiments). **B)** PARC processing as a result of calpain activity is Ca^{2+}-dependent. OV2008 cell lysates were incubated as above with increasing $CaCl_2$ concentrations. Calpain-mediated PARC processing was complete at 250 μM $CaCl_2$. α-fodrin cleavage occurred at lower $CaCl_2$ concentrations and was used as an indicator calpain activity (n = 3 replicate experiments). **C)** CDDP-induced PARC down-regulation in chemosensitive OVCA cells is mediated by calpain. Chemosensitive OVCA cells (OV2008) pre-treated with the cell permeable calpain inhibitor calpeptin (50 μM; 1 h; DMSO control) attenuated PARC down-regulation, α-fodrin cleavage and apoptosis induced by CDDP (24 h) [*p<0.05, ***p<0.001 (versus no Calpeptin); +++p<0.001 (versus no CDDP)]. Results are expressed as mean ± SEM (n = 3 replicate experiments). **D)** Ionomycin (Iono; 1 μM, 1 h) induced PARC down-regulation in both chemosensitive (OV2008) and chemoresistant (C13*, HEY and OVCA420) OVCA cells, with increases in apoptosis in all cell types. Ionomycin did not induce PARC down-regulation in the chemosensitive IOSE and chemoresistant OVCA433 cells. *p<0.05, ***p<0.001; Results are expressed as mean ± SEM (n = 3 replicate experiments).







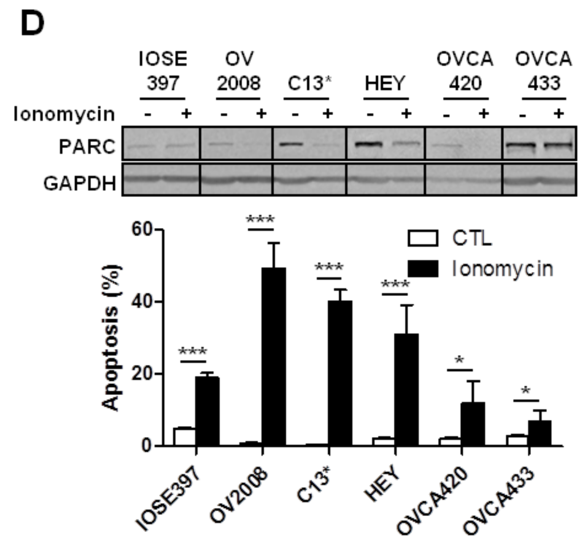


Figure 12

4.9 Caspases and the ubiquitin proteasome pathway are not the primary mediators of CDDP-induced PARC processing in OVCA cells.

The caspase family of cysteine-proteases regulates the process of apoptosis at various levels. Caspase-mediated cell death is generally considered a fast and ordered process where caspase activation is rapidly followed by cellular fragmentation and phagocytic removal (Thornberry, 1998). Since the protein sequence of PARC contains three Asp-X-X-Asp (DXXD) motifs, indicative of potential caspase-3 cleavage sites (Nicholson et al, 1995; Takahashi et al, 1998), we examined the role of caspase-3 in CDDP-induced PARC down-regulation and apoptosis in chemosensitive OVCA cells (OV2008). As shown in **Figure 13A**, although CDDP decreased PARC content, increased PARP cleavage and induced apoptosis, the cell permeable caspase-3 inhibitor Z-DEVD-FMK attenuated CDDP-induced PARP cleavage and apoptosis in a concentration-dependent manner but had no significant effect on PARC cleavage.

Since caspase-7, highly homologous to caspase-3, has very similar substrate specificity and might substitute for caspase-3 in mediating PARC cleavage, the above experiments were extended to include the pan-caspase inhibitor Z-VAD-FMK. As shown in **Figure 13B**, although CDDP-induced PARP cleavage and apoptosis in OV2008 cells were attenuated by pre-treatment with Z-VAD-FMK in a concentration-dependent manner, no significant effect on CDDP-induced PARC degradation could be detected. 2-Way ANOVA: Z-DEVD-FMK ($p<0.001$), Z-VAD-FMK ($p<0.001$), CDDP ($p<0.001$) and interaction between each caspase inhibitors with CDDP ($p<0.001$).

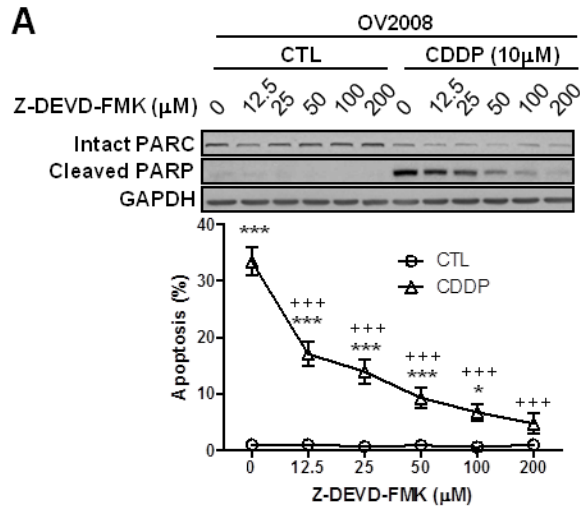
To further determine if PARC is indeed a substrate of caspase-3, the effect of recombinant active caspase-3 on PARC processing in OV2008 cell extracts was

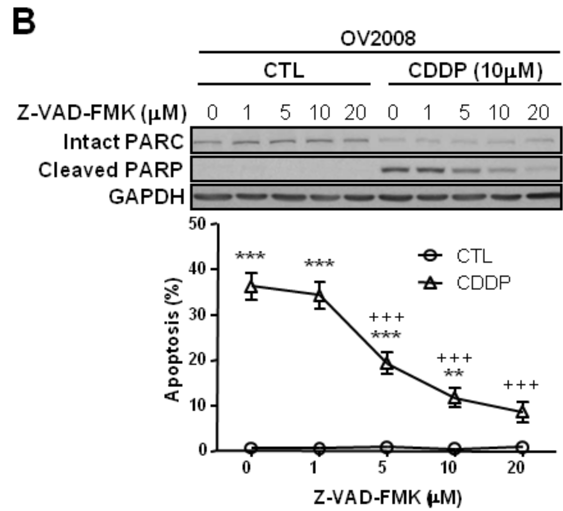
examined *in vitro*. As shown in **Figure 13C**, *in vitro* exposure to active caspase-3 did not significantly decrease PARC content. PARP cleavage was measured to confirm caspase-3 activity which was significant at the lowest caspase-3 concentration used. These results suggest that caspase-3 is not the primary mediator of PARC cleavage.

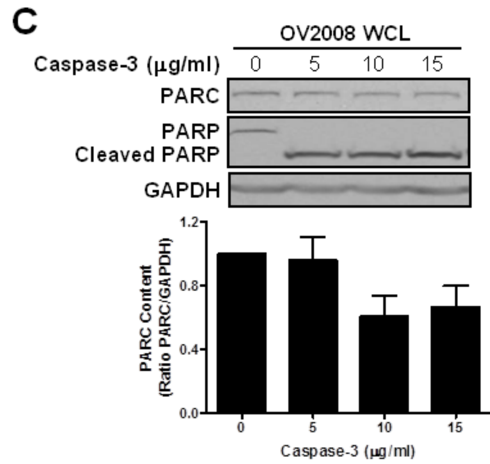
Protein degradation in eukaryotes is mediated predominantly through the ubiquitin pathway (Meng et al, 1999). Although considered a pathway for protein turnover, it is also involved in mediating key processes in apoptosis, cell division and antigen presentation (Ciechanover, 1998; Ciechanover & Schwartz, 1998). Previous reports indicate that PARC is itself an ubituitin E3 ligase and can undergo auto-ubiquitylation *in vitro* (Nikolaev et al, 2003). To explain how CDDP induces PARC down-regulation, and having previously shown that caspases were not involved in mediating PARC processing, we examined the role of the ubiquitin proteasome pathway in this phenomenon. Chemosensitive OV2008 cells were treated with the proteasomal inhibitors Epoxomicin and Lactacystin, followed by CDDP. As shown in **Figure 13D**, CDDP treatment decreased PARC content and induced apoptosis. However, pretreatment with either Epoximicin or Lactacystin attenuated the CDDP-induced apoptosis but failed to exert any significant effect on PARC content. These results suggest that CDDP-induced PARC processing is not mediated by the proteasomal pathway [**Figure 13D**; 2-Way ANOVA; Epoxomycin ($p<0.01$), CDDP ($p<0.01$), interaction ($p<0.001$); Lactacystin ($p<0.001$), CDDP ($p<0.001$), interaction ($p<0.001$)].

Figure 13. Caspases and the ubiquitin proteasome pathway are not the primary mediators of CDDP-induced PARC processing.

CDDP-induced PARC down-regulation is caspase-independent. OV2008 cells were pre-treated for 2 h with the caspase-3 specific inhibitor **A)** Z-DEVD-FMK and the pan-caspase inhibitor **B)** Z-VAD-FMK, followed by CDDP (24 h; DMSO as control). Pre-treatment with both drugs prevented CDDP-induced caspase activity (indicated by PARP cleavage) and apoptosis in a concentration-dependent manner [*p<0.05, **p<0.01, ***p<0.001 (versus no CDDP); +++ p< 0.001 (versus no caspases inhibitor); **A** & **B**], but failed to inhibit CDDP-induced PARC down-regulation compared with control. Results are expressed as mean ± SEM (n = 6 replicate experiments). **C)** Purified active caspase-3 incubated with OV2008 whole cell lysate (WCL; 30 min, 30°C) resulted in complete PARP cleavage at 5 μg/ml with no significant effect on PARC content even at the highest caspase-3 concentration tested (20 μg/ml); p>0.05. Results are expressed as mean ± SEM (n = 3 replicate experiments). **D)** CDDP-induced PARC down-regulation is not mediated by the ubiquitin proteasome pathway. OV2008 cells pre-treated with the proteasome inhibitors Epoxomicin (20 nM; 30 min; DMSO control) and Lactacystin (4 μM; 30 min; DMSO control) did not prevent CDDP-induced (24 h) PARC down-regulation, but attenuated apoptosis [**p<0.01, ***p<0.001 (versus no CDDP); +++ p< 0.001 (versus no proteasome inhibitor)]. Results are expressed as mean ± SEM (n = 3 replicate experiments).







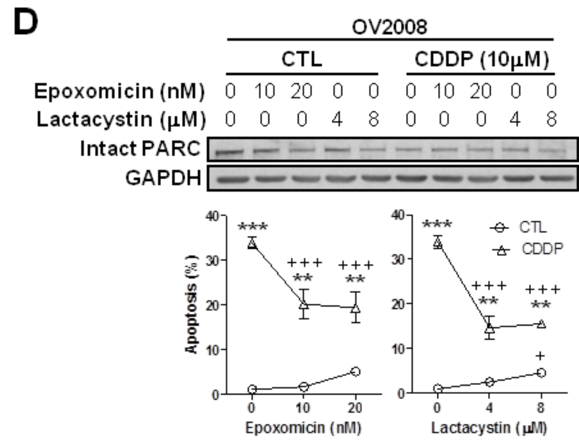


Figure 13

CHAPTER 5: GENERAL DISCUSSION

5.1 Overview and significance

Ovarian cancer is the most lethal of the gynecological cancers and is the seventh most common cause of cancer death in women world-wide. The standard treatment consists of a combination of surgical debulking and platinum-based chemotherapy alone or in combination with paclitaxel. The 5-year survival rates for stages I, II, III, and IV are 74%, 58%, 30% and 19% respectively (Steele et al, 1994). Unfortunately, most patients present with advanced stage disease (stage III and IV) and as a result of late diagnosis and the development of chemoresistance, 55%-75% of women who respond to first-line therapy relapse within two years of completing treatment. The clinical limitation for cisplatin use is even more significant as tumours are frequently cross-resistant to diverse and unrelated drugs. Clinically active analogs such as carboplatin and iproplatin are ineffective against cisplatin-resistant tumours (Kelland, 1993). Furthermore, the benefits of second-line chemotherapy diminish substantially, and patients eventually succumb to their disease (Gore et al, 1995; Ozols, 1992). The mechanisms involved in the development of chemoresistance remain unclear, however evidence suggests that failure of drug-induced apoptosis is a major underlying factor.

Studies of the cellular response to CDDP and other anticancer drugs have frequently focused on the critical role of the tumour suppressor p53. P53-mediated apoptosis is a key determinant of chemosensitivity in ovarian cancer (Fraser et al, 2008; Yang et al, 2006). Its transcription-dependent and -independent roles are both inhibited by enhanced cytoplasmic sequestration (Nikolaev et al, 2003). In cancer types where

there is confinement of p53 in the cytoplasm, there is decreased responsiveness to genotoxic stress induced by radiotherapy and chemotherapy (Nikolaev et al, 2003). As such, it is important to understand the molecular mechanisms by which p53 subcellular localization is regulated. P53-associated, Parkin-like cytoplasmic protein (PARC) has been identified as an important regulator of p53 subcellular localization and function. In this thesis, I examined the cellular and molecular mechanisms that contribute to the development of chemoresistance in human OVCA. I present evidence for p53 regulation by PARC and Akt and examine the role of PARC in regulating p53 subcellular localization and CDDP sensitivity. I also present evidence of the post-translational processing of PARC by the Ca^{2+}-dependent cysteine proteinase calpain.

5.2 Role of PARC in p53 subcellular trafficking and cisplatin-induced apoptosis

Confinement of p53 to the cytoplasm decreases responsiveness of cancer cells to genotoxic stress induced by radiotherapy and chemotherapy (Moll et al, 1995; Nikolaev et al, 2003; Yang et al, 2006). Therefore, it is important to understand the molecular mechanisms by which p53 subcellular localization is regulated. In the present thesis I have shown that PARC regulates p53 subcellular localization and CDDP sensitivity, two responses dependent on Akt status of the cells (Fraser et al, 2008; Yang et al, 2006). I have also demonstrated that CDDP-induced post-translational processing of PARC is mediated by the Ca^{2+}-dependent cysteine protease calpain.

Translocation of p53 to the mitochondria is an early event in p53-dependent apoptosis (Erster et al, 2004; Marchenko et al, 2000; Yang et al, 2006). P53 promotes outer mitochondrial membrane permeabilization and the release of mitochondrial death

proteins by forming inhibitory complexes with the anti-apoptotic Bcl-2 family of proteins (Tomita et al, 2006), events that are thought to initiate and amplify the slower transcription-based response. Furthermore, reports also indicate that expression of mutant p53 is not always the equivalent to p53 loss, as mutant p53 may lose wt-p53 tumour suppressor activity but show gain of functions that contribute to transformation and metastasis (Muller et al, 2009). Due to the pleiotropic role of p53, it is possible that cytoplasmic sequestration of p53 by PARC represents the most basic form of regulation. As such, disturbances in PARC regulation and/or function may impact the ability of p53 to execute its intended role.

PARC content is present in both chemosensitive and resistant OVCA cells, and with higher levels present in resistant ones. CDDP decreased PARC content in the sensitive but not resistant cells, suggesting that higher levels of PARC may be important in conferring CDDP resistance. This idea is further supported by the identification of a PARC-silenced, p53-wt, chemosensitive OVCA cell line (A2780s) whereas PARC is highly expressed in the chemoresistant A2780cp counterpart [**Appendix 7.3** (**Figure A2A**)]. The PARC-silenced phenotype in the A2780s cells appears to be associated with DNA methylation, as 5-Aza-2'-deoxycytidine induced PARC expression [**Appendix 7.3** (**Figure A2B**)]. This represents, to my knowledge the first demonstration of a physiologic condition where PARC content is altered.

A major determinant of chemoresistance in OVCA cells is the activation of the PI3K/Akt pathway. Although no modified or mutated Akt genes have been reported in humans, Akt gene amplification is common in high-grade aggressive ovarian tumours, corresponding with elevated protein content and high kinase activity in various OVCA

cell lines. Our lab has previously demonstrated that CDDP promotes Akt cleavage in a caspase-3 dependent fashion in chemosensitive but not resistant OVCA cells (Asselin et al, 2001a).

Akt activation attenuates CDDP-induced p53 phosphorylation, localization and apoptosis (Fraser et al, 2008; Yang et al, 2006). Here I demonstrate that CDDP decreased Akt activity which corresponded with an increase in p53 phosphorylation (ser15) and apoptosis, in OV2008 cells but not in the resistant C13* counterparts. Inhibition of Akt activity with the Akt specific inhibitor API-2 increased p-p53 (ser15) content and apoptosis in chemoresistant C13* cells. These results are consistent with reports implicating Akt activation as an important determinant in chemoresistance in OVCA (Asselin et al, 2001a; Shayesteh et al, 1999; Yang et al, 2004; Yang et al, 2006).

In chemoresistant OVCA cells, CDDP failed to induce p53-mediated apoptosis, and this is partially attributed to the inability of p53 to accumulate to the mitochondria and/or nucleus as a result of high Akt activity (Fraser et al, 2008; Yang et al, 2006). I demonstrate that PARC and p53 interact in OVCA cells and that this interaction is unaffected by CDDP in chemoresistant OVCA cells. The use of chemoresistant C13* cells for future studies involving PARC/p53 interaction is attractive as it would not require ectopic expression of mutated-p53 to avoid wt-p53-dependent apoptosis, as C13* cells express high basal wt-p53 and PARC levels and are resistant to CDDP. In contrast, investigation of PARC/p53 interaction in chemosensitive OV2008 cells revealed various complications as basal p53 content is maintained at a low level, and CDDP is required to induce p53 up-regulation and would subsequently result in PARC down-regulation, p53 translocation and apoptosis. The cellular mechanisms regulating PARC/p53 interaction

is unclear and may involve modifications to PARC, p53 or both. Possible modifications would include site-specific phosphorylation and monoubiquitination as both have been reported to influence p53 translocation (Marchenko et al, 2007; Muscolini et al, 2011; Yang et al, 2006). The role of Akt in these processes also requires further investigation.

PARC down-regulation enhanced CDDP-induced apoptosis in chemosensitive (OV2008) but not resistant (C13*) OVCA cells. Interestingly, inhibition of Akt is required to sensitize chemoresistant cells to CDDP in a p53-dependent manner, an effect enhanced by PARC-down-regulation. Although p53 is free to move to the various subcellular compartments, I propose that elevated Akt activity in the chemoresistant cells inhibits p53 phosphorylation and function (Fraser et al, 2008). Phosphorylation of p53 on ser15 and/or ser20 is required for p53-induced apoptosis (Unger et al, 1999). CDDP-induced phosphorylation of ser15 in OVCA cells appears to be a critical residue for apoptosis as mutations at this site attenuates CDDP-induced apoptosis (Fraser et al, 2008). The mechanisms by which ser15 phosphorylation affects the pro-apoptotic capacity of p53 is unclear, however evidence suggest it is not mediated by p53-dependent transactivation (Fraser et al, 2008). The possibility that phosphorylation of p53 may affect PARC/p53 interaction requires further investigation.

It has recently been reported that PARC functions as a tumour suppressor and promotes p53-dependent apoptosis in mice(Pei et al, 2011). These results are contrary to myresults and those of others (Mulhall et al; Nikolaev et al, 2003; Vitali et al, 2008). Pei *et al.* (2011) examined the effect of PARC knock-out in gamma-irradiated murine thymus and thymocytes, although PARC expression is very low in human thymus (Nikolaev et al, 2003). Although it is interesting that their PARC knock-out mice exhibited decreased

survival times and developed spontaneous tumours, previous PARC-null mice demonstrated no physical deformities, growth defects or health problems (Skaar et al, 2005). Although the reasons for these discrepancies are not immediately apparent, whether it is related to the stress signal and/or species/cell type used remains to be determined.

5.3 PARC processing in cisplatin-induced apoptosis

CDDP-induced PARC down-regulation was not a result of decreased mRNA content. Although PARC contains three Asp-X-X-Asp (DXXD) motifs, indicative of potential caspase-3 cleavage sites, PARC processing was caspase-independent. Importantly, the results demonstrate that CDDP induced PARC down-regulation is not merely a consequence of apoptosis, as caspase inhibition significantly attenuated cell death but was unable to reverse PARC down-regulation.

In addition, PARC has been shown to self-ubiquitylate *in vitro* and highly ubiquitylated PARC have been detected in human cells where PARC and ubiquitin were ectopically overexpressed (Nikolaev et al, 2003; Skaar et al, 2007). Whether or not PARC ubiquitylation occurs *in vivo* and results in degradation via the proteasomal pathway remains to be determined. However, we demonstrate that CDDP-induced PARC down-regulation is not mediated by the ubiquitin proteasome pathway (UPP). Pre-treatment of chemosensitive OVCA cells with the proteasomal inhibitors Epoxomicin and Lactacystin failed to prevent CDDP-induced PARC down-regulation but did increase p53 content as expected (data not shown). Although many tumour cells are sensitive to proteasome inhibitors, CDDP-induced apoptosis was reduced with treatment. Various

cell lines and primary cultures of quiescent and differentiated cells do not undergo apoptosis on exposure to proteasome inhibitors (Grassilli et al, 1998). The mechanism of resistance is unclear but may involve the differential actiivty of the pro-apoptotic kinase JNK. Furthermore, although Lactacystin is one of the most selective proteasome inhibitors available, it can inhibit cathepsin A and thiol proteases such as calpains, which are implicated in mediating CDDP-induced apoptosis in OVCA cells (Orlowski, 1999).

Substrates modified by Lys48-linked polyubiquitin chains are best characterized as being targeted to the proteasome(Boname et al, 2010), whereas monoubiquitylation or chains conjugated to other lysine residues such as Lys63 and Lys29, provide non-proteolytic signals (Boname et al, 2010; Hicke, 2001; Hoeller et al, 2006). It is possible that the previously reported ubiquitylated PARC species were not of the Lys48-type but were modifications controlling PARC function or localization. Those studies did not examine the ubiquitin chain configurations modifying PARC, as well as the corresponding functional outcomes. PARC regulation by post-translational modification has been proposed and may involve the covalent modification by NEDD8 (Hicke, 2001; Skaar et al, 2007) (related to ubiquitin; also known as Rub1) which is a regulatory modification controlling ligase activity (Hochstrasser, 2000). Interestingly, although PARC fails to ubiquitylate p53, p53 activity can be inhibited by neddylation (Xirodimas et al, 2004). If and how PARC is modified by ubiquitylation or neddylation in OVCA and its significance in CDDP sensitivity remains to be determined.

In the present thesis, I provide evidence implicating calpain as the mediator of CDDP-induced PARC down-regulation. This represents to my knowledge, the first demonstration of a signaling pathway that regulates PARC processing. Calpains cleave

their target proteins rather than completely degrading them (Goll et al, 2003). They are involved in the regulation of various cellular processes such as cell proliferation and differentiation, apoptosis and necrosis. The role of calpains in apoptosis is reflected in a growing list of substrates including p53, Bcl-2, Bax, AIF, caspases and cytoskeletal proteins (Kubbutat & Vousden, 1997; Liu et al, 2009; Mandic et al, 2002; Tan et al, 2006; Wood et al, 1998). Calpain activity is Ca^{2+}-dependent and calpain activation in response to Ca^{2+} signaling is an early event in CDDP-induced apoptosis (Splettstoesser et al, 2007). For instance, calpain-mediated Bid cleavage is one of the earliest pro-apoptotic events involved with CDDP-induced apoptosis (Liu et al, 2008), and CDDP-induced calpain activation precedes AIF release and caspase-9/-3 activation (Liu et al, 2009) and directly regulates both caspase-dependent and AIF-mediated, caspase-independent apoptosis (Liu et al, 2009).

Pre-treatment of chemosensitive OV2008 cells with the specific calpain inhibitor Calpeptin significantly attenuated CDDP-induced PARC down-regulation, α-fodrin cleavage and apoptosis, suggesting that CDDP-induced PARC processing is dependent on calpain cleavage. Although the inhibitor was able to prevent CDDP-induced PARC processing, it only decreased apoptosis to 18% even at the highest effective concentration (25 μM), demonstrating the possible involvement of calpain-independent pathways in CDDP-induced apoptosis. Furthermore, *in vitro* experiments using C13* whole cell lysates demonstrated that PARC processing occurs only in the presence of purified calpain I and not purified active caspase-3 and that calpain-mediated PARC cleavage was Ca^{2+}-dependent. Since calpain is expressed at the same levels in both chemosensitive and resistant cells, my results suggest that this difference to CDDP sensitivity involves the

Ca^{2+} response (Al-Bahlani et al, 2011). Dysregulation of Ca^{2+} signaling in OVCA cells could therefore prevent PARC processing and promote chemoresistance. In fact, my lab recently reported that CDDP induces an increase in intracellular calcium concentration in chemosensitive but not resistant OVCA cells (Al-Bahlani et al, 2011). By inducing a Ca^{2+}-influx in chemosensitive and chemoresistant OVCA cells with Ionomycin, PARC cleavage and apoptosis was induced in both cell types. Ionomycin treatment did not induce PARC processing in the IOSE397 sensitive immortalized ovarian surface epithelial cells nor in the chemoresistant OVCA433 cells, possibly due to different Ca^{2+} regulatory mechanisms (Aagaard-Tillery & Jelinek, 1995; Splettstoesser et al, 2007). These differences may include regulation of Ca2+ influx and expression and function of calpains and its endogenous inhibitors.

In addition to regulating calpain activation, Ca^{2+} is shown to inhibit Akt activity (Smith & Dodd, 2007) and therefore presents itself as an important regulator of apoptosis and CDDP sensitivity. Calpain activation inhibits Akt signaling by cleaving HSP90 (Smith & Dodd, 2007). HSP90 is a molecular chaperone that forms a chaperone-client protein complex with Akt, an association that is essential for proper Akt function (Stalker et al, 2003). Whether or not dysregulated Ca^{2+}/calpain activation affects HSP90-mediated regulation of Akt activation in chemoresistant OVCA cells requires further investigation.

5.4 Experimental value of matched pair ovarian cancer cell lines

Human ovarian cancer cell lines were used to investigate the molecular mechanisms associated with the development to cisplatin resistance. The cell lines used

in this study with reference to descriptions of the tumour of origin can be found in **Appendix 7.1** (**Table 1**). Furthermore, when possible, experiments involved the use of matched pairs of chemosensitive parental cell lines (OV2008 and A2780s) with their isogenic resistant counterparts (C13* and A2780cp, respectively). The chemosensitive cell lines were established from patients with serous cystadenocarcinoma of the ovary, and the resistant cells were selected by chronic exposure to increasing concentrations of CDDP. As such, the resistant cells are in theory, genotypically identical to the parental line except for the acquired characteristics that confers cisplatin resistance. Although selection of resistant cells automatically implies heterogeneity within the original parental cell population, comparisons between paired matched subclones would minimize the background variability one would expect to find in unrelated chemosensitive and resistant OVCA cells. This also has clinical relevance as increased cycles of chemotherapy are associated with the progressive emergence of chemotherapy-resistant disease which decreases overall survival time of patients (Bristow & Chi, 2006). To maximize the relevance of our observations, experiments also included the use of unrelated cell lines.

Interestingly, of all the cell lines examined, PARC protein content was not detected in the chemosensitive A2780s OVCA cells, although was highly expressed in the A2780cp resistant variant. These findings are consistent with the idea that PARC is anti-apoptotic and therefore plays a minimal role in regulating CDDP-mediated events in chemosensitive OVCA cells. Our results suggest that the PARC-silenced phenotype in these cells is a function of DNA methylation, as treatment with 5'-Aza-2'-deoxycytidine induced PARC expression. These cells were invaluable for the process of developing our PARC overexpression vector, and provided evidence to demonstrate the anti-apoptotic

role of PARC in CDDP-induced apoptosis [**Appendix 7.3** (**Figure A2**)].

5.5 Determination of apoptosis

Throughout this thesis, we have discussed the importance of the apoptotic response in the context of cisplatin sensitivity and provided evidence that chemoresistance is in large part a result of a dysregulation of this complex physiological response. Although there are various experimental approaches to detect and quantitate relative apoptotic responses, we have chosen the morphologic method with Hoechst nuclear staining as our primary technique to measure apoptosis. Hoechst 33258 is a DNA-specific fluorochrome that increases in fluorescence upon binding to adenine-thymine-rich portions of DNA (Latt & Stetten, 1976). The DNA-Hoechst 33258 complex is very stable, and is mostly unaffected by common laboratory salts, detergent solutions, or biological materials associated with DNA (Cesarone et al, 1979). The excitation and emission wavelengths of the fluorescent DNA-Hoechst 33258 complex are 360 and 450 nm, respectively (Cesarone et al, 1979). This technique to measure apoptosis relies on visual detection by fluorescence microscopy of cellular and nuclear features associated with apoptosis, such as membrane blebbing, and nuclear condensation and fragmentation. Although this technique is more subjective compared to other techniques such as flow cytometry and MTT assays, it is also more sensitive in that cells are individually examined, avoiding positive signals from cell fragments and being able to identify the markers of late phase of apoptosis as reflected in the changes to nuclear morphology. Furthermore, subjectivity is minimized by standardized experimental conditions. Each assay involves counting, at the minimum, 400 cells from randomly

selected fields, and when possible, the counter is blinded to the experimental treatment to avoid experimental bias. These safeguards have enabled us to provide highly reproducible results, further demonstrating the usefulness and acceptance of this technique as a method to detect apoptosis (Al-Bahlani et al, 2011; Asselin et al, 2001a; Grusch et al, 2002; Sasaki et al, 2000).

To provide further evidence of the reported apoptotic response, the results obtained by Hoechst staining were supported by detecting cleavage of the enzyme poly (ADP-ribose) polymerase (PARP) from a full-length 116 kDa peptide into its cleaved forms (89 kDa and 24 kDa polypeptides) by Western blot (Mullen, 2004). PARP expression is induced by DNA damage and is a substrate for caspase-3 in apoptosis. This enzyme is involved with DNA damage repair both by catalyzing the synthesis of poly (ADP-ribose) and by direct binding to DNA-strand breaks and modifying nuclear proteins. This method again looks at late-stage apoptosis after the activation of caspase-3. Specific proteolytic cleavage of PARP is considered a reliable marker of apoptosis in numerous cell models (Mullen, 2004).

5.6 Manipulation of Akt activity

In this thesis, a dominant-negative Akt construct was used and it consisted of an Akt1 cDNA with alanine mutations at Thr308 and Ser473. These mutations prevent the phosphorylation of these sites, events that are required for Akt activation. Furthermore, this construct contained a third alanine mutation at Lys179 in the kinase domain, which is required for phosphate transfer. This construct is HA-tagged at its C-terminus and was cloned into an adenoviral expression vector that expresses Green Fluorescent Protein

(GFP). As such, this system allowed for simple confirmation of DN-Akt expression by Western blot against the HA epitope, and from detection of the GFP by fluorescence microscopy using a FITC filter. Infection efficiency at an MOI of 80 was greater than 95% in C13*. The dominant-negative effects of this Ala308/Ala473/Ala179-Akt ('triple A'-Akt) have been extensively studied (Fraser et al, 2008; Yang et al, 2006).

To further investigate the role of Akt, API-2, a highly specific pharmacological Akt inhibitor, was used. Use of this drug was advantageous in that: 1) it provided a second mechanism of action in modulating Akt activity, offering further evidence of the contribution of Akt activation in our experimental system, and 2) in experiments requiring multiple gene expression manipulations, use of this drug eliminated the required treatment process associated with the DN-Akt adenoviral infection, a process that when combined with siRNA transfection(s), non-specifically promoted cellular stress, up-regulation of p53 and apoptosis, endpoints being examined. The inhibitory effects of API-2 are highly specific to Akt, having no effect on the upstream activators and showing no inhibitory effects on other members of the AGC kinase family (Yang et al, 2004). Furthermore, API-2 was effective in reducing ovarian cancer tumour growth in xenografts with elevated Akt (Yang et al, 2004).

5.7 Future directions

5.7.1 *In vitro* studies

5.7.1.1 Regulation of PARC/p53 interaction.

The precise mechanism by which Akt regulates p53 subcellular trafficking and chemosensitivity in OVCA cells is unclear. In this thesis, down-regulation of Akt

activity sensitizes chemoresistant cells to CDDP in a p53-dependent manner, and PARC is involved in mediating cytoplasmic p53 retention, an event that attenuates CDDP-induced apoptosis. How Akt affect PARC/p53 interaction is unknown. Since PARC does not have an Akt phosphorylation consensus motif, as determined by sequence analysis, it is possible that PARC/p53 interaction may be regulated by Akt via downstream effectors that modify PARC, p53, or both. To assess the role of Akt in the regulation of PARC/p53 interaction, experiments will be performed using C13* chemoresistant cells which offer the following benefits to IP/co-IP experiments: a) C13* cells express high Akt activity; b) they express high basal PARC and p53 content; c) PARC/p53 interaction is unchanged in C13* cells with CDDP treatment.

By down-regulating Akt activity, using DN-Akt or API-2, the role of Akt on PARC/p53 interaction could be assessed. It is expected that down-regulation of Akt activity in chemoresistant OVCA cells would decrease PARC/p53 interaction and promote apoptosis in both CDDP-treated and untreated cells.

Akt activation prevents CDDP-induced mitochondrial p53 accumulation, mitochondrial death protein release and apoptosis (Yang et al, 2006). Down-regulation of Akt activity can sensitize chemoresistant OVCA cells to CDDP in a p53-dependent manner, requiring the activation and phosphorylation of p53. p53 contains multiple phosphorylation sites, however, phosphorylation of ser15 and ser20 is essential for CDDP-induced apoptosis in OVCA cells (Fraser et al, 2008) and is required for apoptosis in other cellular models (Bulavin et al, 1999; Li et al, 2006; Shono et al, 2002; Unger et al, 1999). It is possible that p53 phosphorylation promotes chemosensitivity through a mechanism that decreases PARC/p53 interaction. To assess if p53 phosphorylation

(ser15 and ser20) influences PARC/p53 interaction, chemosensitive A2780s cells (PARC-silenced) expressing a V5-tagged PARC construct, would be transfected with expression constructs for HA-tagged wt-p53 and its mutants (S15A and S20A) and then treated with CDDP. If p53 phosphorylation attenuates PARC-p53 interaction, it is expected that PARC pull-down would result in reduced co-IP of HA-wt-p53 compared with HA-ser15A-p53 or HA-ser20-p53. Potential complications resulting from CDDP-induced calpain-mediated PARC down-regulation could be avoided by extending these experiments to include pre-treatment of cells with the calpain inhibitor Calpeptin, or the design and expression of a calpain-resistant PARC expression construct. Furthermore, as CDDP-induced phosphorylation of p53 (ser15) occurs prior to detection of PARC down-regulation; use of an optimal CDDP treatment time may circumvent this issue.

Other cellular mechanisms unrelated to p53 phosphorylation may regulate PARC/p53 interaction. Structure/function analysis of the RING-IBR-RING domains of Parkin offers an alternative mechanism that may influence the protein-protein interaction ability of PARC. Similar to Parkin, PARC contains the same RING-IBR-RING domains. These domains are clustered with conserved cysteine residues and are likely to be S-nitrosylated by nitric oxide (NO) (Beasley et al, 2007; Meng et al, 2011). S-nitrosylation of parkin's RING-IBR-RING domain significantly impairs parkin's E3 ligase activity, resulting in abnormal ubiquitination and accumulation of endogenous substrates which contributes to cytoplasmic inclusion formation, and cell death (Meng et al, 2011). Whether NO affects PARC's structure/function in the same manner is unknown. Interestingly, differences in NO production in ovarian cancer cells can mediate CDDP sensitivity, an effect dependent on p53 (Leung et al, 2008). It is possible that NO-

dependent signaling may affect PARC/p53 interaction, and dysfunction of this pathway promotes cytoplasmic p53 retention and chemoresistance.

5.7.1.2 The role and regulation of PARC in fallopian tube epithelial cells.

With the emerging evidence that high grade serous ovarian tumours originate from serous epithelial cells from the fallopian tube, it would be essential to investigate the findings of this thesis in chemosensitive and resistant cell lines or primary cultures derived from fallopian tube epithelial cells. Interestingly, epithelial cells from pre-cancerous lesions located in the fimbrai of the fallopian tube, are characterized by a 'p53 signature'. This signature can be described as strong p53 immunostaining throughout the cytoplasm, reminisent of the high p53 content present in chemoresistant cells examined in this thesis. High cellular p53 content and decreased apoptotic capacity in these cells, also common to other cellular models resistant to DNA-damaging agents, may implicate a role for PARC the pathogenesis of HGSOC with fallopian tube origin and requires further investigation.

5.7.1.3 The role and regulation of PARC in the development of resistance to other chemotherapeutic agents in OVCA cells.

Although other drugs are employed in combination with CDDP as first- or second-line chemotherapeutics in OVCA, the experiments presented in this thesis is limited to an examination of the effects of CDDP to provide in-depth mechanistic information on its action. However, it may prove useful to determine the overall

regulation of apoptosis in OVCA cells by diverse clinically-relevant agents such as paclitaxel or carboplatin. As tumours often become cross-resistant to diverse and unrelated drugs (Gore et al, 1995; Kelland, 1993; Ozols, 1992), it would be interesting to investigate the possible commonalities between these systems as it relates to p53-dependent apoptosis and the role of PARC in determining cell fate. As such, key studies involving changes in CDDP-induced apoptosis will be extended to include the evaluation of the effects of paclitaxel, with/without CDDP, and carboplatin in chemoresistant and sensitive OVCA cells. The role and regulation of PARC in these cells will be evaluated.

5.7.2 *In vivo* studies

The use of cell lines offers a convenient, informative and often highly reproducible approach to study the mechanisms of chemoresistance, and the signaling pathways involved. However, a disadvantage of using cell lines involve potential changes associated with continuous and extended passage resulting in a phenotype far removed from the original tumour of origin. Although we have provided evidence that establishes a role for calpain-mediated PARC processing in p53-dependent apoptosis and chemosensitivity *in vitro*, whether or not this applies to an *in vivo* model requires investigation.

5.7.2.1 Xenograft model

Xenografts of CDDP-sensitive and resistant OVCA cell lines could be established in female Swiss nude mice as previously described (Shaw et al, 2004). Intraperitoneal inoculation of these OVCA cell lines (except C13*) form tumours that distribute within

the peritoneum and possess histological features similar to those of the tumours from which the cells were derived (Rose et al, 1997; Shaw et al, 2004). Subcutaneous cell inoculation would first be selected since tumour growth can be easily monitored. Once the role of PARC is established, the more physiological intraperitoneal route would be used.

To assess the regulation of PARC and its influence on CDDP sensitivity, A2780s (PARC-silenced) and A2780-PARC (constitutive PARC expression) cells would be inoculated in nude mice (Shaw et al, 2004). The effect of CDDP (1.5 mg/kg, intraperitoneal injection, clinical dose equivalent) on tumour volume would be determined and apoptotic (TUNEL and DNA ladder) and mitotic activity assessed. It is expected that CDDP would induce apoptosis, suppress mitotic activity and growth of tumours developed from the A2780s, but not A2780-PARC cells, suggesting that PARC expression can contribute to CDDP resistance. PARC and p53 content and localization would be determined by Western blot and immunohistochemistry. It is expected that PARC expression would promote cytoplasmic p53 retention and attenuate CDDP-induced apoptosis in tumour cells.

To demonstrate the functional role of p53 in PARC-mediated CDDP resistance *in vivo*, the p53 mutant A2780cp and A2780cp-shPARC (stable siPARC with shRNA lentivector) cells could be used to develop variants in which wt-p53 expression could be turned on via the Tet-On System (BD Biosciences). This system has been used successfully to drive transgene expression for up to 11 weeks (Pluta et al, 2005) and would be suitable for our system. As elevated Akt activation is shown to attenuate CDDP-induced apoptosis in these cells, mice will also be treated with API-2 (1

mg/kg/day; intraperitoneal injection; DMSO vector control) to decrease Akt activity. API-2 has been shown to inhibit Akt signalling and induce apoptosis in cancer cells with elevated levels of Akt. Furthermore, API-2 was shown effective in reducing tumour volume in mouse xenograft models of OVCA (Yang et al, 2004). If down-regulation of PARC can overcome CDDP resistance in a p53-dependent manner, we expect that siPARC will have no effect on CDDP-induced responses in 53 mutants unless wt-p53 is reconstituted.

5.7.2.2 Clinical Samples

To determine the applicability of these findings to the *in vivo* human situation, key studies that were completed in cell lines would be extended to include primary cultures of OVCA cells from ascites fluids and solid tumours from chemosensitive and resistant patients with stage III/IV ovarian carcinomas. Differences in PARC, p53, p-p53 (ser15), Akt and p-Akt (ser473) contents and subcellular localization of p53 and apoptosis, in CDDP-treated and untreated cells would be assessed using the methods previously described. *TP53* mutational status (exons 5-9; DNA Binding Domain) will be confirmed by direct PCR-based sequencing. These findings would be correlated with clinical outcome data from the Ottawa Ovarian Tumour Bank.

Furthermore, the influence of Akt inhibition and PARC down-regulation on CDDP-induced p-p53 (ser15) content, p53 subcellular accumulation and apoptosis could be examined in OVCA cells obtained from recurrent tumours. It is expected that non-responsive cells will display high cytoplasmic p53 and PARC content and Akt activity relative to responsive ones.

While p53 inactivation by cytoplasmic retention is common to various cancer types and these cells often show reduced response to genotoxic stress, whether this is the case in resistant ovarian tumours is not clear. To test this possibility, protein levels of PARC, p-p53 (ser15), p53, Akt, and p-Akt (473) in human ovarian tumours (Western blot) would be correlated with outcome and response to chemotherapy *in vivo* (CDDP/taxol). It would also be important to determine if these changes were accompanied by alterations in PARC, p53 and Akt mRNA content (qRT-PCR and *in situ* hybridization). To address the potential complications in these measurements as a result of the heterogeneity of cell types comprising the tumour, specific tumour cells could be obtained using laser capture microdissection. Furthermore, frozen and fixed tumours pairs obtained from same patients during the chemosensitive and resistant phases of treatment could be examined. Only paired chemosensitive and chemoresistant tumours (serous ovarian carcinoma) from patients at stages III and IV would be examined.

5.8 Conclusions

In summary, I have demonstrated that CDDP-induced PARC processing and apoptosis is regulated differently in chemosensitive and chemoresistant human OVCA cells (Woo et al, 2012). Processing of PARC is mediated by the Ca^{2+}-dependent cysteine protease calpain, although CDDP-induced apoptosis may involve the interaction of different proteolytic pathways. Determining the molecular mechanisms controlling PARC processing may contribute to the current understanding of p53 function and apoptosis and ultimately of the underlying mechanisms of chemoresistance in human ovarian cancer.

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CHAPTER 7: APPENDICES

7.1 Characterization of experimental cell lines.

In the present thesis, seven human ovarian cancer cell-lines and one immortalized ovarian surface epithelial cell-line was used to investigate the role and regulation of PARC in the development of CDDP resistance in human ovarian cancer. Supplemental information in reference to the tumours of origin, p53 status and characterization of tumour formation from intraperitoneal xenograft models are listed in **Table 1**.

Table 7.1

Cell Line	Tumour Origin	p53 Status	IP Tumours from Xenograph (nude mice)
OV2008	Serous cystadenocarcinoma (1,2)	WT (3)	Endometrioid adenocarcinomas-squamous differentiation (4)
C13*	Serous cystadenocarcinoma (5)	WT (3)	Failed to form IP tumour (4)
A2780s	Serous cystadenocarcinoma (1)	WT (3,6)	Undifferentiated tumour (4,7)
A2780cp	Serous cystadenocarcinoma (1)	Mutant (3,6)	Undifferentiated tumour (4)
OVCA420	Serous cystadenocarcinoma (8)	WT (9,10)	No Data
OVCA433	Papillary serous cystadenocarcinoma (11-13)	WT (9,10)	Failed to form IP tumour (4)
HEY	Moderately differentiated papillary cystadenocarcinoma (14)	WT (10)	Undifferentiated tumour (4)
IOSE397	Normal ovarian surface epithelial cell transfected with SV40 large T antigen; exhibits no tumorigenic behavior (15)	WT (16)	Not tested

WT: wild-type; IP: intraperitoneal

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7.2 Optimization of OVCA cell attachment using Poly-D-Lysine-coated glass chamber slides.

To quantitate the effects of our treatment conditions on p53 subcellular localization by IF/confocal microscopy, cells were categorized based on p53 co-localization with the mitochondrial and nuclear markers, TOM20 and DAPI, respectively. The results were presented as cell fractions of the total cell population as seen in the method published by Sengupta et al., 2000 (Sengupta et al, 2000). To promote cell attachment and to minimizing the loss of apoptotic cells during the slide preparation, we optimized the concentration of Poly-D-Lysine used to coat the chamber slides prior to cell culture.

Figure A1 demonstrates the effect of coating an 8-chamber glass chamber slide with Poly-D-Lysine (0, 0.01, 0.05, 0.1% w/v; 1 h), to promote cell attachment of OV2008 cells treated with CDDP (10 μM; 24 h; DMSO as control). Based on visual inspection, chamber slides coated with 0.05% PDL was sufficient to promote cell attachment and prevent healthy and apoptotic cells from being rinsed off during the processes of cell fixation slide preparation for immunofluorescence assays.

Figure A1. Optimization of OVCA cell attachment using Poly-D-Lysine-coated glass chamber slides.

8-well glass chamber slides coated with Poly-D-Lysine (PDL) (0, 0.01, 0.05, 0.1% in water; 1 h) were used to plate chemosensitive (OV2008) OVCA cells treated for 24 h with CDDP (10 μM; DMSO as vehicle control). Cells were rinsed 1x with PBS and fixed in paraformaldehyde (4%, 1 h, RT). Cell attachment was determined by bright field analysis on a Leica DMLB fluorescent microscope (200X magnification). The optimum PDL concentration was determined to be 0.05% (n = 2 replicate experiments).

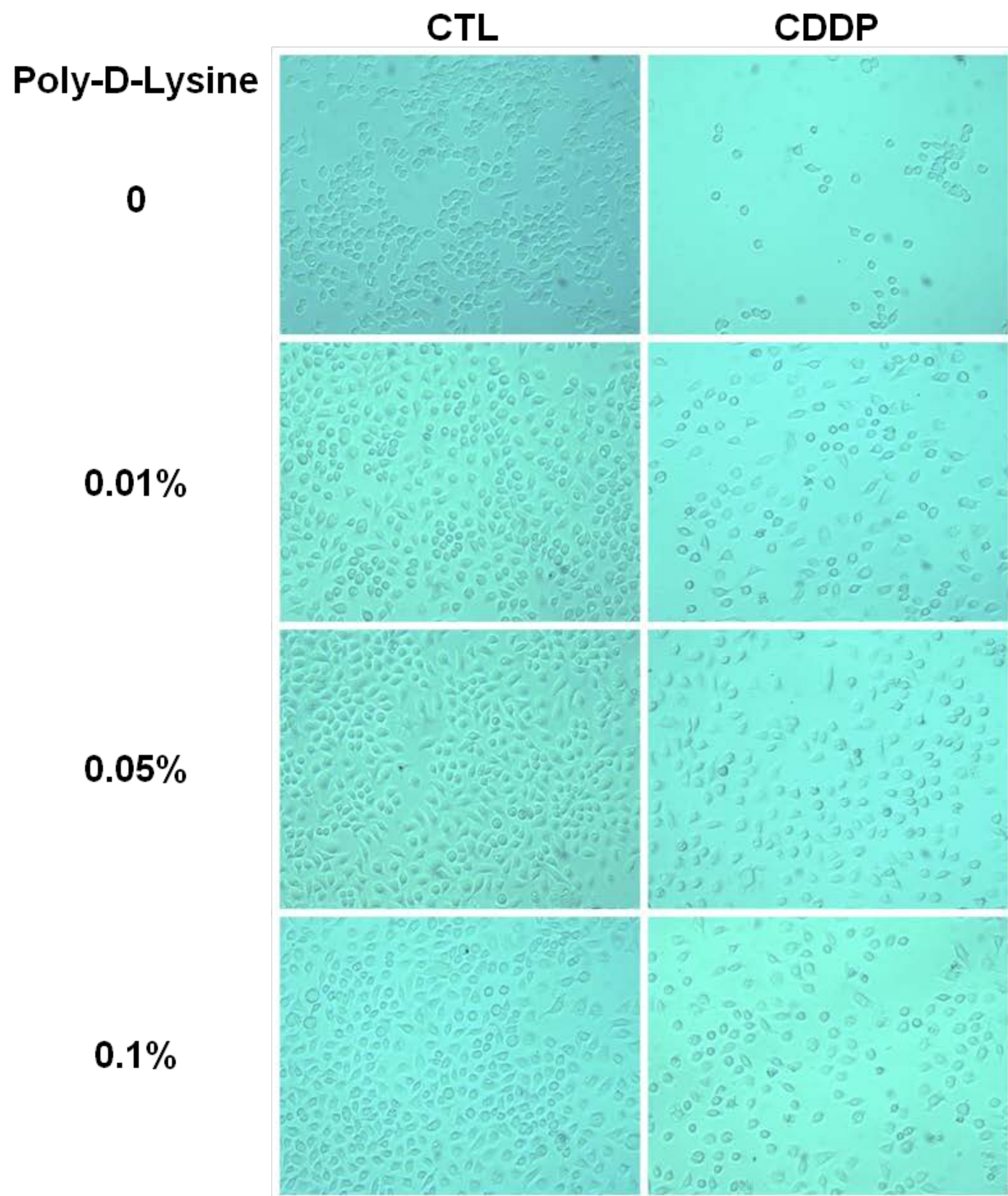


Figure A1

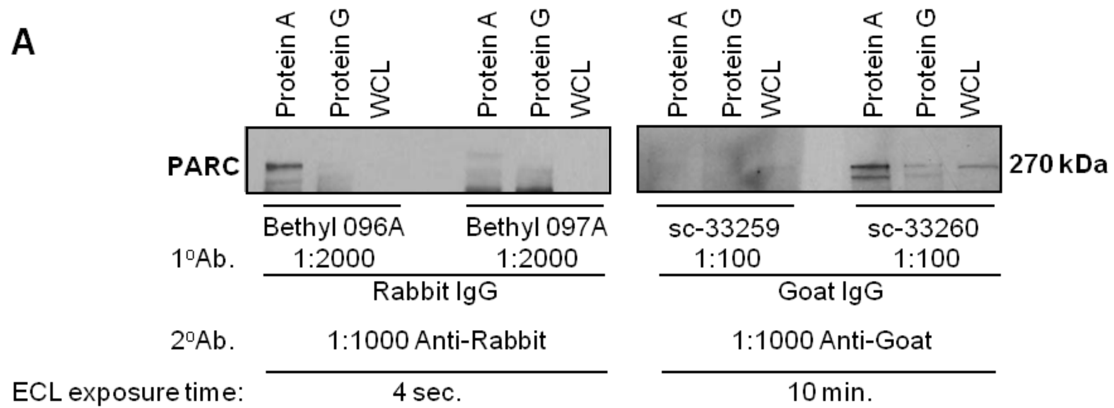
7.3 Optimization of PARC and p53 immunoprecipitation (IP)/co-IP protocol.

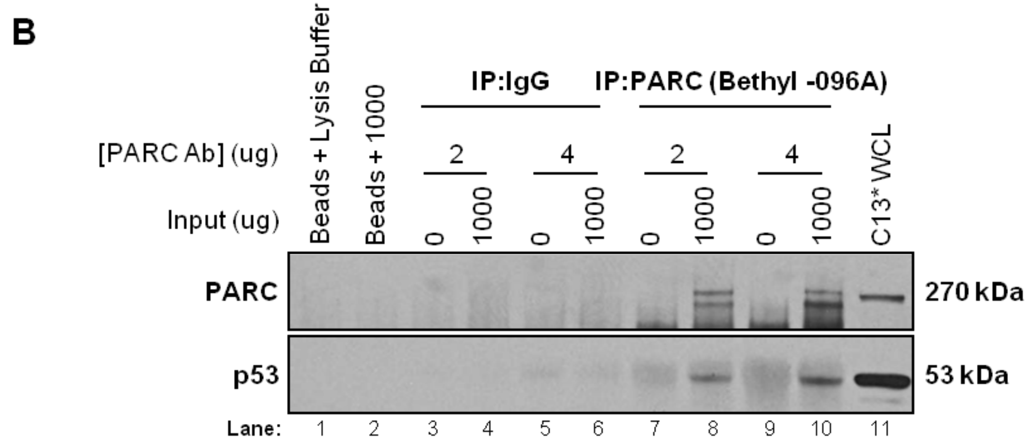
Before proceeding with our experimental plans to examine PARC/p53 interaction by immunoprecipitation, conditions had to be optimized. PARC is a large protein (~270 kDa) (Nikolaev et al, 2003), making it relatively difficult to pull-down. Chemoresistant C13* cells have high basal PARC and p53 content, therefore C13* whole cell lysate (WCL) was used for most of these optimization experiments. Use of C13* cells to examine PARC/p53 interaction is especially attractive as the high basal p53 content does not induce apoptosis avoiding the necessity to rely on exogenous expression of mutant or non-functional p53 (Nikolaev et al, 2003). **Figure A2A** demonstrates the optimization of PARC immunoprecipitation and Western blot detection using Protein A and G Dynabeads incubated with PARC antibody (2 μg; 1 h; Bethyl A300-096A). PARC detection was best using the same antibody used in the IP (Bethyl A300-096A). Next, we determined the optimal concentration of PARC antibody to coat our IP beads to achieve successful PARC pull-down while minimizing non-specific signals detected by Western blot. **Figure A2B** demonstrates strong PARC pull-down and p53 co-IP using the antibody concentration of 2 μg/mg lysate compared to 4 μg/mg lysate. As such, this condition was applied to all subsequent experiments. The successful PARC IP is confirmed by the lack of signal in the IP:IgG controls (Lanes 3-6) and the IP:PARC with no input (Lanes 7 and 9). A positive Western blot control for PARC is represented in Lane 11, consisting of C13* WCL (25 μg). **Figure A2C** examines the use of the IP specific blocking reagent ReliaBLOT (Bethyl; as per manufacturer's instructions; Lanes 1-3), compared to the standard 5% skim milk in TBST (Lanes 4-6), in reducing the non-specific and IgG signal from the Western blot analysis of the IP samples. ReliaBLOT

decreased the non-specific and IgG signals present in the 5% skim milk treated membranes (Lane 2 vs. Lane5). A positive Western blot control for PARC was included (Lanes 3 & 6, C13* WCL: 25 μg). These conditions were applied to all subsequent experiments. More importantly, these early experiments confirmed the interaction of PARC and p53 in OVCA cells.

Figure A2. Optimization of PARC and p53 immunoprecipitation protocol.

A) Whole cell lysates (WCL) of chemoresistant (C13*) OVCA cell (500 µg) were subjected to PARC immunoprecipitation using Protein A or Protein G Dynabeads incubated with PARC antibody (Bethyl A300-096; 2 µg; 1 h; RT). PARC content was detected by Western blot using the various PARC antibodies (Bethyl A300-096A, A300-097A and Santa Cruz sc-33259, sc-33260). PARC immunoprecipitation and detection was best using Protein A Dynabeads and probing with the Rabbit Bethyl A300-096A antibody compared to the other IP and WB conditions examined (n = 2 replicate experiments). **B)** Protein A Dynabeads were incubated with different amounts of PARC antibody (Bethyl, A300-096A; 2 & 4 µg; 1 h; RT) and subsequently incubated with C13* WCL (1 mg; 2 h; 4°C). Protein A Dynabeads incubated with 2 µg of PARC antibody was sufficient to provide clear PARC and p53 IP/co-IP. Also shown are controls for potential non-specific bands originating from the beads (Lanes 1 & 2), antibody IgG (Lanes 3-6), and the lysis buffer (Lanes 3, 5, 7 & 9). A positive PARC WB control (C13* WCL; 25 µg) is loaded in Lane 11 (n = 2 replicate experiments). **C)** Protein A Dynabeads were incubated with PARC antibody (Bethyl, A300-096A; 2 µg; 1 h) and C13* whole cell lysate (1000 µg; 2 h). PARC immunoprecipitation (IP) was determined (WB). Membranes were blocked with ReliaBLOT (Bethyl) or 5% skim milk powder in TBST. The primary and secondary antibodies were also diluted in the same respective blocking buffer. The IP/WB of PARC was strongest using the PARC (Bethyl) antibody for Western blot, with minimal IgG background using ReliaBLOT. IgG controls were included for each condition (n = 2 replicate experiments).





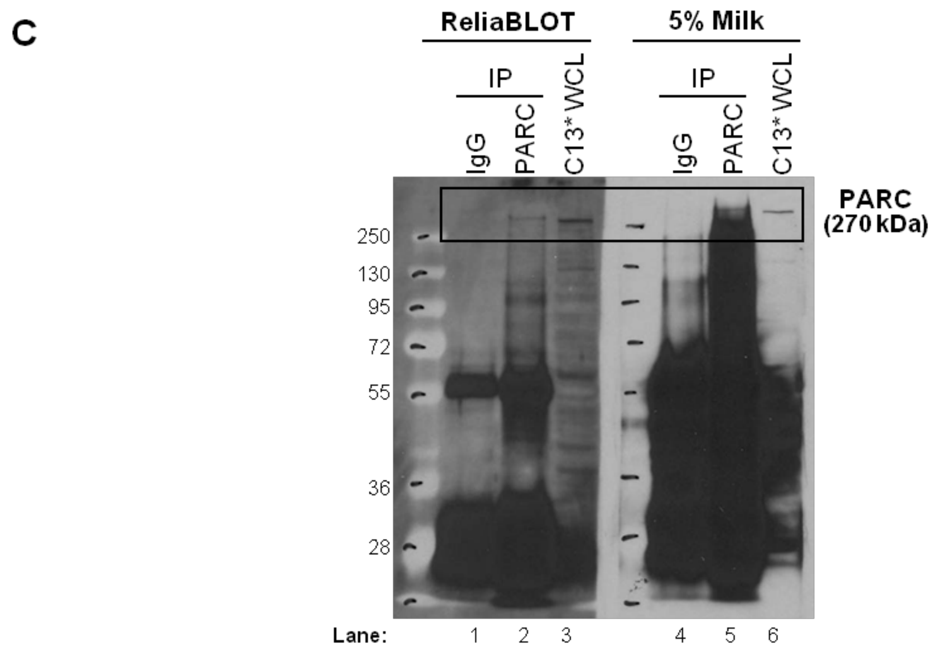


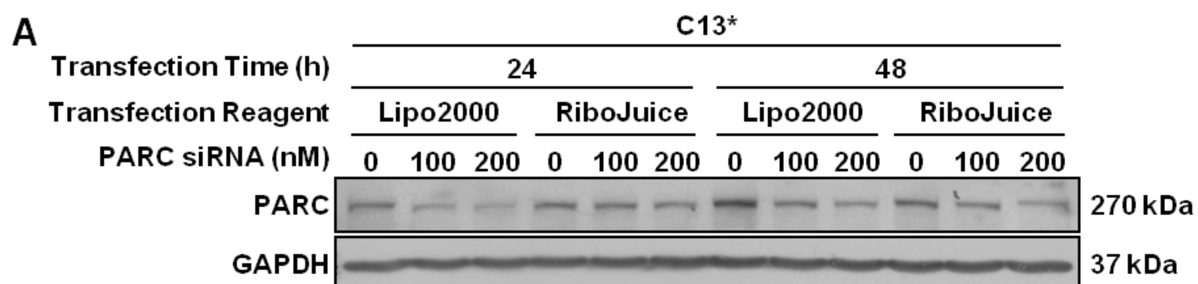
Figure A2

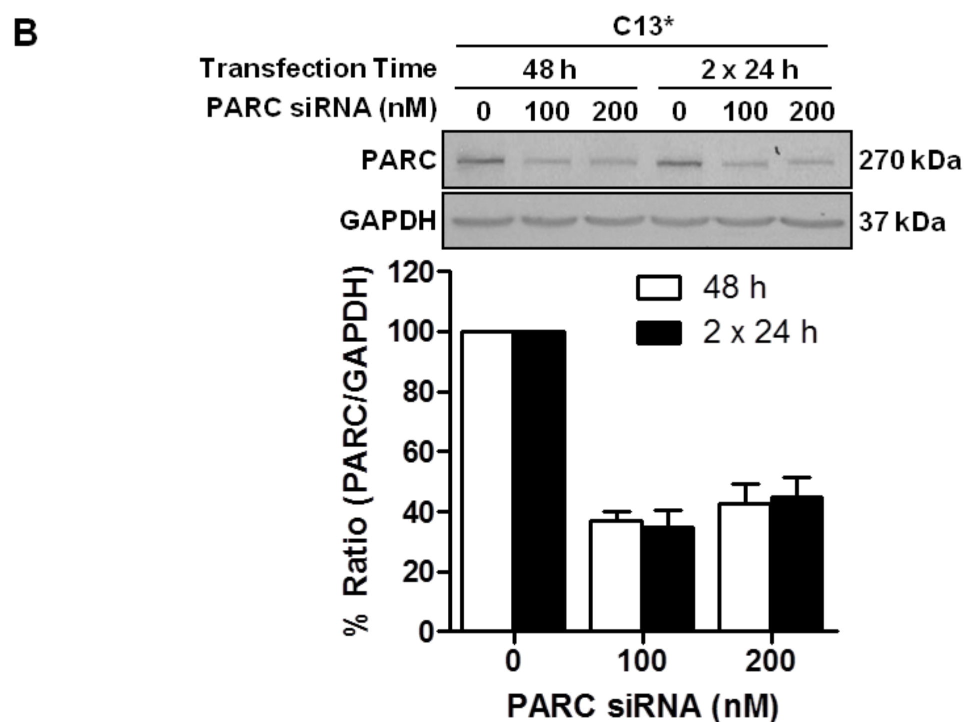
7.4 Optimization of PARC siRNA transfection protocol in OVCA cells.

Before proceeding with our experimental plan to investigate the physiological role of PARC in OVCA and chemoresistance, conditions for PARC down-regulation by siRNA was optimized. Chemoresistant (C13*) OVCA cells were transfected with PARC siRNA (0, 100, 200 nM; 24 & 48 h; S.Seq. as control) using both Ribojuice and Lipofectamne 2000 transfection reagents (Invitrogen). As shown in **Figure A3A**, use of Lipofectamine 2000 compared with Ribojuice, was more effective at decreasing PARC protein content, with greater effect after 48 h versus 24 h transfection. As shown in **Figure A3B**, use of multiple transfections versus a single transfection for 48 h was also examined. C13* cells transfected with fresh PARC siRNA (Lipofectamine 2000) every 24 h for a total of 48 h versus a single 48 h transfection alone, did not result in a significant difference in PARC content at both the 100 nM and 200 nM siRNA concentrations. Optimal PARC siRNA concentration was determined to be 100 nM (**Figure A3C**). These conditions were adapted as standard protocol for all subsequent studies involving PARC down-regulation.

Figure A3. Optimization of PARC siRNA transfection protocol in OVCA cells.

A) Chemoresistant (C13*) OVCA cells were transfected with PARC siRNA (0-200 nM; 24 h & 48 h; S.Seq. as control) using Lipofectamine 2000 or RiboJuice transfection reagents. Western blot analysis of PARC and GAPDH content show greater efficiency of the Lipfectamine 2000 versus RiboJuice protocol to down-regulate PARC content at both 24 and 48 h transfection time (n = 2 replicate experiments). **B)** Chemoresistant (C13*) cells were transfected for 48 h with PARC siRNA using Lipofectamine 2000 (0-200 nM; S.Seq. as control) either as a 1x 48 h or a 2x 24 h sequential treatment, and PARC and GAPDH content was assessed (WB). There were no significant differences in PARC down-regulation when comparing the 2 transfection methods (n = 3 replicate experiments). **C)** Chemosensitive (OV2008) and resistant (C13*) OVCA cells were treated with PARC siRNA (0, 50, 100 and 200 nM; 48 h; S.Seq. as control) followed by CDDP (10 μM; 24 h; DMSO as control) and PARC and GAPDH content were assessed (WB; n = 2 replicate experiments).





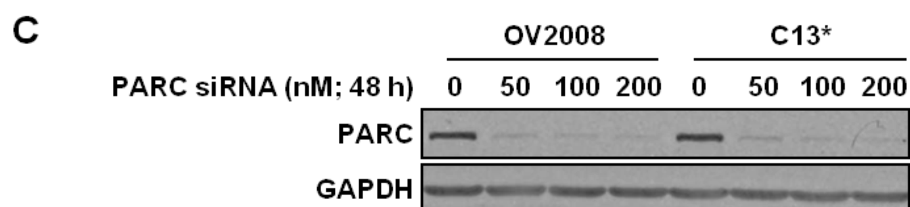


Figure A3

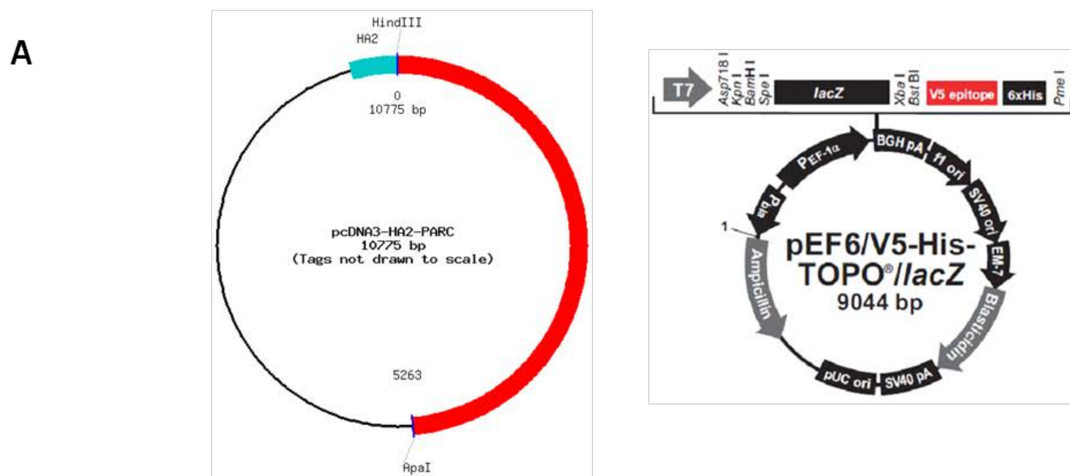
7.5 Construction and optimization of PARC over-expression plasmid.

To determine the physiological role of PARC in the regulation of p53 subcellular localization and CDDP sensitivity, experiments were proposed to over-express PARC in chemosensitive cells treated with CDDP or DMSO control. To accomplish this, we constructed and optimized a PARC plasmid to express a recombinant human PARC-V5-His fusion protein. Since the N-terminus of PARC interacts with the C-terminus of p53 (Nikolaev et al, 2003), the recombinant PARC protein was designed to express a V5-His tag at the C-terminus to avoid potential interference problems with the p53 interaction site. **Figure A4A** demonstrates the pcDNA3-HA2-PARC plasmid construct obtained commercially from Addgene, and the PARC construct was cloned into a pEF6/V5-His-TOPO/lacZ expression plasmid. Proper ligation into the plasmids was confirmed by size in 1% agarose gels stained with ethidium bromide. The presence of the PARC open reading frame (ORF) for each plasmid construct was confirmed by RT-PCR (**Figure A4B**). PARC-silenced human chemosensitive (A2780s) OVCA cells were transfected with the expression vector (1 μg; 24 h) and the PARC fusion protein was detected using PARC antibodies specific to the N-, C- and middle regions of the PARC protein (**Figure A4C**).

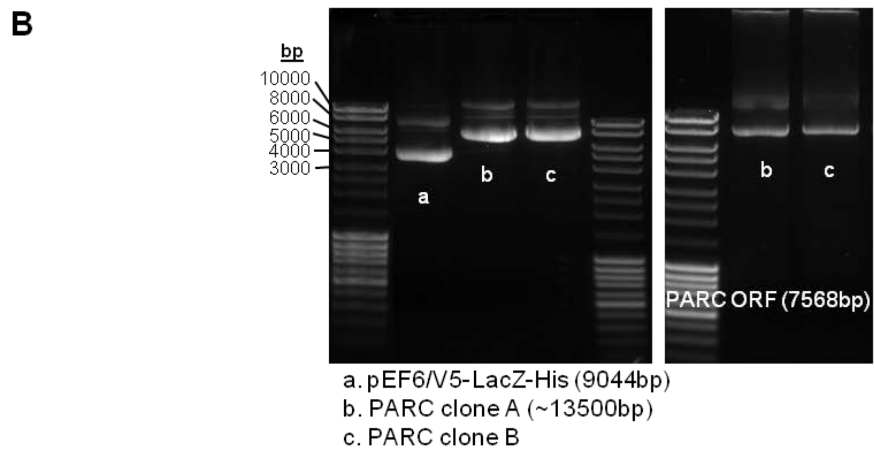
Figure A4. Construction and optimization of PARC over-expression plasmids (PEF6-His-PARC).

A) The pcDNA3-HA2-PARC plasmid construct was obtained commercially from Addgene, and the PARC construct was cloned into a pEF6/V5-His-TOPO/lacZ expression vector (Invitrogen). **B)** Proper ligation into the plasmid was confirmed by size in 1% agarose gels stained with ethidium bromide. The presence of the PARC open reading frame (ORF) for each plasmid construct was confirmed by RT-PCR. **C)** PARC-silenced chemosensitive (A2780s) OVCA cells were transfected with the pEF6-PARC (pEF6-V5-LacZ as control) expression vector and the recombinant PARC protein was detected by Western blot (positive control: C13* WCL; negative control: pEF6-V5-LacZ empty vector transfected and mock transfection with Lipofectamine and PLUS reagent). Site-specific PARC antibodies (left) were able to recognize the N- and C-terminus and middle parts of the recombinant PARC protein [A300-096A, A300-097A (1:10000, Bethyl Laboratories); 1:1000, DR1028 (Calbiochem); 1:100, SC-33259, SC-33260 (Santa Cruz); V5 (1:1000, Invitrogen)].

A



B



a. pEF6/V5-LacZ-His (9044bp)
b. PARC clone A (~13500bp)
c. PARC clone B

C

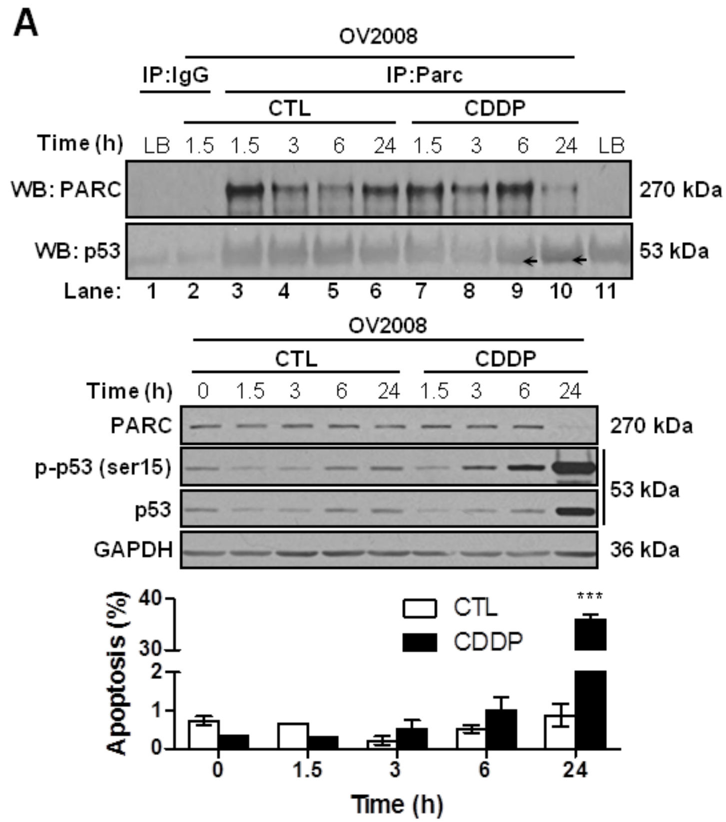
Figure A4

7.6 The effect of CDDP on PARC and p53 interaction in chemosensitive OVCA cells.

The experiments conducted in section 4.7 (**Figure 11**) were extended to include chemosensitive (OV2008) OVCA cells. CDDP induced PARC down-regulation and increased p53, p-p53 (ser15) content and apoptosis [**Figure A5A**, **bottom**; 2-Way ANOVA: CDDP ($p<0.001$), time ($p<0.001$) and CDDP x time interaction ($p<0.001$)]. IP:PARC and co-IP:p53 results were difficult to interpret (**Figure A5A**, **top**) as CDDP induced changes to both PARC and p53 content at 24 h, and at earlier time points, p53 content was relatively low. As a result of low p53 content, co-IP of p53 was difficult to detect [**Figure A5A**, **top**, **Lanes 9 & 10** (arrows)] against the high IgG heavy chain background (~55 kDa). To confirm the presence of p53 in relation to the IgG heavy-chain background, p53 detection was confirmed in IP:PARC, co-IP p53 experiments using whole cell lysates of OV2008 cells treated with or without p53 siRNA (**Figure A5B**; **Lanes 5-8**).

Figure A5. The effect of CDDP on PARC and p53 interaction in chemosensitive OVCA cells.

A) CDDP induced a decrease in PARC pull-down after 24 h (Lane 10) but resulted in an increase in the co-IP of p53 at 6 and 24 h (arrows) compared to the earlier time-points. Also shown are controls for potential non-specific bands involving antibody IgG (Lanes 1 & 2), and the lysis buffer (LB; Lanes 1 & 11). To confirm the effectiveness of the CDDP treatment, PARC, p53, and p-p53 (ser15) content from the whole cell lysate was examined (WB) and apoptosis was measured (Hoechst) [***$p<0.001$ (versus CTL)]. Results are expressed as mean ± SEM (n = 3 replicate experiments). **B)** Chemosensitive (OV2008) OVCA cells were treated with p53 siRNA or scrambled sequence control (100 nM; 48 h) prior to CDDP treatment (10 uM; 24h). Whole cell lysates were subjected to IP:PARC and co-IP/Western blot of p53 is shown. IP of normal rabbit IgG (Lanes 1-3) and input of lysis buffer (Lanes 1 & 4) are included as controls. Whole cell lysates were included to demonstrate the efficacy of p53 down-regulation by siRNA treatment (Lanes 9-12; n = 1).



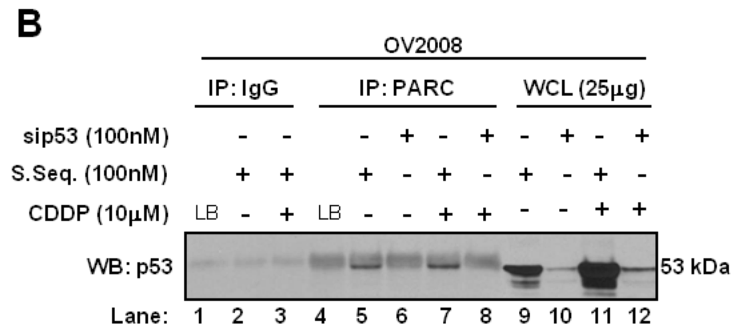


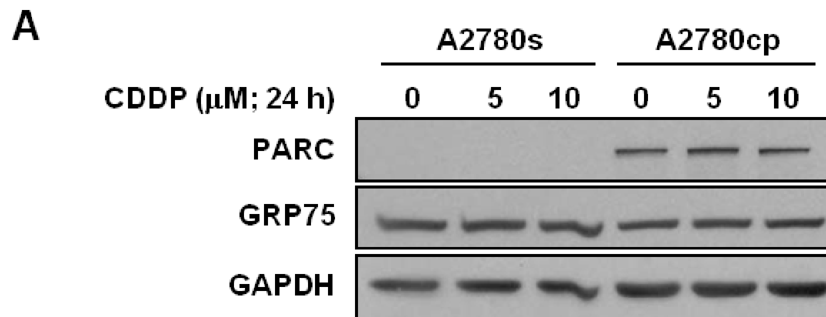
Figure A5

7.7 PARC expression is regulated by DNA methylation in chemosensitive (A2780s) but not chemoresistant (A2780cp) OVCA cells.

Investigation of PARC content in the CDDP-sensitive A2780s OVCA cell line, and its resistant isogenic counterpart (A2780cp) revealed a PARC-silenced phenotype in the A2780s cells (**Figure A6A**). Although unexpected, the lack of PARC protein content in the chemosensitive cells but not the resistant cells is consistent with the idea that PARC is a determinant of chemoresistance, and its role and regulation may impact CDDP sensitivity in OVCA cells. To our knowledge, this is the first identified PARC-silenced cell line, and it has proven to be a valuable tool for our PARC overexpression studies. In addition, with reports that differences in mitochondrial biology may be implicated in the development of chemoresistance, we also examined levels of GRP75 (mitochondrial HSP-70; mitochondria loading control). **Figure A6A** demonstrates that GRP75 content is similar in both cell lines and did not change as a result of CDDP treatment. The PARC-silenced phenotype of these cells is dependent on DNA methylation, as 5-Aza-2'-deoxycytidine induced PARC expression (**Figure A6B**). A decrease in PARC content at the higher concentrations may reflect possible drug toxicities that resulted in PARC degradation. This represents, to our knowledge, the first demonstration of a physiologic condition where PARC content is altered.

Figure A6. PARC expression is regulated by DNA methylation in chemosensitive (A2780s) but not chemoresistant (A2780cp) OVCA cells.

A) PARC protein levels were undetectable in A2780s cells in contrast to the A2780cp cells where PARC content was relatively elevated and unchanged as a result of CDDP-treatment (0-10 μM; 24 h). GRP75 was present in both cell lines and did not change with CDDP treatment (n = 3 replicate experiments). **B)** Treatment of A2780s cells with 5'-Aza-3'deoxycytidine, an inhibitor of DNA methylation (0-10 μM; 72 h), resulted in an increase in PARC content as determined by Western blot (n = 3 replicate experiments).



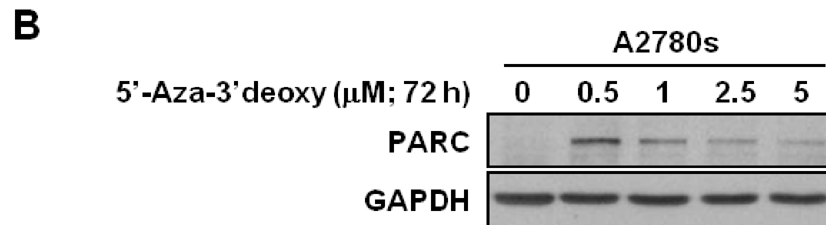


Figure A6