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Regulation of p53-Dependent Apoptosis: Implications for Cell Fate Determination and Cisplatin Resistance in Human Ovarian Cancer Cells *in vitro*

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Regulation of p53-Dependent Apoptosis: Implications for Cell Fate Determination and Cisplatin Resistance in Human Ovarian Cancer Cells *in vitro*

Mr. Michael Fraser

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For Genevieve.

Contribution of Co-Authors

All studies were carried out under the supervision of Dr. Benjamin Tsang. All experimental work was conducted by Mr. Michael Fraser, unless otherwise noted.

Chapter 4:

Title: p53 is a Determinant of Xiap/Akt-Mediated Chemoresistance in Human Ovarian Cancer Cells

Journal: Cancer Research 63 (21): 7081-8, 2003.

Authors: Michael Fraser, Brendan M. Leung, Xiaojuan Yan, Han C. Dan, Jin Q. Cheng, and Benjamin K. Tsang

Mr. Brendan Leung performed approximately 40% of the experiments documented in this chapter, particularly those in figures 1-4. Dr. Xiaojuan Yan performed the immunocytochemistry for MDM2 (not shown) and was consulted on the writing and editing of the manuscript. Drs. Jin Q. Cheng and Han C. Dan were involved in experimental design, and also contributed to the editing of the final manuscript.

Chapter 6:

Title: Regulation of p53 and Suppression of Apoptosis by the Soluble Guanylyl Cyclase/cGMP Pathway in Human Ovarian Cancer Cells

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Authors: Michael Fraser[1], Siu L.Chan[1,2], Sarah S.L. Chan[1], Ronald R. Fiscus[2]

and Benjamin K. Tsang

Ms. Siu Lan Chan initiated the project and performed the experiments detailed in figure 2B with regards to the effects of XIAP over-expression on ODQ-induced apoptosis. Ms. Sarah Chan was an undergraduate summer student in Dr. Tsang's laboratory, under the direct supervision of Mr. Michael Fraser. She performed numerous Western blots and assisted with cell culture. Dr. Ronald R. Fiscus was Ms. Siu Lan Chan's M.Sc. supervisor, and provided support for experimental design and data interpretation, as well as in editing the final manuscript. His laboratory also performed the cGMP assays shown in Figure 1A.

Abstract

Programmed cell death (apoptosis), is a normal physiological process which involves a distinct, well-characterized signaling cascade. However, dysregulation of the apoptotic machinery is commonly observed in many pathophysiological conditions. In particular, human cancer is commonly associated with a reduced capacity of cells to undergo apoptosis in response to cell stress, leading to an abnormal accumulation of damaged cells.

Human ovarian cancer is the most lethal gynecological malignancy, a statistic that is due, in part, to the phenomenon a chemoresistance, by which tumour cells evade the cytotoxic effects of chemotherapeutic agents used to eradicate the disease. Cisplatin (CDDP) and paclitaxel are first-line chemotherapeutics for human ovarian cancer, but resistance to these agents is common, and significantly attenuates positive clinical outcomes. Recent evidence suggests that aberrant regulation of the apoptotic cascade may be a causative factor for chemoresistance. To that end, gene products implicated in the regulation of apoptosis, including the PI3K/Akt and Inhibitor of Apoptosis Protein family are frequently over-expressed and/or dysregulated in chemoresistant cells.

Activation of the *TP53* tumor suppressor gene product, p53, is critical for DNA-damage-induced apoptosis in many cell types, and mutation of this gene is the most frequently observed abnormality in all of human cancer. p53 mutations are commonly, but not always, associated with poor prognosis and chemoresistance. Since the aberrant regulation of p53 may have critical implications for the clinical management of human ovarian cancer, it is of paramount importance to understand the molecular and cellular

mechanisms by which p53 is regulated in these cells, and if and how dysregulated p53 activation may play a role in the etiology of chemoresistance.

Cultured human ovarian cancer cells were used to establish the *in vitro* regulation of p53 and to assess the requirement for p53-dependent apoptosis for CDDP-induced apoptosis. We observed that CDDP induced apoptosis in chemosensitive ovarian cancer cells, but not in their chemoresistant variants. In addition, we demonstrated that over-expression of X-Linked Inhibitor of Apoptosis Protein (XIAP) induces chemoresistance in ovarian cancer cells, while down-regulation of XIAP sensitizes chemoresistant ovarian cancer cells to CDDP-induced apoptosis. Attenuation of Akt signalling using a dominant-negative Akt attenuated XIAP-mediated chemoresistance, suggesting a functional link between XIAP and Akt with respect to the regulation of chemosensitivity. Akt activation, a frequently observed event in human ovarian cancer, inhibited CDDP-induced apoptosis in chemosensitive cells, while dominant-negative Akt up-regulated p53 and sensitized chemoresistant cells to CDDP in a p53-dependent manner, suggesting a functional link between Akt activation and p53-mediated apoptosis. Akt-mediated chemoresistance was also associated with ablated CDDP-induced down-regulation of the anti-apoptotic protein XIAP.

We further demonstrated that CDDP induced the up-regulation of the p53-responsive gene product PUMA, and induced apoptosis in a PUMA-dependent manner, although PUMA expression was not sufficient to confer a chemosensitive phenotype. CDDP also induced phosphorylation of p53 on numerous N-terminal residues in chemosensitive, but not chemoresistant cells. Activation of Akt inhibits the CDDP-induced phosphorylation of p53, while inhibition of Akt function induces p53

phosphorylation. Phosphorylation of Ser15 and Ser20, but not Ser37, was required for p53-dependent apoptosis but not for p53-dependent up-regulation of PUMA.

Moreover, we showed that basal p53 levels are maintained, in part, by the constitutive activation of the soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway, and inhibition of this pathway depletes basal cGMP levels, up-regulates p53 content, stability, and phosphorylation, and induces apoptosis in a partially p53-dependent manner.

These results suggest that: (a) p53 is a critical determinant of cell fate in human ovarian cancer cells, and is regulated by diverse signaling cascades including the Akt and sGC/cGMP pathways, (b) CDDP-induced apoptosis proceeds via a p53-dependent mechanism involving PUMA and p53 phosphorylation, (c) Akt confers resistance to ovarian cancer cells, in part, by suppressing the CDDP-induced activation and phosphorylation of p53. Akt also contributes to chemoresistance by attenuating the CDDP-induced down-regulation of XIAP.

The current study significantly extends our understanding of how the p53 pathway is regulated, and how dysregulation of p53 activation may have profound effects upon the sensitivity of ovarian cancer cells to stress-induced apoptosis. This, in turn, may have important effects upon our understanding of the pathophysiology of chemoresistance in human ovarian cancer; a phenomenon that significantly attenuates sucessful treatment outcomes for this disease.

Contribution of Co-Authors iii
Abstract v
LIST OF ABBREVIATIONS xi
Acknowledgements xvii

Chapter 1 - Introduction 1

Introduction 1
Human Ovarian Cancer – Background 1
Ovarian Cancer Stages 1
Ovarian Cancer Subtypes 2
Risk Factors and Molecular Mechanisms for Ovarian Oncogenesis 5
Ovulation 5
Gonadotropins 6
Genetic Determinants of Familial Ovarian Cancer 7
Molecular Determinants of Sporadic Ovarian Tumourigenesis 8
The Phosphoinositol-3-kinase/AKT pathway 9
p53 10
The Role of p53 in Tumourigenesis 11
Interactions between p53 and BRCA 13
Retinoblastoma (RB) 13
KRAS 14
Other Potential Mediators 15
Chemotherapy for Ovarian Cancer 16
Cisplatin (CDDP) 16
Direct Cellular Response to CDDP (ATM/ATR) 17
Ataxia Telangiectasia-Mutated (ATM) 18
ATM-and-Rad3-Related (ATR) 18
Molecular Targets of ATM/ATR in Response to CDDP 19

Chapter 2 – Apoptosis and Chemoresistance in Human Ovarian Cancer 21

Apoptosis 21
Historical Perspective 21
Apoptosis in Mammalian Cells 22
Initiation and Execution of Apoptosis 22
Apoptosis – Intrinsic Pathway 23
Apoptosis – Extrinsic Pathway 26
Molecular Regulators of Apoptosis 27
The Bcl-2 Family 27
Inhibitor of Apoptosis Proteins (IAPs) 28
Regulation of XIAP Expression 29
Additional functions of XIAP 30
Flice-Like Inhibitory Protein 32

The PI3K/Akt Pathway 33
Akt 34
Soluble Guanylyl Cyclase/cyclic guanosine monophosphate (cGMP) 38
p53 41
Regulation of p53 content and function 41
Transcription-Dependent p53-Mediated Apoptosis 42
Transcription-Independent p53-Mediated Apoptosis 44
Direct Mitochondrial Actions of p53 48
Cytosolic p53 48
Regulation of Apoptosis by p53 Phosphorylation 49
Serine 15 49
Serine 20 53
Serine 46 53
Mechanisms of Chemoresistance in Human Ovarian Cancer 54
Multidrug Resistance 'Pumps' 54
Drug Detoxification 56
Enhanced DNA damage repair 57
Suppression of drug-induced apoptosis 58
Involvement of p53 in Determining CDDP Sensitivity 59

Chapter 3 –Objectives and Hypotheses 61

Objectives and Hypotheses 61
Overall Objectives 61
Specific Hypotheses 61
Specific Objectives 62

Chapter 4 – Cancer Research 63 (21): 7081-8, 2003. 63

p53 is a Determinant of Xiap/Akt-Mediated Chemoresistance in Human Ovarian Cancer Cells 63
Appendix to Chapter 4 92

Chapter 5 – Submitted for publication to Oncogene 93

Akt Promotes Cisplatin Resistance in Human Ovarian Cancer Cells Through Inhibition of p53 Phosphorylation and Nuclear Function 93

Chapter 6 – Oncogene 2006 Apr 6;25(15):2203-2212. 139

Regulation of p53 and Suppression of Apoptosis by the Soluble Guanylyl Cyclase/cGMP Pathway in Human Ovarian Cancer Cells 139

Chapter 7 – Unpublished Data 174

Regulation of Integrin-Linked Kinase by XIAP 174
Regulation of the CDDP-Induced Mitochondrial Accumulation of p53 177
Confirmation of the Inhibitory Effects of Dominant-Negative Akt 185
Phosphorylation does not Regulate p53 Mitochondrial Accumulation 188

Chapter 8 – General Discussion 194

General Discussion 194
Overview and Significance 194
Apoptosis as a Determinant of CDDP Sensitivity 194
Experimental Value of Matched Pair Ovarian Cancer Cell Lines 195
XIAP as a Determinant of Chemoresistance in Ovarian Cancer Cells 197
Interaction between XIAP and AKT in the Determination of CDDP Sensitivity 198
Mechanism of XIAP-Induced Phospho-AKT Up-Regulation 200
Akt as an Independent Determinant of CDDP Resistance 201
Akt as a Therapeutic Target 202
Regulation of CDDP-Induced XIAP Down-Regulation by AKT 204
Involvement of p53 in Chemoresistance 206
Functional Interaction Between AKT and p53 206
Involvement of p53 in CDDP-Induced Apoptosis 208
Paradoxical Findings Regarding the Involvement of p53 in Chemoresistance .. 209
Involvement of PUMA in CDDP-Induced, p53-Mediated Apoptosis 211
Regulation of p53 Nuclear Function 212
Implication of Altered p53 Phosphorylation in Chemoresistance 214
Regulation of Nuclear p53 Function by Akt 217
Regulation of p53 Phosphorylation by Akt 218
Possible Implication of ATR in the Akt-Dependent Regulation of p53 Phosphorylation 219
The Soluble Guanylyl Cyclase/cGMP Pathway as a Cell Fate Determinant 220
Future Directions 222
Xenograft Models 222
Examination of Clinical Samples 224
Conclusions 226

Chapter 9 – References 229

Chapter 10 – Curriculum Vitae 249

LIST OF ABBREVIATIONS

8-Br-cGMP	8-Bromo-Cyclic Guanosine Monophosphate
ANP; ANF	Atrial Natriuretic Peptide/Factor
APAF-1	Apoptotic Protease Activating Factor-1
ATM	Ataxia Telangiectasia-Mutated
ATP	Adenosine Triphosphate
ATR	ATM-and Rad3-Related
ATRIP	ATR Interacting Protein
BAD	Bcl-2-Antagonist of Cell Death
BAX	Bcl-2-Associated X Protein
BCL-2	B-Cell Lymphoma-2
BH	Bcl-2 Homology
BID	BH3-Interacting Domain Death Agonist
BIM	Bcl-2-Like 11
BIR	Baculovirus Inhibitor of Apoptosis Repeat
BMP	Bone Morphogenic Protein
cGMP	Cyclic Guanosine Monophosphate
CAD	Caspase-Activated Deoxyribonuclease
CARD	Caspase-Associated Recruitment Domain
CBP	cAMP Responsive Element Binding (CREB) Binding Protein

CDDP	Cis-diamminedichloroplatinum (II)
CHK1/2	Checkpoint Kinase 1/2
DIABLO	Direct IAP Binding Protein With Low pI
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DN-Akt	Dominant-Negative Akt
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
EGF	Epidermal Growth Factor
eNOS	Endothelial NOS
ER	Estrogen Receptor
FADD	Fas-Associated Death Domain
FasL	Fas Ligand
FIGO	International Federation of Gynecology and Obstetrics
FITC	Fluorescein Isothiocyanate
FLICE	FADD-Like ICE
FLIP	FLICE-Like Inhibitory Protein
FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde Phosphate Dehydrogenase

GMP	Guanosine Monophosphate
GTP	Guanosine Triphosphate
HA	Hemagglutinin
HIAP1/2	Human Inhibitor of Apoptosis Protein-1/2
HRP	Horseradish Peroxidase
IAP	Inhibitor of Apoptosis Protein
ICE	Interleukin 1β-Converting Enzyme
IgG	Immunoglobulin G
ILK	Integrin-Linked Kinase
iNOS	Inducible NOS
IR	Ionizing Radiation
JNK	c-Jun N-Terminal Kinase
LPS	Lipopolysaccharide
kD	Kilo-Dalton
LH	Luteinizing Hormone
LOH	Loss of Heterozygosity
MAPK	Mitogen-Activated Protein Kinase
MAPKAPK2	MAPK-Activated Protein Kinase-2
MDM2	Murine Double-Minute-2

MOI	Multiplicity of Infection
NAIP	Neuronal Apoptosis Inhibitory Protein
NES	Nuclear Exclusion Signal
NF-κB	Nuclear Factor kappa B
NLS	Nuclear Localization Signal
nNOS	Neuronal NOS
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
ORF	Open Reading Frame
OSE	Ovarian Surface Epithelium
P53AIP1	p53-Regulated Apoptosis Inducing Protein-1
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly(Adenosine Diphosphate) Polymerase
PCR	Polymerase Chain Reaction
PDK-1	Phosphotidylinositol-Dependent Protein Kinase-1
PFT	Pifithrin-alpha Hydrobromide
PFU	Plaque-Forming Unit
PI3K	Phosphoinositol 3-OH kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate

PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein Kinase B
PKG	Protein Kinase G; cGMP-Dependent Protein Kinase
PH	Pleckstrin Homology
PMSF	Phenylmethylsulfonyl Fluoride
PTEN	Phosphatase and Tensin Homologue
PUMA	p53-Upregulated Mediator of Apoposis
RB	Retinoblastoma
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcriptase
SDS	Sodium Dodecylsulfate
SEM	Standard Error of the Mean
SHIP	Src Homology 2-Containing Inositol Phosphatase
SMAC	Second Mitochondria-Derived Activator of Caspases
SMAD	Mothers Against Decapentaplegic Homologue
SNP	Single Nucleotide Polymorphism
TAE	Tris-Acetate-EDTA

tBID	Truncated BID
TBS	Tris-Buffered Saline
TBS-T	TBS-Tween
TE	Tris-EDTA
TGF-β	Transforming Growth Factor-beta
TNF-α	Tumour Necrosis Factor-alpha
TP53	Tumour Protein 53
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TUNEL	Terminal Transferase dUTP Nick End Labeling
uPA	Urokinase Plasminogen Activator
UTR	Untranslated Region
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
X-GAL	5-Bromo-4-Chloro-3-Indolyl-β-D-galactoside
XAF1	XIAP-Associated Factor-1
XIAP	X-Linked Inhibitor of Apoptosis Protein
XP	Xeroderma Pigmentosa

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"So many people today—and even professional scientists—seem to me like someone who has seen thousands of trees but has never seen a forest. A knowledge of the historic and philosophical background gives that kind of independence from prejudices of his generation from which most scientists are suffering. This independence created by philosophical insight is—in my opinion—the mark of distinction between a mere artisan or specialist and a real seeker after truth."

- Albert Einstein

Chapter 1 - Introduction

Introduction

Human Ovarian Cancer – Background

Human ovarian cancer is the sixth most commonly diagnosed cancer in Canadian women, and is the most lethal of the gynecological malignancies, resulting in the death of approximately 1500 women per year in Canada (2006 Canadian Cancer Statistics, Canadian Cancer Society/National Cancer Institute of Canada). Due to the lack of early symptoms, ovarian cancers are usually diagnosed after the cancer cells have spread beyond the ovary and local tissues (FIGO Stage III or IV), by which point the disease is largely incurable.

The age-adjusted incidence rate for human ovarian cancer has fallen to about 83% of its 1976 level (from 13.9 vs. 11.6 per 100 000 women; 2005 Canadian Cancer Society Statistics). By contrast, the overall rate of cancer incidence in women over the same time period has increased by approximately 20% (294.9 vs. 355.8 cases per 100 000 women; 2005 Canadian Cancer Society Statistics). However, the percentage of women diagnosed with ovarian cancer who ultimately succumb to the disease has been largely invariant, from approximately 65% in 1976 to 63% in 2005. Taken together, these data suggest that little progress has been made in improving the success of treatment for those diagnosed with the disease.

Ovarian Cancer Stages

The high mortality rate for human ovarian cancer can be primarily explained by two factors: late diagnosis and therapeutic resistance. Ovarian cancer is typically

diagnosed when it has reached stage III or IV, by which time it has spread beyond the abdomen. Stage I and II ovarian cancers are readily curable, with 5-year survival approaching 90% for stage I and 65% for stage II ovarian cancers (Nguyen et al., 1993). By contrast, stage III or IV disease is typically more difficult to treat successfully, and 5-year survival rates range from 39% for stage III ovarian cancer to about 25% for stage IV (Holschneider & Berek, 2000). In large part, the late diagnosis of ovarian cancer is due to the fact that early stage disease is symptomatically silent (Vanderhyden et al., 2003). As such, the disease goes unnoticed until it has advanced and spread throughout the abdomen. Indeed, FIGO stage at the time of diagnosis is the most important prognostic factor for ovarian cancer outcomes (Holschneider & Berek, 2000).

Ovarian cancer is staged according to the following criteria (Kaku et al., 2003):

Stage I: Growth limited to the ovary;

Stage II: Growth involving one or both ovaries with pelvic extension;

Stage III: Tumour involving one of both ovaries with peritoneal implants outside the pelvis; tumour is limited to the pelvis, but with malignant extension to the small bowel or omentum;

Stage IV: Growth involving one or both ovaries with distant metastasis;

The initial therapeutic treatment for ovarian cancer is cytoreductive surgery to remove the bulk of the tumour. If the tumour is confined to the ovary, surgery may be curative, although for the majority of patients, follow-up treatment with combination chemotherapy is generally employed.

Ovarian Cancer Subtypes

While the majority of ovarian cancers derive from the epithelial lining of the ovary (85-90%), a small fraction of ovarian tumours arise from stromal and germ cells (Weiss et al., 1977). Epithelial ovarian cancers are further classified into the following histological subtypes: serous, mucinous, endometrioid, clear cell, transitional cell, mixed epithelial, and undifferentiated. Significant histological differences exist between these tumour types, and these are described below. In addition, specific molecular differences often characterize the various tumour types, and these are discussed within the pertinent sections below.

Serous ovarian tumours are usually large, frequently bilateral (Kaku et al., 2003), and often invade the ovarian capsule, growing on the ovarian surface. The formation of papillae and spaces within the tumour is common, and the lining of the tumour consists of stratified serous-type cells. Serous tumours are the most frequently diagnosed ovarian cancer subtype, and are most frequenly diagnosed in stage III or IV (~75% of cases).

Mucinous tumours, which are also frequently diagnosed, are generally the largest tumour subtype (Kaku et al., 2003), generally unilateral (Hart, 2005), and are predominantly solid. Microscopically, mucinous tumours contain glands and cysts, which are lined by stratified mucinous epithelial cells. The majority of mucinous ovarian cancers are diagnosed in stage I (71%) without stromal invasion, although these tumours can be uncharacteristically aggressive and lethal (Ludwick et al., 2005). In a recent study, mucinous ovarian cancer showed a reduced amenability to combination chemotherapy relative to serous ovarian cancer (Pectasides et al., 2005), although survival times did not differ between the two subtypes. Mucinous ovarian cancer is often diagnosed earlier than serous ovarian cancer (Pieretti et al., 2002).

Endometrioid tumours are characterized by the presence of elements of both the epithelium and stroma, and, as the name suggests, resemble adenocarcinoma of the endometrium (Kaku et al., 2003). Endometrioid tumours are highly cystic, with cysts filled with 'chocolate-coloured' fluid (Kaku et al., 2003). Like mucinous tumours, endometrioid tumours generally present in stage I (48%).

Clear cell ovarian cancer is characterized by the presence of cells containing cytoplasmic glycogen (Kaku et al., 2003). Interestingly, while clear cell ovarian cancer represents only 5-10% of ovarian cancers in Western countries, they comprise a much higher proportion in Japanese women (~30%) (Kaku et al., 2003). Cells from clear cell ovarian cancers are typically polygonal, and may form multiple papillae. More than 60% of these tumours are discovered in stage I.

Columns of transitional epithelial cells characterize *transitional cell tumours*, which are also known as Brenner tumours. Transitional cell ovarian cancers are usually found with significant infiltration of the stroma, often with calcification. Once again, nearly 50% of transitional cell ovarian cancers are identified in stage I, with stage I or stage II diagnoses occurring in nearly 80% of all cases (Kaku et al., 2003).

Mixed epithelial ovarian cancers consist of a mixture of the above epithelial subtypes, along with stromal components. Greater than 10% of the total cell population should be from a second subtype for an appropriate diagnosis of a mixed epithelial tumour to be made (Kaku et al., 2003).

Undifferentiated ovarian cancer shows only minor differentiation (Silva et al., 1991), and shows rapid growth and particularly poor prognosis (Kaku et al., 2003).

Risk Factors and Molecular Mechanisms for Ovarian Oncogenesis

It has proven exceedingly difficult to ascertain the molecular mechanisms underlying the development of ovarian cancer, in large part because most cases of ovarian cancer are discovered at a late stage, by which time the tumour cell biology may have been substantially altered relative to the initial stages of oncogenesis. While the majority of ovarian cancers are sporadic (i.e. non-hereditary), a family history of ovarian cancer is an important risk factor, and heredity accounts for approximately 5-10% of ovarian cancers (Holschneider & Berek, 2000). As such, it is critical to understand the molecular determinants of ovarian tumourigenesis.

Ovulation

Ovulation results in apoptotic cell death of the ovarian surface epithelial (OSE) cells in the immediate vicinity of the ovulation event (Ackerman & Murdoch, 1993). The wound created by this cell death is remodeled via proliferation of surrounding OSE cells. The 'incessant ovulation hypothesis' suggests that the continuous requirement for this wound healing process increases the risk of malignant transformation of these cells (Fathalla, 1971). In support of this hypothesis, late menopause slightly increases the risk of developing ovarian cancer (Franceschi et al., 1991), as does nulliparity (Negri et al., 1991), both of which increase the total number of lifetime ovulations. Similarly, a correlation between early menarche and increased risk of developing ovarian cancer has been noted for some time, although other studies have disputed this claim (Franceschi et al., 1991; Purdie et al., 1995). In addition, multiparity and prolonged use of oral contraceptives, which decrease the total number of ovulations, provide a strong protective effect against the development of the disease (Gross & Schlesselman, 1994; Hankinson et

al., 1995; Risch et al., 1994; Schlesselman, 1995). However, pregnancies that result in spontaneous or induced abortion, which also lower the total number of ovulations, do not appear to provide a protective advantage (Negri et al., 1991; Risch et al., 1994), suggesting that there may be additional protective advantages of both oral contraceptive use and higher parity unrelated to the reduction in the number of ovulations.

While the ovarian surface is generally smooth, invaginations of the surface do occur over time, and may develop into inclusion cysts within the ovarian stroma (Nicosia, 1987). Inclusion cysts are frequently observed in women with a strong hereditary predisposition to ovarian cancer, and microscopic tumours have been shown to arise from these cysts (Deligdisch & Gil, 1989; Salazar et al., 1996; Scully, 1995), suggesting that their development may be an important causative factor in the development of the disease.

Gonadotropins

In addition, a role for excessive gonadotropin (LH and FSH) production in the etiology of ovarian cancer has been suggested based upon the fact that ovarian cancers are most frequently diagnosed in the postmenopausal period when serum FSH and LH levels reach their peak, and because both normal ovarian surface epithelial cells and ovarian cancer cells express FSH and LH receptors (Konishi et al., 1999; Zheng et al., 1996). Both LH and FSH enhance the proliferation and invasiveness of of cultured human OSE cells. However, animals with manipulated production or biological function of these hormones do not show an increased propensity for the development of ovarian cancer. As such, the precise role of gonadotropins in ovarian epithelial tumours remains unclear, although granulosa cell and stromal cell tumours develop in mice with LH

hypersecretion and inhibin deficiency, respectively (Keri et al., 2000; Matzuk et al., 1992; Nilson et al., 2000).

Genetic Determinants of Familial Ovarian Cancer

The single greatest risk factor for ovarian cancer is a family history (Holschneider & Berek, 2000). Indeed, while hereditary ovarian cancers account for only about 5-10% of all cases (Ford & Easton, 1995; Ford et al., 1995; Prat et al., 2005), a family history of the disease increases the estimated lifetime risk of developing ovarian cancer from approximately 1.6% to 9.4%; an approximately 6-fold risk increase (Prat et al., 2005). While no gene has been shown to increase the risk of developing ovarian cancer *specifically*, the identification of the *BRCA1* breast and ovarian cancer susceptibility gene (OMIM# 113705) on chromosome 17q21 (Bowcock, 1993; Miki et al., 1995) provided the first partial molecular explanation for the strong association between ovarian cancer risk and the incidence of ovarian cancer in first- and second-degree relatives. A second gene, identified in 1995 on chromosome 13q12-13 and termed *BRCA2* (OMIM# 600185), also has strong linkage to the development of ovarian cancer (Wooster et al., 1995). Both *BRCA1* and *BRCA2* encode large proteins with nuclear expression, which are involved in DNA damage recognition and/or repair (Scully et al., 2000; Yoshida & Miki, 2004). This implicates these gene products in the control of cell cycle progression, DNA repair, and apoptosis. Interestingly, it has been suggested that *BRCA* gene product function is dependent upon the function of the p53 tumour suppressor (discussed in detail below), whereby BRCA-mediated signaling results in p53-dependent cell cycle arrest and/or apoptosis, and that disruptions in p53 interfere with this function. To that end, at least one report has observed a higher frequency of *TP53* mutation in ovarian tumours with

germline *BRCA1* or *BRCA2* mutations compared with sporadic cancers (Ramus et al., 1999).

BRCA1 and BRCA2 mutations increase the risk of developing the disease by up to 30-fold (Prat et al., 2005). While the incidence of mutations is high in founder populations such as Ashkenazi Jews (~2.3%) (Struewing et al., 1997), the mutation rate in the general population is estimated to be less than 0.1% (Ford et al., 1995). These mutations are highly variable, ranging from simple point mutations and small deletions/insertions, up to large-scale genetic rearrangements (Mazoyer et al., 1998; Montagna et al., 2003; Prat et al., 2005; Puget et al., 1997; Unger et al., 2000). Indeed, more than 250 individual mutations in these genes have now been identified. *BRCA* mutations are rare in sporadic ovarian cancer (Foster et al., 1996), although loss of heterozygosity (LOH) at the *BRCA* loci has been identified in sporadic tumours, as has down-regulation of nuclear *BRCA1* protein content (Foster et al., 1996; Garcia et al., 2000), suggesting that loss of *BRCA* gene product function may have a role in sporadic ovarian tumourigenesis.

While *BRCA* mutations account for a large proportion of hereditary ovarian cancer cases, other hereditary forms exist, such as Lynch II syndrome, which is caused by mutations of components of the DNA mismatch repair pathway, and is associated with increased risk of colorectal, ovarian, endometrial, and gastric cancers, among others (Lynch et al., 1966a; Lynch et al., 1966b; Lynch et al., 2004).

Molecular Determinants of Sporadic Ovarian Tumourigenesis

While sporadic ovarian cancer constitutes the vast majority of all diagnosed cases, the eludication of the molecular mechanisms involved has proved difficult, in part due to

the fact that late diagnosis precludes a detailed molecular analysis of the early events in ovarian tumourigenesis. However, a number of candidate molecules have been implicated in this process.

The Phosphoinositol-3-kinase/AKT pathway

Phosphoinositol-3-OH-kinase (PI3K) is a lipid kinase implicated in cytokine and growth factor signaling (detailed below). PI3K activates protein kinase B (PKB), commonly known as Akt, thereby promoting cell proliferation, cell cycle progression, and protecting cells from apoptosis. In one study, the *PIK3CA* gene (OMIM# 171834), which encodes the p110α subunit of PI3K, showed increased copy number in 89% (8/9) of human ovarian cancer cell lines and in 100% (5/5) of cells from human ovarian tumour ascites fluid (Shayesteh et al., 1999). In addition, 58% (7/12) of primary human ovarian tumours showed increased *PIK3CA* copy number. By contrast, none of the human breast cancer or melanoma cell lines examined (0/5) showed increased *PIK3CA* copy number, nor did normal ovarian epithelial cells (0/4). Moreover, increased copy number was associated with higher rates of *PIK3CA* transcription, protein content, and PI3K activity. In addition, ovarian cancer cell lines with high PI3K activity showed increased sensitivity to growth arrest induced by the PI3K inhibitor LY294002, relative to cells with normal PI3K activity. While the sample sizes used in this study were small, these results do suggest that dysregulation of *PIK3CA* may be an important event in ovarian tumourigenesis.

Additionally, mRNA of the PI3K target Akt2 is amplified 30- to 45-fold in human ovarian cancer cell lines, relative to normal ovarian epithelial cells, this is associated with amplification of the *AKT2* gene (OMIM# 164731), and *AKT2* copy number was elevated

in two of fifteen primary ovarian tumours (Cheng et al., 1992). Subsequent work demonstrated amplification of Akt2 in 12.1% (16/132) ovarian cancers, whereas no Akt2 alterations were detected in 24 benign or borderline tumours (Bellacosa et al., 1995). By contrast, Akt2 was amplified in only 2.8% (3/106) of breast cancers. Akt2 was subsequently shown to be over-expressed in 36% (33/91) and activated in 47% (20/43) of ovarian cancers, wheras the related kinase Akt1 was activated in 10% (4/43) (Yuan et al., 2000). A further study showed that Akt1 is amplified in 72% (8/11) of ovarian cancers (Sun et al., 2001b), although the small sample size complicates the interpretation of these data. Importantly, overexpression of Akt2 transforms NIH3T3 cells and ablation of Akt2 using antisense technology reduces tumourigenicity and invasiveness in pancreatic carcinoma cells (Cheng et al., 1996). These data, taken together, suggest that the PI3K/Akt pathway is frequently over-expressed/amplified in human ovarian cancer and may be an important contributor to ovarian tumourigenesis.

p53

The *TP53* (OMIM# 191170) gene product, p53, is a transcription factor implicated in the regulation of genes involved in cell cycle progression, DNA repair, and apoptosis (discussed below). p53 has been termed the 'guardian of the genome' (Lane, 1992), since one of its principle roles appears to be the identification of DNA lesions and the transduction of a DNA damage response cascade involving the regulation of the aforementioned processes. Basal p53 protein content is low in most cells due to negative feedback facilitated by the p53-responsive gene product murine double minute-2 (MDM2; OMIM# 164785). MDM2 facilitates the degradation of p53 via the 26S proteasome pathway by ubiquitinating p53 (Kubbutat et al., 1997). Cell stress, including

DNA damage, induces post-translational modification of p53, including phosphorylation (detailed below), which inhibits MDM2-mediated p53 ubiquitination by repressing the binding of MDM2 to the p53 N-terminal (Shieh et al., 1997). Once up-regulated, p53 performs its biological function as a transcriptional regulator, although novel functions for this protein have also been proposed (see below). When the *TP53* gene is mutated, giving rise to an altered gene product, there is a loss of negative feedback against p53, due to diminished p53-mediated MDM2 up-regulation. For this reason, over-expression of p53 is often taken as a surrogate measure of *TP53* mutation, although this correlation is not absolute (Pieretti et al., 2002).

The Role of p53 in Tumourigenesis

An overwhelming amount of data suggests that dysregulation of the *TP53* gene is a critical determinant of tumourigenesis. *TP53* knockout mice develop normally but tumours occur at numerous sites by six months of age (Donehower et al., 1992) and loss of one *TP53* allele increases susceptibility to spontaneous and carcinogen-induced tumourigenesis (Harvey et al., 1993). Mutation of *TP53* is the most frequent genetic aberration in human tumours, and ovarian cancer shows a particularly high rate of *TP53* mutation of approximately 50-60% (Aunoble et al., 2000). By contrast, *TP53* alterations are uncommon in benign ovarian tumours (Skilling et al., 1996a), and the prevalence of *TP53* mutation is positively correlated with increasing disease stage; approximately 58% of stage III/IV disease versus 37% in stage I/II disease (Shelling et al., 1995). These data suggest that alterations in this gene may contribute to the progression of the disease, and possibly to its lethality. In support of this hypothesis, survival times are significantly shorter in patients with tumours harbouring *TP53* mutations, versus patients with wild-

type *TP53* tumours (Hogdall et al., 2006). This may, in part, be due to the effects of attenuated p53 function on currently utilized chemotherapeutic regimens (see below).

LOH at the *TP53* locus has been frequently detected in ovarian cancer (Gallion et al., 1995), suggesting that loss of p53 expression may be an important determinant of ovarian tumourigenesis. However, many ovarian tumours and cell lines possess normal *TP53* alleles, suggesting that loss of p53 is not required for tumourigenesis (Skilling et al., 1996b). However, it is likely that loss of *TP53* contributes to this process in conjunction with alterations in other tumour suppressors and oncogenes. In support of this hypothesis, targeted disruption of *TP53* in mice ovaries using adenoviral Cre-LoxP-mediated gene inactivation produces tumours in less than 15% of mice (Flesken-Nikitin et al., 2003). However, simultaneous disruption of *TP53* and the *RB1* tumour suppressor gene (see below), which is also aberrantly regulated in ovarian cancer (Gras et al., 2001), produces ovarian tumours in nearly 100% (33/34) of targeted mice (Flesken-Nikitin et al., 2003). These tumours were primarily of the serous subtype.

The role of *TP53* as a determinant of ovarian carcinogenesis may also be related to ovarian cancer subtype, since *TP53* mutations are particularly frequent in serous ovarian carcinoma. Mutation or over-expression of *TP53* has been found in serous ovarian cancers in 58% and 59% of cases, respectively, whereas the percentage of endometrioid, mucinous, and clear-cell ovarian tumours showing *TP53* mutation was only 28%, 16%, and 10%, respectively (Skilling et al., 1996a). A subsequent study found that *TP53* over-expression is more frequent in serous ovarian cancer (63%) than mucinous ovarian cancer (22%), and is positively correlated to the malignant potential of serous tumours (Morita et al., 2000). However, since serous ovarian cancer is diagnosed

far more frequently than the other histological subtypes, sample bias may partially explain this discrepancy. This is supported by a recent study, which showed no significant difference in the incidence of *TP53* mutation in 124 ovarian cancer patients with respect to histological subtype (Hogdall et al., 2006). As such, it remains unclear whether *TP53* mutations are, on the whole, more frequent in serous ovarian cancer than in other ovarian tumour types.

Interactions between p53 and BRCA

As mentioned above, *TP53* alterations are found more frequently in tumours with *BRCA* alterations than in sporadic, *BRCA*-independent tumours (Schuyer & Berns, 1999), suggesting that loss of p53 function may be an important component of *BRCA*-mediated tumourigenesis. In support of this hypothesis, p53 and BRCA1 physically associate within the cell, and wild-type BRCA1 facilitates p53-mediated gene expression, whereas mutant BRCA1 reduces p53 function (Ouchi et al., 1998; Zhang et al., 1998). However, results from a recent study showed no difference in the frequency of *TP53* over-expression in *BRCA1*-associated and sporadic ovarian carcinomas (Aghmesheh et al., 2004), thereby casting doubt upon the precise interrelationship between these two factors with respect to ovarian tumourigenesis.

Therefore, while *TP53* is critical for tumourigenesis in other tumour types, the precise role of *TP53* mutation in ovarian carcinogenesis is difficult to assess, although the significantly higher frequency of *TP53* in malignant versus benign ovarian tumours does support a role for this gene in the development and/or progression of the disease.

Retinoblastoma (RB)

The retinoblastoma tumour suppressor gene (*RB1*; OMIM# 180200) encodes a protein (RB) involved in preventing G1-S cell cycle progression, via direct binding to the E2F transcription factor (Zhang et al., 1999), which is implicated in the up-regulation of genes involved in cell cycle progression, including cyclin E and cyclin D1 (Ohtani et al., 1995). RB is phosphorylated by cyclin-dependent kinases, leading to an inhibition of its E2F binding function, thereby promoting cell cycle progression (Akiyama et al., 1992). LOH at the *RB1* locus is frequenty observed in ovarian cancer (18-30%) (Gras et al., 2001; Li et al., 1991). *RB1* mutations have been detected in ovarian cancer cell lines (Yaginuma et al., 1997) and in primary ovarian cancer cells (Liu et al., 1994). Although the functional role of *RB1* in ovarian carcinogenesis has not been directly established, the most compelling evidence for a contributory role of *RB1* mutation in ovarian tumourigenesis is the fact that combined inactivation of *RB1* and *TP53* in mice by intrabursal adenoviral Cre potently induced the formation of ovarian tumours that resembled human ovarian tumours (Flesken-Nikitin et al., 2003).

KRAS

The product of the *KRAS* proto-oncogene (OMIM# 190070), KRAS, is a member of the small GTPase superfamily. *KRAS* mutations in human cancers occur preferentially at codon 12 (Yanez et al., 1987), leading to the production of a constitutively active gene product. Activated KRAS binds to numerous downstream proteins, including PI3K (Deora et al., 1998; Espada et al., 1999; Kiss & Crilly, 1995), thereby activating the tumourigenic PI3K/Akt pathway, and RAF (McCormick, 1999), which is implicated in the activation of the mitogen-activated protein (MAP) kinase/ERK pathway, thereby leading to enhanced gene expression of critical cell cycle activators such as Cyclin D1.

Activated KRAS also up-regulates genes involved in metastasis (urokinase plasminogen activator; uPA) and angiogenesis (vascular endothelial growth factor; VEGF) (Janulis et al., 1999; Rak et al., 1995; Silberman et al., 1997). Constitutive activation of KRAS transforms NIH3T3 cells and rat intestinal epithelial cells (Oldham et al., 1996), suggesting that dysregulated KRAS function may be an important component of tumourigenesis in human cancer. Amplification of the *KRAS* oncogene in ovarian cancer was first identified in 1985 in a patient with serous ovarian carcinoma (Filmus & Buick, 1985). Subsequent work has shown that activating mutations in *KRAS* are frequent in mucinous ovarian carcinoma (~75%), but not in other ovarian cancer types (14%), although the overall frequency of *KRAS* alterations is ~27% (Enomoto et al., 1990). This pattern of *KRAS* mutation in mucinous ovarian cancer has been borne out in other studies (Scambia et al., 1997), although in this study, the bulk of the *KRAS* mutations were present in benign ovarian tumours. Morita et al found 24% of mucinous ovarian cancers showing *KRAS* mutation, while only 15% of serous tumours showed mutation of this gene (Morita et al., 2000). A larger study also showed a higher frequency of *KRAS* mutation in mucinous ovarian cancer (50%; 13/26) than serous ovarian cancer (23%; 15/64) (Suzuki et al., 2000). Cells from ascites fluid of patients with ovarian cancer show a high frequency of *KRAS* mutation (47%), and this is positively correlated with disease stage (Dokianakis et al., 1999). As such, mutation of *KRAS* may be a critical factor in ovarian carcinogenesis, particularly in mucinous ovarian cancer.

Other Potential Mediators

Other potential mediators of ovarian carcinogenesis include the *OVCA1* and *OVCA2* tumour suppressor genes (OMIM# 603527 and 607896, respectively) (Phillips et

al., 1996; Schultz et al., 1996), which are down-regulated in a large proportion of ovarian tumours and cell lines. OVCA1 over-expression inhibits the growth of ovarian cancer cells (Bruening et al., 1999) and, importantly, OVCA1 mRNA expression is induced by BRCA1 (Atalay et al., 2002), suggesting that dysregulation of OVCA1 may be an important factor in ovarian carcinogenesis induced by loss of *BRCA1* function.

Mutation of the *PTEN* gene (OMIM# 601728), a negative regulator of PI3K/Akt signaling (see below), is common in endometrioid ovarian cancer (Gomes & Andrade, 2006; Sato et al., 2000), although only rarely occurs in other forms of the disease, suggesting that it may preferentially contribute to carcinogenesis of the endometrioid subtype.

As such, while several potential mediators of ovarian carcinogenesis have been identified, the molecular mechanisms underlying this process are highly variable, and likely to be due to concomitant alteration of numerous genes, including, but not limited to, those described above.

Chemotherapy for Ovarian Cancer

Combination chemotherapy is a standard treatment regimen for advanced ovarian cancer, and this typically involves the use of a platinum agent, such as cisplatin or carboplatin, and a taxane, typically paclitaxel (McGuire, 2003; McGuire & Markman, 2003).

Cisplatin (CDDP)

The anti-tumour activities of cisplatin (*cis*-Pt(II)$(NH_3)_2Cl_2$; *cis*-diaminodichloroplatinum; CDDP) were first described in 1969 by Rosenberg et al, who

showed that several platinum compounds, including CDDP, which were previously shown to inhibit cell division in Gram-negative bacteria, also inhibited the formation of sarcomas and leukemias in mice (Rosenberg et al., 1969). Since that time, CDDP has become a routinely employed anti-cancer agent, used in the treatment of numerous solid tumour types, including ovarian, testicular, and head and neck carcinoma.

CDDP consists of a central platinum ion bound to two amino groups (NH_3) and two chloride ions (Cl) in the *cis* configuration. Interestingly, the *trans-* isomer does not possess anti-tumour properties, suggesting that the configuration about the central platinum is critical to the biological activities of the molecule (Plooy et al., 1984).

CDDP is uncharged in its native state, and thus readily crosses the cell membrane. Upon entering the cell, where the chloride ion concentration is far lower than in the extracellular environment, the chloride ions are released, thereby creating a positively charged platinum core (Ise et al., 2005). This strong electrophilic compound readily interacts with negatively charged cellular molecules, including DNA and proteins. CDDP forms inter- and intra-strand DNA crosslinks (Perez, 1998), often between adjacent guanines (Munchausen & Rahn, 1975), thereby distorting the DNA double helix, and initiating a cellular DNA damage response.

Direct Cellular Response to CDDP (ATM/ATR)

One of the primary cellular responses to DNA damage, such as that induced by CDDP, is the activation of an evolutionarily conserved pathway involving members of the ataxia telangiectasia-mutated (ATM) family, including ATM and ATR (ATM and Rad3 Related), a related protein. Both ATM and ATR are members of the

phosphoinositol-3-kinase family (Cimprich et al., 1996; Savitsky et al., 1995a; Savitsky et al., 1995b), and both are critical mediators of the cellular response to DNA damage.

Ataxia Telangiectasia-Mutated (ATM)

Mutations in the *ATM* gene (OMIM# 607585) give rise to ataxia telangiectasia (AT) (Kapp et al., 1992; Savitsky et al., 1995a; Savitsky et al., 1995b). Cells from AT patients are hypersensitive to ionizing radiation, and AT patients show an increased propensity for the development of cerebral ataxia and numerous malignancies, particularly lymphomas. ATM is primarily responsible for sensing double stand breaks (DSBs), such as that induced by ionizing radiation (Bakkenist & Kastan, 2003). The ultimate trigger for ATM activation appears to be altered chromatin structure, rather than direct binding to DSBs, since ATM is rapidly activated by agents that alter chromatin structure, such as the histone deacetylase inhibitor trichostatin A (Bakkenist & Kastan, 2003).

ATM-and-Rad3-Related (ATR)

ATR was cloned by Cimprich et al, who described a 301 kDa protein with a high degree of homology to the phosphoinositol kinases, including PI3K (Cimprich et al., 1996). Mutations in the *ATR* gene (OMIM# 601215) give rise to Seckel syndrome (O'Driscoll et al., 2003), which is characterized by hematological abnormalities, including pancytopenia, chromosomal instability, and the frequent occurrence of Acute Myeloid Leukemia (Butler et al., 1987; Hayani et al., 1994). *ATR* deficiency results in early embryonic death (Brown & Baltimore, 2000), suggesting *ATR* is critical for normal cell function. ATR is responsible for sensing stalled replication forks (McGowan & Russell, 2004), although there is some overlap between ATM and ATR with respect to

the specific lesions that give rise to their activation. ATR is constitutively bound to another protein, ATR-Interacting Protein (ATRIP), which is required for binding to a DNA binding protein called replication protein A (RPA) (Zou & Elledge, 2003; Zou et al., 2003). ATR-ATRIP is recruited to sites of DNA damage via binding to RPA. The subsequent recruitment of ATR substrates to this complex facilitates downstream signaling. ATR signaling also requires a protein complex consisting of Rad9, Husl, and Radl (Parrilla-Castellar et al., 2004), which is termed the '9-1-1 complex' (Weiss et al., 2002). The 9-1-1 complex is required for initial DNA damage sensing, and this, in combination with RPA binding to DNA, leads to the recruitment and activation of ATR. ATR signaling is induced by numerous cell stresses, including ionizing radiation (IR) (Cortez et al., 2001), ultraviolet radiation (Abraham, 2001) and CDDP (Damia et al., 2001). Interestingly, ATR enzymatic activity appears to be unaffected by genotoxic agents, including ultraviolet or ionizing radiation (Abraham, 2001), suggesting an indirect mechanism of activation of ATR signaling, possibly through alterations in ATR subcellular localization or interaction with specfic co-factors.

Molecular Targets of ATM/ATR in Response to CDDP

ATR phosphorylates numerous downstream targets such as the checkpoint kinases CHK1 (Liu et al., 2000) and CHK2 (Xu et al., 2001), both of which play key roles in initiating cell cycle arrest, DNA repair, and/or programmed cell death (apoptosis) (Gonzalez et al., 2003; Hirao et al., 2002; Peters et al., 2002; Takai et al., 2000).

p53 is a target of ATM/ATR-mediated phosphorylation and is directly phosphorylated on Ser15 by both ATM (Canman et al., 1998; Delia et al., 2000) and ATR (Tibbetts et al., 1999). ATM/ATR also indirectly promotes the phosphorylation of

p53 on Ser20 (Chehab et al., 2000) through activation of CHK1 and CHK2 (Chehab et al., 2000; Hirao et al., 2002; Shieh et al., 2000). Phosphorylation of these sites on p53 is required for protection of p53 from MDM2-mediated degradation, activation of p53-dependent gene transcription (Dumaz & Meek, 1999), and/or p53-induced apoptosis (Unger et al., 1999b) (discussed in detail below). Interestingly, BRCA1 is required for ATM/ATR-dependent p53 phosphorylation (Foray et al., 2003), and BRCA1 may itself be an ATM/ATR substrate providing further evidence that p53 is implicated in BRCA1 signaling. CHK2 can also promote p53-independent apoptosis via activation of the promyelocytic leukemia (PML) protein (Yang et al., 2002), although the mechanisms of PML-mediated apoptosis remain unclear, but may be related to the coordination of protein-protein interactions within the nucleus (Dellaire & Bazett-Jones, 2004).

Chapter 2 – Apoptosis and Chemoresistance in Human Ovarian Cancer

Apoptosis

Historical Perspective

Programmed cell death is an evolutionarily conserved process by which cells are eliminated from an organism, via an ATP-dependent biochemical cascade. The term 'apoptosis' was first used by Kerr et al to describe a form of cell death occurring naturally during development, distinct from necrosis which arises during acute injury (Kerr et al., 1972). However, the existence of 'programmed cell death' was identified several years earlier by Lockshin and Williams (Lockshin & Williams, 1965). Kerr noted that this type of cell death was characterized by a series of morphological and structural changes to the cell, including the condensation of chromatin into pyknotic bodies and the presence of plasma membrane blebbing.

Research using the nematode *Caenorhabditis elegans* provided the bulk of the early evidence for the existence of apoptosis. It was noted that while the adult *C. elegans* consists of 959 cells, a further 131 cells are generated during development but are not present in the adult (Kimble & Hirsh, 1979; Sulston & Horvitz, 1977; Sulston et al., 1983). Furthermore, because the loss of these cells was entirely reproducible with respect to which cells die and at what time, it was reasoned that there must be genetic control over these processes.

The first *C. elegans* gene to be definitively shown to participate in apoptosis was the *nuc-1* gene, which encodes a DNA endonuclease (Sulston, 1976). Loss of *nuc-1* causes defective DNA degradation in cells undergoing apoptosis. It was subsequently discovered that two genes, named *ced-1* and *ced-2* (for *ce*ll *d*eath abnormal), are required

for the phagocytosis of the remnants of apoptotic cells by neighbouring cells (Hedgecock et al., 1983), while a third gene, *ced-3*, is required for the initiation of apoptosis (Xue et al., 1996). Further work in *C. elegans* defined a large subset of genes involved in activating, inhibiting, and propagating apoptosis, and many of these genes have direct homologues in higher organisms.

Apoptosis in Mammalian Cells

Mammalian cell apoptosis is a highly regulated process involving multiple activation and inhibition steps, which integrates signaling from stimuli arising both within the cell and in the extracellular environment. There are two principle pathways of mammalian apoptosis:, the intrinsic, or mitochondrial pathway, and the extrinsic, or death receptor-mediated pathway, although recent evidence suggest that the endoplasmic reticulum may also play an important role in apoptosis induced by some cellular stressors (Szegezdi et al., 2003). Apoptosis is initiated by a triggering event, such as DNA damage, which ultimately leads to the activation of a family of cysteine-aspartic acid proteases known as caspases (detailed below). Caspase activation is ultimately responsible for the execution of apoptosis. Upstream of caspase activation, the signaling cascade is activated by pro-apoptotic molecules such as Fas, and Tumour Necrosis Factor-alpha (TNF-α).

Initiation and Execution of Apoptosis

DNA damage is sensed by a complex network of proteins, and results in numerous potential outcomes, including cell cycle arrest, DNA repair, and, if the damage is particularly severe, the induction of apoptosis.

Apoptosis – Intrinsic Pathway

The intrinsic apoptosis pathway is activated by the release of pro-apoptotic proteins, such as cytochrome c, Smac/DIABLO, HTR/Omi, and AIF, from the mitochondrial matrix to the cytosol (Du et al., 2000; Susin et al., 1996; Suzuki et al., 2001), ultimately leading to the activation of the caspase-9/caspase-3 cascade (**Figure 1**). The release of these proteins from the mitochondria is facilitated by the actions of pro-apoptotic members of the Bcl-2 family of proteins, such as Bax, Bad, Bak, and Bid, by promoting the loss of mitochondrial membrane potential and the release of the aforementioned proteins from the mitochondrial matrix (detailed below). Bax, for example, is normally present in the cytosol, and is activated by numerous cell stressors, which promote its translocation to the mitochondria where Bax-Bax homodimers are an integral component of a mitochondrial membrane pore, through which ions and small proteins are released from the mitochondria (Antonsson et al., 2001). The formation of Bax-Bax homodimers is inhibited by anti-apoptotic Bcl-2 family members such as Bcl-2 (the mammalian homologue of the *C. elegans ced-9* gene product, CED-9) (Hengartner & Horvitz, 1994) and Bcl-XL, which prevent Bax dimerization, thereby preventing the formation of membrane pores (Antonsson et al., 1997; Antonsson et al., 2001).

Adding a further level of complexity, Bcl-2 is itself inhibited by BH3-only members of the Bcl-2 family (see below), which allow the activation and dimerization of Bax (O'Connor et al., 1998; Yu et al., 2001).

Figure 1 - Mammalian Cell Apoptosis – The Intrinsic and Extrinsic Pathways

Cell stress, such as DNA damage, activates BH3-only Bcl-2 family members, such as PUMA and Bad, which attenuate the tonic inhibitory interaction between Bcl-2 and Bax, thus facilitating mitochondrial Bax-Bax homodimerization. Bax dimerization leads to the formation of pores in the outer mitochondrial membrane through which ions and small molecules, such as cytochrome c (cyt c), Smac, AIF (not shown), HtrA2/Omi, and APAF-1, escape the mitochondrial matrix into the cytosol. Cytochrome c and APAF-1 complex with ATP and pro-caspase-9, inducing its cleavage into active caspase-9. Caspase-9 similarly activates pro-caspase-3. Activated caspase-3 is a key effector of apoptosis, leading to the cleavage of numerous substrates including Poly(ADP)Ribose Polymerase (PARP), Inhibitor of Caspase-Activated DNase (ICAD), and key structural proteins such as actin. Smac and Omi facilitate apoptosis by inhibiting the Inhibitor of Apoptosis Proteins, such as XIAP, while AIF translocates to the nucleus and serving as a caspase-independent endonuclease (not shown). This results in DNA fragmentation, nuclear condensation, and loss of structural integrity. Activation of cell death receptors by extracellular ligands such as FasL and TRAIL (not shown) leads to the recruitment of pro-caspase-8 to the activated receptor, via the Fas-Activated Death Domain (FADD) protein, inducing pro-caspase-8 cleavage and activation. This in turn promotes the activation of caspase-3. Crosstalk between the extrinsic and intrinsic pathways is achieved through caspase-8-mediated trunctation of the Bcl-2 family member Bid (into tBid), which translocates to and promotes the activation of the mitochondria. FLIP, which attenuates caspase-8 cleavage and activity, and XIAP, which blocks caspase-9 cleavage and caspase-3 activity, attenuate these cascades, thus inhibiting apoptosis.

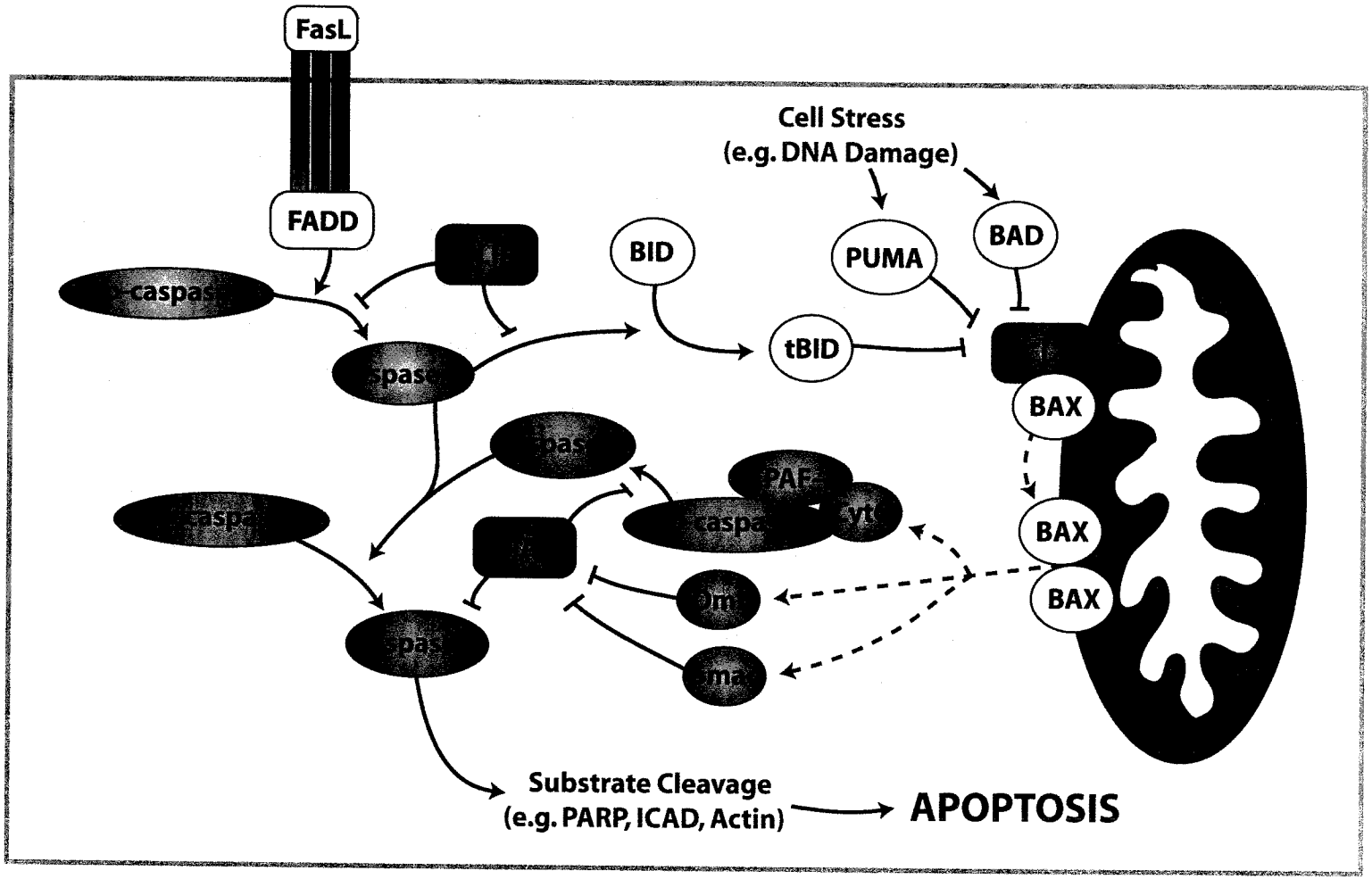
FasL
FADD
Cell Stress
(e.g. DNA Damage)
BID
PUMA
BAD
tBID
BAX
BAX
BAX
Substrate Cleavage
(e.g. PARP, ICAD, Actin)
APOPTOSIS

Figure 1

Once the mitochondria have been activated, the released proteins activate an intricate signaling cascade that ultimately leads to the activation of full-fledged apoptosis. For instance, cytochrome c and APAF-1 (the mammalian homologue of the *C. elegans ced-4* gene product, CED-4) (Zou et al., 1997) participate in the formation of a complex known as the apoptosome (Li et al., 1997). The apoptosome contains cytochrome c, APAF-1, and pro-caspase-9, the zymogen form of the initator caspase, caspase-9. The formation of this complex leads to the ATP-dependent cleavage of pro-caspase-9 into active caspase-9 (Li et al., 1997). Caspase-9 then propagates the apoptotic signal by catalysing the cleavage of pro-caspase-3 into the active executioner, caspase-3 (Fernandes-Alnemri et al., 1996; Li et al., 1997). Caspase-3, along with the related caspase-7, is responsible for the ultimate execution of apoptosis, which is facilitated by the cleavage, at consensus DXXD motifs, of various proteins involved in maintaining cell structure and the integrity of DNA and the nucleus. One important caspase-3 target is Inhibitor of Caspase-Activated DNase (ICAD), which is a tonic inhibitor of Caspase-Activated DNase (CAD) (Enari et al., 1998; Sakahira et al., 1998). CAD is an endonuclease involved in the internucleosomal cleavage of DNA, producing DNA fragments of 185-bp (and multiples of 185-bp, the internucleosomal distance) (Wyllie et al., 1984). Caspase-3 also cleaves and inactivates the DNA repair protein Poly(ADP)Ribose Polymerase (PARP) (de Murcia & Menissier de Murcia, 1994; Tewari et al., 1995), as well as members of the Inhibitor of Apoptosis Protein (IAP) family (discussed below), including X-Linked Inhibitor of Apoptosis Protein (XIAP) (Johnson et al., 2000). Caspase-3 activation is thought of as the 'point-of-no-return' for the induction

of apoptosis, although recent evidence suggests that active caspase-3 can be attenuated by inhibitory proteins such as XIAP (Deveraux et al., 1998).

Other proteins released from the mitochondria include Second Mitochondria-Derived Activator of Caspases (Smac), also known as DIABLO, and HtrA2/Omi, which are both direct inhibitors of IAP proteins (see below) (Du et al., 2000; Suzuki et al., 2001), and Apoptosis Inducing Factor (AIF), which is a DNA endonuclease that can faciliate DNA fragmentation and apoptosis independently of caspase activation (Susin et al., 1996).

Apoptosis – Extrinsic Pathway

The extrinsic pathway (**Figure 1**) involves the binding of a ligand, which is either free in the extracellular environment or bound to the extracellular surface of a neighbouring cell, to a receptor, thereby initiating an outside-in signaling cascade. The Fas/FasL and TNFα/TNFR are two such ligand/receptor systems. Binding of a death ligand, such as Fas Ligand (FasL), to a death receptor, such as Fas, results in receptor oligomerization (Kischkel et al., 1995) leading to binding of the Fas-Associated Death Domain (FADD) protein to the activated receptor (Chinnaiyan et al., 1995; Chinnaiyan et al., 1996). FADD activation induces the proteolytic cleavage of pro-caspase-8 (and pro-caspase-10) into active caspase-8 (and caspase-10) (Fernandes-Alnemri et al., 1996; Muzio et al., 1996; Wang et al., 2001). Other members of the death ligand/receptor family include TNF-α-related Apoptosis Inducing Ligand (TRAIL)/TRAIL-R, and DR5, which also serves as a receptor for TRAIL (MacFarlane et al., 1997; Wiley et al., 1995).

Caspase-8, like caspase-9, induces the cleavage of pro-caspase-3 into its active form (Fernandes-Alnemri et al., 1996), and has additional functions such as the activation

of Bid (Luo et al., 1998), a pro-apoptotic member of the Bcl-2 family, thus linking the activation of the extrinsic and intrinsic pathways. Caspase-8 activation is attenuated by the endogenous inhibitor Flice-Like Inhibitory Protein (FLIP; discussed below) (Irmler et al., 1997; Thome et al., 1997; Wu Xiao et al., 2002). Importantly, the extrinsic pathway is activated by chemotherapeutic agents, including CDDP (Schneiderman et al., 1999), suggesting possible role of this pathway in the pro-apoptotic response to these agents.

Molecular Regulators of Apoptosis

The mammalian cell has evolved intricate mechanisms to regulate the induction of apoptosis, and this process is stimulated and inhibited by numerous cellular mediators. While a complete discussion of the intricate mechanisms of mammalian apoptosis is beyond the scope of this thesis, several of the most important mediators are described below.

The Bcl-2 Family

Bcl-2 (*B cell lymphoma*) was identified as the mammalian counterpart of the *C. elegans* CED-9 protein (Hengartner & Horvitz, 1994). Bcl-2 is a causative factor for follicular lymphoma, where it was first identified at the breakpoint of the 14;18 chromosomal translocation, characteristic of that disease (Pegoraro et al., 1984; Tsujimoto et al., 1985; Tsujimoto et al., 1984). Since the discovery of Bcl-2, numerous related proteins have been characterized, and can be broadly categorized as "anti-apoptotic" or "pro-apoptotic". Anti-apoptotic members display homology in their Bcl-2 Homology (BH) domains, BH1-4 (Scorrano & Korsmeyer, 2003), and include Bcl-2, Bcl-X_L, and CED-9. By contrast, the pro-apoptotic family members lack homology in the BH4 domain, and can be broady categorized as "multi-domain" or "BH3-only".

Upon the initiation of a death signal (e.g. DNA damage), BH3-only proteins, such as PUMA, NOXA, Bad, and Bid, are activated and/or up-regulated by diverse processes including transcriptional activation (e.g. PUMA and NOXA) (Nakano & Vousden, 2001; Oda et al., 2000a), dephosphorylation (e.g. Bad) (Basu et al., 1998), or cleavage (e.g. Bid) (Luo et al., 1998). Activation of BH3-only proteins induces their mitochondrial translocation, where they initiate the activation of the mitochondria through at least three distinct mechanisms; (a) the direct permeabilization of the outer mitochondrial membrane, thus permitting the release of pro-apoptotic molecules such as cytochrome c, such as that facilitated by Bid, (Kluck et al., 1999); (b) the direct inhibition of anti-apoptotic Bcl-2 family members, such as that induced by Bim and PUMA (O'Connor et al., 1998; Yu et al., 2001); and (c) the activation of multi-domain, pro-apoptotic Bcl-2 family members, such as the activation of Bax through direct interaction with PUMA (Liu et al., 2003). Activated multi-domain proteins form channels within the outer mitochondrial membrane, leading to membrane permeabilization (Antonsson et al., 2001; Gross et al., 1998; Wei et al., 2000) and the release of pro-apoptotic molecules from the mitochondrial matrix to the cytosol (Deng et al., 2002; Gross et al., 1998).

Bcl-2 is an integral membrane protein, which constitutively resides at the outer mitochondrial membrane (Janiak et al., 1994). Bcl-2 prevents the formation of Bax oligomers (Antonsson et al., 1997; Antonsson et al., 2001), although this may not result from direct Bcl-2/Bax interaction (Mikhailov et al., 2001). As mentioned above, activated BH3-only proteins bind Bcl-2, thus reducing its ability to prevent the formation of Bax oligomers, and to prevent mitochondrial activation.

Inhibitor of Apoptosis Proteins (IAPs)

IAPs were first characterized as the mammalian homologues of baculovirus proteins involved in the prevention of cell death in infected host cells (Crook et al., 1993; Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996). The IAP family consists of at least six members, including X-Linked Inhibitor of Apoptosis Protein (XIAP), Human Inhibitor of Apoptosis Protein-1 and -2 (HIAP1 and HIAP2), Neuronal Apoptosis Inhibitory Protein (NAIP), Livin, and Survivin (Ambrosini et al., 1997; Duckett et al., 1996; Kasof & Gomes, 2001; Liston et al., 1996; Roy et al., 1995; Uren et al., 1996) (**Figure 2**). Each of the IAP proteins contain at least one Baculovirus Inhibitory Repeat (BIR) domain, which contributes to the anti-apoptotic properties of the molecule (Riedl et al., 2001; Takahashi et al., 1998). XIAP, for example, contains three BIR domains, the most important of which are BIR2 and BIR3, which are responsible for the direct inhibition of caspase-3 and caspase-9, respectively (Deveraux et al., 1999; Riedl et al., 2001), although other studies have suggested that the BIR domains are dispensable for XIAP-mediated caspase inhibition (Chai et al., 2001). XIAP inhibits the conversion of pro-caspase-9 to caspase-9 (Takahashi et al., 1998), and blocks the activity of cleaved caspase-3 (Deveraux et al., 1998; Takahashi et al., 1998). Because of this property, XIAP attenuates both the intrinsic (Finucane et al., 1999; Perkins et al., 1998) and extrinsic (Takahashi et al., 1998) pathways, making XIAP an extraordinarily potent anti-apoptotic protein.

Regulation of XIAP Expression

The expression of the *BIRC4* gene (OMIM# 300079), which encodes XIAP, is up-regulated by activation of the NF-κB signaling pathway (Stehlik et al., 1998; Wang et al., 2002), which is itself activated by cell stresses such as TNF-α and lipopolysaccharide

(LPS) (Stehlik et al., 1998) and cell survival pathways such as the MAPK and PI3K/Akt pathway (Kane et al., 1999; Park & Levitt, 1993). This provides close integration between pro-survival signaling cascades and one of the key effectors of apoptosis inhibition.

Additional functions of XIAP

While the BIR domains appear to be critical for the anti-apoptotic effects of XIAP, other data suggests that these domains may be partially dispensable for this function (Silke et al., 2001). XIAP also contains a RING-Zinc Finger domain, which possesses E3 ubiquitin ligase activity (Yang et al., 2000). XIAP induces its' own ubiquitination in response to apoptotic stress, including treatment with various chemotherapeutic agents (Dan et al., 2004; Yang et al., 2000). However, the E3 ligase activity of XIAP may also play a role in XIAP-mediated cell survival, since XIAP catalyzes the ubiquitination of caspase-3, thus facilitating its' proteasomal degradation, and mutation of the RING domain attenuates the anti-apoptotic effects of the molecule. SMAC is also ubiquitinated by XIAP (MacFarlane et al., 2002), which may help to prevent apoptosis in response to minimal mitochondrial activation.

Figure 2 - The IAP Family

The IAP family consists of at least six members, all of which possess at least one BIR domain. The IAPs are endogenous caspase inhibitors, an activity that requires the BIR domains. Additionally, several members of the family contain RING-Zn Finger domains, which possess E3 ubiquitin ligase activity. This activity is responsible for the ubiquitination (and subsequent proteasomal degradation) of numerous proteins, including caspase-3 and Smac. XIAP, the most potent member of the family, attenuates the activation of caspase-9 through the BIR3 domain, and inhibits caspase-3 activity through the BIR2 domain. XIAP has also been shown to participate in TGF-β/BMP signaling through binding to members of the TGF-β receptor superfamily, and this appears to be BIR domain-dependent (Lewis et al., 2004).

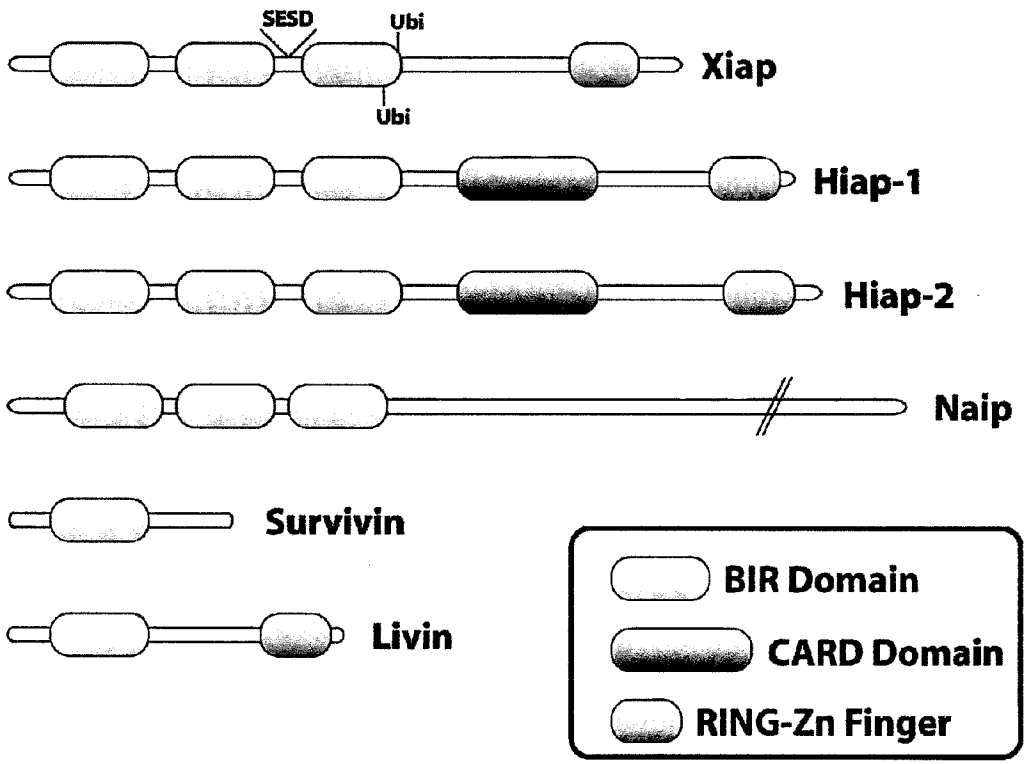
SESD
Ubi
Ubi
Xiap
Hiap-1
Hiap-2
Naip
Survivin
Livin
BIR Domain
CARD Domain
RING-Zn Finger

Figure 2

Recent evidence suggests that the anti-apoptotic properties of XIAP may also involve its ability to regulate signaling through other pathways, including the SMAD pathway, by binding to members of the TGF-β receptor family and facilitating their activation (Birkey Reffey et al., 2001; Sanna et al., 2002a; Sanna et al., 2002b; Yamaguchi et al., 1999) and the PI3K/Akt pathway, by up-regulating the phosphorylation of Akt (Asselin et al., 2001a; Asselin et al., 2001b), although the precise contribution of the modulation of these pathways to XIAP-mediated cell survival is not clear.

Flice-Like Inhibitory Protein

Flice-Like Inhibitory Protein (FLIP), which is the product of the *CFLAR* gene (OMIM# 603599) is an endogenous inhibitor of caspase-8 activation, and thus potently inhibits the extrinsic cell death pathway (Irmler et al., 1997; Thome et al., 1997). FLIP exists in two splice variant isoforms (Irmler et al., 1997; Park et al., 2001): FLIP-long (FLIP-L) and FLIP-short (FLIP-S), both of which contain two death-effector domains (DED), with FLIP-L possessing an additional inactive C-terminal caspase-like domain (Irmler et al., 1997). FLIP can suppress apoptosis induced by the death receptor ligands Fas and TRAIL (Thome et al., 1997; Yang et al., 2003), and attenuates DNA damage-induced apoptosis (Abedini et al., 2004; Kamarajan et al., 2003; Kinoshita et al., 2000).

FLIP inhibits apoptosis by blocking the cleavage and activation of pro-caspase-8, and this is achieved via distinct mechanisms by FLIP-L and FLIP-S. FLIP-S blocks the initial cleavage of intact pro-caspase-8 into the 43 kDa intermediate form, whereas FLIP-L blocks the cleavage of p43 pro-caspase-8 into the 20 kDa active caspase-8 (Wajant, 2003).

Interestingly, like XIAP, FLIP is also an NF-κB-responsive gene product (Micheau et al., 2001), and NF-κB protects cells from apoptosis induced by a variety of cellular insults, including CDDP (Lu et al., 2006; Tsou et al., 2003). Paradoxically, NF-κB can also facilitate cell death, including that induced by CDDP in head and neck squamous carcinoma cells (Kim et al., 2006), although other studies have found the opposite in cervical carcinoma cells, suggesting that NF-κB activation may elicit cell-specific effects with regards to the regulation of apoptosis.

The PI3K/Akt Pathway

The PI3K/Akt pathway is implicated in the regulation of cell cycle progression and cell fate. Its primary cellular role is as a transducer of growth factor and cytokine signaling. Growth factors, such as epidermal growth factor (EGF), bind to their specific receptors, inducing receptor autophosphorylation or phosphorylation of specific receptor substrates, such as insulin receptor substrate-1 (IRS-1) (Burgering & Coffer, 1995; Hadari et al., 1992). Class I and II PI3Ks, which consist of p85 and p110 subunits, are activated through binding of the p85 Src-Homology-2 (SH2) domain to phosphotyrosine residues on the phosphorylated receptor. This causes PI3K-mediated phosphorylation of the membrane phospholipid phosphotidylinositol-4,5-bisphosphate (PIP2) at the 3' position of the inositol ring, producing phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Cheng et al., 2002). PIP3 is required for recruitment of proteins containing a plekstrin homology (PH) domain to the cell membrane (Haslam et al., 1993; Mayer et al., 1993). Three such proteins are Akt (also known as protein kinase B and Rac) (Konishi et al., 1994), phosphatidylinositol-dependent kinase-1 (PDK1) (Alessi et al., 1997), and integrin-linked kinase (ILK) (Hannigan et al., 1996) (**Figure 3**). PDK1, which is

constitutively recruited to the cell membrane by virtue of its strong affinity for PIP2 and PIP3 (Currie et al., 1999), catalyses the phosphorylation of Akt on a threonine residue at amino acid 308 (T308) (Alessi et al., 1997), which lies inside its kinase domain. ILK is activated by binding to PIP3 through its PH domain, and phosphorylates Akt on a serine residue at amino acid 473 (S473) (Delcommenne et al., 1998). These phosphorylations are dependent upon the integrity of the PH domain of Akt, which is required for its own recruitment to the cell membrane (Konishi et al., 1994).

Activation of the PI3K/Akt pathway is inhibited by the actions of two lipid phosphatases, Phosphatase and Tensin Homology (PTEN) (Stambolic et al., 1998), which converts PIP3 to PIP2, and SH2-Containing Inositol Phosphatase (SHIP) (Aman et al., 1998), which converts PIP3 to phosphatidylinositol-3,4-bisphosphate. Both PTEN and SHIP block Akt activation by preventing its PIP3-mediated membrane recruitment (Carver et al., 2000).

Akt

Akt was identified as the mammalian homologue of a retroviral oncogene, v-akt (Bellacosa et al., 1991), and exists as three highly similar isotypes: Akt1, Akt2, and Akt3, which share >85% protein homology (Cheng et al., 2002). Although these isotypes appear to share considerable functional overlap (Cheng et al., 2002), functional studies have suggested that isotype-specific effects may exist. For instance, $Akt2^{-/-}$ mice develop normally but display hyperinsulinemia and show symptoms of human type 2 diabetes (Cho et al., 2001a). Along these lines, Akt2 is highly expressed in insulin-responsive tissues (Altomare et al., 1995), suggesting a possible mechanism by

Figure 3 - Activation and Regulation of the PI3K/Akt Pathway

PI3K binds to phosphotyrosine on activated receptors (e.g. EGFR) via its p85 subunit, initiating a conformational change in its p110 catalytic subunit, thereby activating its lipid kinase activity. PI3K phosphorylates the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2) at the 3' position of the inositol ring, forming phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 recruits proteins containing a pleckstrin homology (PH) domain, such as PDK1, ILK, and Akt. Akt is phosphorylated on Thr308 by PDK1, and on Ser473 by ILK (and others, not shown). The pathway is negatively regulated by PTEN and SHIP (not shown), lipid phosphatases that convert PIP3 into PIP2 and phosphatidylinositol-3,4-bisphosphate, respectively. Akt phosphorylates the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1/CIP1}$ on Thr145 (not shown) (Rossig et al., 2001), facilitiating cell cycle progression. Akt phosphorylates Estrogen Receptor-alpha (ERα) on Ser167 (Sun et al., 2001a), resulting in the up-regulation of cyclin D1 (Castoria et al., 2001; Dupont & Le Roith, 2001) and S-phase progression. Akt contributes to the activation of NF-κB-mediated gene transcription through phosphorylation of IκB kinase (IKKα) on Thr23 (Ozes et al., 1999). Similarly, Akt directly phosphorylates the pro-apoptotic Bcl-2 family member Bad on Ser136 and Ser112 (Datta et al., 1997; del Peso et al., 1997) and indirectly activates Bad Ser112 phosphorylation through activation of PAK1 (Tang et al., 2000). Akt also down-regulates FasL through phosphorylation of members of the forkhead family of transcription factors, including FKHRL1, FKHR and AFX, which are transcriptional activators of FasL expression (Biggs et al., 1999; Brunet et al., 1999; Ciechomska et al., 2003; Kops et al., 1999). Akt phosphorylates MDM2 (Gottlieb et al., 2002; Mayo &

Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001), promoting the degradation of p53, which both attenuates p53-mediated apoptosis, and promotes cell cycle progression.

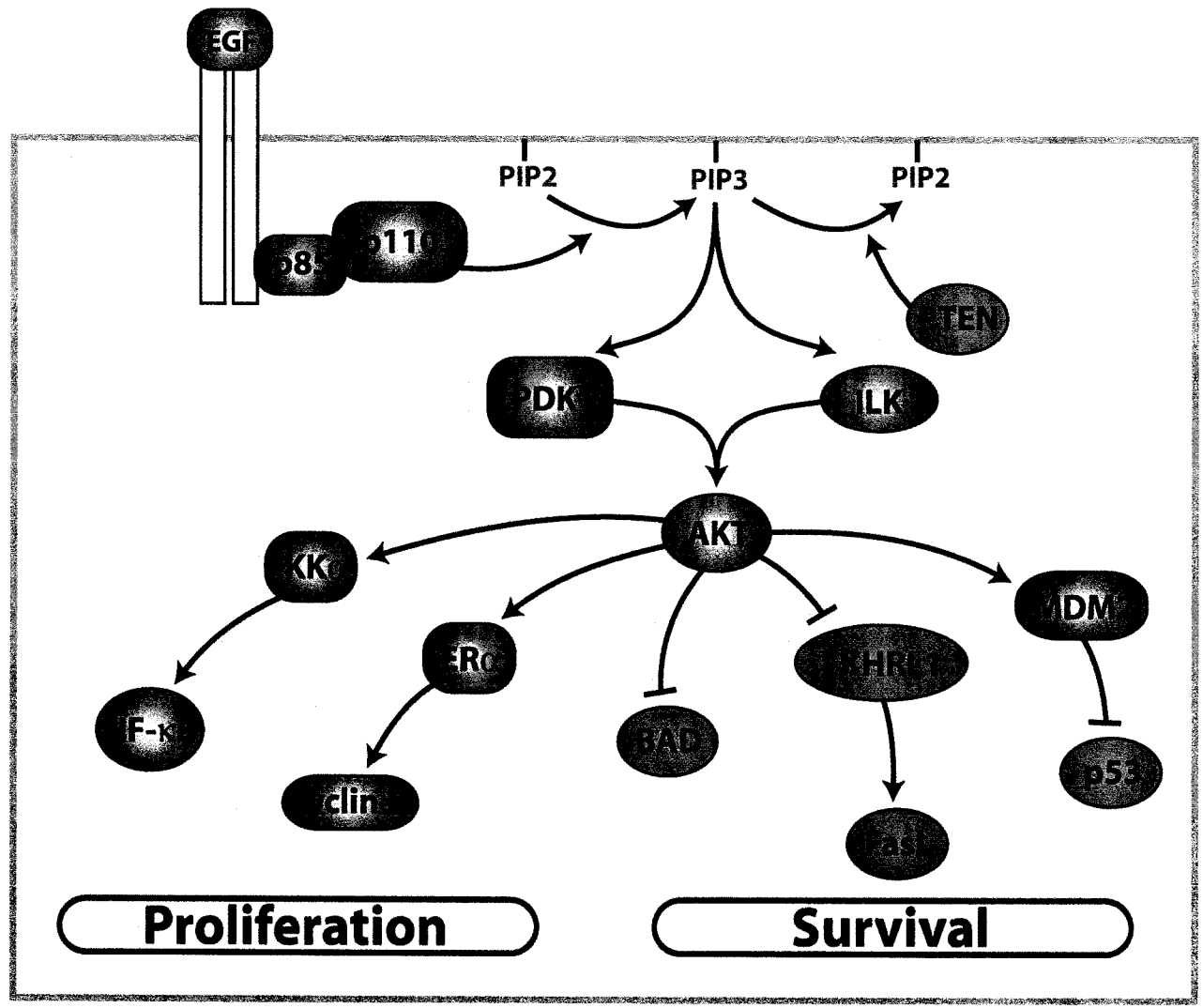

Figure 3

which the critical role for Akt2 in blood glucose regulation is mediated. In contrast, $Akt1^{-/-}$ mice are reduced in size, relative to the wild-type littermates, but do not show a diabetic phenotype (Cho et al., 2001b). Whether these effects are due to differential expression patterns between isotypes or due to the phosphorylation of different substrates by each isotype remains unknown.

Phosphorylation of both T308 and S473 is required for activation of Akt (Alessi et al., 1996). Activated Akt phosphorylates substrates within an RXRXXS/T consensus (Cheng et al., 2002) (**Figure 3**). Akt promotes cell cycle progression through phosphorylation of the c-myc proto-oncoprotein (Ahmed et al., 1997), and stimulates the expression of NF-κB-responsive gene products through phosphorylation of IκB, the tonic negative regulator of NF-κB signaling (Kane et al., 1999). Akt can also activate NF-κB-mediated gene transcription independently of IκB (Wang et al., 2002), although the mechanisms underlying this phenomenon are unclear.

Akt is a direct inhibitor of apoptosis, which is facilitated by phosphorylation-mediated inhibition of pro-apoptotic proteins. The forkhead-family transcription factor FKHRL1 promotes the activation of the extrinsic apoptosis pathway via the transcriptional activation of the *FASLG* gene (OMIM# 134638) (Brunet et al., 1999), which encodes FasL. Akt phosphorylates FKHRL1 on Thr32 and Ser253, which causes nuclear exclusion of FKHRL1, thereby inhibiting its activity as a transcriptional regulator (Brunet et al., 1999). Akt also directly phosphorylates Bad on Ser112 and Ser136 (Datta et al., 1997; del Peso et al., 1997), which attenuates its ability to directly bind to and inhibit Bcl-2 (Scheid et al., 1999). Akt can also phosphorylate and inactivate caspase-9, thereby suppressing the intrinsic cell death pathway (Cardone et al., 1998), although this

appears to be limited to human caspase-9, since mouse caspase-9 lacks the Ser196 phosphorylation site found in human caspase-9 (Fujita et al., 1999).

Soluble Guanylyl Cyclase/cyclic guanosine monophosphate (cGMP)

A small, but growing amount of evidence suggests that the basal activity of soluble guanylyl cyclase (sGC) is required for the viability of mammalian cells (Fiscus, 2002). sGC is activated by nitric oxide (NO), and the sGC/cGMP pathway is responsible for the vasodilatory effects of NO (Fiscus et al., 1983; Griffith et al., 1985; Ignarro et al., 1987; Murad et al., 1987; Murad et al., 1986) (**Figure 4**). Nitric oxide is produced by the enzyme nitric oxide synthase, which exists in three isoforms: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) (Xu et al., 2002). NOS converts the amino acid L-arginine into NO (Moncada et al., 1988; Palmer et al., 1988a; Palmer et al., 1988b), which in turn binds to and activates sGC, thus elevating cGMP levels. cGMP binds to and activates protein kinase G (PKG), which phosphorylates substrates within the consensus $(R/K)_{2-3}$-X_{1-2}-S/T (Kwan et al., 2004). One of the primary physiological roles of sGC/cGMP/PKG activation is smooth muscle relaxation, which is mediated by PKG-dependent reductions in intracellular calcium levels (reviewed in (Lincoln et al., 2001)), although the precise molecular mechanisms underlying this phenomenon are unclear. The system is inhibited by the actions of phosphodiesterases (PDEs), which catalyse the conversion of cGMP into GMP (Beltman et al., 1995). In particular, PDE5 plays an important role in the regulation of cellular cGMP levels, and this has been exploited for the clinical management of penile erectile dysfunction, using specific PDE5 inhibitors such as sildenafil (Boolell et al., 1996).

Figure 4 – Regulation of cGMP Production

Binding of Atrial Natriuretic Peptide (ANP) to its receptor enhances the activity of the associated enzyme, particulate guanylyl cyclase (pGC). pGC converts GTP to cGMP. Likewise, binding of nitric oxide, which is produced from L-arginine by nitric oxide synthase (NOS), to soluble guanylyl cyclase (sGC), produces cGMP. sGC also has basal activity in the absence of NO, and is responsible for the maintenance of basal cGMP levels. cGMP in turn activates a cGMP-dependent protein kinase, protein kinase G (PKG), which phosphorylates substrates at a $(R/K)_{2-3}$-X_{1-2}-S/T consensus sequence. Amongst the known celullar targets for PKG is the inositol 1,4,5-trisphophate (IP_3) receptor, the phosphorylation of which inhibits calcium release from the sarcoplasmic reticulum (Schlossmann et al., 2000), promoting a reduction in intracellular calcium levels and attenuating myosin light chain (MLC) contraction, leading to smooth muscle relaxation, which is a principle physiological function of NO. PKG also directly phosphorylates myosin light chain kinase (van Riper et al., 1997), thereby lowering MLC phosphorylation and contraction. Nitric oxide also activates Akt (Ha et al., 2003), suggesting a possible mechanism by which this pathway promotes cell survival.

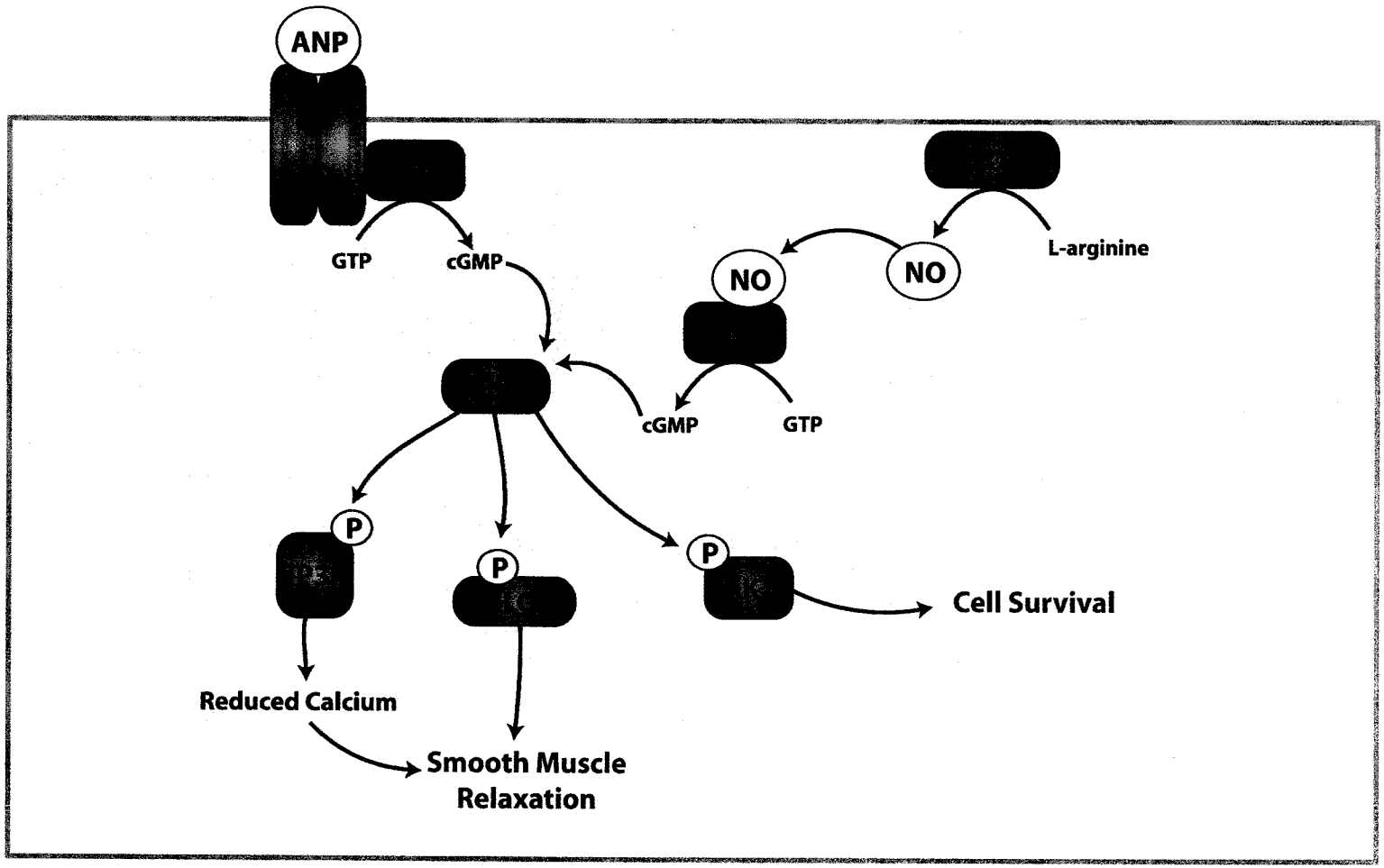
ANP
GTP
cGMP
NO
NO
L-arginine
cGMP
GTP
P
P
P
Cell Survival
Reduced Calcium
Smooth Muscle
Relaxation

Figure 4

While sGC is activated by NO, the enzyme also has basal activity, and through this function sGC maintains a basal, steady-state level of cGMP. While the mechanisms that underlie sGC/cGMP-mediated cell survival remain largely unknown, activation of this pathway using pharmacological agents such as the stable cGMP analog 8-Br-cGMP protects PC12 prostate cancer cells from serum-deprevation-induced apoptosis (Fiscus et al., 2001), suggesting an important pro-survival role for this pathway. This effect is also achieved using atrial natriuretic peptide (ANP), which is an activator of particulate guanylyl cyclase (pGC), which also produces cGMP. Another cGMP analog, dibutyryl-cGMP, prevents apoptosis in chick embryo motor neurons (Weill & Greene, 1990). A physiological role for this pathway in the prevention of spontaneous apoptosis came with the observation that exposure of rat cerebellum to sGC inhibitors causes progressive destruction of differentiating cells (Garthwaite et al., 1988). Similarly, the specific sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), have demonstrated a requirement of this enzyme for protection against spontaneous apoptosis (Estevez et al., 1998b; Fiscus, 2002; Fiscus et al., 2002). The protective effects of cGMP appear to be mediated through activation of PKG, since the specific PKG inhibitor KT5823 also induces spontaneous apoptosis in uterine epithelial cells (Chan & Fiscus, 2003). Interestingly, the cGMP/PKG pathway can activate Akt, which suggests a potential mechanism by which cGMP-mediated cell survival proceeds (Ha et al., 2003). By contrast, other studies have shown that the sGC/cGMP/PKG pathway facilitates apoptosis, since activation of PKG induces apoptosis in colon cancer cells (Deguchi et al., 2004; Liu et al., 2001). However, whether this pathway is implicated in the regulation of human cancer cell fate has not been evaluated.

p53

p53 was originally characterized in 1979 as a binding protein of the SV40 T antigen (Lane & Crawford, 1979), and subsequently as a protein expressed in human tumour cell lines but not in normal human cells (Crawford et al., 1981). Murine p53 was cloned in 1983 by Oren and Levine (Oren & Levine, 1983). The human *TP53* gene is located on chromosome 17p13 (Isobe et al., 1986), and encodes a 393 amino acid phosphoprotein.

Regulation of p53 content and function

The p53 protein consists of several functional domains, including an N-terminal transactivation domain, a central DNA binding domain, and a carboxy terminal nuclear localization and nuclear export sequences (Oren, 2003) (**Figure 5**). p53 is a transcription factor that regulates the expression of numerous genes implicated in the initiation and propagation of cell cycle arrest, DNA repair, and apoptosis. p53 binds to a consensus nucleotide sequence, two copies of 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0-13 bases (el-Deiry et al., 1992), in the promoter region of these genes, thereby facilitating or attenuating their expression. p53-mediated gene transcription is enhanced by its binding to additional co-activators, including the acetyltransferase CBP/p300 which catalyses the acetylation of histone proteins (Lee et al., 1998; Ogryzko et al., 1996; Scolnick et al., 1997), thus relaxing chromatin structure permitting the active transcription of the specific gene. CBP/p300 also directly acetylates p53 on its carboxy terminal, thereby enhancing its transactivation function (Gu & Roeder, 1997).

p53 levels in unstressed cells are normally low, and this is due to a negative-feedback loop initiated by the p53-dependent up-regulation of murine double minute-2

(MDM2) (**Figure 6**). MDM2 binds p53 on its N-terminus between amino acids 17-27 (Chen et al., 1993; Kussie et al., 1996; Picksley et al., 1994). MDM2 catalyzes the ubiquitination of p53, which promotes its proteasomal degradation (Kubbutat et al., 1997). This system functions as a prototypical negative feedback loop, since p53 transcriptionally up-regulates MDM2, and this may be accomplished through p53-mediated activation of an internal promoter downstream of MDM2 exon 1 (Barak et al., 1993; Juven et al., 1993). MDM2 is induced by p53, which, given the ubiquitin ligase activity of MDM2 toward p53, serves to tightly control steady-state p53 levels. For this reason, basal p53 levels are typically very low in unchallenged cells. p53 mutation, which is commonly observed in human tumours, attenuates the up-regulation of MDM2, resulting in elevated levels of p53 through loss of negative feedback. This characteristic is frequently exploited to indirectly measure the presence of mutant p53 in human tumours by examining the expression of p53 protein by immunohistochemistry. While this is useful for missense mutations, other mutations (nonsense, deletions, addition) do not result in a viable protein, and thus cannot be detected in this manner (Casey et al., 1996). By contrast, the occurrence of false positives by this method (i.e. over-expression without detectable mutation) is ~15% (Righetti et al., 1996), and both immunohistochemical and direct sequencing are required to assess the full spectrum of p53 mutations (Casey et al., 1996). MDM2 can also inhibit p53-mediated transactivation, and this appears to be distinct from its role as an E3 ubiquitin ligase for p53 (Chen et al., 1995; Momand et al., 1992).

Transcription-Dependent p53-Mediated Apoptosis

Homozygous deletion of p53 in mice results in a loss of sensitivity of thymocytes from these animals to undergo apoptosis in response to radiation and the chemotherapeutic agent etoposide (Clarke et al., 1993), thus implicating p53 as a critical mediator of DNA damage-induced apoptosis. Indeed, while some cellular insults, such as glucocorticoid and high intracellular calcium levels, induce apoptosis in a p53-independent manner (Clarke et al., 1993), it has been frequently observed that DNA damage-induced apoptosis is largely dependent upon p53 function.

p53 up-regulates numerous genes involved in both the intrinsic and extrinsic pathways, and down-regulates a number of genes involved in the direct repression of apoptosis (**Table 1**). Bax was one of the earliest gene products shown to be transcriptionally regulated by p53 (Miyashita et al., 1994; Selvakumaran et al., 1994), and Bax is required for apoptosis induced by numerous stimuli (Deckwerth et al., 1996; Knudson et al., 1995; Sakakura et al., 1996). However, homozygous deletion of Bax does not completely recapitulate the phenotype of p53 $^{-/-}$ mice with respect to apoptosis (Yu et al., 2005; Zhang et al., 2000), suggesting that other mediators can compensate for the loss of Bax, and are likely important for the propagation of DNA damage-induced, p53-dependent apoptosis.

p53-upregulated mediator of apoptosis (PUMA) is a BH3-only member of the Bcl-2 family, and is up-regulated in a p53-dependent manner (Nakano & Vousden, 2001). Knockout of PUMA confers resistance to DNA damage-induced apoptosis to a similar extent as knockout of p53, suggesting that PUMA is a critical mediator of p53-dependent apoptosis (Villunger et al., 2003). Indeed, in neuronal cells, over-expression of PUMA is sufficient to induce apoptosis (Cregan et al., 2004). A related BH3-only protein, NOXA,

is also regulated by p53 (Oda et al., 2000a), although the role of NOXA in p53-dependent apoptosis is not clear since NOXA$^{-/-}$ mice remain moderately sensitive to DNA damage-induced, p53-dependent apoptosis (Villunger et al., 2003).

Transcription-Independent p53-Mediated Apoptosis

p53-mediated apoptosis can also proceed in a transcription-independent mechanism, since expression of a temperature-sensitive p53 mutant induces apoptosis at the permissive temperature, and this is insensitive to both protein and RNA synthesis inhibitors and since p53 mutants that do not show DNA binding activity and/or p53 transactivational capacity can induce apoptosis (Bissonnette et al., 1997; Caelles et al., 1994). It was originally hypothesized that p53 may inhibit the transcription of factors involved in the suppression of apoptosis. Indeed, Bcl-2 is transcriptionally repressed by p53, as is survivin, a member of the IAP family of anti-apoptotic proteins (Haldar et al., 1994; Hoffman et al., 2002) (**Table 1**).

Figure 5 – A Schematic Representation of p53

p53 consists of an N-terminal MDM2 binding domain, which overlaps or lies adjacent to several key phosphorylation sites (Ser15, Thr18, and Ser20), which are implicated in the inhibition of MDM2-p53 binding. The MDM2 binding site also lies within the p53 transactivation domain, which is required for p53-mediated gene activation. A central DNA binding domain is required for recognition of the p53 consensus sequence (5'-PuPuPuC(A/T)(T/A)GPyPyPy-3') within the promoters of p53-responsive genes. The p53 C-terminal contains nuclear import and export sequences (NLS and NES, respectively), as well additional phosphorylation and acetylation sites, which are implicated in the activation of the molecule.

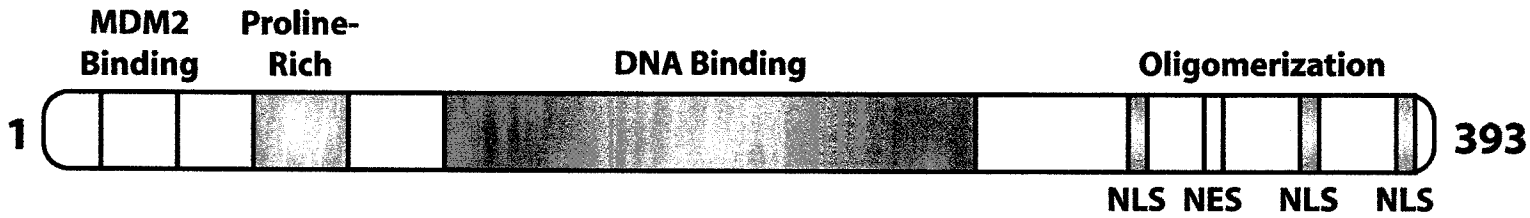
MDM2
Binding
Proline-
Rich
DNA Binding
Oligomerization
1
393
NLS
NES
NLS
NLS

Figure 5

Figure 6 – The MDM2-p53 Negative Feedback Loop

In most unstressed cells, p53 levels are low or undetectable. This is primarily due to the p53-responsive gene product MDM2, which catalyzes the ubiquitination of p53. This targets p53 for proteasomal degradation, leading to a reduction in its steady-state levels. As such, MDM2 and p53 exist in a classical negative feedback loop. However, following cell stress such as DNA damage, p53 is up-regulated through inhbition of MDM2-mediated ubiquitination. This is believed to be mediated through phosphorylation of p53 on specific N-terminal residues, including Ser15, Thr18, and Ser20, all of which lie within or adjacent to the MDM2 binding site. Phosphorylated p53 has a reduced affinity for MDM2, leading to diminished MDM2 binding, and consequently to increased p53 stability. As such, p53 accumulates and intitiates its cellular responses.

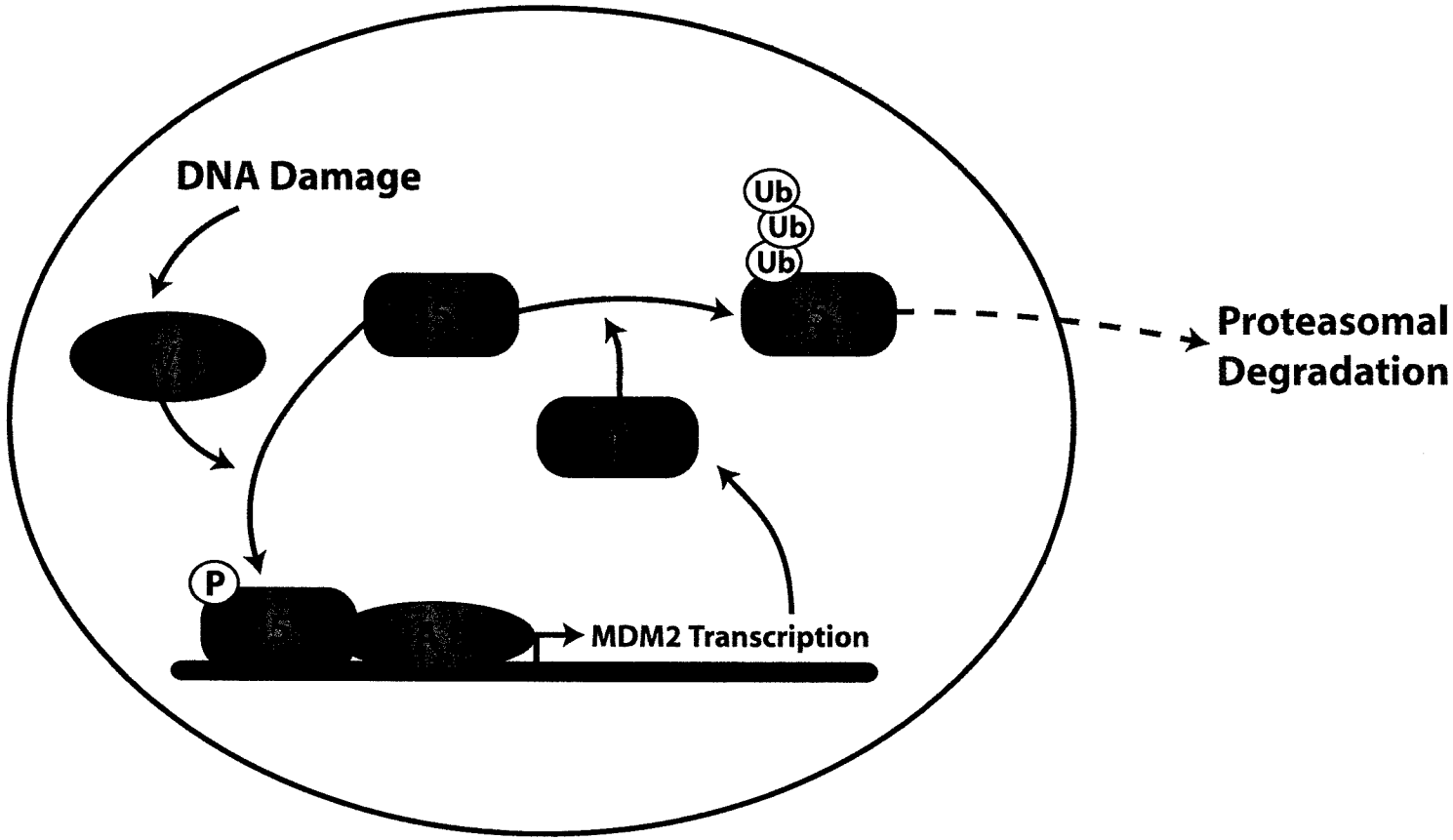
DNA Damage
Ub
Ub
Ub
Proteasomal
Degradation
P
MDM2 Transcription

Figure 6

Table 1 - Partial list of p53-responsive genes implicated in apoptosis

Gene Name	Gene Product	Effects of p53	Reference
BAX	Bax	up-regulated	(Miyashita et al., 1994)
BBC3	PUMA	up-regulated	(Nakano & Vousden, 2001)
PMAIP1	NOXA	up-regulated	(Oda et al., 2000b)
P53AIP1	p53AIP1	up-regulated	(Oda et al., 2000a)
TP53INP1	p53DINP1	up-regulated	(Okamura et al., 2001)
APAF1	APAF1	up-regulated	(Fortin et al., 2001)
FAS	Fas	up-regulated	(Owen-Schaub et al., 1995)
PTEN	PTEN	up-regulated	(Stambolic et al., 2001)
BCL2	Bcl-2	down-regulated	(Haldar et al., 1994)
BIRC5	Survivin	down-regulated	(Hoffman et al., 2002)

Direct Mitochondrial Actions of p53

Additionally, recent evidence suggests that p53 can directly activate the intrinsic cell death pathway through translocation to the mitochondria (Marchenko et al., 2000) (**Figure 7**). Following cell stress, a fraction of the total cellular p53 protein rapidly associates with the mitochondria, and this precedes the up-regulation of early p53-responsive gene products such as PUMA (Erster et al., 2004). Subsequent work has demonstrated that mitochondrial p53 interacts with both Bcl-2 and Bcl-XL, and plays a similar role to that of the pro-apoptotic proteins Bad and Bik, sequestering Bcl-2/Bcl-XL and preventing their inhibitory interactions with pro-apoptotic molecules such as Bax and inducing apoptosis (Mihara et al., 2003). This effect of p53 is dependent upon its DNA binding domain, and mutations of this domain block the ability of p53 to bind to Bcl-2/Bcl-XL and to activate the mitochondria. While this effect was originally shown to be unique to p53-mediated apoptosis and to be absent during p53-mediated cell cycle arrest, subsequent work has shown that p53 can accumulate at the mitochondria in the absence of apoptosis (Essmann et al., 2005). For this reason, and because of the enormous volume of data supporting the role of transcriptional activation as a central component of p53-mediated apoptosis, the relative contribution of the direct mitochondrial effects of p53 to p53-mediated apoptosis remains unclear.

Cytosolic p53

Very recent work has shown that p53 also promotes apoptosis in a transcription-independent manner through its actions in the cytosol (**Figure 7**). This occurs due to the actions of cytoplasmic p53, which is normally bound by Bcl-XL. Following cell stress, nuclear p53 up-regulates PUMA, which binds cytoplasmic Bcl-XL, thereby releasing p53,

and inducing Bax activation (Chipuk et al., 2005). Once again, the contribution of this phenomenon to p53-mediated apoptosis is unclear.

Regulation of Apoptosis by p53 Phosphorylation

p53 is highly phosphorylated at both its amino and carboxy terminus in response to cell stress (Jay et al., 1981; Lees-Miller et al., 1992; Meek & Eckhart, 1988; Patschinsky et al., 1992; Shieh et al., 1997) (**Figure 8**). Phosphorylation is mediated by a wide variety of protein kinases, dependent upon the cellular context and the type of cell stress. While the function of many of these sites has been examined in detail, with respect to the regulation of apoptosis, phosphorylation of Ser15, Ser20, and Ser46 appear to have the most relevance, and the role and regulation of these phosphorylations are summarized below.

Serine 15

p53 is directly phosphorylated on Ser15 by both ATM (Banin et al., 1998; Canman et al., 1998; Delia et al., 2000) and ATR (Tibbetts et al., 1999). Other molecules proposed to directly phosphorylate this site include the mitogen activated protein kinase ERK (Persons et al., 2000) and double-stranded DNA-dependent protein kinase (DNA-PK) (Lees-Miller et al., 1992). While some studies have indicated that phosphorylation of Ser15 is required for p53-mediated transactivation (Dumaz & Meek, 1999), others have, using site-directed mutagenesis, shown no requirement for Ser15 in this process (Fuchs et al., 1995; Unger et al., 1999b). Those studies that have ascribed this function to Ser15 have also suggested that this occurs through increased affinity of p53 for the transcriptional co-activator p300 (Dumaz & Meek, 1999; Lambert et al., 1998). Thus, Ser15 phosphorylation may have a tissue-specific role in the regulation of p53-mediated

gene expression. Additionally, numerous studies have supported a role for Ser15 phosphorylation in p53-induced apoptosis (Borges et al., 2004; Bulavin et al., 1999; Li et al., 2006; Unger et al., 1999b), although the precise mechanisms underlying this process remain unclear, particularly because some groups have failed to observe a marked change in the expression of pro-apoptotic genes induced by wild-type p53 versus p53 mutants which cannot be phosphorylated at Ser15 (Unger et al., 1999b). Similarly, phosphorylation of Ser15 is required for stabilization of p53 following DNA damage in some systems (Shieh et al., 1997), while in others, mutational analysis indicates that Ser15 is dispensable for p53 stabilization following DNA damage (Ashcroft et al., 1999). Ser15 phosphorylation is also required for p53-induced cell cycle arrest (Fiscella et al., 1993). Importantly, Ser15 phosphorylation may depend upon the previous phosphorylation of other sites, including Ser33 and Ser46 (Bulavin et al., 1999), suggesting that p53 phosphorylation may be coordinately regulated. Taken together, these observations suggest that Ser15 phosphorylation is an important component of the biological function of p53, although the specific effects of Ser15 phosphorylation may be context-specific.

Figure 7 - Mitchondrial and Cytoplasmic Actions of p53

During apoptosis, a fraction of total p53 translocates to and accumulates at the mitochondria. Mitochondrial p53 binds to Bcl-2 and Bcl-XL, attenuating their inhibitory role against pro-apoptotic molecules such as Bax. The presence of p53 at the mitochondria is sufficient to induce apoptosis, and preceeds up-regulation of pro-apoptotic, p53-responsive genes products such as PUMA and Bax. Additionally, p53 is constitutively bound to Bcl-XL in the cytosol. Following cell stress, PUMA is up-regulated and liberates p53 from Bcl-XL, thus promoting the direct p53-induced activation of Bax.

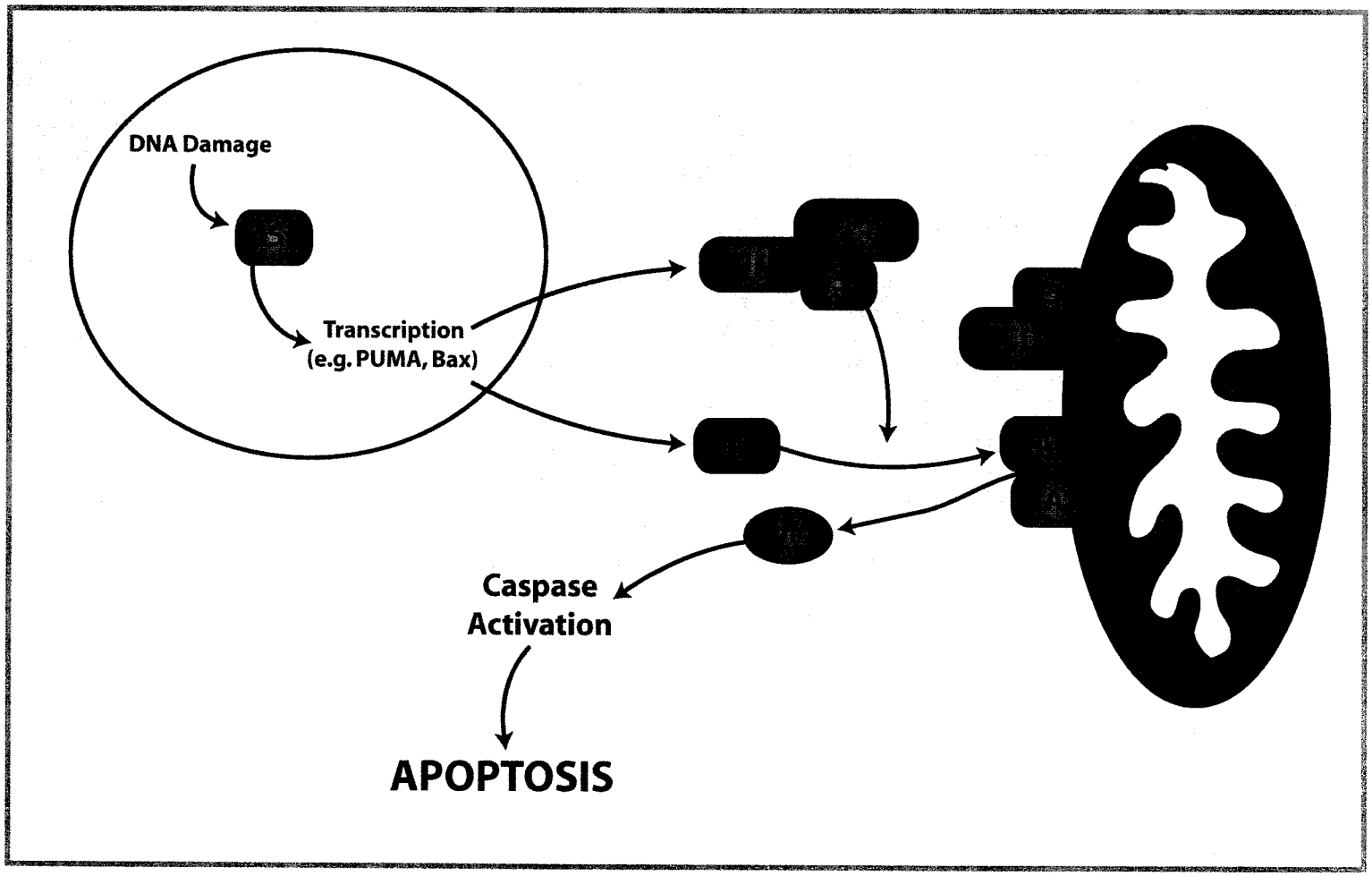
DNA Damage
Transcription
(e.g. PUMA, Bax)
Caspase
Activation
APOPTOSIS

Figure 7

Figure 8 - Regulation of p53 N-Terminal Phosphorylation

p53 is phosphorylated on numerous amino and carboxy terminal residues, many of which have been implicated in the regulation of p53 stability, p53-DNA binding and p53-mediated transcriptional activation, p53-induced cell cycle arrest, and p53-induced apoptosis. Phosphorylation of p53 also increases the affinity for transcriptional co-activators such as CBP/p300, which binds to p53 and promotes p53-mediated transcription. Importantly, phosphorylation of several amino terminal residues is linked to the attenuation of MDM2-p53 binding, which in turn inhibits the MDM2-mediated ubiquitination of p53, thus increasing p53 stability. Phosphorylation of p53 is performed by numerous kinases, including members of the ATM/ATR family, which are activated by DNA damage and directly phosphorylate Ser15 and Ser37, and promote the phosphorylation of Ser20 through activation of the checkpoint kinases, CHK1 and CHK2. p53 is also phosphorylated by members of the MAPK family, including ERK (not shown), p38, and JNK (Buschmann et al., 2001; Persons et al., 2000; Sanchez-Prieto et al., 2000), and by numerous other kinases including double-stranded DNA-dependent protein kinase (DNA-PK), homeodomain-interacting protein kinase-2 (HIPK2), and protein kinase C (Di Stefano et al., 2004; Yoshida et al., 2006). Ser6 and Ser9 are substrates of casein kinase I and II (CK1/II) (Herrmann et al., 1991; Higashimoto et al., 2000; Meggio et al., 1994; Muller & Scheidtmann, 1995).

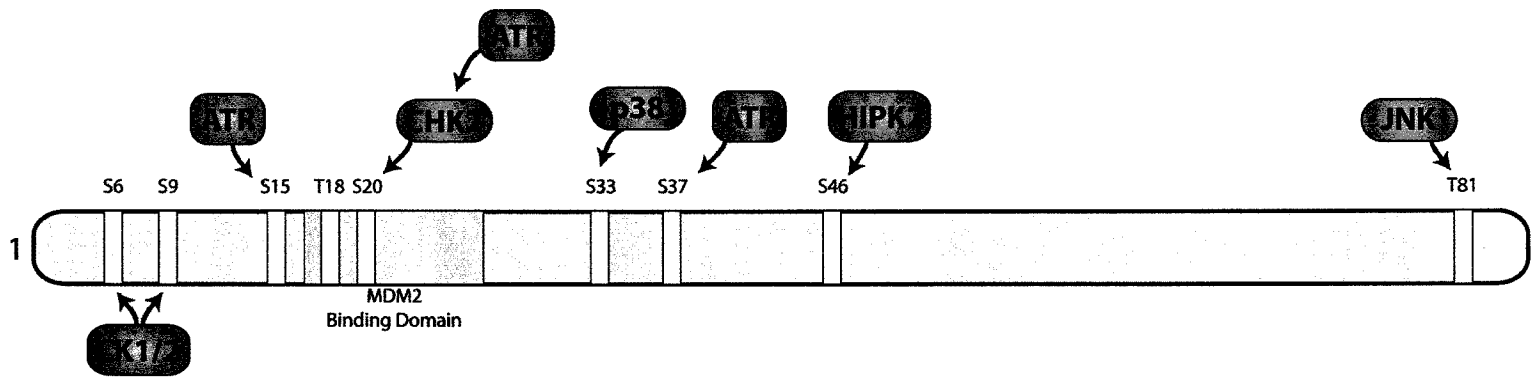
ATR
ATR
CHK2
p38
ATM
HIPK2
JNK
S6
S9
S15
T18
S20
S33
S37
S46
T81
1
MDM2
Binding Domain
CK1

Figure 8

Serine 20

ATM and ATR also promote the phosphorylation of Ser20 through activation of the CHK1 and CHK2 kinases (Chehab et al., 2000; Chehab et al., 1999; Hirao et al., 2002; Shieh et al., 2000). Phosphorylation of Ser20 appears to be required for protection of p53 from MDM2-mediated degradation (Jabbur et al., 2001; Unger et al., 1999a). This may be due to the fact that Ser20 lies within the MDM2 binding site on p53, amino acids 17-27 (Picksley et al., 1994). Ser20 phosphorylation is also required for the p53-mediated induction of $p21^{WAF1/CIP1}$ and p53-induced apoptosis (Jabbur et al., 2001; Unger et al., 1999b), although this does not appear to be universal, since thymocytes from mice bearing a mutation at Ser23 (the murine equivalent of human Ser20) respond to ionizing radiation and ultraviolet radiation treatment with normal induction of gene expression and apoptosis (Wu et al., 2002). However, very recent data suggests that mouse Ser18 and Ser23 (the equivalents of human Ser15 and Ser20) are required for p53-dependent apoptosis in thymocytes, although neither appears to mediate this effect on its own (Chao et al., 2006). This suggests that phosphorylation of Ser20 may contribute to p53-dependent apoptosis in a manner that is dependent upon the phosphorylation of other sites, including Ser15.

Serine 46

Ser46 phosphorylation has been observed following ultraviolet radiation, CDDP, and adriamycin (Bulavin et al., 1999; Di Stefano et al., 2004; Yoshida et al., 2006), and is required for the p53-dependent up-regulation of the pro-apoptotic, mitochondrial protein p53-regulated apoptosis inducing protein-1 (p53AIP1) (Oda et al., 2000b).

Phosphorylation of Ser46 is mediated by protein kinase C and homeodomain-interacting protein kinase-2 (Di Stefano et al., 2004; Yoshida et al., 2006).

Mechanisms of Chemoresistance in Human Ovarian Cancer

Following cytoreductive surgery, the majority of ovarian cancer patients undergo combination chemotherapy with CDDP plus taxol. This regimen is more efficacious than the combination of CDDP with other agents, including cyclophosphamide (McGuire WP et al, 1996). Most patients (~80%) show a substantial response to this treatment regimen and enter disease remission (McGuire et al., 1996a; McGuire et al., 1996b). However, in a large proportion of those patients, the tumours will recur, and recurrent tumours are almost invariably resistant (~10-30% response rates) to further chemotherapeutic intervention (Judson et al., 1999). This gives rise to a clinical phenomenon known as chemoresistance, which is now identified as a major cause of treatment failure in human ovarian cancer.

Ovarian cancer is a multi-factorial phenomenon. Indeed, as has been discussed, the molecular mechanisms of ovarian tumourigenesis are varied, and numerous molecular abberations may give rise to the development of an ovarian tumour. Likewise, chemoresistance is a multi-factorial phenomenon, and the outcome of clinical chemotherapy may depend upon a multitude of factors, including disease stage and tumour type. However, while numerous theories have been espoused to explain chemoresistance in ovarian cancer, several common determinants have been identified and summarized below.

Multidrug Resistance 'Pumps'

Often, the ability of the drug to enter and remain within the cell is compromised by the presence of so-called 'drug efflux' pumps, such as *p*-glycoprotein. By exporting chemotherapeutic agents from the cell, these ATP-dependent integral membrane proteins limit the access of the drug to its target, thereby eliminating its biological effects.

A recent study has show that down-regulation of *p*-glycoprotein, the product of the *ABCB1* gene (OMIM# 171050), sensitized two ovarian cancer cell lines to CDDP (Zhang et al., 2005), suggesting that enhanced efflux of CDDP from the ovarian cancer cell may be an important component of CDDP resistance. However, others have suggested that platinum agents are not substrates for these efflux pumps (Lautier et al., 1996), and work conducted with chemosensitive ovarian cancer cells and their resistant variants suggest that CDDP uptake and DNA platination, while slightly reduced in the chemoresistant variant cells, cannot completely account for the large different in CDDP sensitivity in these cells (Mansouri et al., 2003). A very recent study provided data showing that primary cultured ovarian cancer cells over-express *ABCB1* following recovery from CDDP treatment (Metzinger et al., 2005), suggesting that while CDDP itself may not be a substrate of these drug efflux pumps, it may influence the cellular response to other drugs that are substrates, such as taxol (see below). Indeed, CDDP has been shown to induce resistance to taxol *in vitro* (Judson et al., 1999), and cross-resistance to these agents is commonly observed in clinical chemotherapy (McGuire et al., 1996a; McGuire et al., 1996b). More recent evidence has also supported the hypothesis that other drug efflux pumps, including MRP2, may be directly implicated in CDDP resistance since over-expression of MRP2 induces a marked resistance to CDDP while down-regulation of MRP2 increases CDDP sensitivity (Cui et al., 1999; Koike et al.,

1997), although other studies have failed to demonstrate a role for MRP2 in the determination of CDDP sensitivity (Shen et al., 2000). As such, the precise contribution of drug efflux pumps, particularly *p*-glycoprotein and MRP2, to chemoresistance remains unclear.

In contrast, taxol is a known substrate for these drug efflux pumps, and pharmacologic inhibition of *p*-glycoprotein enhances taxol-induced cytotoxicity (Mechetner & Roninson, 1992), suggesting that this may be one mechanism by which clinical taxol resistance is manifested. In support of this hypothesis, a single nucleotide polymorphism (SNP; G2677T/A) in the *ABCB1* gene product was correlated with treatment outcome following taxol-based chemotherapy for ovarian cancer (Green et al., 2006). Importantly, in one recent study, these polymorphisms were observed in over 60% of ovarian cancer patients (Nakajima et al., 2005), although other studies have observed no correlation between *p*-glycoprotein expression and overall disease survival (Ozalp et al., 2002). These results suggest that aberrant *ABCB1* function may be an important mechanism of taxol resistance in human ovarian cancer.

Drug Detoxification

CDDP can become covalently linked to glutathione (γ-glutamylcysteinylglycine; GSH) either non-enzymatically, or via the activity of the enzyme glutathione-*S*-transferase (GST) (Ishikawa & Ali-Osman, 1993). GSH-CDDP complexes are removed from cells via the ATP-dependent transporter GS-X (Zhang et al., 2001). Indeed, CDDP resistance in ovarian cancer is associated with elevated GSH levels, and targeted down-regulation of GST or cellular depletion of GSH increases CDDP-induced cytotoxicity (Juvekar et al., 2000; Zhang et al., 2001; Zhang et al., 2005). GSH levels are associated

with multidrug resistance in a panel of human ovarian cancer cells and GSH levels are elevated in primary cultured ovarian cancer cells taken from the same patient pre- and post-chemotherapy (Hamaguchi et al., 1993; Wolf et al., 1987), suggesting that this may be an important determinant of chemoresistance in this disease.

Enhanced DNA damage repair

CDDP-induced DNA damage activates the nucleotide excision repair (NER) pathway (Gunz et al., 1996). The NER pathway is exceptionally complex, and involves the activation of at least thirty proteins (Bernstein et al., 2002). Platinated DNA is sensed by members of the xeroderma pigmentosa (XP) family, including XPC and XPE, which in turn recruit proteins such as XPB and XPD, which form part of the transcription factor complex TFIID. XPB and XPD serve as helicases, which unwind the DNA double helix to allow access of other molecules, such as XPA and RPA (see above), to the site of lesion. Finally, XPF/ERCC1 and XPG bind to these complexes, thereby excising the damaged section of DNA, followed by *de novo* synthesis of the excised portion by DNA polymerases (reviewed in (van Steeg, 2001)).

Enhanced DNA repair may promote resistance to DNA damaging agents such as CDDP by reversing the platination of DNA, and thus attenuating the downstream propagation of the DNA damage signal. Indeed, reconstitution of Chinese hamster ovary cells deficient in ERCC1 produces marked resistance to CDDP, relative to the parental cells (Lee et al., 1993). Over-expression of ERCC1 is associated with CDDP resistance in non-small cell lung carcinoma (Rosell et al., 2003), although XPA expression has been associated with improved clinical outcome in ovarian cancer (Stevens et al., 2005). Moreover, down-regulation of ERCC1 in cultured ovarian cancer cells sensitizes the cells

to CDDP-induced cytotoxicity (Selvakumaran et al., 2003). However, if and how these factors are dysregulated in ovarian tumours and ovarian cancer cells remains unclear, since increases in gene copy number are infrequent (Yu et al., 2000), although increases in mRNA levels have been detected in several studies (Dabholkar et al., 1992; Dabholkar et al., 1994; States & Reed, 1996).

Suppression of drug-induced apoptosis

The induction of apoptosis is an important component of the response to chemotherapy and closely correlates with sensitivity to CDDP in ovarian cancer cells (Gibb et al., 1997; Sato et al., 1999), suggesting that loss of apoptotic capacity may be an important determinant of chemoresistance in human ovarian cancer. In agreement with this hypothesis, chemoresistant ovarian cancer cells often show a decreased ability to undergo apoptosis in response to chemotherapeutic agents, and aberrant regulation of key component of the apoptosis signaling cascades, including Bcl-2, Bax, and p53, is frequently associated with poor response to chemotherapy in human ovarian cancer (Kupryjanczyk et al., 2003; Murata et al., 2004; Sato et al., 1999; Skirnisdottir et al., 2002). The IAP family member survivin inhibits taxol-induced apoptosis and protein levels of survivin are correlated with a reduced remission rate following taxol-based chemotherapy in ovarian cancer patients (Song et al., 2003; Zaffaroni et al., 2002). Likewise, XIAP attenuates CDDP-induced apoptosis in cultured ovarian cancer cells (Li et al., 2001; Sasaki et al., 2000) while restoration of p53 function sensitizes p53-mutant, chemoresistant cells to CDDP *in vitro* and in *in vivo* xenograft models of ovarian cancer (Adler et al., 1997; Fraser et al., 2003; Song et al., 1999; Song et al., 1997). These data suggest that apoptosis is critical for sensitivity to chemotherapy in human ovarian cancer.

Involvement of p53 in Determining CDDP Sensitivity

In particular, the loss of components of the p53-induced apoptosis pathway is frequently associated with chemoresistance in human ovarian cancer cells. Expression of a temperature-sensitve mutant of p53 in human ovarian cancer cells conferred resistance to CDDP-induced apoptosis and cell cycle arrest, while over-expression of Bcl-2 similarly attenuated CDDP-induced apoptosis, suggesting that these mediators of apoptosis may be critical for sensitivity to CDDP (Eliopoulos et al., 1995). Likewise, over-expression of Bax sensitizes ovarian cancer cells to CDDP-induced apoptosis (Tsuruta et al., 2001). A subsequent study demonstrated that mutation of p53 is associated with resistance to CDDP, and this is accompanied by dramatic reductions in CDDP-induced apoptosis (Perego et al., 1996). Moreover, mutant p53 ovarian cancer cells are sensitized to CDDP by re-introduction of wild-type p53 and, importantly, re-introduction of wild-type p53 into xenografts of human ovarian cancer cells expressing mutant p53 reduces tumour volume and prolongs animal survival when given in combination with CDDP (Song et al., 1999; Song et al., 1997). This result has been observed by other independent groups with additional ovarian cancer cell line-derived xenografts (Heise et al., 2000; Kigawa et al., 2001). Clinically, the survival of patients whose tumours show high levels of p53, as detetcted by immunohistochemistry, is significantly shorter than those with low p53 staining and non-responders to CDDP-based chemotherapy have a higher frequency of p53 mutation than responders (Gadducci et al., 2002; Herod et al., 1996; Kigawa et al., 2001; Reles et al., 2001; Righetti et al., 1996). As such, there is considerable evidence to support a role for p53-mediated apoptosis as an important determinant of CDDP sensitivity in human ovarian cancer.

However, this finding is not universal, and several studies have shown that p53 is not required for CDDP-induced apoptosis or, paradoxically, attenuates CDDP sensitivity. Expression of the human papilloma virus E6 protein (HPV-E6), which ubiquitinates and catalyzes the degradation of p53, sensitizes ovarian cancer cells to CDDP (Pestell et al., 2000), although the same group earlier reported a direct correlation between wild-type p53 status and CDDP sensitivity (Pestell et al., 1998). In addition, ovarian cancer cells stably transfected with MDM2 are more sensitive to CDDP-induced cytotoxicity than controls (Mi & Ni, 2003). As such, there may exist both p53-dependent and p53-independent mechanisms by which CDDP induces its cytotoxic effects in ovarian cancer cells. This is supported by evidence from an examination of a panel of cancer cell lines, many of which expressed mutant p53 yet retained sensitivity to CDDP (Fan et al., 1997). These data support a model in which p53 is but one component of a larger, more intricate pathway that ultimately mediates the apoptotic response to CDDP.

By contrast, taxol is known to induce apoptosis in a p53-independent manner, and it is now widely accepted that wild-type p53 interferes with taxol-induced apoptosis (Cassinelli et al., 2001) and patients with mutant p53 ovarian tumours respond better to taxol versus patients with wild-type p53 tumours (Laframboise et al., 2000; Lavarino et al., 2000).

Chapter 3 –Objectives and Hypotheses

Objectives and Hypotheses

Overall Objectives

The overall objective of the current study is to improve our understanding of the cellular and molecular determinants of cell fate determination in ovarian cancer cells. In particular, we are interested in elucidating the mechanisms regulating apoptosis in these cells, and in establishing if and how the aberrant regulation of apoptosis contributes to the phenomenon of CDDP resistance in these cells. A better understanding of these mechanisms may ultimately lead to improved outcomes in the treatment of human ovarian cancer.

Specific Hypotheses

We hypothesize that,

(a) chemoresistance is, in part, a consequence of reduced CDDP-induced apoptosis,

(b) p53 is required for CDDP-induced apoptosis in ovarian cancer cells,

(c) p53-dependent CDDP sensitivity is related to CDDP-induced p53 phosphorylation and nuclear activation,

(d) Akt activation is a determinant of chemoresistance, which is related to the suppression of p53-mediated apoptosis, and the stabilization of XIAP, which is itself a key determinant of chemoresistance, and

(e) the sGC/cGMP pathway promotes survival in ovarian cancer cells through attenuation of p53 activation.

Specific Objectives

We wish to establish the role and regulation of the p53 tumour suppressor in ovarian cancer. Specifically, we wish to investigate the contribution of p53 to apoptosis in these cells, and to determine if and how abberant regulation of p53 is implicated in CDDP resistance. Additionally, we are interested in the regulation of p53 by additional cell fate determinants, including XIAP, Akt, and the sGC/cGMP pathway, and in determining how these pathways themselves regulate apoptosis in ovarian cancer cells. Importantly, we wish to establish the mechanisms of p53-mediated apoptosis in ovarian cancer cells, including the involvement of CDDP-induced p53-mediated gene transactivation and p53 phosphorylation.

Chapter 4 – Cancer Research 63 (21): 7081-8, 2003.

p53 is a Determinant of Xiap/Akt-Mediated Chemoresistance in Human Ovarian Cancer Cells

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Running Title: p53 in Xiap/Akt-mediated chemoresistance in ovarian cancer.

Keywords: Xiap; Akt; p53; chemoresistance; cisplatin

List of Abbreviations:

IAP: Inhibitor of Apoptosis Protein, **Xiap**: X-Linked Inhibitor of Apoptosis Protein, **PI3K**: Phosphoinositol-3-OH-Kinase, **DN-Akt**: Dominant-Negative Akt, **HA:** Hemagglutinin, **HA-Myr-Akt2**: Hemagglutinin-tagged, Myristoylated Akt2, **MOI**: multiplicity of infection, **Xiap-as**: adenoviral Xiap antisense cDNA, **Xiap-s**: adenoviral Xiap sense cDNA, **EGTA**: [Ethylenebis(oxyethylenenitrilo)] tetraacetic acid, **DMSO**: dimethyl sulfoxide, **PFT**: pifithrin-α-hydrobromide, **CDDP**: cis-platinum (II) diamine dichloride/cisplatin, **DMEM**: Dulbecco's Modified Eagle Medium, **X-Gal**: 5-Bromo-4-chloro-3-indolyl-beta-D-galactoside, **RPMI-1640**: Roswell Park Memorial Institute 1640, **FasL:** Fas Ligand, **MDM2**: Murine Double Minute-2

Abstract

We previously established that X-Linked Inhibitor of Apoptosis Protein (Xiap) is a determinant of cisplatin resistance in human ovarian cancer cells and that downregulation of Xiap sensitizes cells to cisplatin in the presence of wild-type p53. Furthermore, Xiap upregulates the PI3K/Akt pathway by increasing Akt phosphorylation. However, the precise relationships between Xiap, Akt, and p53 in chemoresistance are unknown. Here we show that both Xiap and Akt can modulate cisplatin sensitivity individually, but that Xiap requires Akt for its full function. Furthermore, dominant-negative Akt sensitizes ovarian cancer cells to cisplatin (10 μM), an effect that is absent in cells expressing mutant p53 or treated with the p53 inhibitor PFT (30 μM), but restored by exogenous wild-type p53. Cisplatin increased p53, decreased Xiap content and induced apoptosis in OV2008 cells, but not in the resistant counterpart (C13*). However, dominant-negative Akt restored all of these characteristics to C13* cells. Expression of a constitutively active Akt2 prevented cisplatin-mediated downregulation of Xiap and apoptosis in A2780s cells. Akt2-mediated chemoresistance could not be reversed by Xiap downregulation. These results suggest that while Xiap, Akt2, and p53 are important mediators of chemoresistance in ovarian cancer cells, Akt2 may be an important regulator of both Xiap and p53 contents following cisplatin challenge. Inhibition of Xiap and/or Akt expression/function may be an effective means of overcoming chemoresistance in ovarian cancer cells expressing either endogenous or reconstituted wild-type p53.

Introduction

While chemotherapy remains a major treatment modality for human ovarian cancer, chemoresistance is a clinical problem that severely limits treatment success. Cisplatin is a first-line chemotherapeutic agent in the treatment of ovarian cancer. It is now widely accepted that the apoptotic capacity of the cancer cell is pivotal in determining the response to chemotherapeutic agents. Indeed, several genes that regulate apoptosis, namely the p53, Akt, and phosphoinositol-3-OH-kinase (PI3K) family genes are frequently altered in cancer cells (Lasky & Silbergeld, 1996; Yuan et al., 2000). These observations suggest that inhibition of apoptosis by survival proteins is a key step in the development of chemoresistance.

The Inhibitor of Apoptosis Proteins (IAPs), originally identified in baculovirus, are potent endogenous inhibitors of programmed cell death. X-linked Inhibitor of Apoptosis Protein (Xiap) is an inhibitor of the execution caspase-3 and -7, and directly suppresses the mitochondrial apoptotic pathway by inhibiting caspase-9 (Deveraux et al., 1997). Our laboratory has previously demonstrated that cisplatin decreases Xiap protein level, induces Akt cleavage, and induces apoptosis in chemosensitive, p53 wild-type ovarian epithelial cancer cells (Li et al., 2001). These responses were not observed in chemoresistant, p53 wild-type ovarian epithelial cancer cells (C13*). However, down-regulation of Xiap by adenoviral antisense in C13* cells increased cisplatin sensitivity (Sasaki et al., 2000). By contrast, Xiap down-regulation was unable to induce apoptosis or increase cisplatin sensitivity in cisplatin-resistant, mutant p53 ovarian cancer cells (A2780cp), suggesting that p53 status is a determinant of Xiap-mediated chemoresistance. Taken together, these results suggest that Xiap regulates chemosensitivity by protecting

cells from cisplatin-induced apoptosis, and that Xiap may manifest some of its effects through modulation of a p53-mediated pathway. In addition, Xiap overexpression induces chemoresistance and Akt phosphorylation, indicative of Akt activation, effects which were attenuated by the PI3K inhibitor LY294002 (Asselin et al., 2001a). Together, these data suggest that Xiap-mediated chemoresistance may be in part due to the activation of the PI3K/Akt pathway. It has been shown in a number of cell types that Akt1 (Akt), Akt2, and Akt3 promote cell survival and suppress apoptosis induced by a variety of stimuli. A major downstream target of these kinases is Bad; a pro-apoptotic member of the Bcl-2 family of apoptotic regulators (Datta et al., 1997; del Peso et al., 1997; Jiang et al., 2000; Tang et al., 2000). The enzymes also phosphorylate and inactivate members (FKHR/FKHRL1) of the Forkhead transcription factor family (Brunet et al., 1999; Suhara et al., 2002), which are involved in the regulation of Fas ligand (FasL) transcription. Studies have demonstrated alterations of Akt2 at the DNA or mRNA levels in 15-20% of human ovarian cancers, as well as Akt2 activation and overexpression in primary ovarian carcinomas (Cheng et al., 1992; Yuan et al., 2000). However, the relationship between Akt2, Xiap, and other mediators of chemoresistance is unknown.

In the current study, we demonstrate that both Xiap and Akt2 can modulate cisplatin sensitivity, but that Akt2 is involved in regulating Xiap content in the presence of cisplatin. Furthermore, we demonstrate that functional p53 is absolutely required for the chemosensitizing effects of Xiap and/or Akt downregulation. Overall, our results are consistent with the notion that modulation of Xiap or Akt function may be a useful

therapeutic strategy in overcoming chemoresistance in tumours expressing wild-type p53, or in tumours supplemented with exogenous p53.

Materials and Methods

Reagents

RPMI 1640 medium or D-MEM/F-12 medium (GIBCO/BRL) was used for cell cultures. Both media were supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), penicillin (100 units/ml), fungizone (0.625 μg/ml) and 1% non-essential amino acids (all GIBCO/BRL). Cisplatin and DMSO were from Sigma. Pifithrin-α-hydrobromide was from Tocris Inc. Adenoviral constructs with Xiap-sense, -antisense, wild-type p53 and LacZ cDNA were provided by Dr. Ruth Slack (Adenovirus Core Facility, Neuroscience Research Institute, University of Ottawa). Adenoviral construct containing hemagglutinin (HA)-tagged, triple-A mutated (K179A, T308A and S473A) kinase-dead (dominant-negative) DN-Akt was a generous gift from Dr. Kenneth Walsh (Cardiovascular Research, St. Elizabeth's Medical Centre, Boston). All adenovirus stock solutions were CsCl purified. Primary antibodies were anti-Xiap rabbit polyclonal IgG (Trevigen), anti-p53 mouse monoclonal IgG (Transduction Labs), anti-$p21^{WAF1/CIP1}$ mouse monoclonal IgG (Cell Signaling), and anti-HA mouse monoclonal IgG (Roche). Appropriate dilutions were determined empirically using the manufacturer's instructions as a starting point.

Cell Culture

Cisplatin-sensitive (OV2008 and A2780s) and -resistant (C13* and A2780cp) cell lines were cultured as previously reported (Asselin et al., 2001a). Prior to each experiment, 1.8×10^5 cells were plated on uncoated 6-well plates in the appropriate medium with 10% fetal bovine serum for 12-18 hours for proper attachment.

Creation of Stably Transfected Cell Lines

A2780s cells were stably transfected with pcDNA3 vector (Invitrogen) containing constitutively active HA-Myr-Akt2 or pcDNA3 alone as previously reported in (Yuan et al., 2003b).

Adenovirus infection and Cisplatin treatment

After 12-18 hours of plating, cells were infected with adenoviral Xiap sense, antisense, wild-type p53, DN-Akt and/or LacZ control at various viral doses (multiplicity of infection; MOI) as previously reported (Asselin et al., 2001a). Total MOI was maintained constant for all treatment groups. Adenovirus infection efficiency (MOI = 5; 24 h) was greater than 90%, as determined by an X-gal staining assay against LacZ construct infected cells. Unless otherwise specified, 10 μM cisplatin was dissolved in DMSO and added to the cells in culture medium with 2% fetal bovine serum at 24 hours prior to harvest.

Determination of Apoptosis

Following treatment, cells were harvested as described in (Asselin et al., 2001a) and percent apoptosis was determined by nuclear staining with Hoechst 33248 stain (12.5 ng/ml; Sigma), as reported in (Sasaki et al., 2000).

Protein Extraction and Western Immunoblotting

Cells were sonicated in lysis buffer containing 50mM Hepes, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 100mM Sodium Fluoride, 10mM sodium pyrophosphate, 10% v/v glycerol, 1% v/v Triton X-100, 1mM Phenylmethylsulfonylfluoride, 10 μg/μl aprotinin and 1mM $Na_3(VO_4)$. Proteins were isolated and quantified according to previously published protocols (Sasaki et al., 2000). Equivalent amounts of total protein were loaded onto acrylamide gels (8-10%) and separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes according to previously published protocols (Sasaki et al., 2000). Ponceau-S staining was used to confirm even loading between groups. The membranes were blocked for 1 hour in blotto (5% skim milk in Tris-buffered saline-Tween), and subsequently incubated for 12h at 4°C in primary antibodies diluted in blotto (α-Xiap 1:2000; α-p53 1:1000; α-p21 1:2000, and α-HA 1:1000). For the detection of primary antibodies, membranes were incubated with horseradish peroxidase conjugated goat IgG raised against the proper species (goat-anti-rabbit for α-Xiap and goat-anti-mouse for α-HA, α-p53, α-p21; BioRad) diluted 1:2000 in blotto for 1h at room temperature. Horseradish peroxidase activity was visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) and the signals were recorded on HyperFilm MP (Amersham Pharmacia Biotech) and developed in a Kodak X-Omat film developer. Results were scanned and densiometrically analysed using Scion Image software (Scion Inc).

Statistical Analysis

Experimental results are expressed as the mean of at least three independent experiments. Data were analysed using one- or two-way analysis of variance with Tukey

post-test or two-tailed t-tests to assess differences between experimental groups (PRISM 3.0, GraphPad Software Inc). Statistical significance was inferred at $p<0.05$.

Results and Discussion

The Akt Pathway is Involved in Xiap-Mediated Chemoresistance

In order to form the basis upon which our subsequent studies were carried out, and to confirm our previously report that Xiap is implicated in cisplatin resistance in ovarian cancer cells, cisplatin sensitive, p53 wild-type OV2008 cells were infected with adenovirus carrying Xiap sense (Xiap-s) cDNA or LacZ at various MOIs (MOI = 0, 1 and 5) for 48 h. Twenty-four hours post-infection, cells were treated with 10 μM cisplatin or DMSO. Western blotting analysis demonstrated an increase in Xiap protein level with increasing Xiap-s dose. Infection with LacZ alone did not increase Xiap protein level (not shown). Whereas DMSO induced similar cellular morphologies and apoptotic responses in both Xiap-s and LacZ infected groups, overexpression of Xiap (MOI = 5) prior to the addition of cisplatin decreased apoptosis 2.5 fold compared to LacZ ($p<0.01$). The protective effect of Xiap was also dose-dependent, with Xiap-s at MOI = 5 of showing a 1.6 fold decrease in apoptosis relative to the Xiap-s group at MOI = 1 ($p<0.05$) (**Figure 1A**). To further assess the role of Xiap in chemoresistant cells, C13* cells were infected with adenovirus carrying Xiap antisense (Xiap-as) cDNA at various MOIs (0, 10, 20, 30 and 40) for a total of 48h. At 24h post-infection, cells were treated with 10 μM cisplatin or DMSO control. Western analysis confirmed down-regulation of Xiap by Xiap-as. While down-regulation of Xiap alone did not induce apoptosis, sensitivity toward cisplatin increased significantly as a function of Xiap-as MOI ($p<0.05$) (**Figure**

1B). These results confirm the involvement of Xiap in chemoresistance, as previously reported by our laboratory (Asselin et al., 2001a; Li et al., 2001; Sasaki et al., 2000).

Figure 1: Xiap is a Determinant of Chemoresistance in Human Ovarian Cancer Cells.

A. Effects of overexpression of Xiap on cisplatin-induced cell death in chemosensitive OV2008 cells using an adenoviral vector (Xiap-s; (MOI=0-5; LacZ control; 24h infection)). Overexpression was confirmed by Western analysis. **: $p<0.01$, relative to LacZ control, +: $p<0.05$, relative to Xiap-s MOI=1 group. **B.** Effects of downregulation of Xiap on cisplatin-induced cell death in chemoresistant C13* cells using an adenoviral Xiap antisense cDNA (Xiap-as; MOI=0, 10, 20, 30, 40; LacZ control; 48h infection). Western analysis confirmed the downregulation of Xiap with increasing doses of Xiap-as. *: $p<0.05$, relative to CTL group.

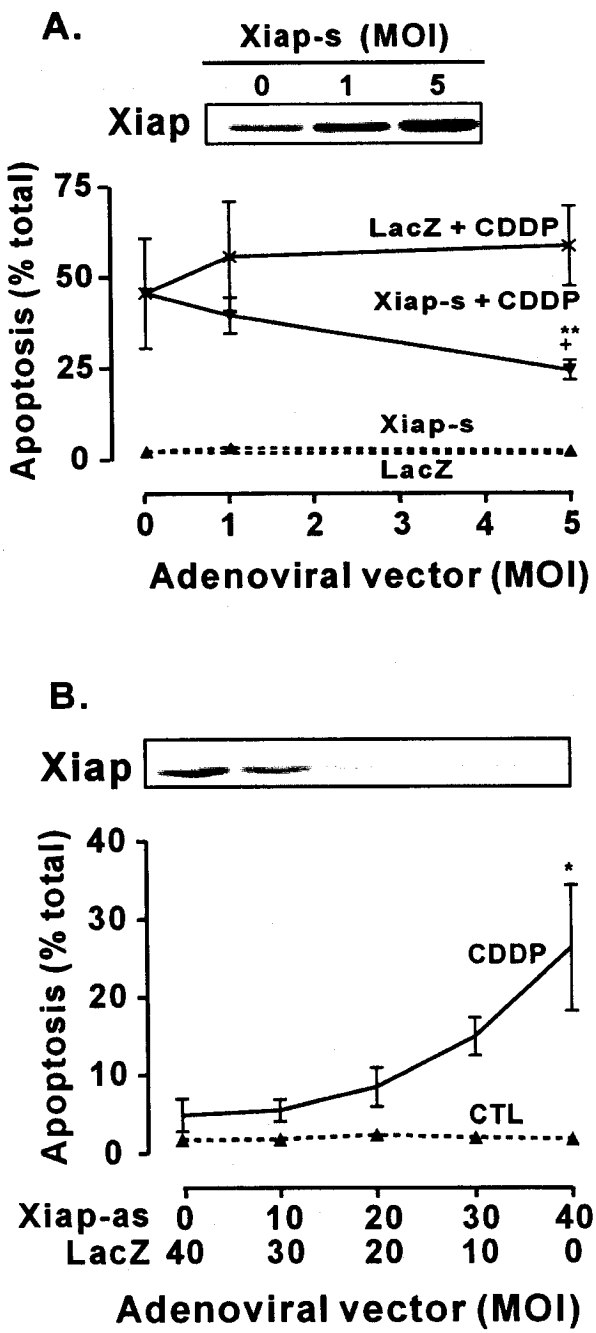
A.
Xiap-s (MOI)
0
1
5
Xiap
Apoptosis (% total)
75
50
25
0
LacZ + CDDP
Xiap-s + CDDP
**
+
Xiap-s
LacZ
0
1
2
3
4
5
Adenoviral vector (MOI)
B.
Xiap
Apoptosis (% total)
40
30
20
10
0
*
CDDP
CTL
Xiap-as 0 10 20 30 40
LacZ 40 30 20 10 0
Adenoviral vector (MOI)

Figure 1

In addition, our laboratory has previously reported that overexpression of Xiap increases Akt phosphorylation and renders ovarian cancer cells resistant to the cytotoxic actions of cisplatin, an effect that is partially attenuated by LY294002 (Asselin et al., 2001a). Thus, to determine the role, if any, of Akt in Xiap-mediated chemoresistance, chemosensitive ovarian cancer cells (A2780s and OV2008) were infected with DN-Akt (0, 10, 20 and 40 MOI). The dominant-negative effects of this Akt mutant have previously been demonstrated (Krieg et al., 2003). At 1h post-infection, Xiap-s (MOI = 5) was added to each group (total MOI equalized with LacZ). The cells were treated with 10 μM cisplatin or DMSO, 48h post-infection. An extra group of cells infected with 45 MOI of LacZ was included as a control. Cisplatin (10 μM) induced a significant apoptotic response ($p<0.01$) in both A2780s (**Figure 2A**) and OV2008 cells (**Figure 2B**) when infected with LacZ (MOI = 45). Xiap-s induced resistance toward cisplatin compared to LacZ control in both A2780s ($p<0.01$) and OV2008 cells ($p<0.05$). In the absence of cisplatin, replacement of LacZ with DN-Akt in A2780s cells induced apoptosis in a dose-dependent manner (MOI=0, 10, 20 and 40) despite the presence of Xiap-s (MOI=5; data not shown). However, no significant change in apoptotic response was observed in OV2008 under the same conditions, suggesting that the A2780s cell line is more dependent upon the integrity of Akt for its survival than the OV2008 cell line. However, in both A2780s and OV2008 cells, replacement of LacZ with DN-Akt attenuated the protective effect of Xiap. Cisplatin-induced apoptotic response was restored up to 70% of control (LacZ, MOI = 45) in cells co-expressing Xiap (MOI = 5) and DN-Akt (MOI = 40). Taken together, these results support our hypothesis that Xiap is a determinant of

cisplatin sensitivity in ovarian cancer cells and that Akt is a downstream intermediate of Xiap-mediated chemoresistance.

Akt is a Determinant of Chemoresistance

A recent study has demonstrated that an ovarian cancer cell line expressing a constitutively active PI3K catalytic subunit p110α is resistant to paclitaxel, relative to the parental control cells, an effect that was reversed by the PI3K inhibitor LY294002 (Hu et al., 2002). Moreover, data from our own laboratory has demonstrated that Xiap-mediated chemoresistance is partially attenuated by LY294002 (Asselin et al., 2001a). In addition, a recent time-course study has demonstrated that expression of an active Akt2 in ovarian cancer cells renders the cells resistant to cisplatin (Yuan et al., 2003b). In investigating further the mechanisms of Akt-mediated chemoresistance, we have extended these observations with a concentration-response study using chemosensitive ovarian cancer cells (A2780s), stably transfected with pcDNA3 expression vector carrying constitutively active Akt2 (A2780s-AAkt2). Responses to cisplatin (0, 2.5, 5, 10 and 20 μM) were compared to A2780s cells stably transfected with empty pcDNA3 vector (A2780s-PMH6). While cisplatin induced apoptosis in a concentration-dependent manner in A2780s-PMH6 cells ($p<0.01$), the presence of constitutively activated Akt2 reduced the sensitivity of cells towards cisplatin ($p<0.01$) (Figure 3A). These data are consistent with results demonstrated by Yuan et al (Yuan et al., 2003b).

Figure 2 - Akt is Implicated in Xiap-Mediated Chemoresistance.

Xiap was overexpressed in A2780s (**A**) and OV2008 (**B**) cells by Xiap-s (MOI=5) in the presence of DN-Akt (MOI=0-40), followed by 24h of cisplatin (10 μM) challenge (DMSO as CTL). Cisplatin-induced apoptosis was assessed by nuclear morphology. **: $p<0.01$, relative to CTL. ++: $p<0.01$, relative to LacZ + CDDP group. +: $p<0.05$, relative to LacZ + CDDP group. #: $p<0.05$, relative to Xiap-s (MOI=5) alone + CDDP group.

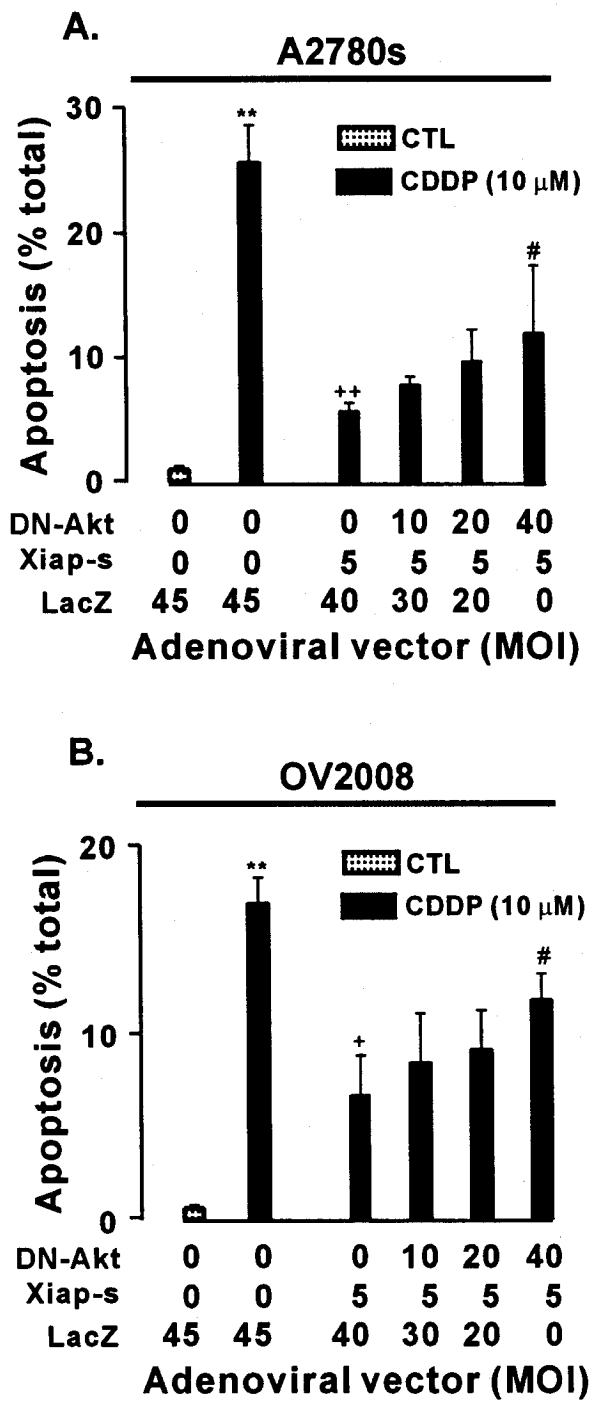
A.
A2780s
Apoptosis (% total)
30
20
10
0
CTL
CDDP (10 μM)
**
++
#
DN-Akt 0 0 0 10 20 40
Xiap-s 0 0 5 5 5 5
LacZ 45 45 40 30 20 0
Adenoviral vector (MOI)
B.
OV2008
Apoptosis (% total)
20
10
0
CTL
CDDP (10 μM)
**
+
#
DN-Akt 0 0 0 10 20 40
Xiap-s 0 0 5 5 5 5
LacZ 45 45 40 30 20 0
Adenoviral vector (MOI)

Figure 2

To further investigate the involvement of Akt in chemoresistance, p53 wild-type chemoresistant cells (C13*) were infected for 48h with DN-Akt (MOI = 0, 10 20 and 40). The DN-Akt construct was tagged with a hemagglutinin (HA) epitope polypeptide and the presence of HA signal on Western blot was indicative of DN-Akt expression. 48h post-infection, cells were treated with 10 μM cisplatin or DMSO. A significant difference between the cisplatin treated group and control ($p<0.01$) was observed. Furthermore, the effect of DN-Akt on the cisplatin sensitivity of C13* cells was concentration-dependent ($p<0.05$) (**Figure 3B**). Taken together, these data confirm that Akt modulates the sensitivity of human ovarian cancer cells to cisplatin.

Figure 3 - Akt is a Determinant of Chemoresistance in Human Ovarian Cancer Cells.

A. CDDP-sensitive A2780s cells were stably transfected with pcDNA3 vector containing an activated form of Akt2 (A2780s-AAkt2; marked 'Active-Akt2'), or empty vector (A2780s-PMH6; marked 'Control') and treated with cisplatin (10 μM) or DMSO (CTL). Cisplatin-induced apoptosis was assessed by nuclear morphology. **: $p<0.01$, relative to cisplatin-free group. ++: $p<0.01$, relative to A2780s-AAkt2 cells. **B.** C13* cells were infected for 48h with adenovirus containing dominant-negative Akt cDNA (DN-Akt, MOI=0-40; LacZ control) and treated with cisplatin (10 μM) or DMSO (CTL) for 24h. Western blot using anti-HA antibody confirmed expression of DN-Akt. **: $p<0.01$, relative to CTL group. +: $p<0.05$, relative to DN-Akt MOI=0.

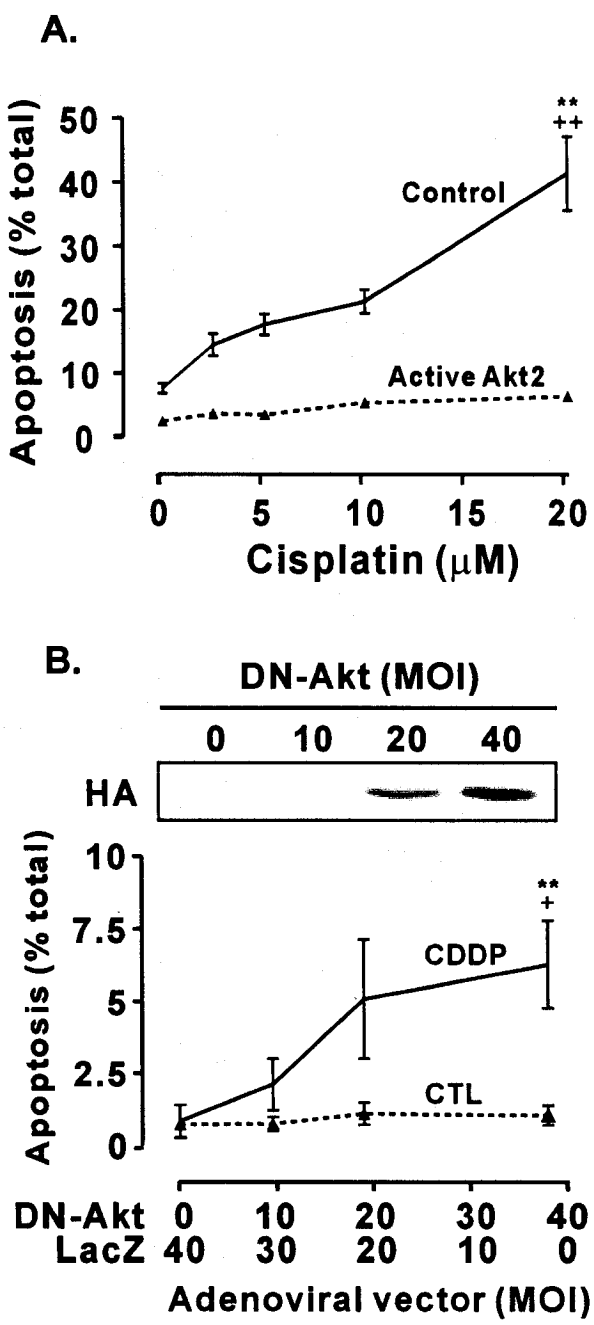
A.
Apoptosis (% total)
50
40
30
20
10
0
**
++
Control
Active Akt2
0
5
10
15
20
Cisplatin (μM)
B.
DN-Akt (MOI)
0
10
20
40
HA
Apoptosis (% total)
10
7.5
5
2.5
0
**
+
CDDP
CTL
DN-Akt 0 10 20 30 40
LacZ 40 30 20 10 0
Adenoviral vector (MOI)

Figure 3

p53 Function is Required For Sensitization to Cisplatin Through Suppression of Akt Activity

Previous studies from our laboratory have established that Xiap downregulation sensitizes chemoresistant, p53 wild-type (C13*), but not p53 mutant (A2780cp) ovarian cancer cells to cisplatin (Sasaki et al., 2000). However, concomitant reintroduction of wild-type p53 induced apoptosis and permitted the cisplatin-sensitizing effects of Xiap downregulation in these p53 mutant cells. To determine whether p53 was also required for the chemosensitizing effects of Akt activity downregulation, A2780cp cells were infected with DN-Akt (MOI= 0, 10, 20 and 40) for 48h, followed by subsequent treatment with 10 μM cisplatin for 24h. DN-Akt failed to sensitize A2780cp cells to cisplatin (data not shown), suggesting that a wild-type p53 is required for the pro-apoptotic effects of downregulation of Akt activity. To determine whether p53 status is indeed a determinant of Akt-mediated chemoresistance, A2780cp cells were co-infected with DN-Akt (MOI= 0, 10, 20 and 40) and wild-type p53 (MOI=10). The presence of reconstituted wild-type p53 sensitized the cells to cisplatin in a DN-Akt-dependent manner (cisplatin $p<0.001$; DN-Akt $p<0.01$; Interaction $p<0.05$) (**Figure 4A**). This effect was also dependent upon the concentration of wild-type p53 when DN-Akt dose was held constant (All effects, $p<0.0001$; **Figure 4B**). The combined results demonstrate that while suppression of Akt sensitizes chemoresistant cells to cisplatin, this effect requires the presence of a wild-type p53.

Figure 4 - Wild-Type p53 is Required for Sensitization of Chemoresistant Cells to Cisplatin.

A2780cp cells (p53 mutant) were infected with DN-Akt (MOI=0-40), wild-type p53 (MOI=0-20), and/or LacZ (to equalize total MOI) adenovirus and treated with 10 μM cisplatin (black bars) or DMSO as control (white bars). Dose-dependent effects of DN-Akt (**A**) and wild-type p53 (**B**) on cisplatin sensitivity were assessed. ***: $p<0.0001$, relative to wild-type p53 MOI=0. +++: $p<0.0001$, relative to CDDP-free group at same viral conditions. ++: $p<0.01$, relative to CDDP-free group at same viral conditions.

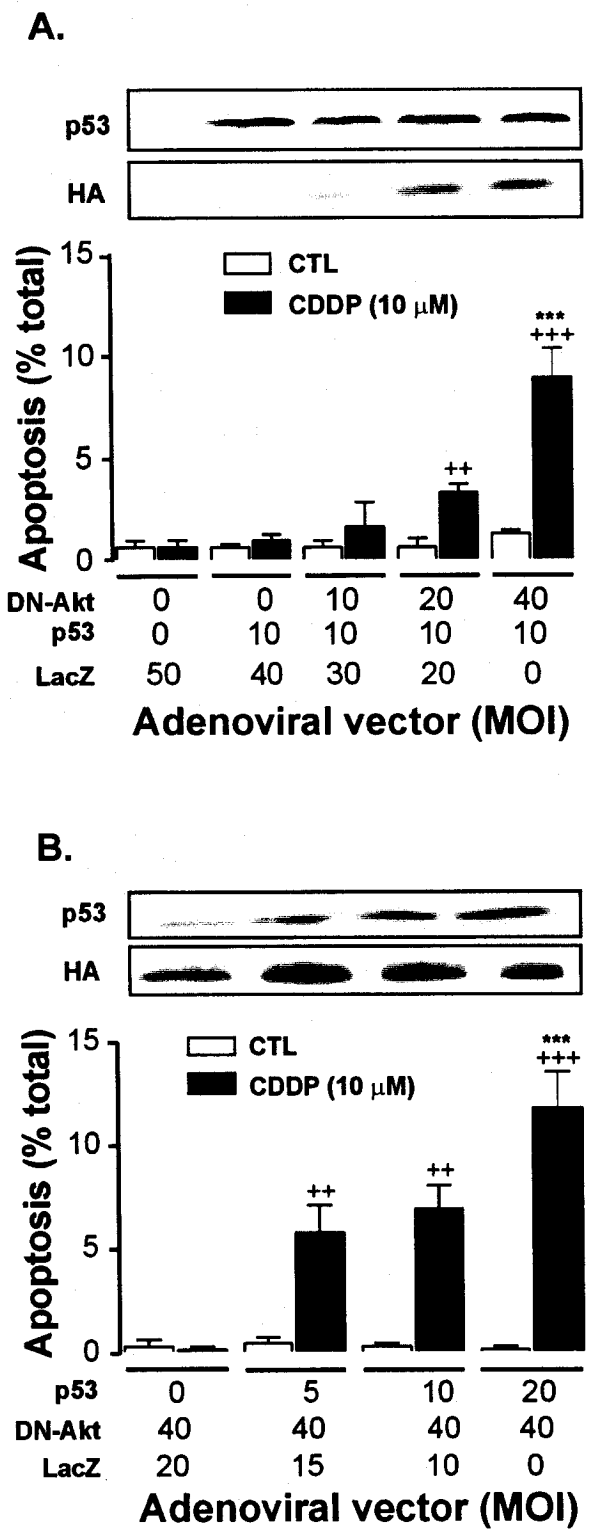
A.
p53
HA
CTL
CDDP (10 μM)
Apoptosis (% total)
15
10
5
0
***
+++
++
DN-Akt 0 0 10 20 40
p53 0 10 10 10 10
LacZ 50 40 30 20 0
Adenoviral vector (MOI)
B.
p53
HA
CTL
CDDP (10 μM)
Apoptosis (% total)
15
10
5
0
***
+++
++
++
p53 0 5 10 20
DN-Akt 40 40 40 40
LacZ 20 15 10 0
Adenoviral vector (MOI)

Figure 4

To determine if the involvement of Akt in Xiap-mediated chemoresistance depends on p53 function, we employed the specific p53 inhibitor, pifithrin-α-hydrobromide (PFT). The inhibitory effects of this compound on p53 function have been previously reported (Arango et al., 2001; Komarov et al., 1999; Zhang et al., 2003). OV2008 cells co-infected with Xiap-s (MOI=5) and DN-Akt (MOI = 0, 40, 80) were incubated with PFT (0, 10, 20 and 40 μM) and cisplatin (10 μM). In the absence of PFT, DN-Akt reversed Xiap-induced chemoresistance in a dose-dependent manner ($p<0.05$). However, PFT dose-dependently attenuated the effects of DN-Akt on Xiap-induced chemoresistance up to 20 μM ($p<0.01$; **Figure 5A**). Interestingly, at 40 μM, PFT increased overall cell death in all groups, indicative of non-specific PFT toxicity; we subsequently determined the maximum tolerable dose of PFT to be 30 μM (data not shown), and this concentration was used for all subsequent experiments.

To further confirm the requirement of p53 in DN-Akt-mediated chemosensitization, A2780cp cells were infected with DN-Akt (MOI= 40) and wild-type p53 (MOI= 0, 5, 10, 20, 40), and treated with cisplatin (10 μM) in the presence or absence of PFT (30 μM; **Figure 5B**). While DN-Akt failed to sensitize these cells to cisplatin, introduction of wild-type p53 dose-dependently sensitized the cells to cisplatin ($p<0.0001$). Addition of PFT significantly attenuated apoptosis overall ($p<0.01$), and significantly ablated the effects of wild-type p53 ($p<0.05$). To confirm the functionality of the exogenous wild-type p53, we determined p21 content. While p21 content was undetectable in the absence of exogenous p53, reintroduction of wild-type p53 increased p21 content in a dose-dependent manner, an effect that was attenuated by the presence of PFT **(Figure 5B)**.

To confirm that the actions of PFT were, at least in part, specific to p53, C13* cells were infected with DN-Akt (MOI= 0, 40, 80) and wild-type p53 (MOI=0, 5, 10, 20) and treated with 10 μM cisplatin in the presence and absence of PFT (30 μM). While expression of DN-Akt sensitized the cells to cisplatin in a dose-dependent manner ($p<0.0001$), this effect was attenuated by pre-treatment with PFT ($p<0.01$). However, the effects of PFT were reversed by overexpression of wild-type p53 ($p<0.0001$), suggesting that the effects of PFT are, at least in part, mediated through p53 (**Figure 5C**). Finally, there was a significant interaction between DN-Akt and wild-type p53 ($p<0.05$), suggesting that Akt downregulation is more effective at sensitizing the cells to cisplatin when p53 content is high.

Thus, wild-type p53 function is required for the pro-apoptotic effects of either Xiap downregulation (Sasaki et al., 2000) or Akt suppression; these effects are inhibited by pharmacological inhibitors of p53 or by endogenous mutant p53, and stimulated by the introduction of wild-type p53. As such, modulation of Xiap and/or Akt function may be a viable option in overcoming chemoresistance in tumours expressing wild-type p53. One recent review has suggested that there is an approximately 51% overall incidence of p53 mutation in epithelial ovarian cancer (Kmet et al., 2003). However, in tumours that express mutant p53, modulation of Xiap and/or Akt could conceivably be coupled to replacement of functional p53 by gene therapy, a process that was viable in at least one recent study (Wen et al., 2003).

Figure 5 - DN-Akt-mediated Sensitization is Attenuated by a Specific p53 Inhibitor

A. OV2008 cells were infected with Xiap-s, DN-Akt, and/or LacZ adenovirus and treated with 10 μM cisplatin and increasing doses of PFT (0-20 μM). Apoptosis was determined by nuclear morphology. *: $p<0.05$, relative to Xiap-s MOI=5, DN-Akt MOI=0 group. **: $p<0.01$, relative to PFT-free control. **B.** A2780cp cells were infected with DN-Akt (MOI=40) and/or wild-type p53 (MOI=0-40) and treated with 10 μM cisplatin in the presence (black bars) or absence (white bars) of PFT (30 μM). Cisplatin-induced apoptosis was assessed by nuclear morphology. p21 content was tracked via Western blot as a marker of p53 function. ***: $p<0.0001$ relative to wild-type p53 MOI=0. ++: $p<0.01$, relative to 30 mM PFT group. **C.** C13* cells were infected with DN-Akt (MOI=0-80) and/or wild-type p53 (MOI=0-20) and treated with 10 μM cisplatin in the presence or absence of PFT (30 μM). Apoptosis was assessed by nuclear morphology. ***: $p<0.0001$, relative to DN-Akt (MOI=0) + CDDP. ++: $p<0.01$, relative to DN-Akt infection without PFT. ###: $p<0.0001$, relative to wild-type p53 (MOI=0) + PFT.

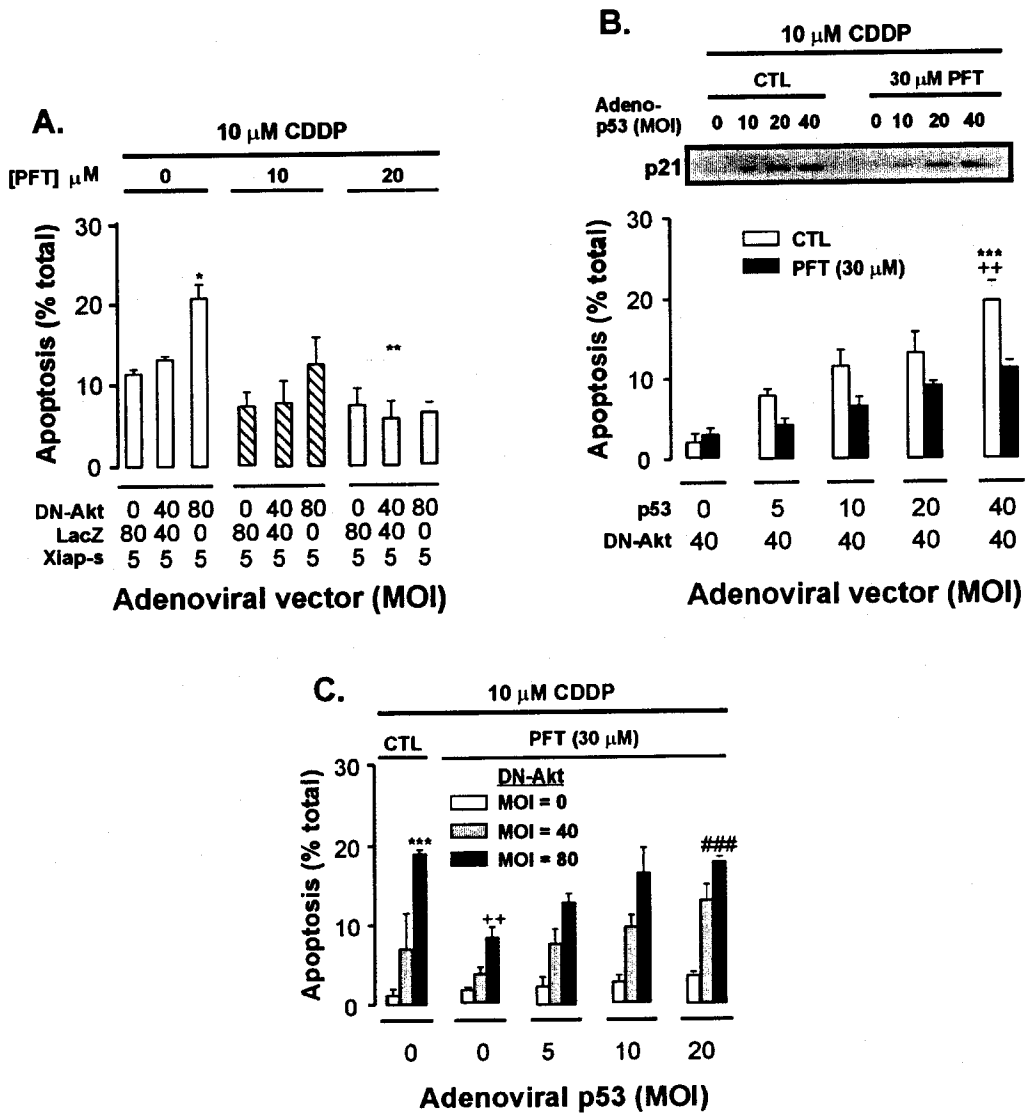
A.
10 μM CDDP
[PFT] μM
0
10
20
Apoptosis (% total)
30
20
10
0
*
**
DN-Akt 0 40 80 0 40 80 0 40 80
LacZ 80 40 0 80 40 0 80 40 0
Xiap-s 5 5 5 5 5 5 5 5 5
Adenoviral vector (MOI)
B.
10 μM CDDP
CTL
30 μM PFT
Adeno-p53 (MOI)
0 10 20 40
0 10 20 40
p21
Apoptosis (% total)
CTL
PFT (30 μM)
***
++
p53 0 5 10 20 40
DN-Akt 40 40 40 40 40
Adenoviral vector (MOI)
C.
10 μM CDDP
CTL
PFT (30 μM)
DN-Akt
MOI = 0
MOI = 40
MOI = 80
Apoptosis (% total)
***
++
###
0 0 5 10 20
Adenoviral p53 (MOI)

Figure 5

Akt regulates the cisplatin-mediated induction of p53

The relative contribution of the p53 pathway to the modulation of chemosensitivity by Akt is not known. To test the relationship between Akt and p53, we cultured OV2008 and C13* cells with increasing doses of cisplatin (0, 2.5, 5, 10 μM). Whereas cisplatin induced a concentration-dependent increase in p53 in the chemosensitive OV2008 cell line, p53 was not detectable in C13* cells at any of the cisplatin doses ($p<0.00001$, Cell Line; $p<0.01$, cisplatin; $p<0.01$, interaction, **Figure 6A**).

Because the chemoresistance observed in C13* cells appears to be Akt-dependent, we next wanted to determine the relationship, if any, between Akt and cisplatin-induced upregulation of p53. We downregulated Akt activity with DN-Akt (MOI=0, 40, 80) in the presence of 10 μM cisplatin. Whereas cisplatin alone (LacZ MOI=80) failed to induce p53 content, cisplatin upregulated p53 content in the presence of DN-Akt in a manner dependent on the concentration of DN-Akt ($p<0.0001$, **Figure 6B**). Our finding that Akt regulates p53 content is consistent with the reports of Ogawara et al (Ogawara et al., 2002), where basal p53 levels could be controlled by Akt activation through activation, but not nuclear translocation, of MDM2. Similarly, we observed that the subcellular localization of MDM2 was Akt-independent (data not shown). Whereas cisplatin has previously been shown to increase p53 content (Qin & Ng, 2002), to our knowledge, our report represents the first to demonstrate that this process is negatively regulated by Akt. We are currently investigating the precise mechanisms by which Akt regulates the cisplatin-mediated induction of p53.

Figure 6 - Akt Regulates Xiap and p53 Content in the Presence of Cisplatin.

A. OV2008 and C13* cells were cultured and treated with cisplatin at increasing doses (0–10 μM). Western analysis shows effects of cisplatin on p53 and Xiap. **B.** C13* cells were infected with DN-Akt (MOI=0-80) and/or LacZ, followed by treatment with 10 μM cisplatin for 24h. Western analysis shows effects of cisplatin plus DN-Akt on p53 and Xiap. A2780s-PMH6 (marked 'Control') and A2780s-AAkt2 (marked 'Active Akt2') cells were treated for 24h in the absence or presence of 10 μM cisplatin. Xiap content was tracked by Western blot (**C**) and is expressed graphically as fold of CTL (A2780s-PMH6 cells without CDDP) (**D**). The percentage of cells undergoing apoptosis was determined by nuclear morphology and is expressed graphically (**E**). **: $p<0.05$, relative to A2780s-PMH6, -CDDP group (white bar, marked CTL). ***: $p<0.001$, relative to A2780s-PMH6, -CDDP group (white bar).

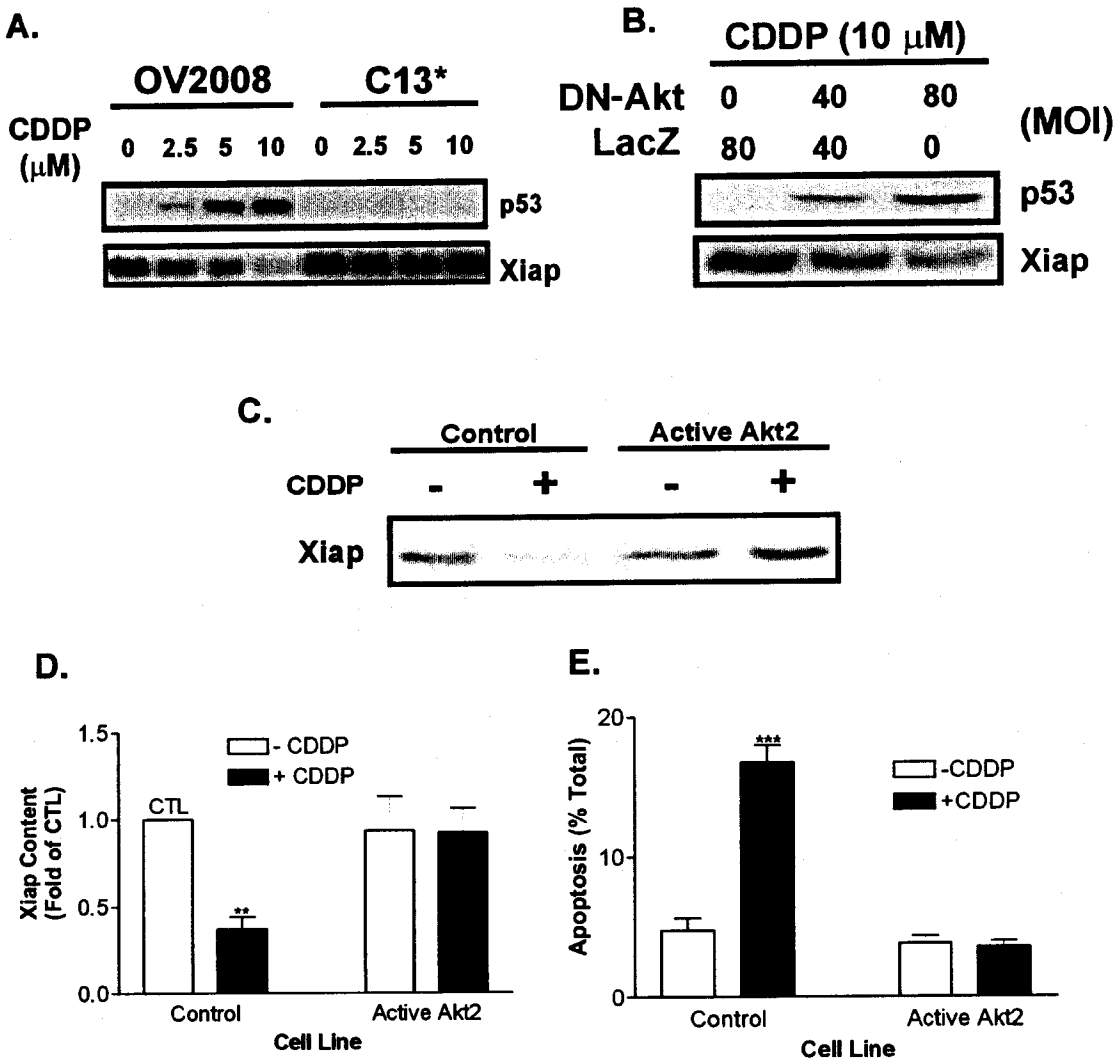
A.
OV2008
C13*
CDDP (μM)
0 2.5 5 10 0 2.5 5 10
p53
Xiap
B.
CDDP (10 μM)
DN-Akt 0 40 80
LacZ 80 40 0
(MOI)
p53
Xiap
C.
Control
Active Akt2
CDDP - + - +
Xiap
D.
Xiap Content (Fold of CTL)
1.5
1.0
0.5
0.0
- CDDP
+ CDDP
CTL
**
Control
Active Akt2
Cell Line
E.
Apoptosis (% Total)
20
10
0
***
-CDDP
+CDDP
Control
Active Akt2
Cell Line

Figure 6

Akt2 protects against cisplatin-induced downregulation of Xiap

Previous studies from our laboratory have demonstrated that cisplatin decreases Xiap content in chemosensitive, but not chemoresistant, human ovarian cancer cells (Li et al., 2001). However, if and how Akt is implicated in this process is not known. In accordance withour earlier data (Li et al., 2001), cisplatin (0-10 μM) decreased Xiap content in a concentration-dependent manner in OV2008 cells, but not in C13* cells ($p<0.05$, all effects; **Figure 6A**). This difference was most evident at 10 μM cisplatin, where OV2008 cells expressed only about 50% of basal (control) Xiap, compared with C13* cells which showed no difference from the control cells ($p<0.001$). However, when C13* cells were infected with DN-Akt (MOI=0-80) and treated with 10 μM cisplatin, Xiap was downregulated in a DN-Akt dependent manner ($p<0.01$, **Figure 6B**).

As mentioned, Akt2 has been demonstrated to modulate cellular cisplatin sensitivity in ovarian cancer cells (Yuan et al., 2003b). To examine if Akt2 could regulate Xiap content, we utilized the A2780s-AAkt2 (active Akt2) and A2780s-PMH6 (control) cell lines. While cisplatin (10 μM) induced significant apoptosis ($p<0.001$, relative to control, **Figure 6E**) and decreased Xiap content (60% decrease at 10 μM, $p<0.05$ overall, relative to control, **Figure 6C and E**) in A2780s-PMH6 cells, it failed to elicit either effect in A2780s-AAkt2 cells ($p<0.0001$, compared to A2780s-PMH6, all effects), suggesting a possible role for Akt2 in the protection of Xiap against cisplatin-induced downregulation.

To test whether this protection of Xiap plays a significant role in the anti-apoptotic effects of Akt2 activation, we downregulated Xiap using an adenoviral Xiap antisense cDNA (Xiap-as; MOI=0-40; 48h infection) in A2780s-AAkt2 cells, followed

by treatment with 10 μM cisplatin (24h). While Western blot analysis confirmed the downregulation of Xiap by Xiap-as, cisplatin-induced apoptosis was minimal and not significantly different in the two treatment groups (data not shown), suggesting that maintenance of Xiap content is a functionally minor downstream event in the anti-apoptotic effects of Akt2 activation.

Thus, Akt2 regulates the cisplatin-mediated downregulation of Xiap. Interestingly, it has been reported that Xiap is an *in vitro* substrate for cleavage by a number of caspases, including caspase 3, 6, 7, and 8 (Deveraux et al., 1999). In the same study, it was reported that Fas-induced apoptosis was associated with cleavage of Xiap. Thus, it is possible that Xiap is cleaved by cisplatin-dependent activation of caspases, an effect which is inhibited by Akt2. In such a case, the failure of cisplatin to decrease Xiap content in chemoresistant cells may be secondary to aberrant regulation of the Akt2 pathway. Interestingly, Akt is known to phosphorylate and inactivate FKHRL1 and FKHR1 (Brunet et al., 1999), both key regulators of FasL expression. Our laboratory has previously demonstrated that cisplatin can upregulate both Fas and FasL in OV2008 and A2780s cells, but only Fas in C13* and neither Fas nor FasL in A2780cp cells, suggesting that deregulation of this system may be an important determinant of chemoresistance in ovarian cancer cells (Schneiderman et al., 1999). Whether activation of the Fas/FasL system by cisplatin is an important event in the subsequent downregulation of Xiap, and whether this phenomenon is Akt2-dependent, remains unclear.

Xiap is also known to act as its own E3 ubiquitin ligase (Yang et al., 2000). It is possible, therefore, that aberrations in the ability of Xiap to become ubiquitinated and

degraded in the 26S proteasome may underlie the observed maintenance of Xiap content in chemoresistant, but not chemosensitive cells, following cisplatin challenge. Whether Akt2 modulates the process of Xiap ubiquitination is not known. However, it is clear that Akt2 is a central modulator of the cellular response to cisplatin, both through its ability to inhibit the expression and function of pro-apoptotic proteins such as Bad and p53, and, interestingly, through its ability to protect anti-apoptotic proteins such as Xiap from cisplatin-induced downregulation.

Note that Akt2 activation did not increase basal Xiap levels, as has been demonstrated in our laboratory in rat granulosa cells (Wang et al., 2002). Moreover, downregulation of Xiap did not attenuate Akt2-mediated chemoresistance. Thus, while Xiap is not required for the anti-apoptotic effects of Akt2 activation, Akt2 *is* required for full implementation of Xiap-mediated chemoresistance. We are currently investigating the mechanism(s) by which Xiap is downregulated in response to cisplatin and by which Akt2 functions to inhibit this process.

In addition, our work provides evidence that reversal of chemoresistance can be achieved by inhibiting the function of either Xiap and/or Akt, but that this process requires a functional p53 to be effective. It is likely, therefore, that suppression of Akt (or Xiap) sensitizes cells to p53-mediated apoptosis following cisplatin challenge. Thus, in cells expressing mutant p53 or treated with PFT, Akt or Xiap downregulation has no effect on cell death because p53 is non-functional. In tumours expressing wild-type p53, suppression of Akt or Xiap could lead to reversal of chemoresistance. However, our data suggests that modulation of Akt activity may be a more effective mechanism of

overcoming chemoresistance, since Akt2 appears to control Xiap levels but can exert its anti-apoptotic effects in the absence of Xiap.

Thus, our work has demonstrated that Akt is a key regulator of cisplatin sensitivity, both through its ability to inhibit cisplatin-induced apoptosis by itself, and through its implication as a downstream intermediate of Xiap-mediated chemoresistance. Furthermore, we have shown that p53 is an important determinant of the response to Akt downregulation in these cells, and we provide evidence that the increase in p53 content due to cisplatin is negatively regulated by Akt. In addition, we show that Akt2 can regulate the effects of cisplatin on Xiap content and that the failure of Xiap to be downregulated by cisplatin in chemoresistant cells is an Akt2-dependent phenomenon.

In conclusion, our work provides evidence that p53 is an important determinant of Akt-mediated chemoresistance and that Xiap and Akt are important, intimately related regulators of the cellular response to cisplatin. Both p53 mutation and chemoresistance occur at extremely high frequencies in ovarian cancer. Our data suggests that overcoming chemoresistance by modulation of Xiap and/or Akt will, in some way, involve the restoration of p53 function in p53 mutant or null cells.

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List of References

1. Yuan ZQ, Sun M, Feldman RI, et al. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. Oncogene 2000;19(19):2324-30.
2. Lasky T, Silbergeld E. P53 mutations associated with breast, colorectal, liver, lung, and ovarian cancers. Environ Health Perspect 1996;104(12):1324-31.
3. Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. Nature 1997;388(6639):300-4.
4. Li J, Feng Q, Kim JM, et al. Human ovarian cancer and cisplatin resistance: possible role of inhibitor of apoptosis proteins. Endocrinology 2001;142(1):370-80.
5. Sasaki H, Sheng Y, Kotsuji F, Tsang BK. Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. Cancer Res 2000;60(20):5659-66.
6. Asselin E, Mills GB, Tsang BK. XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. Cancer Res 2001;61(5):1862-8.
7. Datta SR, Dudek H, Tao X, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91(2):231-41.
8. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 1997;278(5338):687-9.
9. Jiang K, Coppola D, Crespo NC, et al. The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. Mol Cell Biol 2000;20(1):139-48.
10. Tang Y, Zhou H, Chen A, Pittman RN, Field J. The Akt proto-oncogene links Ras to Pak and cell survival signals. J Biol Chem 2000;275(13):9106-9.
11. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999;96(6):857-68.
12. Suhara T, Kim HS, Kirshenbaum LA, Walsh K. Suppression of Akt signaling induces Fas ligand expression: involvement of caspase and Jun kinase activation in Akt-mediated Fas ligand regulation. Mol Cell Biol 2002;22(2):680-91.
13. Cheng JQ, Godwin AK, Bellacosa A, et al. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci U S A 1992;89(19):9267-71.
14. Yuan ZQ, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ. AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: implication of AKT2 in chemoresistance. J Biol Chem 2003;278(26):23432-40.
15. Krieg T, Landsberger M, Alexeyev MF, Felix SB, Cohen MV, Downey JM. Activation of Akt is essential for acetylcholine to trigger generation of oxygen free radicals. Cardiovasc Res 2003;58(1):196-202.

16. Hu L, Hofmann J, Lu Y, Mills GB, Jaffe RB. Inhibition of phosphatidylinositol 3'-kinase increases efficacy of paclitaxel in in vitro and in vivo ovarian cancer models. Cancer Res 2002;62(4):1087-92.
17. Arango D, Corner GA, Wadler S, Catalano PJ, Augenlicht LH. c-myc/p53 interaction determines sensitivity of human colon carcinoma cells to 5-fluorouracil in vitro and in vivo. Cancer Res 2001;61(12):4910-5.
18. Komarov PG, Komarova EA, Kondratov RV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 1999;285(5434):1733-7.
19. Zhang M, Liu W, Ding D, Salvi R. Pifithrin-alpha supresses p53 and protects cochlear and vestibular hair cells from cisplatin-induced apoptosis. Neuroscience 2003;120(1):191-205.
20. Kmet LM, Cook LS, Magliocco AM. A review of p53 expression and mutation in human benign, low malignant potential, and invasive epithelial ovarian tumors. Cancer 2003;97(2):389-404.
21. Wen SF, Mahavni V, Quijano E, et al. Assessment of p53 gene transfer and biological activities in a clinical study of adenovirus-p53 gene therapy for recurrent ovarian cancer. Cancer Gene Ther 2003;10(3):224-38.
22. Ogawara Y, Kishishita S, Obata T, et al. Akt enhances Mdm2-mediated ubiquitination and degradation of p53. J Biol Chem 2002;277(24):21843-50.
23. Qin LF, Ng IO. Induction of apoptosis by cisplatin and its effect on cell cycle-related proteins and cell cycle changes in hepatoma cells. Cancer Lett 2002;175(1):27-38.
24. Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. Embo J 1999;18(19):5242-51.
25. Schneiderman D, Kim J-M, Senterman M, Tsang BK. Sustained suppression of Fas ligand expression in cisplatin-resistant human ovarian surface epithelial cancer cells. Apoptosis 1999;4(4):271-82.
26. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 2000;288(5467):874-7.
27. Wang Y, Chan S, Tsang BK. Involvement of inhibitory nuclear factor-kappaB (NFkappaB)-independent NFkappaB activation in the gonadotropic regulation of X-linked inhibitor of apoptosis expression during ovarian follicular development in vitro. Endocrinology 2002;143(7):2732-40.

Appendix to Chapter 4

All data presented in this section is reported as the mean ± standard error of the mean (SEM).

Statistical analysis of the data presented in figures 1 and 2 was performed as described in Materials and Methods. However, due to space constraints, it is not possible to show the results of all possible statistical comparisons. Certain comparisons were included to highlight particularly important results, such as that between control and CDDP-treated cells, or cells infected with XIAP sense adenovirus vs. control (LacZ) adenovirus. We have attempted to show the most relevant statistical information.

Both the A2780-AAkt2 and A2780-PHM6 cell lines were derived as G418-resistant clones of the parental A2780s cell line, which possesses wild-type p53 (see chapter 7).

Chapter 5 – Submitted for publication to Oncogene

Akt Promotes Cisplatin Resistance in Human Ovarian Cancer Cells Through Inhibition of p53 Phosphorylation and Nuclear Function

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Running Title: Regulation of p53 by Akt in Ovarian Cancer Cells

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Key Words: ovarian cancer, apoptosis, p53, Akt, cisplatin

Abbreviations Used

TP53, tumour protein 53; CDDP, cis-diaminedichloroplatinum; IAP, inhibitor of apoptosis protein; GAPDH, glyceraldehyde phosphate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RNAi, RNA interference; siRNA, small inhibitory RNA; DMEM, Dulbecco's Modified Eagle Medium; ECL, enhanced chemiluminescence; PFT, pifithrin-alpha hydrobromide; PBS, phosphate-buffered saline; Xiap, X-Linked Inhibitor of Apoptosis Protein; DN-Akt, dominant-negative Akt; MDM2, murine double minute-2; PUMA, p53-Upregulated Modulator of Apoptosis; RPMI, Roswell Park Memorial Institute; PI3K, Phosphatidylinositol-3-OH-kinase; M-MuLV, Moloney Murine Leukemia Virus; RT-PCR, Reverse Transcriptase Polymerase Chain Reaction

Abstract

Resistance to cisplatin-based chemotherapy is a major cause of treatment failure in human ovarian cancer. Wild-type *TP53* status is often, but not always, associated with cisplatin sensitivity, suggesting that additional factors may be involved. Overexpression/activation of the phosphatidylinositol-3-kinase/Akt pathway is commonly observed in ovarian cancer and Akt activation is a determinant of chemoresistance in ovarian cancer cells, an effect that may result from its inhibitory actions on p53-dependent apoptosis. To that end, we examined the role and regulation of p53 in chemosensitive ovarian cancer cells, as well as in their chemoresistant counterparts, and investigated if and how Akt influences this pathway. Cisplatin induced apoptosis in chemosensitive, but not chemoresistant cells, and this was inhibited by down-regulation of p53. Cisplatin up-regulated PUMA in a p53-dependent manner, and the presence of PUMA was necessary, but not sufficient for cisplatin-induced apoptosis. p53 was phosphorylated on numerous N-terminal residues, including Ser15, Ser20, and Ser37 in response to cisplatin in chemosensitive, but not chemoresistant cells. Furthermore, activation of Akt inhibited the cisplatin-induced up-regulation of PUMA, and suppressed cisplatin-induced p53 phosphorylation, while inhibition of Akt increased total and phospho-p53 contents and sensitized p53 wild-type, chemoresistant cells to cisplatin-induced apoptosis. Finally, mutation of Ser15 and/or Ser20, but not of Ser37, to alanine significantly attenuated the ability of p53 to facilitate CDDP-induced apoptosis, and this was independent of PUMA expression. These results support the hypothesis that p53 is a determinant of CDDP sensitivity, and suggest that Akt contributes to chemoresistance, in part, by attenuating p53-mediated PUMA up-regulation and

phosphorylation of p53, which are essential, but independent determinants of sensitivity to CDDP-induced apoptosis.

Introduction

Resistance to cisplatin (CDDP)-based chemotherapy is a major cause of treatment failure in human ovarian cancer. While the molecular mechanisms underlying chemoresistance are poorly understood, induction of apoptosis is an integral effect of CDDP chemotherapy (Sato et al., 1999), and alterations in the apoptotic capacity of the cancer cell are frequently observed, and are key determinants of chemosensitivity (Dan et al., 2004; Fraser et al., 2003; Sasaki et al., 2000).

The *TP53* gene product p53 is a tumour suppressor, the function of which has been associated with chemosensitivity and improved clinical prognosis in human ovarian cancer (Fraser et al., 2003; Perego et al., 1996; Righetti et al., 1996; Song et al., 1999). Indeed, our previous data suggests that p53 is a determinant of CDDP sensitivity in ovarian cancer cells. However, wild-type *TP53* status alone is not a universal predictor of chemotherapeutic response (Fraser et al., 2003; Sasaki et al., 2000), suggesting that additional mechanisms, unrelated to *TP53* genotype, play important roles in regulating CDDP sensitivity.

p53-mediated apoptosis occurs via transcriptional activation of target gene products such as Bax (Miyashita et al., 1994), p53-Upregulated Modulator of Apoptosis (PUMA), and NOXA (Villunger et al., 2003; Wong et al., 2005), transcriptional repression of gene products such as Bcl-2 (Miyashita et al., 1994) and survivin (Hoffman et al., 2002; Mirza et al., 2002), and via a transcription-independent mechanism whereby p53 directly binds to Bcl-2 and Bcl-XL at the mitochondria (Erster et al., 2004; Mihara et al., 2003), thus inhibiting their anti-apoptotic effects. Recent evidence suggests that up-regulation of PUMA is an important mechanism of CDDP-induced apoptosis (Jiang et al.,

2006). However the mechanisms by which p53-induced PUMA up-regulation is regulated, and whether PUMA is sufficient for CDDP-induced apoptosis have not been addressed. Additional transcription-independent mechanisms of p53-induced apoptosis, such as direct activation of Bax in the cytosol, have also been proposed (Chipuk et al., 2005). p53-mediated apoptosis is dependent upon the phosphorylation of several N-terminal residues, including Ser15, Ser20, and Ser37 (Bulavin et al., 1999; Shono et al., 2002; Unger et al., 1999b). Thus, p53 phosphorylation status may profoundly affect the apoptotic capacity of the cancer cell.

Akt is a serine/threonine protein kinase that is activated in a PI3K-dependent manner by growth factors and cytokines, and is implicated in cell proliferation and survival. The PI3K/Akt pathway is frequently over-expressed/activated in human ovarian cancer (Bellacosa et al., 1995; Cheng et al., 1992; Sun et al., 2001b; Yuan et al., 2000), and we and others previously demonstrated that activation of Akt promotes a chemoresistant phenotype and that inhibition of Akt sensitizes chemoresistant cells to CDDP-induced apoptosis (Asselin et al., 2001a; Dan et al., 2004; Fraser et al., 2003; Yuan et al., 2003a). However, our data also suggests that the ability to sensitize resistant cells to CDDP in this way depends upon the presence of a functional p53 (Fraser et al., 2003), suggesting a functional relationship between Akt and p53 with respect to the determination of chemosensitivity.

While Akt can modulate p53 content by phosphorylating MDM2, thus promoting the ubiquitin/proteasome-dependent proteolysis of p53 (Gottlieb et al., 2002; Mayo & Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001), other evidence suggests that Akt can regulate p53 function, independently of changes in its content or sub-cellular

localization (Yamaguchi et al., 2001). Since phosphorylation of p53 is required for its pro-apoptotic effects, it is possible that Akt may influence this process, thereby attenuating p53-mediated apoptosis.

We investigated the hypothesis that activated Akt negatively regulates CDDP-induced apoptosis and confers CDDP resistance in cultured ovarian cancer cells by attenuating p53 phosphorylation and activation of p53-responsive gene products. We show that p53 is required for CDDP-induced apoptosis in human ovarian cancer cells and that this is dependent upon the induction of PUMA. Moreover, CDDP induces phosphorylation of p53 on multiple residues in chemosensitive ovarian cancer cells, but not the respective chemoresistant variants. The up-regulation of both PUMA and p53 phosphorylation was attenuated by activation of Akt. Finally, we show that Ser15 and Ser20 are required for maximal CDDP-induced apoptosis, an effect that was independent of the regulation of PUMA.

Taken together, these data have important implications for our understanding of the molecular mechanisms of chemoresistance in human ovarian cancer cells, and in particular, the role of Akt in this process. Since chemoresistance is a major hurdle to the success of clinical therapy for this disease, it is critical to understand precisely how resistant cells may evade the normal execution of apoptosis.

Results

p53 is required for CDDP-Induced Apoptosis in Human Ovarian Cancer Cells

Wild-type *TP53* genotype is frequently associated with chemosensitivity in human ovarian cancer cells and tumours. However, ovarian tumors and cell lines bearing wild-type p53 often possess a chemoresistant phenotype, suggesting that p53 may be

necessary, but not sufficient for chemosensitivity, and that additional mechanisms of chemoresistance in addition to altered *TP53* genotype likely exist (Fraser et al., 2003; Righetti et al., 1996; Sasaki et al., 2000).

To determine whether CDDP-induced apoptosis involves p53-mediated events, p53 wild-type, chemosensitive (OV2008) human ovarian cancer cells were transfected with p53-specific siRNA (0-100 nM; 24h) or a control siRNA, and then treated with CDDP (0, 5 μM; 24h). As shown in figure 1A, in the presence of the control siRNA, CDDP up-regulated p53 and the p53-responsive gene product PUMA (lane 1 vs. lane 2), a BH3-only antagonist of the anti-apoptotic Bcl-2 family members which is a critical intermediate of p53-mediated apoptosis (Villunger et al., 2003; Wong et al., 2005), and significantly increased the percentage of cells showing apoptotic morphology, as assessed by Hoescht 33258 nuclear staining (1.24 ± 0.25% vs. 29.9 ± 3.97%, $p<0.001$). By contrast, transfection of p53 siRNA markedly down-regulated p53 and attenuated the CDDP-induced up-regulation of PUMA. Furthermore, CDDP-induced apoptosis was significantly inhibited by p53 siRNA ($p<0.05$). We next repeated the above experiment in the chemosensitive A2780s cell line. As expected, p53 siRNA down-regulated p53 in both control and CDDP-treated cells, and significantly inhibited CDDP-induced apoptosis (10.6 ± 0.63% vs. 3.89 ± 0.54%, $p<0.05$; figure 1B).

The A2780cp cell line is a variant of the A2780s cell line, which expresses a heterozygous mutation in the p53 gene. While Skilling et al originally reported a V173F mutation in this cell line (Skilling et al., 1996b), direct sequencing of this site in our cell

line demonstrated the presence of a V172F mutant (data not shown), which is consistent with the results published by Siddik et al (Siddik et al., 1998).

Fig 1. Regulation of CDDP-Induced PUMA Up-Regulation and Apoptosis by p53. (A) Chemosensitive OV2008 cells were transfected with p53-specific or control siRNA (0-100 nM) for 48h, followed by treatment with CDDP (0, 5 μM; DMSO control) for a further 24h. p53, PUMA, and GAPDH contents were assessed by Western blotting, and apoptosis was determined by Hoescht 33258 staining and UV microscopy; (B) Chemosensitive A2780s cells were transfected with p53-specific or control siRNA (0-50 nM) for 24h, followed by treatment with CDDP (0, 5 μM) for a further 24h. p53 and GAPDH contents were assessed by Western blotting, and apoptosis was determined as above; (C) Wild-type p53 A2780s cells and the p53 mutant, chemoresistant variant cell line A2780cp were treated with CDDP (0-10 μM) for 24h. p53, PUMA, and GAPDH contents were assessed by Western blot, and apoptosis was determined as above. * - $p<0.05$, *** - $p<0.0001$

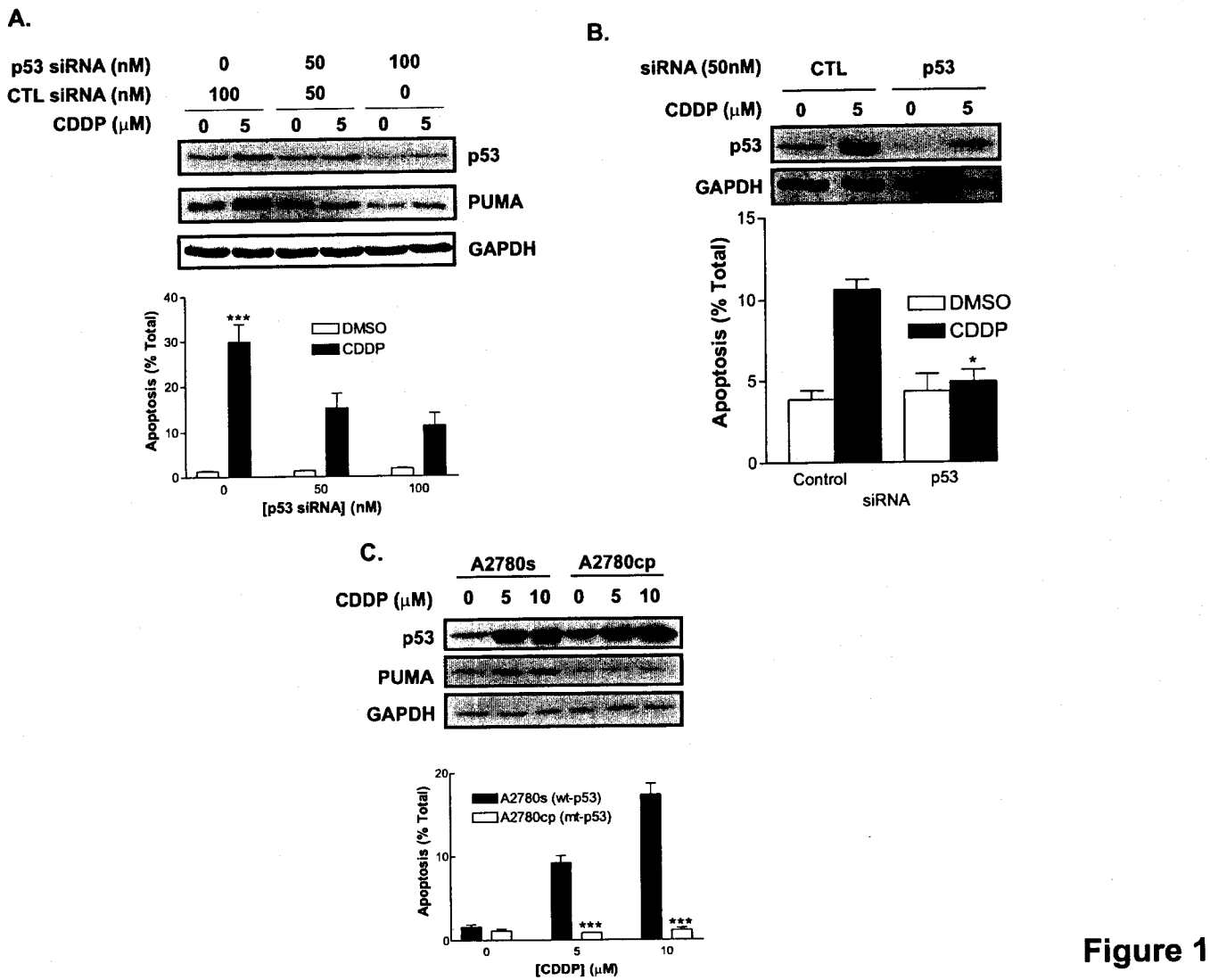

A.
p53 siRNA (nM) 0 50 100
CTL siRNA (nM) 100 50 0
CDDP (μM) 0 5 0 5 0 5
p53
PUMA
GAPDH
Apoptosis (% Total)
DMSO
CDDP
***
[p53 siRNA] (nM)
B.
siRNA (50nM) CTL p53
CDDP (μM) 0 5 0 5
p53
GAPDH
Apoptosis (% Total)
DMSO
CDDP
*
Control
p53
siRNA
C.
A2780s A2780cp
CDDP (μM) 0 5 10 0 5 10
p53
PUMA
GAPDH
A2780s (wt-p53)
A2780cp (mt-p53)
Apoptosis (% Total)
***
***
[CDDP] (μM)

Figure 1

To further examine the role of p53 in CDDP sensitivity, A2780s and A2780cp cells were cultured in the presence of CDDP (0, 5, 10 μM; 24h). Apoptosis was determined as above. Moreover, p53 and PUMA contents were examined by Western blot. As shown in figure 1C, CDDP up-regulated p53 and PUMA and induced apoptosis in the A2780s cells, whereas this was significantly attenuated in the A2780cp cells ($p<0.001$). Consistent with the presence of mutant p53 in the latter cell line, total p53 levels were higher in the absence of CDDP compared to the wild-type A2780s cells, likely due to loss of MDM2-mediated negative feedback against p53 (Honda et al., 1997; Sasaki et al., 2000).

Taken together, these results demonstrate that p53 is critical for sensitivity to CDDP-induced apoptosis in ovarian cancer cells

PUMA is necessary but not sufficient for CDDP-induced apoptosis

Since PUMA is a critical intermediate of p53-mediated apoptosis, and is up-regulated by CDDP in a p53-dependent manner in ovarian cancer cells, we next wanted to determine its importance for sensitivity to CDDP-induced apoptosis. A2780s cells were transfected with siRNA targeted to PUMA (0-100 nM; 48h), and then treated with CDDP (0, 10 μM; 24h). The effect of the siRNAs on the levels of PUMA was confirmed by Western blot. As shown in figure 2A, down-regulation of PUMA significantly attenuated CDDP-induced apoptosis ($18.1 \pm 0.48\%$ vs. $4.68 \pm 0.81\%$, $p<0.001$, all effects), demonstrating that PUMA is critical for sensitivity to CDDP-induced apoptosis.

Both the OV2008 chemosensitive ovarian cancer cell line and its resistant variant counterpart, C13*, express wild-type p53 (Sasaki et al., 2000). To further examine the effects of CDDP on p53-mediated transactivation and apoptosis, these cells were treated

Fig 2. PUMA is Necessary, But Not Sufficient, for CDDP-Induced Apoptosis.

(A) A2780s cells were transfected with PUMA-specific or control siRNA (0-50 nM) for 24h, followed by treatment with CDDP (0, 10 μM; DMSO control) for a further 24h. PUMA, p53, and GAPDH contents were assessed by Western blot, and apoptosis was assessed by Hoescht 33258 staining and UV microscopy; **(B)** Chemosensitive OV2008 cells and their p53 wild-type, chemoresistant variant cell line C13* were treated with CDDP (0-10 μM) for 24h. p53, PUMA, and GAPDH contents were assessed by Western blot, and apoptosis was determined as above; **(C)** OV2008 and C13* cells were treated with CDDP (0-10 μM) for 24h. Nuclear lysates were obtained, normalized for protein content, and p53-DNA binding was assessed as detailed in Materials and Methods. p53 contents in nuclear lysates were also determined by Western blot, using C23 (nucleolin) as a loading control. *** - $p<0.0001$

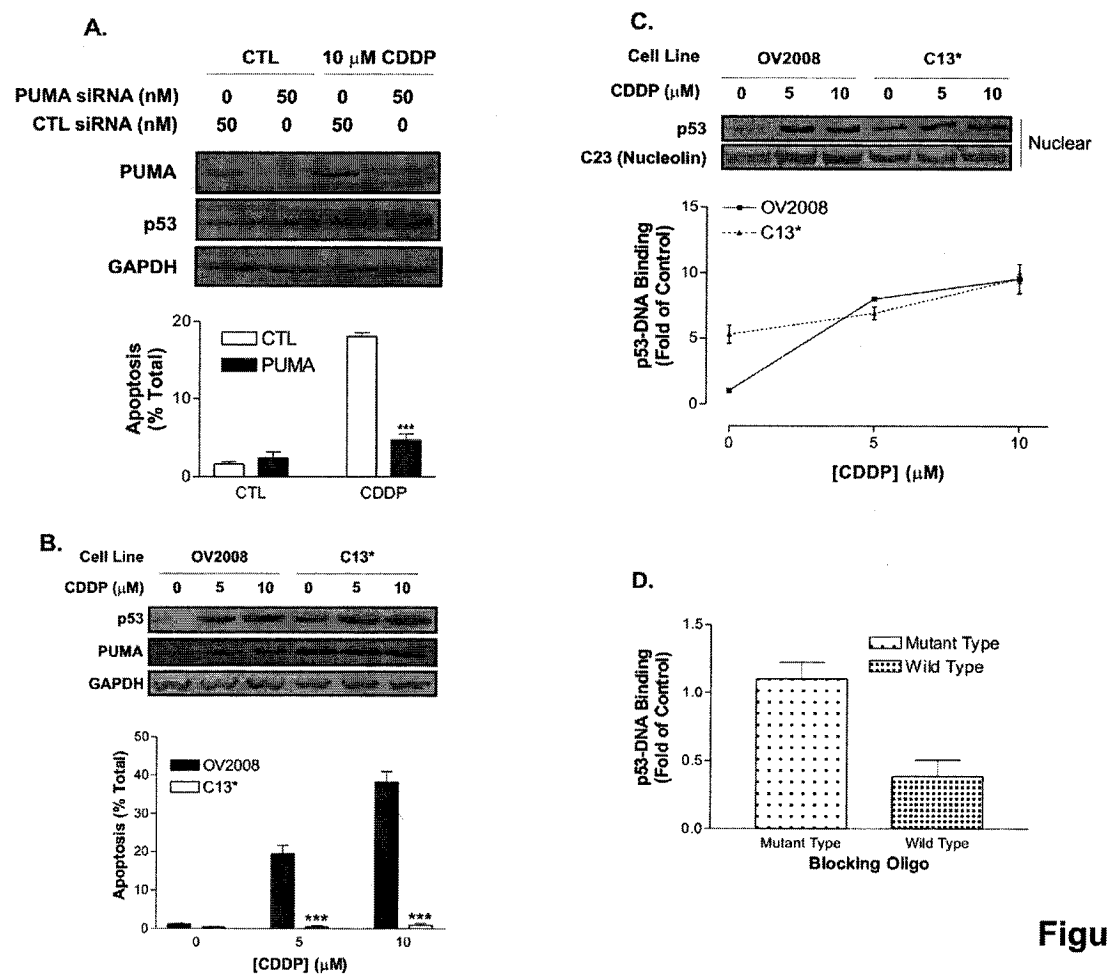

A.
CTL
10 μM CDDP
PUMA siRNA (nM) 0 50 0 50
CTL siRNA (nM) 50 0 50 0
PUMA
p53
GAPDH
Apoptosis (% Total)
CTL
PUMA
CTL
CDDP
B.
Cell Line
OV2008
C13*
CDDP (μM) 0 5 10 0 5 10
p53
PUMA
GAPDH
Apoptosis (% Total)
OV2008
C13*
[CDDP] (μM)
C.
Cell Line
OV2008
C13*
CDDP (μM) 0 5 10 0 5 10
p53
C23 (Nucleolin)
Nuclear
p53-DNA Binding (Fold of Control)
OV2008
C13*
[CDDP] (μM)
D.
p53-DNA Binding (Fold of Control)
Mutant Type
Wild Type
Mutant Type
Wild Type
Blocking Oligo

Figure 2

with CDDP (as above) and the protein contents of p53 and PUMA were examined by Western blot. As shown in figure 2B, p53 content was markedly higher in the resistant C13* cell line compared to the sensitive OV2008 cell line in the absence of CDDP. Furthermore, CDDP markedly up-regulated p53 in OV2008 cells, but only slightly up-regulated p53 in the resistant C13* cells. Consistent with these results, while CDDP up-regulated PUMA in the chemosensitive OV2008 cells, C13* cells expressed much higher basal levels of PUMA, but these levels were refractory to CDDP. This suggests that the expression and up-regulation of PUMA is insufficient to induce apoptosis or to support a chemosensitive phenotype. To help confirm whether these PUMA content data were associated with the biological function of p53, p53-DNA binding was determined using the TransAM p53 kit as described in Materials and Methods. Nuclear p53 content was also examined by Western blot. As shown in figure 2C, p53 accumulated in the nucleus of both OV2008 and C13* cells in response to CDDP ($p<0.05$), although basal nuclear p53 content was higher in the C13* cells ($p<0.05$). Furthermore, basal p53-DNA binding was greater than 5-fold higher in the C13* cells relative to the OV2008 cells ($p<0.01$), which may explain the high basal levels of PUMA in these cells. However, CDDP up-regulated p53-DNA binding more effectively in the chemosensitive OV2008 cells than in the resistant variant C13* cells ($p<0.01$), which is consistent with the pattern of CDDP-induced PUMA up-regulation in these cells. The specificity of the assay for p53 was confirmed by introduction of free wild-type p53 consensus oligonucleotide, which competed out p53 binding to the immobilized oligo. Mutated p53 consensus oligonucleotide did not affect p53 binding to the immobilized oligo (figure 2D). Thus, C13* cells have high basal p53-DNA binding activity, but are unable to mount an

effective increase in this response to CDDP. These results suggest that the presence of p53 nuclear function and PUMA *alone* is insufficient to support a CDDP sensitive phenotype, suggesting the existence of additional mechanisms of CDDP-induced, p53-dependent apoptosis in these cells.

Activation of Akt Attenuates CDDP-Induced p53 Nuclear Function and Apoptosis

Activation of the PI3K/Akt pathway is observed in a high percentage of human ovarian cancers (Bellacosa et al., 1995; Cheng et al., 1992; Sun et al., 2001b; Yuan et al., 2000), and we and others have previously demonstrated that activation of Akt is a determinant of chemoresistance in human ovarian cancer cells (Asselin et al., 2001a; Dan et al., 2004; Fraser et al., 2003; Yuan et al., 2003a). Furthermore, our previous data suggests that Akt-mediated chemoresistance may, in part, be mediated through alterations in p53-mediated apoptosis (Fraser et al, 2003). To that end, we next examined the effects of Akt activation on CDDP-induced p53-mediated gene expression using the A2780s cell line, stably transfected with empty pcDNA3 vector (A2780-CTL) or pcDNA3 containing constitutively active Akt2 (A2780-AAkt2). The phenotype of these cells has been extensively characterized (Dan et al., 2004; Fraser et al., 2003; Yuan et al., 2003a). As shown in figure 3A, CDDP markedly up-regulated p53 and PUMA and induced apoptosis in the control-transfected cells. By contrast, CDDP only slightly up-regulated p53 in the A2780-AAkt2 cells, although basal p53 levels were higher in the presence of active Akt2. Moreover, despite the presence of ample p53, active Akt2 attenuated the CDDP-induced up-regulation of PUMA, and significantly inhibited CDDP-induced apoptosis (15.5 ± 0.97% vs. 1.53 ± 0.22% at 10 μM CDDP, $p<0.001$). To evaluate the kinetics of CDDP-induced PUMA up-regulation, and the effects of Akt activation on this parameter, we

Fig 3. Akt Inhibits the CDDP-Induced Activation of p53 Nuclear Function.

(A) A2780s cells stably transfected with control vector (A2780-CTL; Ctl) or constitutively activated Akt2 (A2780-AAkt2; Akt) were treated with CDDP (0-10 μM; DMSO control) for 24h. p53, PUMA, and GAPDH contents were examined by Western blot, and apoptosis was determined by Hoescht 33258 staining and UV microscopy; **(B)** A2780-CTL and A2780-AAkt2 cells were treated with CDDP (10 μM) for various durations (0-24h). p53, PUMA, MDM2, and GAPDH contents were assessed by Western blot; **(C)** A2780-CTL and A2780-AAkt2 cells were treated with CDDP (10 μM or DMSO; 0-6h). Total RNA was extracted and reverse transcribed, and PUMA and β-actin mRNA contents were assessed by endpoint RT-PCR as indicated in Materials and Methods; **(D)** A2780-CTL and A2780-AAkt2 cells were treated with CDDP (0-10 μM) for 24h. Nuclear lysates were obtained, normalized for protein content, and p53-DNA binding was assessed as detailed in Materials and Methods. p53 contents in nuclear lysates were also determined by Western blot, using C23 (nucleolin) as a loading control. * - $p<0.05$, *** - $p<0.0001$

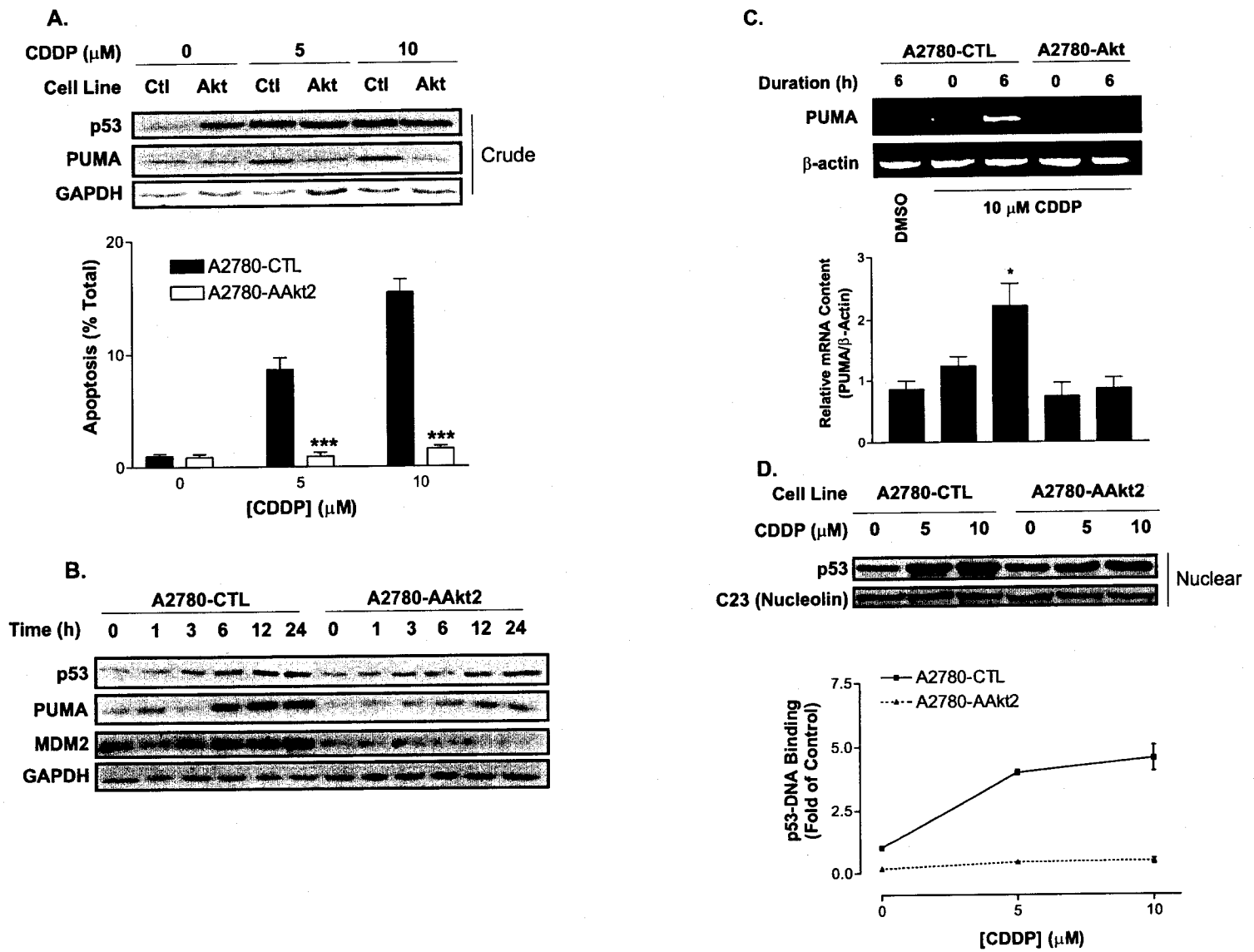

A.
CDDP (μM)
0
5
10
Cell Line
Ctl
Akt
Ctl
Akt
Ctl
Akt
p53
PUMA
GAPDH
Crude
Apoptosis (% Total)
A2780-CTL
A2780-AAkt2
***
***
[CDDP] (μM)
B.
A2780-CTL
A2780-AAkt2
Time (h)
0 1 3 6 12 24 0 1 3 6 12 24
p53
PUMA
MDM2
GAPDH
C.
A2780-CTL
A2780-Akt
Duration (h)
6 0 6 0 6
PUMA
β-actin
DMSO
10 μM CDDP
Relative mRNA Content (PUMA/β-Actin)
*
D.
Cell Line
A2780-CTL
A2780-AAkt2
CDDP (μM)
0 5 10 0 5 10
p53
C23 (Nucleolin)
Nuclear
p53-DNA Binding (Fold of Control)
A2780-CTL
A2780-AAkt2
[CDDP] (μM)

Figure 3

next performed a time course analysis PUMA protein content in response to CDDP in A2780-CTL or A2780-AAkt2 cells. We also examined the protein content of MDM2 over the same time course. As shown in figure 3B, PUMA up-regulation occurred between 3-6h post-CDDP in the A2780-CTL cells. Up-regulation of MDM2, the principle negative regulator of p53, occurred even earlier, with marked up-regulation within 1-3h post-CDDP. As expected, the up-regulation of these gene products was markedly lower in the A2780-AAkt2 cells, which is consistent with an inactivation of p53-mediated gene transcription in these cells. The reduction in basal MDM2 content in A2780-AAkt2 cells (0h; lane 1 vs. lane 7) may help to explain why basal p53 levels are elevated in these cells relative to the control-transfected cells.

To provide further evidence that the effects of Akt on CDDP-induced PUMA content were due to changes in its p53-dependent transcription, we next evaluated the effects of Akt activation on PUMA mRNA content by RT-PCR. A2780-CTL and A2780-AAkt2 cells were cultured in the presence of CDDP (10 μM) for 6h, and PUMA and β-actin mRNA contents were assessed (as per Materials and Methods). As expected, 6h of CDDP treatment significantly up-regulated the mRNA content of PUMA in A2780-CTL cells (figure 3C; $p<0.05$), but not in the A2780-AAkt2 cells. Six hours of culture in the absence of CDDP (DMSO alone) in A2780-CTL cells did not change PUMA mRNA content, relative to the 0h group (lane 1 vs. lane 2). β-actin mRNA content was not affected by Akt activation, CDDP treatment, or duration of culture. This result is consistent with the hypothesis that Akt attenuates the CDDP-induced up-regulation of PUMA gene transcription.

To further evaluate the effects of Akt activation on p53 biological function, we next determined the nuclear accumulation and DNA binding function of p53 in A2780-AAkt2 cells and their control-transfected counterparts. CDDP significantly increased nuclear p53 localization ($p<0.05$) and this was not significantly affected by activation of Akt. Furthermore, CDDP induced p53 binding to its consensus oligonucleotide in the A2780-CTL cells, and this was significantly attenuated in the A2780-AAkt2 (figure 3D, $p<0.001$). Taken together, these data suggest that Akt may confer resistance, in part, through down-regulation of p53-dependent PUMA gene expression.

CDDP-induced p53 Phosphorylation is Attenuated in Chemoresistant Cells

While p53-mediated up-regulation of PUMA is required for CDDP-induced apoptosis, and is attenuated by Akt activation, p53-DNA binding activity and PUMA content is present in chemoresistant, p53 wild-type C13* cells. These data suggest that p53-mediated PUMA up-regulation is necessary, but not sufficient for CDDP-induced apoptosis. To that end, we next evaluated the regulation of p53 phosphorylation, an event that is required for p53-mediated apoptosis (Unger et al., 1999b), in response to CDDP. p53 is phosphorylated on numerous N-terminal residues. However, the precise function of these phosphorylations remains unclear. Several reports suggest that phosphorylation is required for protection of p53 from MDM2-mediated ubiquitination (Ashcroft et al., 1999; Ashcroft et al., 2000; Chehab et al., 1999; Fuchs et al., 1998; Shieh et al., 1997), and phosphorylation of Ser15 and Ser20 plays an important role in the transduction of p53-mediated apoptosis (Unger et al., 1999b), although this finding is not universal, and Ser15/Ser20 phosphorylation is dispensible for p53-dependent apoptosis in some systems (Chao et al., 2006). Thus, we next examined the hypothesis that alterations

in CDDP-induced p53 phosphorylation are important determinants of chemoresistance in ovarian cancer cells. OV2008 and C13* cells were treated with CDDP (as above) and we determined total and phospho-p53 contents by Western blot using phospho-specific antibodies targeted to Ser6, Ser15, Ser20, Ser33, and Ser37. As shown in figure 4, and consistent with results shown in figure 1, basal p53 was higher in C13* cells and CDDP up-regulated p53 in both OV2008 and C13* cells, although the effects were much more pronounced in the chemosensitive OV2008 cells. However, when phospho-p53 content was analysed, we found that while CDDP effectively induced phosphorylation of all examined N-terminal residues except Ser6 in OV2008 cells, phosphorylation on these sites was markedly attenuated in C13* cells (except for Ser6), despite the presence of comparable total p53 levels in both cell lines (figure 4, top panel). This result suggests that CDDP-induced p53 phosphorylation is attenuated in chemoresistant cells, and is consistent with the hypothesis that aberrant regulation of p53 phosphorylation may be a causative factor in chemoresistance in human ovarian cancer cells. To confirm that this was not a cell-type specific effect, we further examined the CDDP-induced phosphorylation of Ser15 and Ser20 in A2780s cells and their chemoresistant counterpart, A2780cp. CDDP effectively induced p53 phosphorylation in the sensitive A2780s cells, but not in A2780cp cells (figure 4, bottom panel). Taken together, these results demonstrate that p53 phosphorylation is attenuated in chemoresistant ovarian cancer cells, and support the hypothesis that CDDP resistance may, in part, be mediated through aberrant regulation of phosphorylation at these sites.

Akt Attenuates CDDP-induced p53 Phosphorylation

While Akt activation can attenuate p53-mediated gene expression and promote a chemoresistant phenotype, whether Akt can affect p53 phosphorylation is unknown. To test these possibilities, we cultured A2780-CTL and A2780-AAkt2 cells in the absence and presence of CDDP (as above) and phospho- and total p53 contents were analysed by Western blot. As shown in figure 5A, while CDDP up-regulated total and phospho-p53 contents (Ser6, Ser15, Ser20, Ser33, and Ser37) in a concentration-dependent manner in the control A2780-CTL cells, phosphorylation at these sites (except Ser6) was markedly attenuated in the A2780-AAkt2 cells. This result demonstrates that Akt activation can inhibit CDDP-induced p53 phosphorylation, thus suggesting a novel mechanism of Akt-mediated cell survival. Furthermore, these findings are consistent with the hypothesis that down-regulation of p53 phosphorylation may, in part, contribute to Akt-mediated chemoresistance in human ovarian cancer cells.

Fig 4. CDDP-Induced p53 Phosphorylation is Attenuated in Chemoresistant Cells. **(A)** OV2008 and C13* or **(B)** A2780s and A2780cp cells were treated with CDDP (0-10 μM) for 24h, and total and phospho-p53 and contents were assessed by Western blot using antibodies directed against total p53 (DO-1) or against specific phosphorylated residues on p53, as indicated.

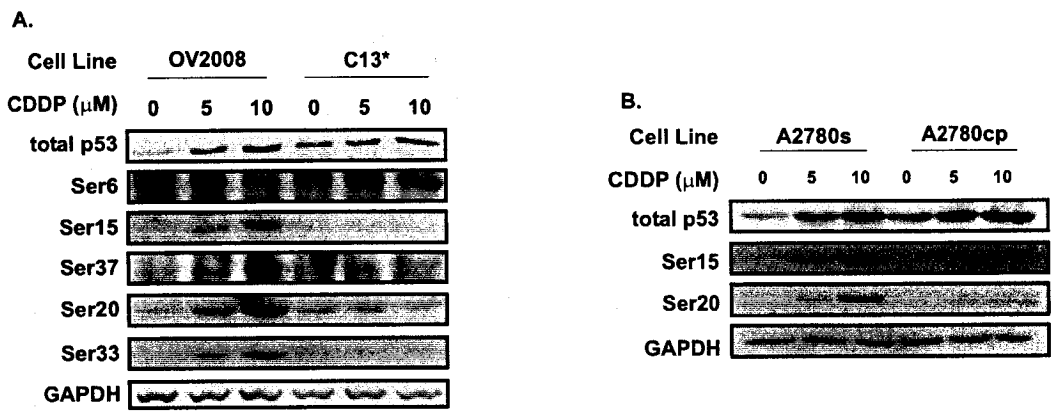

A.
Cell Line
OV2008
C13*
CDDP (μM)
0 5 10 0 5 10
total p53
Ser6
Ser15
Ser37
Ser20
Ser33
GAPDH
B.
Cell Line
A2780s
A2780cp
CDDP (μM)
0 5 10 0 5 10
total p53
Ser15
Ser20
GAPDH

Figure 4

To further examine the role of Akt on p53 phosphorylation, the chemoresistant, p53 wild-type C13* cell line was infected with adenoviral dominant-negative Akt (DN-Akt; MOI = 0-80, 48h; LacZ control) and then treated with CDDP (as above). This adenovirus encodes a kinase-dead mutant form of Akt (K179A), which has been mutated at T308 and S473 (all to alanine), which additionally cannot be activated by phosphorylation (T308A, S473A). We previously demonstrated that expression of DN-Akt sensitizes chemoresistant cells to CDDP-induced apoptosis in a p53-dependent manner (Fraser et al, 2003). Transient expression of DN-Akt was confirmed by Western blot against the HA epitope. Consistent with results from figure 2 and 4, CDDP alone failed to up-regulate p53 or to induce phosphorylation of Ser15, Ser20, or Ser37 in C13* cells infected with LacZ (figure 5B). However, expression of DN-Akt up-regulated total p53, and this p53 was phosphorylated on Ser15, Ser20, and Ser37. Moreover, while DN-Akt up-regulated phospho-p53 (Ser15) by nearly 10-fold (9.8 ± 1.2 at MOI = 80 relative to MOI = 0), total p53 was up-regulated by 1.5-fold (1.5 ± 0.02 at MOI = 80 relative to MOI = 0), which represents an increase in the ratio of phospho/total p53 of nearly 7-fold (6.8 ± 0.84 at MOI = 80 relative to MOI = 0, $p<0.0001$). However, while DN-Akt slightly increased the percentage of apoptotic cells relative to LacZ infection alone, the cells were significantly sensitized to CDDP-induced apoptosis ($p<0.01$).

Fig 5. Akt Attenuates CDDP-Induced p53 Phosphorylation.

(A) A2780-CTL (Ctl) or A2780-AAkt2 (Akt) cells were treated with CDDP (0-10 μM) for 24h, and total and phospho-p53 contents were assessed by Western blot using antibodies directed against total p53 (DO-1) or against specific phosphorylated residues on p53, as indicated; **(B)** C13* cells were infected with adenoviral HA-tagged dominant-negative Akt (DN-Akt) at various concentrations (MOI = 0-80). Adenoviral LacZ was used to normalize adenoviral load in each group. Transient expression of DN-Akt was confirmed by Western blot against the HA epitope. Total and phospho-p53 contents were assessed as above, and apoptosis was determined using Hoescht 33258 staining. Ratios of phospho-p53/total p53 were determined by calculating the fold change in each species, relative to lane 1, and then dividing the phospho-p53 fold change by the total p53 fold change. **(C)** p53 mutant A2780cp cells were infected with DN-Akt (MOI = 0, 80), using adenoviral LacZ to balance adenoviral load in each group. Transient DN-Akt expression was confirmed using Western blot against the HA epitope, and total and phospho-p53 contents were assessed as above. Ratios were calculated as in panel (B) ** - $p<0.01$, *** - $p< 0.0001$.

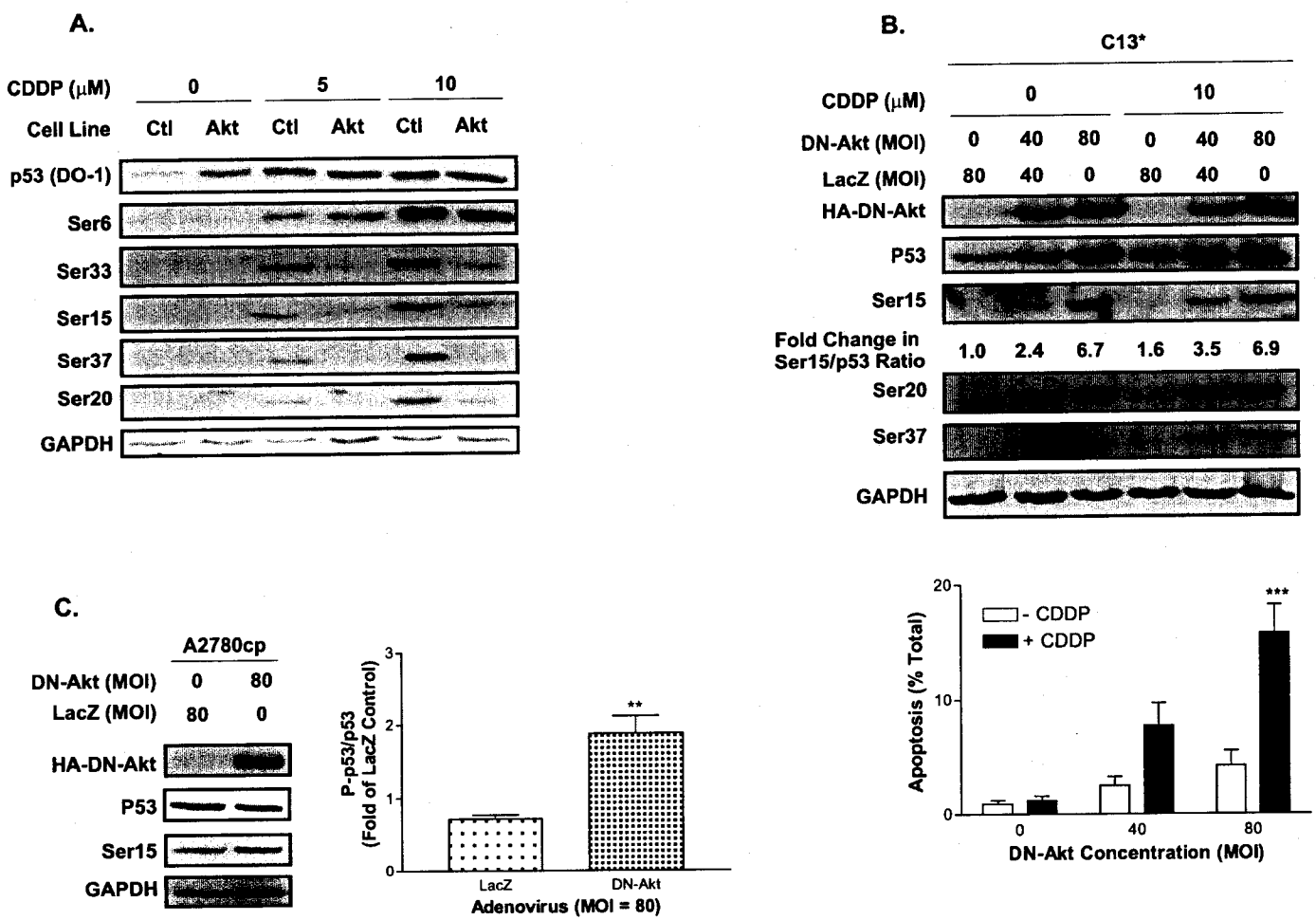

A.
CDDP (μM)
0
5
10
Cell Line
Ctl
Akt
Ctl
Akt
Ctl
Akt
p53 (DO-1)
Ser6
Ser33
Ser15
Ser37
Ser20
GAPDH
B.
C13*
CDDP (μM)
0
10
DN-Akt (MOI)
0 40 80 0 40 80
LacZ (MOI)
80 40 0 80 40 0
HA-DN-Akt
P53
Ser15
Fold Change in Ser15/p53 Ratio
1.0 2.4 6.7 1.6 3.5 6.9
Ser20
Ser37
GAPDH
C.
A2780cp
DN-Akt (MOI)
0
80
LacZ (MOI)
80
0
HA-DN-Akt
P53
Ser15
GAPDH
P-p53/p53 (Fold of LacZ Control)
0
1
2
3
**
LacZ
DN-Akt
Adenovirus (MOI = 80)
Apoptosis (% Total)
0
10
20
- CDDP
+ CDDP
***
0
40
80
DN-Akt Concentration (MOI)

Figure 5

To further confirm the effects of dominant-negative Akt expression on phospho-p53 content, chemoresistant A2780cp cells were infected with DN-Akt (MOI = 80; as above) and total and phospho-p53 (Ser15) contents were evaluated by Western blot. Since Akt can induce an MDM2-dependent down-regulation of p53 (Gottlieb et al., 2002; Mayo & Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001), and since mutant p53 cells contain only minimal MDM2 content (Sasaki et al., 2000), we surmised that DN-Akt should not increase total p53 in p53 mutant cells, thus providing an ideal system to study the effects of Akt inhibition on p53 phosphorylation. Expression of DN-Akt was confirmed by detection of the HA epitope. As expected, DN-Akt did not up-regulate p53 in these cells, but increased phosphorylation of Ser15 was observed in cells infected with DN-Akt, relative to the LacZ-infected control cells (Figure 5C). Indeed, DN-Akt increased the ratio of phospho/total p53 by greater than 2.5-fold, relative to the LacZ-infected cells ($p<0.01$), demonstrating that suppression of Akt activity specifically up-regulates p53 phosphorylation.

Taken together, these results suggest that Akt inhibits the CDDP-induced phosphorylation of p53, and offers a novel paradigm for Akt-mediated chemoresistance and cell survival.

Phosphorylation of Ser15 and Ser20 is required for optimal CDDP-induced, p53-dependent apoptosis

Phosphorylation of Ser15 and Ser20 is critical for the induction of p53-mediated apoptosis (Unger et al., 1999b). Since we observed attenuated CDDP-induced phosphorylation of these residues in chemoresistant cells, relative to their sensitive counterparts, we next determined whether these sites were critical mediators of the

observed apoptotic response using various p53 mutant expression vectors constructed by site-directed mutagenesis (as described in Materials and Methods). The requirement for p53 phosphorylation in the restoration of chemosensitivity was determined using CDDP-resistant, p53 mutant (Yaginuma & Westphal, 1992) OVCAR-3 cells, transiently transfected with HA-tagged WT, S15A, S20A, S37A-p53 or a combined S15A/S20A-p53 (pcDNA3.1 empty vector control) for 24h, treated with CDDP (10 μM) for a further 24h, and assessing apoptosis as above. All of the p53 constructs were expressed to equivalent levels in the absence of CDDP, as measured by Western blot against the HA epitope, and this was not affected by treatment with CDDP. Consistent with their CDDP resistant phenotype, CDDP alone failed to induce apoptosis in OVCAR-3 cells (figure 6A). Expression of the p53 constructs alone likewise did not increase the number of apoptotic cells, relative to the control vector. However, transfection with wild-type p53 significantly sensitized the cells to CDDP-induced apoptosis ($p<0.001$). Furthermore, this effect of wild-type p53 was significantly, although not completely, blocked by mutation of Ser15 or Ser20 to alanine ($p<0.001$), while mutation of Ser37 to alanine had no effect on p53-mediated sensitization ($p>0.05$). This result strongly supports the hypothesis that p53 phosphorylation is required for CDDP sensitivity, and suggests that Ser15 and Ser20 are key amino acids involved. Interestingly, mutation of both Ser15 and Ser20 to alanine did not significantly reduce CDDP-induced apoptosis relative to the S15A mutant ($6.7 \pm 0.85\%$ vs. $4.7 \pm 1.5\%$, $p>0.05$), but did significantly attenuate CDDP-induced apoptosis relative to S20A ($9.4 \pm 1.0\%$ vs. $4.7 \pm 1.5\%$, $p<0.01$), suggesting that both Ser15 and Ser20 are critical for sensitivity to CDDP-induced apoptosis. Furthermore, CDDP alone did not up-regulate PUMA in OVCAR-3 cells,

which is consistent with their p53 mutant genotype. However, all of the exogenously expressed HA-p53 constructs up-regulated PUMA to similar levels, further suggesting that the observed dependence of CDDP-induced, p53-mediated apoptosis on p53 phosphorylation is not mediated through changes in PUMA expression, which is consistent with the results obtained in C13* cells (figure 2).

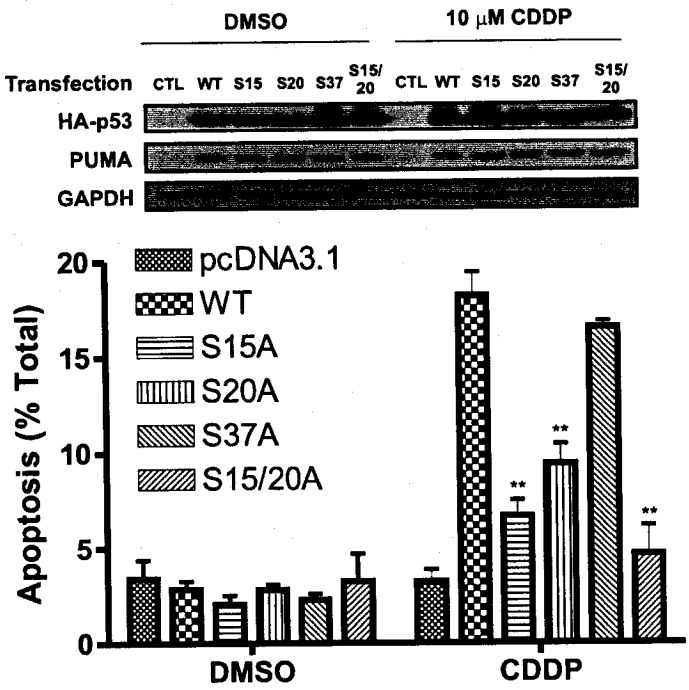
To confirm that these constructs were phosphorylated, and that mutation to alanine prevents these phosphorylations, we transfected OVCAR-3 cells with the HA-p53 constucts followed by treatment with CDDP (10 μM), and then immunoprecipitated HA-p53 using an agarose-immobilized goat polyclonal anti-HA antibody (goat IgG control), followed by Western blot against total and phospho-p53, using specific antibodies. We detected both exogenous and endogenous p53 when the immunoprecipitates were probed for total p53 (figure 6B), suggesting that exogenous HA-tagged p53 and endogenous p53 interact within the cell. While endogenous p53 was phosphorylated on Ser15 and Ser20 in all groups (figure 6B; lower band; lanes 2-6), mutation to alanine blocked phosphorylation in the exogenous p53 (Figure 6B; upper band). This occurred for both Ser15 and Ser20.

Taken together, these data strongly support the hypothesis that phosphorylation of p53 on Ser15 and Ser20 in response to CDDP is required for CDDP-induced apoptosis.

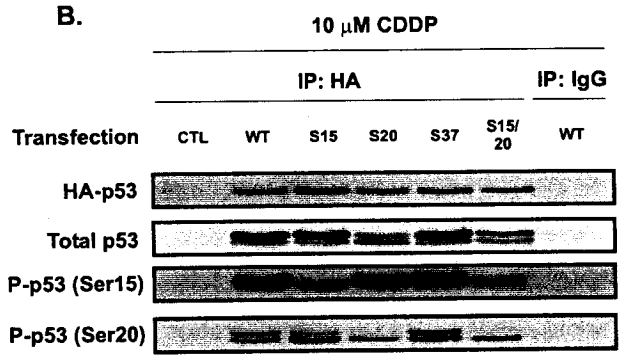
Fig 6. Phosphorylation of Ser15 and Ser20 is Required For CDDP-Induced Apoptosis.

(A) p53 mutant, chemoresistant OVCAR-3 cells were transfected with control vector (pcDNA3.1), HA-tagged wild-type p53, or HA-tagged p53 mutated serine to alanine at Ser15 (S15), Ser20 (S20), Ser37 (S37), or both Ser15 and Ser20 (S15/20). Transient expression of these constructs was confirmed by Western blot against the HA epitope. PUMA and GAPDH contents were assessed by Western blot, and apoptosis was determined using Hoescht 33258 staining and UV microscopy; **(B)** OVCAR-3 cells were transfected as above, and HA-tagged p53 was immunoprecipitated from whole-cell lysates as described in Materials and Methods. HA-p53, total p53, and phospho-p53 (Ser15 and Ser20) contents were assessed by Western blot of the HA immunoprecipitates. ** - $p<0.01$

A.



B.



Upper Bands: exogenous HA-p53
Lower Bands: endogenous p53

Figure 6

Discussion

Our data demonstrates that CDDP-induced, p53-mediated PUMA up-regulation is required for sensitivity to CDDP-induced apoptosis in ovarian cancer cells, and is inhibited by Akt activation, which is a frequently observed event in human ovarian cancer. Moreover, the presence of PUMA is necessary, but not sufficient, for CDDP-induced apoptosis. Furthermore, we provide evidence that CDDP-induced p53 phosphorylation at multiple residues is attenuated in chemoresistant ovarian cancer cells, relative to their sensitive counterparts, and that this, too, is inhibited by Akt activation. Finally, we show that Ser15 and Ser20 are required for maximal sensitivity to CDDP-induced apoptosis, and that this is independent of the regulation of PUMA. Thus, the current study suggests that p53 facilitates CDDP sensitivity possibly via two distinct pathways, one involving up-regulation of PUMA and a second, independent pro-apoptotic pathway involving phosphorylation of Ser15 and Ser20. Our results strongly suggest that Akt-mediated chemoresistance is propagated, at least in part, through inhibition of both mechanisms of p53-induced apoptosis.

Recent evidence suggests that apoptotic capacity is a central determinant of chemosensitivity in human ovarian cancer (Asselin et al., 2001a; Dan et al., 2004; Fraser et al., 2003; Sasaki et al., 2000; Sato et al., 1999). While p53 is an important regulator of apoptosis, the precise role of p53 in the determination of CDDP sensitivity in ovarian cancer cells is unclear. *TP53* mutations are commonly observed in human ovarian cancer, and are frequently associated with chemoresistance in tumours and in cultured cells (Akeshima et al., 2001; Kigawa et al., 2001; Perego et al., 1996; Reles et al., 2001; Righetti et al., 1996; Sato et al., 1999). Our own previous studies have demonstrated that

p53 is a critical regulator of cell fate in ovarian cancer cells (Fraser et al., 2005; Fraser et al., 2003; Sasaki et al., 2000), although a direct correlation between p53 mutation and chemoresistance has not been demonstrated (Lavarino et al., 2000). Thus, it is of great interest to determine if and how p53 activation contributes to the sensitivity of ovarian cancer cells to CDDP.

CDDP markedly up-regulated p53 in chemosensitive ovarian cancer cells and down-regulation of p53 by RNAi significantly attenuated CDDP-induced apoptosis in wild-type p53, chemosensitive ovarian cancer cells, suggesting that endogenous p53 is a key determinant of CDDP-induced apoptosis. These data are consistent with those of Song et al, who showed that expression of exogenous wild-type p53 in mutant p53 ovarian cancer cells sensitized them to CDDP-induced apoptosis (Song et al., 1997). Furthermore, Vasey et al showed that expression of dominant-negative p53 conferred a chemoresistant phenotype (Vasey et al., 1996). However, these studies dealt with the effects of *exogenous* p53, and did not address whether physiological levels of p53 are required for CDDP-induced apoptosis. Thus, the current study builds upon these data and provides evidence that endogenous p53 is required for the sensitivity of ovarian cancer cells to CDDP-induced apoptosis.

CDDP-induced apoptosis was associated with PUMA up-regulation in two unrelated chemosensitive ovarian cancer cell lines (A2780s and OV2008), and was attenuated by RNAi-mediated p53 down-regulation. Furthermore, PUMA was not induced by CDDP in the p53 mutant cell line A2780cp, which is consistent with a p53-dependent mechanism of up-regulation. Down-regulation of PUMA by RNAi significantly attenuated CDDP-induced apoptosis. These data are consistent with

previous studies showing that PUMA knockout mice are resistant to p53-dependent apoptosis and show a similar apoptotic phenotype to p53 knockout mice (Villunger et al., 2003). While our evidence strongly suggests that PUMA is required for CDDP-induced apoptosis, it is possible that activation of other p53-responsive genes, such as Bax, may also be important contributors. However, we did not observe changes in the levels of Bax protein content in the chemosensitive OV2008 cells in response to CDDP (data not shown) suggesting that p53-mediated up-regulation of Bax may not be a major contributing factor to CDDP-induced apoptosis in these cells. Whether other p53-responsive gene products (e.g. p53AIP1, PTEN, Fas) may indeed play a role in these processes is not known.

A very recent study has shown that p53-induced PUMA is required for CDDP-induced apoptosis in renal cells (Jiang et al., 2006), and our data suggests a similar requirement in ovarian cancer cells. Furthermore, our observation that PUMA up-regulation is mediated through p53, and that RNAi against both PUMA and p53 significantly inhibits CDDP-induced apoptosis strongly suggests that p53 is a critical mediator of CDDP-induced apoptosis in ovarian cancer cells, a finding that has been disputed in some studies. However, we have extended these findings by showing that the presence of p53 activity and PUMA expression is not *sufficient* for CDDP-induced apoptosis since chemoresistant C13* cells showed abundant p53-DNA binding activity and PUMA content in the absence of CDDP, although these were only slightly up-regulated by CDDP, yet failed to undergo apoptosis in the presence of CDDP. Conversely, Cregan et al showed that over-expression of PUMA was sufficient to induce apoptosis in neurons (Cregan et al., 2004), suggesting that this effect may be cell type-

specific. We previously showed that sensitization of C13* cells to CDDP-induced apoptosis is dependent on the presence of p53 (Fraser et al., 2003; Sasaki et al., 2000). Since chemoresistance was associated with a failure to further increase the DNA binding activity of p53 or to up-regulate PUMA, one intriguing possibility is that cells expressing high levels of p53 activity are selected during the development of chemoresistance, have adapted to living under these normally lethal conditions, and thus cannot respond to cell stresses such as CDDP by activating p53. This is supported by data showing that p53 levels are generally much higher in chemoresistant cells (Brown et al., 1993). Taken together, these data suggest that while up-regulation of PUMA is a key contributor to the p53-mediated response to CDDP, additional p53-dependent mechanisms likely contribute to CDDP-induced apoptosis in ovarian cancer cells.

We and others have demonstrated that activation of Akt confers resistance of ovarian cancer cells to CDDP-induced apoptosis while suppression of Akt function sensitizes chemoresistant cells to clinically relevant concentrations of CDDP in a p53-dependent manner (Dan et al., 2004; Fraser et al., 2003; Yuan et al., 2003a), suggesting a functional link between Akt-mediated chemoresistance and p53. While several studies have shown that Akt can facilitate p53 degradation via phosphorylation of MDM2 (Gottlieb et al., 2002; Mayo & Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001), we did not observe a reduction in p53 content in cells expressing activated Akt relative to the control cells. By contrast, we observed a slight up-regulation of basal p53 content in the presence of activated Akt, which may have been due to the slight down-regulation of basal MDM2 content in cells expressing activated Akt (figure 4B). However, a marked reduction of CDDP-induced p53-DNA binding capacity, up-regulation of PUMA and

apoptosis was observed in response to Akt activation, which is consistent with the results of Yamaguchi et al (Yamaguchi et al., 2001), and suggests that in some cells, Akt may attenuate p53 function rather than its content. It has recently been demonstrated that Akt activation blocks proteasomal degradation of p300 (Chen et al., 2004), and p300 is an inhibitor of DNA damage-induced p53-dependent PUMA expression and apoptosis (Iyer et al., 2004). Thus, it is possible that p300 stabilization is one mechanism by which Akt inhibits the activation of p53 in these cells, although this hypothesis has not yet been evaluated. Thus, Akt confers resistance to CDDP-induced apoptosis in part through inhibition of CDDP-induced p53-mediated gene activation.

p53 is phosphorylated on its N-terminal in response to various cell stresses, and p53 phosphorylation is involved in the protection of p53 from MDM2-mediated ubiquitination and proteasomal degradation. Phosphorylation of p53 on Ser15 and/or Ser20 is required for p53-induced apoptosis (Unger et al., 1999b), although this does not appear to hold true under all circumstances (Chao et al., 2006). CDDP effectively induced phosphorylation on numerous p53 N-terminal residues in two chemosensitive ovarian cancer cell lines, but not in their respective chemoresistant variant cell lines, suggesting that altered p53 phosphorylation may contribute to the chemoresistance of these cells. Moreover, while expression of wild-type p53 sensitized a p53 mutant, chemoresistant cell line (OVCAR-3) to CDDP-induced apoptosis, substitution of alanine on Ser15 and/or Ser20 attenuated this effect. To our knowledge, this data represents the first demonstration that p53 phosphorylation is altered in chemoresistant cells and is required for CDDP-induced apoptosis, and provides compelling evidence that aberrant regulation of phosphorylation may be a critical determinant of the sensitivity of human

ovarian cancer cells to CDDP-induced apoptosis. While our data provides clear evidence supporting a role for Ser15 and Ser20 p53 phosphorylation in CDDP-induced apoptosis, we cannot exclude the possibility that phosphorylation of other sites may also be implicated in this process, although it appears that at least one of those sites, Ser37, does not play a major role in CDDP-induced apoptosis. Indeed, Ser46 has been identified as an important phosphorylation site for the regulation of p53-induced apoptosis (Oda et al., 2000b). However, Ser46 phosphorylation was not observed in OV2008 cells exposed to CDDP (data not shown), suggesting that this site may not be universally required for CDDP-induced, p53-dependent apoptosis in ovarian cancer cells.

The mechanism by which Ser15/Ser20 phosphorylation alters the apoptotic capacity of p53 remains unclear, though it appears unlikely that alterations in the phosphorylation state of these sites regulates the activation of p53-dependent PUMA gene expression, since p53-induced PUMA up-regulation in OVCAR-3 cells was not altered by their mutation to alanine. While we have not examined in detail the expression of additional pro-apoptotic, p53-responsive genes, such as Bax, Unger et al showed that Bax promoter activation was induced by p53 irrespective of its phosphorylation status (Unger et al., 1999b). However, the possibility that phosphorylation of these sites may alter the expression of additional p53-responsive genes, thereby promoting p53-mediated apoptosis, requires further investigation.

Interestingly, inhibition of p53 Ser15 phosphorylation by mutagenesis blocked the transcriptional activity of p53 without any effect upon p53 content or interaction with MDM2 (Dumaz & Meek, 1999). While our results suggest that Ser15 phosphorylation may not be required for p53-dependent transactivation in ovarian cancer cells, our results

agree with the observation that Ser15 is not required for maintenance of p53 steady-state levels (Dumaz & Meek, 1999). We recently demonstrated that CDDP induces the direct targeting of p53 to the mitochondria in chemosensitive cells, but not in their resistant counterparts (Yang et al., 2006), and that p53 targeted to the mitochondria was able to induce apoptosis more rapidly than nuclear-targeted p53. Thus, it is possible that phosphorylation may be implicated in the mitochondrial translocation of p53. While recent data suggests phosphorylation is not the determining factor for the alternate translocation of p53 to the nucleus or the mitochondria (Nemajerova et al., 2005), whether phosphorylation is *required* for mitochondrial translocation has not been evaluated. p53 also plays a pro-apoptotic role in the cytoplasm by activating Bax, an event that appears to require its PUMA-dependent liberation from a p53-Bcl-XL complex (Chipuk et al., 2005). Thus, it is possible that this process requires phosphorylation of p53 on one or more residues. These hypotheses are currently under investigation in our laboratory.

In addition, Akt activation markedly reduced the CDDP-induced phosphorylation of p53 on several N-terminal residues while dominant-negative Akt could up-regulate p53 and induce the phosphorylation of p53 independently of changes in p53 content. Interestingly, Yamaguchi et al did not observe a change in p53 phosphorylation in response to Akt activation (Yamaguchi et al., 2001). However, they used a [^{32}P]-orthophosphate labeling assay, which may not be sensitive enough to detect changes in phosphorylation at individual amino acids. In contrast, we used phospho-specific antibodies, which may permit a more precise analysis of phosphorylation status at specific residues. To our knowledge, this is the first report on the regulation of p53

phosphorylation by Akt, and suggests a novel mechanism of Akt-mediated cell survival and chemoresistance in human ovarian cancer. However, while our observation that DN-Akt up-regulates phospho-p53 suggests that Akt attenuates CDDP-induced apoptosis through suppression of p53 phosphorylation, the fact that CDDP did not further increase phospho-p53, yet induced greater apoptosis that DN-Akt alone suggests that other factors, unrelated to p53 phosphorylation, may be implicated in CDDP-induced apoptosis. The observation that mutation of Ser15 or Ser20 to Ala did not completely attenuate CDDP-induced apoptosis supports this hypothesis.

The contribution of p53 to clinical chemosensitivity remains unclear, although several studies (Perego et al., 1996; Petty et al., 1998; Reles et al., 2001; Righetti et al., 1996; Sato et al., 1999) have demonstrated a strong correlation between wild-type *TP53* genotype and sensitivity to CDDP chemotherapy and prolonged survival in human ovarian cancer. In addition, we and others have provided evidence that p53 is required for CDDP-induced apoptosis in human ovarian cancer cell culture and xenografts (Fraser et al., 2003; Song et al., 1999; Song et al., 1997). However, a few studies have failed to show a correlation between p53 mutational status and CDDP sensitivity (Mano et al., 1999; Mi & Ni, 2003; Pestell et al., 2000). However, many of these studies have relied upon non-specific ablation of p53 function (e.g. expression of HPV-E6) and/or did not specifically distinguish between CDDP-induced apoptosis and necrosis. This is of primary concern since previous studies have suggested that apoptosis is a primary effect of CDDP-centred chemotherapy in ovarian cancer patients and in xenograft models of human ovarian cancer (Kigawa et al., 2001; Sato et al., 1999). Furthermore, because the spectrum of p53 mutation is wide, and because different p53 mutations may impact p53

function more or less severely, a more detailed analysis of the relationship between specific p53 mutations and the incidence of chemoresistance is warranted, and may help to shed light upon the precise role of p53 in clinical chemoresistance.

The results of the current study suggest a model by which CDDP up-regulates PUMA via activation of p53, thereby facilitating activation of the mitochondrial cell death pathway. Furthermore, CDDP induces the phosphorylation of p53 on numerous N-terminal residues, including Ser15 and Ser20, which is also required for efficient CDDP-induced apoptosis. Akt effectively blocks these processes, thereby conferring resistance to CDDP-induced apoptosis. Under this model, mutation of the *TP53* gene, which inhibits the up-regulation of PUMA, or failure to phosphorylate wild-type p53, or both, results in a chemoresistant phenotype; both events are required for the full apoptotic response to CDDP. This is consistent with the observation that wild-type *TP53* status is not always correlated with chemosensitivity. In addition, it is likely that other p53-independent cellular events, including the down-regulation of Xiap, are required for CDDP-induced apoptosis. However, our evidence suggests that effective activation and phosphorylation of p53 is essential for sensitivity to CDDP. For this reason, it will be of great interest to study the effects of chemotherapy on the levels of total and phospho-p53, as well as PUMA content, in human ovarian tumours with respect to treatment outcomes. Moreover, since Akt attenuates *both* processes, it will be of paramount importance to study the relationship between activation/over-expression of Akt in human tumours and their sensitivity to CDDP-centred chemotherapy.

In summary, we have demonstrated that p53 is essential for CDDP-induced apoptosis in human ovarian cancer cells, and that this is mediated, at least in part, through

the up-regulation of PUMA and the phosphorylation of p53 on Ser15 and Ser20. Furthermore, p53 phosphorylation is attenuated in chemoresistant cells. Activation of Akt confers resistance by blocking p53-mediated transactivation and p53 phosphorylation. A more thorough understanding of the molecular mechanisms underling chemoresistance in human ovarian cancer may ultimately improve treatment outcomes for this disease.

Materials and Methods

Reagents

Cisplatin (CDDP), Hoechst 33258, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), aprotinin were purchased from Sigma (St. Louis, MO, USA). Mouse monoclonal antibodies to p53 (DO-1) and Bax (2D2) were from Santa Cruz Biotechnologies (San Diego, CA, USA). Rat monoclonal anti-HA was purchased from Roche (clone 3F10). Mouse monoclonal anti-MDM2 was from Calbiochem (Ab-1). Mouse monoclonal anti-phospho-p53 (Ser15; clone 16G8), and rabbit polyclonal anti-phospho-p53 (Ser6, Ser20, Ser33, Ser37) were from Cell Signaling Technology, Inc (Beverly, CA, USA). Rabbit polyclonal anti-PUMA was from Sigma. Mouse anti-GAPDH (ab8245) was from Abcam (Cambridge, UK). siRNA to p53 was purchased from Cell Signaling Technology, Inc. siRNA to PUMA was purchased from Santa Cruz Biotechnologies. Control siRNA was from Dharmacon (Lafayette, CO, USA). Ribojuice siRNA transfection reagent was from Novagen (San Diego, CA, USA). Pre-stained SDS-PAGE standards were from BioRad (Hercules, CA, USA). Adenoviral dominant-negative Akt was a generous gift from Dr. Kenneth Walsh (Cardiovascular Research, St. Elizabeth's Medical Centre, Boston). HA-tagged wild-type p53 in pcDNA3.1 was a

generous gift from Dr. Jin Q. Cheng, Moffitt Cancer Center, University of South Florida, Tampa, FL.

Cell Lines and Cell Culture

Chemosensitive ovarian cancer cells (A2780s and OV2008) and their respective chemoresistant variants (A2780cp and C13*) were cultured as previously reported (Asselin et al., 2001a; Fraser et al., 2005; Fraser et al., 2003; Sasaki et al., 2000) in DMEM/F12 and RPMI 1640. OVCAR-3 cells were cultured in RPMI 1640 media. All media were supplemented with 10% FBS, streptomycin (50 g/ml), penicillin (50 units/ml), fungizone (0.625 g/ml; Life Technologies, Inc. BRL, Carlsbad, CA, USA) and non-essential amino acids (1%). Cells were plated at a density of 5 x 10^4 cells/cm^2 on 6-well plates or 60 mm dishes 18h prior to the initiation of treatment. At the time of treatment, cell density was <85%.

Site-Directed Mutagenesis

HA-tagged wild-type p53 in pcDNA3 was used as a template for site-directed mutagenesis, using the QuickChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). Primers used to mutate p53 to alanine at the indicated site(s) were as follows; Ser15: Forward 5'-GTCGAGCCCCCTCTGGCACAGGAAACATTTTCAGACC-3', Reverse 5'-GGTCTGAAAATGTTTCCTGTGCCAGAGGGGGCTCGAC-3'; Ser20: Forward 5'-CTGAGTCAGGAAACATTTGCAGACCTATGGAAACTACTT-3', Reverse 5'-AAGTAGTTTCCATAGGTCTGCAAATGTTTCCTGACTCAG-3'; Ser37: Forward 5'-TGTCCCCCTTGCCGGCACAAGCAATGGATGATTTG-3', Reverse 5'-CAAATCATCCATTGCTTGTGCCGGCAAGGGGGACA; Ser15/Ser20: Forward 5'-GTCGCACAGGAAACATTTGCAGACCTATGGAAACTACTT-3, Reverse: 5'-

AAGTAGTTTCCATAGGTCTGCAAATGTTTCCTGTGCCAG-3'. The template plasmid DNA was amplified using *Pfu* Polymerase (Fermentas, Hanover, MD) according to the manufacturer's instructions for 16 cycles, digested with DpnI (Fermentas) for 1h at 37°C, and then transformed into XL10 Gold cells (Stratagene) and plated onto LB plates containing 100 μg/ml ampicillin. The following day, colonies were picked, amplified overnight at 37°C in LB broth containing 100 μg/ml ampicillin, and plasmid DNA was extracted using the Plasmid Mini Kit from Qiagen (Valencia, CA). The presence of mutations was confirmed by direct sequencing at the OHRI sequencing facility.

Hoechst 33258 Staining

At the end of the culture period, cells attached to the growth surface were removed by trypsin treatment [trypsin (0.05%), EDTA (0.53mM); 37 °C, 1 min]. Attached and floating cells were pooled, pelleted by centrifugation, and re-suspended in phosphate buffered formalin (10%) containing Hoechst 33258 (12.5 ng/ml). Cells were spotted onto slides for microscopy. Nuclear staining was observed using a Zeiss fluorescence microscope (magnification 400X). Cells with typical apoptotic nuclear morphology (nuclear shrinkage, condensation, and fragmentation) were identified and counted as previously reported (Asselin et al., 2001a; Fraser et al., 2003; Sasaki et al., 2002; Sasaki et al., 2000), using randomly selected fields. A minimum of 200 cells were counted in each treatment group. The counter was 'blinded' to sample identity to avoid experimental bias. Data is expressed as the percentage of total cells showing apoptotic morphology.

Protein Extraction and Western Blot Analysis

Cells were pelleted and lysed in ice-cold lysis buffer (pH 7.4) containing 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM NaPPi, 10 % Glycerol and 1% Triton X-100. Protease inhibitors PMSF (1 mM) and aprotonin (10 g/l), as well as 1 mM Na_3VO_4 were added to the lysis buffer freshly. Cell lysates were sonicated briefly, incubated on ice for 1 h and pelleted by centrifugation (15, 000 x g; 20 min). The supernatant was taken as whole-cell lysate and stored at -20 ^{o}C for subsequent analyses. Protein concentration was determined using Bio-Rad DC protein assay kit. Equal amounts of proteins (30-70 μg) were loaded and resolved by 10% SDS-PAGE and electro-transferred (30V, 16 h) onto nitrocellulose membranes (Bio-Rad). Membranes were blocked (room temperature, 1 h) with 5% Blotto [Tris-HCl (10 mM; pH 8.0), NaCl (150 mM), Tween 20 (0.05%, v/v; TBS-Tween 20) containing skim milk (5%; w/v)], then incubated overnight with primary antibodies [p53 (1:1,000), Bax (1:1000), PUMA (1:1000), MDM2 (1:2000), or GAPDH (1:30,000)], and subsequently with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody [1:2,000 in 5% Blotto; room temperature, 1 h; 1:20,000 for GAPDH]. Peroxidase activity was visualized with an ECL kit (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) after three washes (15 min/wash) with TBS-Tween 20. Signal intensity was determined densitometrically using Scion Image software, version 4.02 from Scion Corporation (Frederick, MD, USA). All quantified Western blot data was corrected for loading using the anti-GAPDH blots. Western blots shown in figures are representative of at least three independent experiments.

Immunoprecipitation-Western Blots

Cultured cells were transfected with HA-tagged p53 constructs for 24 hours (as indicated) and then treated with CDDP for a further 24 hours. The cells were lysed in standard lysis buffer (as above) for 1 hour on ice, and then transferred to a 1.5 mL microcentrifuge tube, and centrifuged for 20 minutes at 14,000 x g to remove cellular debris. The Supernatents were analysed for total protein content, and 300 μg of total protein was incubated with 15 μL of agarose-immobilized goat polyclonal anti-HA antibody (Bethyl Laboratories, Montgomery, TX) in a final volume of 300 μL, adjusted with lysis buffer. Immunoprecipitation was carried out with gentle rocking, overnight at 4C. The agarose beads were pelleted by centrifugation at 500 x g for 2 minutes, and then washed three times with 1 mL lysis buffer, with each wash followed by a 2 minute centrifugation at 500 x g. After the final wash, 30 μL of 2x SDS sample buffer was added to the beads, the sample were boiled and then loaded onto 12% SDS-PAGE gels. Following protein transfer to nitrocellulose, phospho-p53, total p53, and exogenous HA-p53 was detected by Western blotting as described above.

RT-PCR

Cultured cells were harvested as above, and cell pellets were stored at -80C until further processing. Total RNA was extracted using the RNeasy Mini Kit from Qiagen. An aliquot of total RNA from each sample was subjected to DNase I treatment to remove genomic DNA contamination using the DNA-free kit from Ambion, Inc (Austin, TX). mRNA was reverse transcribed using oligo(dT) primers with M-MuLV Reverse Transcriptase via the Retroscript kit from Ambion. PCR primers were from Invitrogen (Burlington, ON) as follows: PUMA sense: 5'-TGTGACCACTGGCATTCATT-3'; PUMA antisense: 5'-CCTGTAAGATACTGTATATGCGCTGC-3'; β-Actin sense: 5'-

GGACTTCGAGCAAGAGATGG-3'; β-actin antisense: 5'-CACCTTCACCGTTCCAGTTT-3'. To ensure linearity of the results, the cycle number was optimized by performing PCR reactions at 20-45 cycles. All subsequent PCR reactions were performed within the linear range of amplification. PCRs were performed using HotStarTaq Taq Polymerase from Qiagen Inc. Following a 15 minute activation step at 95C, PCR conditions were: denaturation (94C) for 45 seconds, annealing (PUMA: 56C, β-actin: 54C) for 45 seconds, extension (72C) for 30 seconds, for 37 or 25 cycles for PUMA and β-actin, respectively. 10 μl of each PCR product was separated on a 1.5% agarose-ethidium bromide gel and visualized by ultraviolet trans-illumination using a BioRad GelDoc system.

Genomic DNA Sequencing

To confirm the *TP53* genotype of A2780cp cells, total genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen) and amplified by PCR using HotStarTaq Polymerase (Qiagen). The PCR conditions were as follows: 95°C, 15 minutes, 30 cycles of 94°C for 1 min, 54°C for 45 sec, 72°C for 1 min, followed by a final extension step of 72°C for 10 min. Primers flanked codon 172 as follows: Forward 5'-CAGCCCTGTCGTCTCTCCAG-3', Reverse 5'-TTATCTGTTCACTGGTGCCC-3'. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and extracted using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Purifed product was directly sequenced using the forward PCR primer.

Adenoviral Infection

Cells were infected with an HA-tagged 'triple-A' (K179A, T308A, S473A) dominant-negative Akt (DN-Akt) or LacZ cDNA at various multiplicities of infection

(MOI) as indicated in the text and previously described (Li et al., 2001). As previously reported, adenovirus infection efficiency at MOI of 5, as determined by an X-gal staining assay against LacZ construct infected cells, was >90% (Fraser et al., 2003; Sasaki et al., 2000). DN-Akt expression was confirmed by Western blot analysis against the HA epitope tag.

RNA Interference

Six μl of transfection reagent (Novagen, San Diego, CA) was added to 244 μl of DMEM/F12 without serum. The mixture was vortexed and incubated for 5 minutes at room temperature. Following incubation, 7.5 μl of 10 μM stock siRNA construct was added. The mixture was incubated at room temperature for a further 15 minutes. During this period, the culture media was removed from the cells and the cells were washed once with PBS. The siRNA mixture was added to each well with an additional 1250 μl of complete (10% FBS) media. The cells were returned to the incubator and the media was removed 6 hours later and replaced with fresh, complete media for the duration of the culture (24-48h). Down-regulation was confirmed by Western blot analysis.

Transient Transfection

A2780s cells were cultured overnight in 6-well plates and then transfected with 1 μg of pcDNA3.1-derived vectors (empty vector control) using Lipofectamine Plus (Invitrogen) in 1 ml serum-free medium according to the manufacturer's instructions. 3h post-transfection, each well was supplemented with 1 ml of medium containing 20% FBS. 24h post-transfection, media was removed and the cells were harvested or treated as required for a further 24h.

DNA Binding Assay

To assess p53-DNA binding capacity, nuclear lysates of CDDP-treated cells were obtained using the NE-PER Nuclear/Cytoplasmic Extraction kit from Pierce (Rockford, IL), according to the manufacturer's instructions and as previously reported in our laboratory (Wang et al., 2002) and total nuclear protein contents were determined (as above). To assess p53-DNA binding, we used the p53 TransAM kit from ActiveMotif (Carlsbad, CA). Briefly, equivalent amounts of nuclear protein were incubated for 1h at room temperature in separate wells of a 96-well plate containing an immobilized p53 consensus oligonucleotide (5'-GGACATGCCCGGGCATGTCC-3'). After 1h, plates were washed 3x in fresh 1x wash buffer (supplied with kit), and then incubated for 1h at room temperature with anti-p53 antibody. The plates were washed a further 3x with 1x wash buffer, and then incubated 1h with HRP-conjugated anti-rabbit antibody. After a further wash step, HRP was detected using 1x developing solution (supplied with kit), and the reaction was stopped with 1x stop solution (supplied with kit) when a light blue colour was observed. Plates were immediately read using a UV spectrophotometer at 450nm. In addition to sample wells, the assay was performed on 3 wells containing only lysis buffer as a negative control. Assay specificity was confirmed by pre-incubation with free mutant or wild-type oligonucleotide.

Statistical Analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was carried out by one- or two-way ANOVA or by Student's t-test (where appropriate) using PRISM software (Version 3.0; GraphPad, San Diego, CA, USA). Differences between multiple experimental groups were determined by the Bonferroni or Tukey post-hoc tests. Statistical significance was inferred at $p<0.05$.

References

Akeshima R, Kigawa J, Takahashi M, Oishi T, Kanamori Y, Itamochi H, Shimada M, Kamazawa S, Sato S and Terakawa N. (2001). *Br J Cancer*, **84,** 1551-5.

Ashcroft M, Kubbutat MH and Vousden KH. (1999). *Mol Cell Biol*, **19,** 1751-8.

Ashcroft M, Taya Y and Vousden KH. (2000). *Mol Cell Biol*, **20,** 3224-33.

Asselin E, Mills GB and Tsang BK. (2001). *Cancer Res*, **61,** 1862-8.

Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V and et al. (1995). *Int J Cancer*, **64,** 280-5.

Brown R, Clugston C, Burns P, Edlin A, Vasey P, Vojtesek B and Kaye SB. (1993). *Int J Cancer*, **55,** 678-84.

Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E and Fornace AJ, Jr. (1999). *Embo J*, **18,** 6845-54.

Chehab NH, Malikzay A, Stavridi ES and Halazonetis TD. (1999). *Proc Natl Acad Sci U S A*, **96,** 13777-82.

Chen J, Halappanavar SS, St-Germain JR, Tsang BK and Li Q. (2004). *Cell Mol Life Sci*, **61,** 1675-83.

Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN and Testa JR. (1992). *Proc Natl Acad Sci U S A*, **89,** 9267-71.

Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD and Green DR. (2005). *Science*, **309,** 1732-5.

Cregan SP, Arbour NA, Maclaurin JG, Callaghan SM, Fortin A, Cheung EC, Guberman DS, Park DS and Slack RS. (2004). *J Neurosci*, **24,** 10003-12.

Dan HC, Sun M, Kaneko S, Feldman RI, Nicosia SV, Wang HG, Tsang BK and Cheng JQ. (2004). *J Biol Chem*, **279,** 5405-12.

Dumaz N and Meek DW. (1999). *Embo J*, **18,** 7002-10.

Erster S, Mihara M, Kim RH, Petrenko O and Moll UM. (2004). *Mol Cell Biol*, **24,** 6728-41.

Fraser M, Chan SL, Chan SS, Fiscus RR and Tsang BK. (2005). *Oncogene*.

Fraser M, Leung BM, Yan X, Dan HC, Cheng JQ and Tsang BK. (2003). *Cancer Res*, **63,** 7081-8.

Fuchs SY, Adler V, Pincus MR and Ronai Z. (1998). *Proc Natl Acad Sci U S A*, **95,** 10541-6.

Gottlieb TM, Leal JF, Seger R, Taya Y and Oren M. (2002). *Oncogene*, **21,** 1299-303.

Hoffman WH, Biade S, Zilfou JT, Chen J and Murphy M. (2002). *J Biol Chem*, **277,** 3247-57.

Honda R, Tanaka H and Yasuda H. (1997). *FEBS Lett*, **420,** 25-7.

Iyer NG, Chin SF, Ozdag H, Daigo Y, Hu DE, Cariati M, Brindle K, Aparicio S and Caldas C. (2004). *Proc Natl Acad Sci U S A*, **101,** 7386-91.

Jiang M, Wei Q, Wang J, Du Q, Yu J, Zhang L and Dong Z. (2006). *Oncogene*.

Kigawa J, Sato S, Shimada M, Takahashi M, Itamochi H, Kanamori Y and Terakawa N. (2001). *Hum Cell*, **14,** 165-71.

Lavarino C, Pilotti S, Oggionni M, Gatti L, Perego P, Bresciani G, Pierotti MA, Scambia G, Ferrandina G, Fagotti A, Mangioni C, Lucchini V, Vecchione F, Bolis G, Scarfone G and Zunino F. (2000). *J Clin Oncol*, **18,** 3936-45.

Li J, Feng Q, Kim JM, Schneiderman D, Liston P, Li M, Vanderhyden B, Faught W, Fung MF, Senterman M, Korneluk RG and Tsang BK. (2001). *Endocrinology*, **142,** 370-80.
Mano Y, Kikuchi Y, Yamamoto K, Kita T, Hirata J, Tode T, Ishii K and Nagata I. (1999). *Eur J Cancer*, **35,** 1214-9.
Mayo LD and Donner DB. (2001). *Proc Natl Acad Sci U S A*, **98,** 11598-603.
Mi RR and Ni H. (2003). *Gynecol Oncol*, **90,** 238-44.
Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P and Moll UM. (2003). *Mol Cell*, **11,** 577-90.
Mirza A, McGuirk M, Hockenberry TN, Wu Q, Ashar H, Black S, Wen SF, Wang L, Kirschmeier P, Bishop WR, Nielsen LL, Pickett CB and Liu S. (2002). *Oncogene*, **21,** 2613-22.
Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B and Reed JC. (1994). *Oncogene*, **9,** 1799-805.
Nemajerova A, Erster S and Moll UM. (2005). *Cell Death Differ*, **12,** 197-200.
Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y and Taya Y. (2000). *Cell*, **102,** 849-62.
Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, Masuyama N and Gotoh Y. (2002). *J Biol Chem*, **277,** 21843-50.
Perego P, Giarola M, Righetti SC, Supino R, Caserini C, Delia D, Pierotti MA, Miyashita T, Reed JC and Zunino F. (1996). *Cancer Res*, **56,** 556-62.
Pestell KE, Hobbs SM, Titley JC, Kelland LR and Walton MI. (2000). *Mol Pharmacol*, **57,** 503-11.
Petty R, Evans A, Duncan I, Kurbacher C and Cree I. (1998). *Pathol Oncol Res*, **4,** 97-102.
Reles A, Wen WH, Schmider A, Gee C, Runnebaum IB, Kilian U, Jones LA, El-Naggar A, Minguillon C, Schonborn I, Reich O, Kreienberg R, Lichtenegger W and Press MF. (2001). *Clin Cancer Res*, **7,** 2984-97.
Righetti SC, Della Torre G, Pilotti S, Menard S, Ottone F, Colnaghi MI, Pierotti MA, Lavarino C, Cornarotti M, Oriana S, Bohm S, Bresciani GL, Spatti G and Zunino F. (1996). *Cancer Res*, **56,** 689-93.
Sasaki H, Kotsuji F and Tsang BK. (2002). *Gynecol Oncol*, **85,** 339-50.
Sasaki H, Sheng Y, Kotsuji F and Tsang BK. (2000). *Cancer Res*, **60,** 5659-66.
Sato S, Kigawa J, Minagawa Y, Okada M, Shimada M, Takahashi M, Kamazawa S and Terakawa N. (1999). *Cancer*, **86,** 1307-13.
Shieh SY, Ikeda M, Taya Y and Prives C. (1997). *Cell*, **91,** 325-34.
Shono T, Tofilon PJ, Schaefer TS, Parikh D, Liu TJ and Lang FF. (2002). *Cancer Res*, **62,** 1069-76.
Siddik ZH, Mims B, Lozano G and Thai G. (1998). *Cancer Res*, **58,** 698-703.
Skilling JS, Squatrito RC, Connor JP, Niemann T and Buller RE. (1996). *Gynecol Oncol*, **60,** 72-80.
Song K, Cowan KH and Sinha BK. (1999). *Oncol Res*, **11,** 153-9.
Song K, Li Z, Seth P, Cowan KH and Sinha BK. (1997). *Oncol Res*, **9,** 603-9.
Sun M, Wang G, Paciga JE, Feldman RI, Yuan ZQ, Ma XL, Shelley SA, Jove R, Tsichlis PN, Nicosia SV and Cheng JQ. (2001). *Am J Pathol*, **159,** 431-7.

Unger T, Sionov RV, Moallem E, Yee CL, Howley PM, Oren M and Haupt Y. (1999). *Oncogene*, **18,** 3205-12.
Vasey PA, Jones NA, Jenkins S, Dive C and Brown R. (1996). *Mol Pharmacol*, **50,** 1536-40.
Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ, Adams JM and Strasser A. (2003). *Science*, **302,** 1036-8.
Wang Y, Chan S and Tsang BK. (2002). *Endocrinology*, **143,** 2732-40.
Wong HK, Fricker M, Wyttenbach A, Villunger A, Michalak EM, Strasser A and Tolkovsky AM. (2005). *Mol Cell Biol*, **25,** 8732-47.
Yaginuma Y and Westphal H. (1992). *Cancer Res*, **52,** 4196-9.
Yamaguchi A, Tamatani M, Matsuzaki H, Namikawa K, Kiyama H, Vitek MP, Mitsuda N and Tohyama M. (2001). *J Biol Chem*, **276,** 5256-64.
Yang X, Fraser M, Moll UM, Basak A and Tsang BK. (2006). *Cancer Res*, **66,** 3126-36.
Yuan ZQ, Feldman RI, Sussman GE, Coppola D, Nicosia SV and Cheng JQ. (2003). *J Biol Chem.*
Yuan ZQ, Sun M, Feldman RI, Wang G, Ma X, Jiang C, Coppola D, Nicosia SV and Cheng JQ. (2000). *Oncogene*, **19,** 2324-30.
Zhou BP, Liao Y, Xia W, Zou Y, Spohn B and Hung MC. (2001). *Nat Cell Biol*, **3,** 973-82.

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Regulation of p53 and Suppression of Apoptosis by the Soluble Guanylyl Cyclase/cGMP Pathway in Human Ovarian Cancer Cells

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Abbreviations Used

sGC, soluble guanylyl cyclase; pGC, particulate guanylyl cyclase; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; IAP, inhibitor of apoptosis protein; GAPDH, glyceraldehyde phosphate dehydrogenase; BNP, brain natriuretic peptide; ANF, atrial natriuretic factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RNAi, RNA interference; siRNA, small inhibitory RNA; DMEM, Dulbecco's Modified Eagle Medium; ECL, enhanced chemiluminescence; PFT, pifithrin-alpha hydrobromide; PBS, phosphate-buffered saline; Xiap, X-Linked Inhibitor of Apoptosis Protein; Xiap-s, Adenoviral Xiap sense cDNA; ROS, reactive oxygen species; MDM2, murine double minute-2; NO, nitric oxide; CHX, cycloheximide

Abstract

Dysregulated apoptosis plays a critical role in the development of a number of aberrant cellular processes, including tumorigenesis and chemoresistance. However, the mechanisms that govern the normal apoptotic program are not completely understood. Soluble guanylyl cyclase (sGC) and cGMP promote mammalian cell viability via an unknown mechanism and p53 status is a key determinant of cell fate in human ovarian cancer cells. Whether an interaction exists between these two determinants of cell fate is unknown. We hypothesized that basal sGC activity reduces p53 content and attenuates p53-dependent apoptosis in human ovarian cancer cells. Suppression of sGC activity with the specific inhibitor ODQ lowered cGMP content, and increased p53 protein content and induced apoptosis in three ovarian cancer cell lines, effects which were attenuated by the cGMP analog 8-Br-cGMP and by Atrial Natriuretic Factor, an activator of particulate guanylyl cyclase, which circumvent the inhibition of sGC. ODQ prolonged p53 half-life, induced phosphorylation of p53 on Ser15, and up-regulated the p53-dependent gene products p21, MDM2, and the pro-apoptotic, p53-responsive gene product Bax. ODQ activated caspase-3 and ODQ-induced apoptosis was inhibited by over-expression of X-Linked Inhibitor of Apoptosis Protein. Pre-treatment with the specific p53 inhibitor pifithrin or down-regulation of p53 using a specific siRNA significantly attenuated ODQ-induced apoptosis. Moreover, ODQ-induced up-regulation of p21 and Bax and ODQ-induced apoptosis were significantly reduced in a p53 mutant cell line relative to the wild-type parental cell line. Thus, the current study establishes that basal sGC/cGMP activity regulates p53 protein stability, content, and function,

possibly by altering p53 phosphorylation and stabilization, and promotes cell survival in part through regulation of caspase-3 and p53.

Introduction

Dysregulated apoptosis contributes to the development of a number of pathologies, including cancer (Reap et al., 1995), (Estevez et al., 1998a; Fiscus, 2002; Fiscus et al., 2002) (Liebermann et al., 1995). Tumour growth results, in part, from an imbalance between cell proliferation and apoptosis (Kerr et al., 1994; LaCasse et al., 1998; Levine et al., 1995; Sheets & Yeh, 1997) and our laboratory has been interested in the role of dysregulated apoptosis in the regulation of cell fate in human ovarian cancer cells (Asselin et al., 2001a; Fraser et al., 2003; Sasaki et al., 2000).

Soluble guanylyl cyclase (sGC) is one of the major producers of basal cGMP content in mammalian cells (Fiscus, 2002; Garthwaite et al., 1995; Waldman & Murad, 1987), and is necessary for the survival of several cell types under normal growth conditions, as well as for protection against various apoptotic stimuli (Flamigni et al., 2001). Our previous studies have demonstrated that the basal activities of sGC and a downstream target protein in the cGMP signalling pathway, protein kinase G (PKG), may be important for the protection of uterine epithelial cells (Chan & Fiscus, 2003) and neural cell lines (Fiscus, 2002) against spontaneous apoptosis. However, the precise mechanisms by which sGC/cGMP regulates cell fate are not known.

While p53 status is a determinant of cisplatin resistance in human ovarian cancer cells and failure to properly regulate p53 may be a contributing factor to chemoresistance (Fraser et al., 2003), precisely how p53 is regulated in these cells is unclear. In the current study, the possibility that the constitutive presence of sGC-derived cGMP is an

important negative regulator of p53 content and p53-dependent apoptosis in human ovarian cancer cells is examined. The current study demonstrates that basal sGC-derived cGMP is required for the viability of human ovarian cancer cells, likely by inhibiting the activation of execution caspases such as caspase-3. Furthermore, these findings suggest that sGC/cGMP-mediated survival of these cells proceeds, in part, by lowering basal p53 protein content and phosphorylation and p53-induced apoptosis and that the basal activity of this pathway may alter p53 content and function by promoting p53 degradation.

Results

ODQ depletes cellular cGMP levels

To determine whether sGC is an important regulator of cGMP content in human ovarian cancer cells, OV2008 cells were cultured for 48h, and then treated 20 min with ODQ (0-100 μM), a potent and specific soluble guanylyl cyclase inhibitor (Garthwaite et al., 1995; Moro et al., 1996; Mulsch et al., 1997; Olesen et al., 1998). Cells were harvested and cGMP assays were performed as indicated in Materials and Methods. As shown in figure 1A, ODQ significantly lowered cellular cGMP levels in a concentration-dependent manner ($p<0.0001$). Moreover, at 50 and 100 μM, ODQ lowered cGMP content by 63 and 70%, respectively ($p<0.001$, relative to untreated control). Even at 30 μM, ODQ significantly reduced cGMP levels, relative to control ($p<0.05$). These results demonstrate that inhibition of sGC activity lowers cGMP levels and suggest that sGC is a major regulator of basal cGMP levels in ovarian cancer cells.

Induction of apoptosis by the soluble guanylyl cyclase inhibitor ODQ

ODQ induces apoptosis in immortalized uterine epithelial cells (Chan & Fiscus, 2003) as well as in established neural cell lines (Fiscus, 2002), suggesting that basal levels of sGC activity are important for preventing spontaneous induction of apoptosis. However, it is unclear whether this pathway affects the viability of human ovarian cancer cells, where disruptions of apoptotic mediators is commonly observed. To address this question, A2780s and OV2008 human ovarian cancer cell lines were incubated for 24h in the presence or absence of ODQ (0, 30, 50 μM). DMSO was used as a control and maintained at a concentration of 0.1% (v/v) in all groups. Previous studies demonstrated that ODQ at 40 μM completely inhibited sGC activity, lowered basal levels of cGMP to one-fifth of normal levels and induced apoptosis in neural cell lines (Fiscus, 2002). In the present study, exposure of A2780s or OV2008 cells to ODQ caused a concentration-dependent increase in apoptosis, as measured by Hoechst nuclear staining ($p<0.0001$; **figure 1B**). ODQ-induced apoptosis was also confirmed by TUNEL assay. As expected, ODQ (30 μM) increased the percentage of TUNEL-positive (i.e. apoptotic) A2780s cells ($p<0.0001$), relative to DMSO control (20 ± 1.25% vs. 0.7 ± .15%; **figure 1C**). The pro-apoptotic effects of ODQ were significantly attenuated by the cell-permeable, stable cGMP analog 8-Br-cGMP (OV2008, $p<0.01$; A2780s, $p<0.05$). Furthermore, ODQ-induced apoptosis was significantly attenuated by the particulate guanylyl cyclase (pGC) activator Atrial Natriuretic Factor (ANF; **figure 1D**, $p<0.001$, all effects), suggesting that sGC activity is required for the viability of ovarian cancer cells, and that ODQ-induced apoptosis is specific to its inhibitory effects on sGC.

Figure 1 - Depletion of cGMP and induction of apoptosis by the soluble guanylyl cyclase inhibitor ODQ in human ovarian cancer cells.

(A) OV2008 cells were cultured for 48h and then treated with ODQ (0-100 μM) for 20 min. cGMP assays were performed as indicated in Materials and Methods. Histogram shows mean ± SEM of 5 independent experiments. ODQ significantly reduced cGMP content overall ($p<0.0001$). Moreover, at 50 μM ODQ, cGMP content was reduced by >60%, compared to control ($p<0.001$), while at 100 μM ODQ, cGMP content ~70% lower than control cells ($p<0.001$). Results demonstrate that inhibition of sGC activity lowers cGMP levels, suggesting that sGC is a major regulator of basal cGMP levels in ovarian cancer cells. * - $p<0.05$, ** - $p<0.001$, relative to untreated control cells. *(B)* OV2008 and A2780s human ovarian cancer cells were cultured in the presence of the soluble guanylyl cyclase (sGC) inhibitor ODQ (0-50 μM), in the presence or absence of the stable cGMP analog 8-Br-cGMP (1 mM; top panel). Histograms show mean ± SEM of 4 independent experiments. ODQ significantly increased the number of apoptotic cells in both OV2008 and A2780s cells ($p<0.0001$, both cell lines). Moreover, 8-Br-cGMP significantly attenuated the effects of ODQ ($p<0.01$, OV2008; $p<0.05$, A2780s). *(C)* A2780s cells were cultured for 24h in the presence of ODQ (0, 30 μM). Floating and attached cells were pooled and apoptosis was determined by TUNEL assay as described in Materials and Methods. *(D)* A2780s cells were treated with ODQ (0, 30μM) in the presence of Atrial Natriuretic Factor (ANF, 0-1 μM; bottom panel), an activator of particulate guanylyl cyclase. Histograms show mean ± SEM of 5 independent experiments. ANF significantly attenuated ODQ-induced apoptosis ($p<0.01$). Results suggest that sGC is a major regulator of basal cGMP content in ovarian cancer cells, that

sGC activity is required for basal viability of human ovarian cancer cells and that the effects of ODQ are likely mediated through the cGMP pathway. *** - $p<0.001$, relative to equi-molar ODQ, 8-Br-cGMP-treated cells; ** - $p<0.01$, relative to equi-molar, 8-Br-cGMP-treated cells.

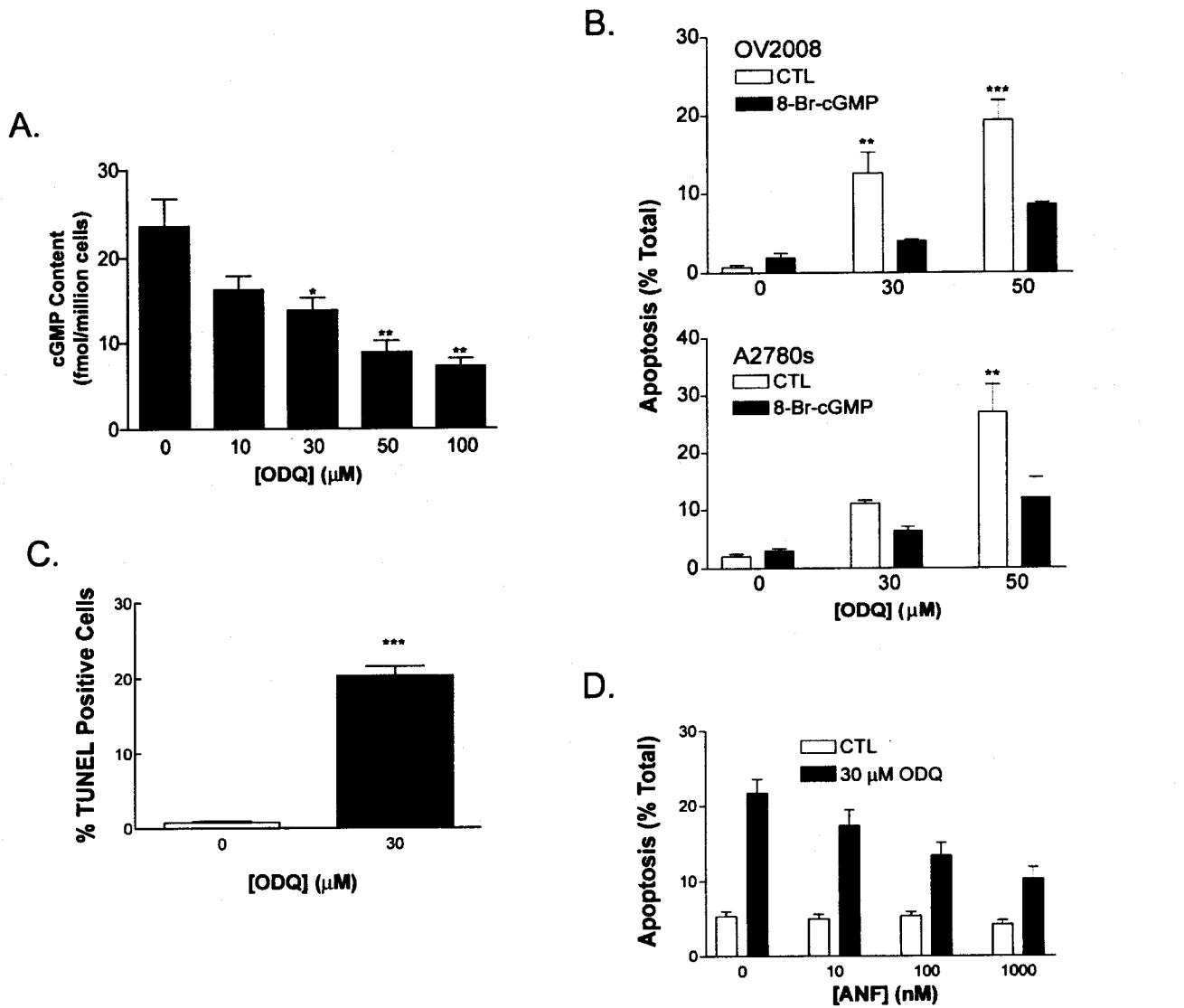

A.
cGMP Content (fmol/million cells)
30
20
10
0
0
10
30
50
100
[ODQ] (μM)
B.
OV2008
CTL
8-Br-cGMP
Apoptosis (% Total)
30
20
10
0
0
30
50
A2780s
CTL
8-Br-cGMP
40
30
20
10
0
0
30
50
[ODQ] (μM)
C.
% TUNEL Positive Cells
30
20
10
0
0
30
[ODQ] (μM)
D.
Apoptosis (% Total)
CTL
30 μM ODQ
30
20
10
0
0
10
100
1000
[ANF] (nM)

Figure 1

ODQ activates caspase-3 and induces Xiap-sensitive apoptosis

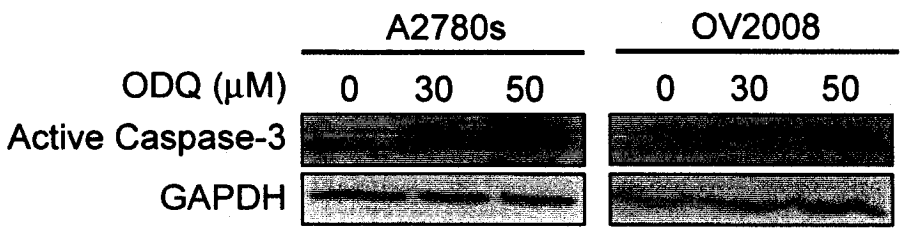
Since activation of the caspase cascade is a critical event for the induction of apoptosis in mammalian cells, whether inactivation of the sGC/cGMP pathway is associated with changes in caspase-3 activity were next determined. A2780s and OV2008 cells were treated with ODQ (0, 30, 50 μM) for 24h and p17 (active) caspase-3 content was determined by Western blotting. As shown in **figure 2A**, ODQ markedly up-regulated the active form of caspase-3 in a concentration-dependent manner in both cell lines, suggesting that suppression of basal sGC activity may result in increased caspase-3 activity.

X-Linked Inhibitor of Apoptosis Protein (Xiap) is a potent endogenous inhibitor of caspase-3, -7, and -9, and is a central regulator of cell fate in human ovarian cancer cells (Asselin et al., 2001a; Li et al., 2001; Sasaki et al., 2000). To determine whether ODQ-induced apoptosis is similarly regulated, Xiap was over-expressed using an adenoviral sense Xiap cDNA (Xiap-s). Cells were infected for 24h with Xiap-s (MOI = 0, 2.5, 5, 10; LacZ control), and then treated with ODQ (0, 100 μM) for a further 24h. As shown in **figure 2B**, ODQ significantly increased the percentage of apoptotic cells, relative to the untreated cells ($p<0.001$). However, while infection with the LacZ adenovirus had no effect on ODQ sensitivity, Xiap-s inhibited ODQ-induced apoptosis in a concentration-dependent manner ($p<0.01$). Over-expression of Xiap was confirmed by Western blot analysis (**figure 2B**, lanes 3, 4, and 5, compared to lane 1). Interestingly, ODQ alone down-regulated Xiap (lane 2 vs. lane 1), suggesting that regulation of Xiap may be involved in ODQ-induced apoptosis. These results

Figure 2 - ODQ-induced apoptosis is associated with activation of caspase-3 and is inhibited by Xiap.

(A) A2780s and OV2008 cells were cultured for 24h in the presence of ODQ (0, 30, 50 μM). p17 active caspase-3 content in whole-cell lysates was determined by Western blotting. Blots are representative of 3 independent experiments. ODQ markedly up-regulated the active form of caspase-3 in a concentration-dependent manner in both cell lines. *(B)* A2780s cells were cultured for ~18h, and then infected with adenovirus containing cDNA for Xiap or LacZ (MOI=0, 2.5, 5, 10). Following 24h of infection, the virus was removed and the cells were treated with ODQ (0, 100μM) for a further 24h. Over-expression of Xiap was confirmed by Western blot analysis (representative of 3 independent experiments). ODQ significantly induced apoptosis ($p<0.001$), relative to the untreated control. However, these effects were inhibited by over-expression of Xiap in a concentration-dependent manner. *** - $p<0.001$, relative to CTL; * - $p<0.05$, ** - $p<0.01$, relative to virus-free, ODQ-treated cells.

A.


A2780s
OV2008
ODQ (μM)
0 30 50
0 30 50
Active Caspase-3
GAPDH

B.

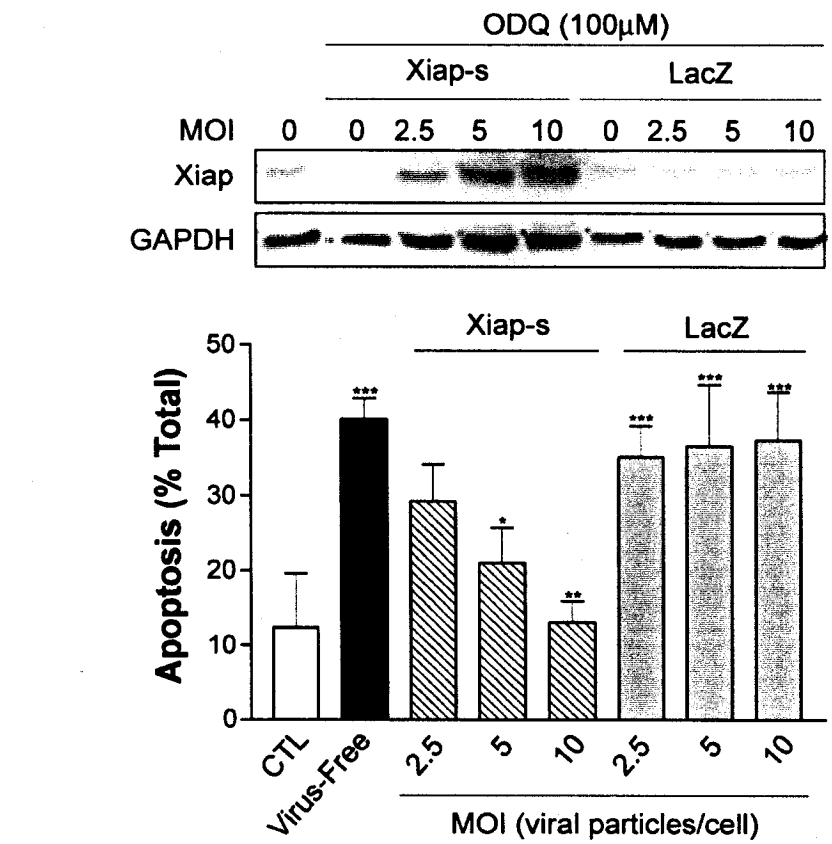

ODQ (100μM)
Xiap-s
LacZ
MOI 0 0 2.5 5 10 0 2.5 5 10
Xiap
GAPDH
Xiap-s
LacZ
Apoptosis (% Total)
50
40
30
20
10
0
***
*
**
CTL
Virus-Free
2.5
5
10
2.5
5
10
MOI (viral particles/cell)

Figure 2

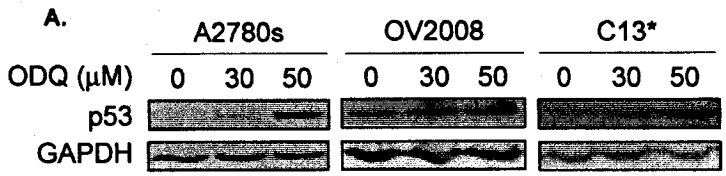
demonstrate that ODQ-induced apoptosis is inhibited by Xiap, and suggest that ODQ-induced apoptosis involves modulation of a Xiap-regulated pathway, such as caspase-3 activation.

p53 is regulated by the sGC/cGMP pathway and is required for maximal ODQ-induced apoptosis

Since p53 is an important regulator of cell fate in human ovarian cancer cells, we next examined whether ODQ-induced apoptosis is associated with changes in p53 content in these cells. A2780s, OV2008 and C13* cell lines were treated with ODQ as above and p53 contents were determined by Western blotting. ODQ markedly up-regulated p53 in all three cell lines (**figure 3A**), suggesting that p53 may be involved in apoptosis induced by sGC inhibition.

Furthermore, while ODQ up-regulated p53 in both A2780s and OV2008 cells (30 and 50 μM, respectively), this was significantly attenuated by 8-Br-cGMP (1 mM; **figure 3B**, $p<0.01$), suggesting that ODQ-induced up-regulation of p53 and induction of apoptosis is secondary to suppression of cellular cGMP levels. 8-Br-cGMP did not have a measurable effect on basal p53 levels, suggesting that in unstressed cells, p53 levels are kept at their lowest possible levels.

Figure 3: Up-regulation of p53 protein content through inhibition of the sGC/cGMP pathway. ***(A)*** A2780s, OV2008, and C13* cells were cultured for 24h in the presence of ODQ (0, 30, 50 μM). p53 protein content was assessed by Western blot analysis using an anti-p53 antibody (A2780s – clone 80; OV2008 – clone DO-1). Results were normalized for protein loading by re-probing the blots with an anti-GAPDH antibody. Western blots are representative of 3 independent experiments. ODQ markedly up-regulated p53 in a concentration-dependent manner in all cell lines. ***(B)*** A2780s (top panel) or OV2008 (bottom panel) cells were cultured for 24h in the presence of ODQ (0, 30 or 50 μM, as indicated) in the presence of 8-Br-cGMP (0, 1 mM). p53 protein content was assessed by Western blot analysis. Protein content was determined by densitometry and is shown as mean ± SEM of 3 independent experiments. ODQ significantly increased p53 content in A2780s cells by over 4 fold and in OV2008 cells by greater than 2-fold, relative to untreated control (***- $p<0.001$, ** - $p<0.01$). These effects were significantly attenuated by 8-Br-cGMP ($p<0.05$). Results suggest that p53 protein content is regulated by the sGC/cGMP pathway. ** - $p<0.01$, relative to equi-molar ODQ, CTL-treated cells.


A.
A2780s
OV2008
C13*
ODQ (μM) 0 30 50 0 30 50 0 30 50
p53
GAPDH

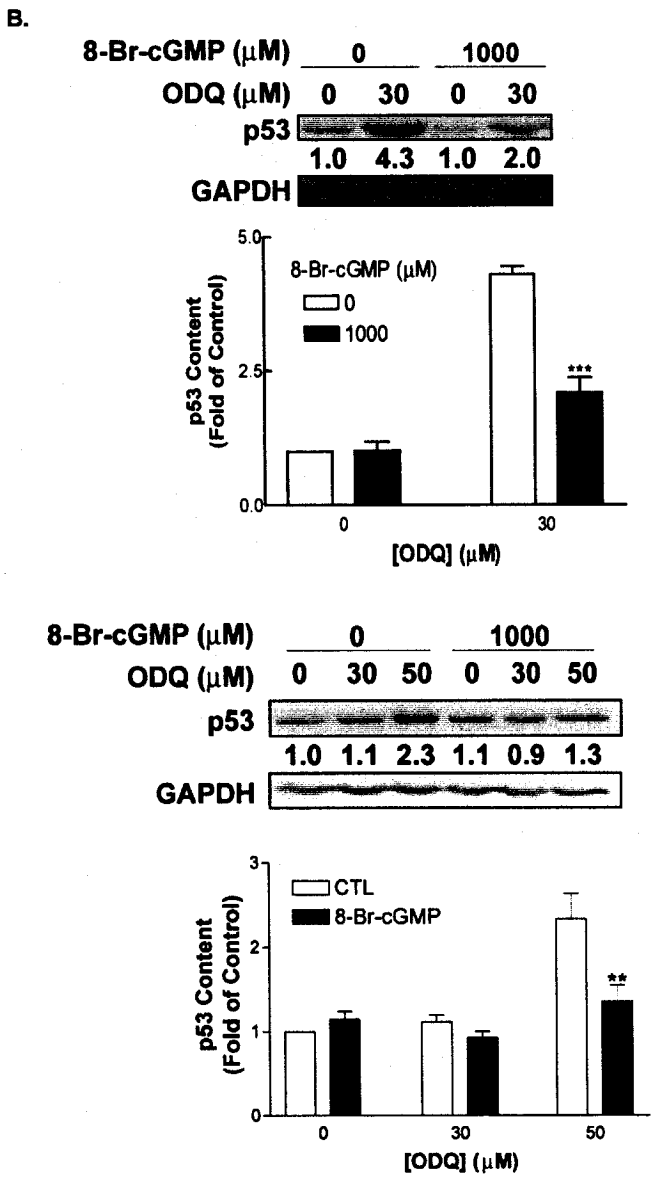

B.
8-Br-cGMP (μM) 0 1000
ODQ (μM) 0 30 0 30
p53
1.0 4.3 1.0 2.0
GAPDH
p53 Content (Fold of Control)
8-Br-cGMP (μM)
0
1000
***
[ODQ] (μM)
8-Br-cGMP (μM) 0 1000
ODQ (μM) 0 30 50 0 30 50
p53
1.0 1.1 2.3 1.1 0.9 1.3
GAPDH
CTL
8-Br-cGMP
**
p53 Content (Fold of Control)
[ODQ] (μM)

Figure 3

To determine whether p53 is required for ODQ-induced apoptosis, we treated A2780s cells with ODQ (30 μM; 24h) in the presence of the specific p53 inhibitor PFT (0-30 μM). PFT is an inhibitor of p53 transactivation and p53-dependent apoptosis, which may function by preventing the nuclear import of p53 following cell stress (Komarov et al., 1999). This inhibitor markedly attenuates the p53-induced expression of $p21^{WAF1/CIP1}$, and its biological effects are reversible by over-expression of p53 (Fraser et al., 2003). As shown in **figure 4A**, while ODQ significantly increased the number of apoptotic cells ($p<0.0001$), PFT significantly, although not completely, attenuated ODQ-induced apoptosis ($p<0.01$), suggesting that p53 function is, at least in part, required for ODQ-induced apoptosis. However, even at 30 μM PFT, ODQ was able to induce significant apoptosis, suggesting either that 30 μM PFT does not completely inhibit the pro-apoptotic effects of p53, or that ODQ may induce both p53-dependent and -independent apoptosis. Furthermore, while the inhibitory effects of PFT on p53-mediated gene transcription are well documented, it is unclear whether PFT inhibits the putative mitochondrial effects of p53 (Mihara et al., 2003). Thus, to further examine the role of p53 in sGC-mediated cell survival, we down-regulated p53 by RNAi in ODQ-treated A2780s cells using a p53-specific siRNA. The cells were transfected for 24h, and then treated with ODQ (0, 30 μM) for a further 24h. The p53 siRNA (50 nM) markedly lowered p53 protein content in the absence and presence of ODQ (**figure 4B**), relative to the control siRNA. Furthermore, while ODQ significantly induced apoptosis ($p<0.0001$), this effect was significantly, although not completely, inhibited by the p53 siRNA ($p<0.05$). Together, these data suggest that up-regulation of p53 is, at least in part,

required for ODQ-induced apoptosis and that the sGC/cGMP pathway contributes to cell survival by promoting a reduction of basal p53 content.

p53 mutant cells show reduced response to ODQ-induced apoptosis

Since mutation of the *TP53* gene is a frequent occurrence in human ovarian cancer (Havrilesky et al., 2003; Leitao et al., 2004), whether p53 mutation affects the requirement for sGC activity for basal survival of ovarian cancer cells was next determined. To accomplish this, A2780s cells (wt p53) and the p53-mutant counterpart cell line, A2780cp, were cultured in the presence of ODQ (as above). The passage number of both cell lines was equal during co-treatment, thereby eliminating the confounding effects of passage across cell lines. A2780cp cells express p53 mutated in helix 1 of the DNA binding domain (Olivier et al., 2002) (p53 mutation database, version R8, June 2003). While A2780cp cells expressed more p53 protein in the absence of ODQ than did the A2780s cells (**figure 4C**, lane 1 vs. lane 4), ODQ only slightly up-regulated p53 in the A2780cp cells while p53 was markedly up-regulated by ODQ in A2780s cells. Furthermore, while ODQ up-regulated the p53-responsive gene products $p21^{WAF1/CIP1}$ and Bax in a concentration-dependent manner in the A2780s cells, these responses were markedly attenuated in A2780cp cells, despite the high p53 content in these cells (**figure 4C**). This suggests that p53 function is markedly attenuated in the p53 mutant A2780cp cell line. Moreover, ODQ-induced apoptosis was significantly attenuated in A2780cp cells, relative to A2780s cells ($p<0.01$), suggesting that modulation of p53-mediated apoptosis is one important mechanism by which the sGC/cGMP pathway regulates cell fate.

Figure 4 - The sGC/cGMP pathway regulates cell viability, in part, through activation of p53

(A), A2780s cells were cultured for 24h in the presence of ODQ (0, 30μM) in the absence or presence of the specific p53 inhibitor pifithrin-alpha hydrobromide (PFT; 0-30 μM). Results shown as mean ± SEM of 3 independent experiments. ODQ significantly increased the number of apoptotic cells ($p<0.0001$). This effect was significantly, though not completely, attenuated by PFT ($p<0.01$). *** - $p<0.001$, relative to equi-molar PFT, ODQ-treated cells; ** - $p<0.01$, relative to equi-molar PFT, ODQ-treated cells. ***(B)***, A2780s cells were transfected with a p53-specific siRNA (0, 50 nM; scrambled sequence as control) for 24h, and subsequently treated for a further 24h with ODQ (0, 30 μM). Protein content and extent of apoptosis were assessed as indicated above. Western blot is representative of and histogram shows mean ± SEM of 3 independent experiments. ODQ significantly increased the number of apoptotic cells ($p<0.0001$). This was significantly, though not completely, attenuated by down-regulation of p53 using the p53 siRNA ($p<0.05$). ***(C)*** A2780s cells and the p53 mutant variant cell line A2780cp were cultured for 24h in the presence of ODQ (0, 15, 30 μM). Histogram shows mean ± SEM of 3 independent experiments. ODQ significantly increased the number of apoptotic cells in both cell lines ($p<0.0001$). However, apoptosis was significantly, though not completely, attenuated in the p53 mutant A2780cp cell line, relative to its wild-type p53 counterpart, A2780s ($p<0.0001$). To confirm the lack of p53 function in the A2780cp cells, we tracked p53, Bax, and $p21^{WAF1/CIP1}$ content by Western blot analysis (representative of 3 independent experiments). ODQ markedly up-regulated p53, p21, and Bax content in the wild-type A2780s cells. Despite the high levels of (mutant) p53 in the A2780cp cells,

only minimal p21 and Bax immunoreactivity was observed in this cell line. Taken together, these data suggest that the up-regulation of p53 plays a role in ODQ-induced apoptosis, and that the sGC/cGMP pathway regulates basal cell viability, at least in part, through down-regulation of p53. *** - $p<0.001$, relative to CTL transfected, CTL-treated cells; * - $p<0.05$, relative to p53 transfected, CTL-treated cells.

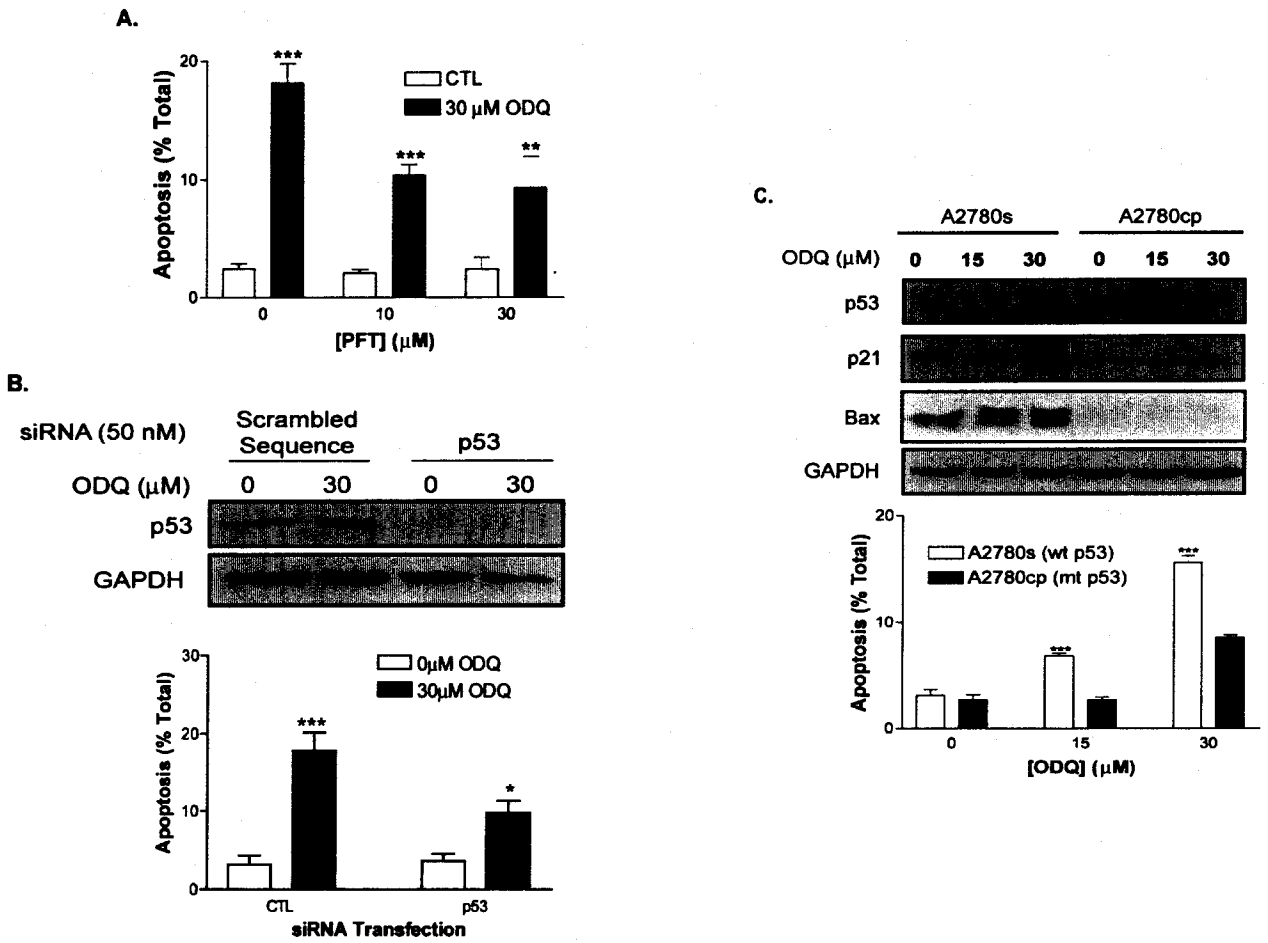

A.
Apoptosis (% Total)
CTL
30 μM ODQ
[PFT] (μM)
B.
siRNA (50 nM)
Scrambled Sequence
p53
ODQ (μM)
p53
GAPDH
0μM ODQ
30μM ODQ
CTL
p53
siRNA Transfection
C.
A2780s
A2780cp
ODQ (μM)
p53
p21
Bax
GAPDH
A2780s (wt p53)
A2780cp (mt p53)
[ODQ] (μM)

Figure 4

ODQ increases p53 stability and induces its phosphorylation on Ser 15

The mechanism by which the sGC/cGMP pathway contributes to low basal p53 content and p53-mediated apoptosis is unclear. Since p53 can be regulated by MDM2-mediated ubiquitination and proteasomal degradation, we next wanted to determine whether inhibition of sGC affects p53 stability. To that end, A2780s cells were 'pulsed' with ODQ (30 μM; DMSO control) for 6h, followed by a 'chase' period (0-240 min) in the presence of 10 μg/ml cycloheximide (CHX) (see Materials and Methods). This concentration of CHX was chosen based on previous studies performed in this cell line (Xiao et al., 2003). Whole-cell lysates were analysed for p53 content by Western blot. Blots were analysed such that p53 content in each treatment group (i.e. ODQ vs. DMSO) was determined within its linear range to avoid the possible confounding effects of ODQ-mediated p53 up-regulation over the 'pulse' period. As shown in **figure 5A**, in the presence of DMSO, p53 was reduced to less than half of its time zero content in <30 minutes post-CHX. By contrast, ODQ markedly extended p53 half-life to 120-240 minutes, suggesting a 4-8 fold increase in p53 half-life.

While MDM2 is a key negative regulator of p53 stability, it is also a p53-responsive gene product. Furthermore, the interaction of MDM2 with p53 is attenuated by phosphorylation of p53 on Ser15. For these reasons, an analysis of MDM2 levels may not be indicative of the role of MDM2 in p53 stabilization. To that end, we next examined the effects of ODQ on MDM2 content and p53 Ser15 phosphorylation. A2780s cells were treated with ODQ (0-30 μM, as above) and whole-cell lysates were analysed for MDM2, total p53, and phospho-p53 (Ser15). As shown in **figure 5B**, ODQ

up-regulated p53, and p53 was phosphorylated on Ser15. Furthermore, MDM2 was also up-regulated by ODQ, which was expected given its p53-responsive nature.

Taken together, these results suggest that inhibition of the sGC/cGMP pathway up-regulates p53 and induces p53-dependent apoptosis via phosphorylation-dependent protein stabilization.

Figure 5 - sGC/cGMP Regulates p53 Stability and Phosphorylation.

(A) A2780s cells were 'pulsed' with ODQ (30 μM) or DMSO (1:1000) for 6h. Media was removed and cells were treated with cycloheximide (10 μg/ml) for different durations as indicated. Whole-cell lysates were analysed for p53 content by Western blotting. Mean p53 contents (fold of zero hour control for each treatment group) of three independent experiments is included beneath the representative Western blot. ***(B)*** A2780s cells were treated with ODQ (30 μM) or DMSO (1:1000) for 24h, and p53, phospho-p53 (Ser15), and MDM2 contents were analysed by Western blotting. Blots shown are representative of 3 independent replicates.

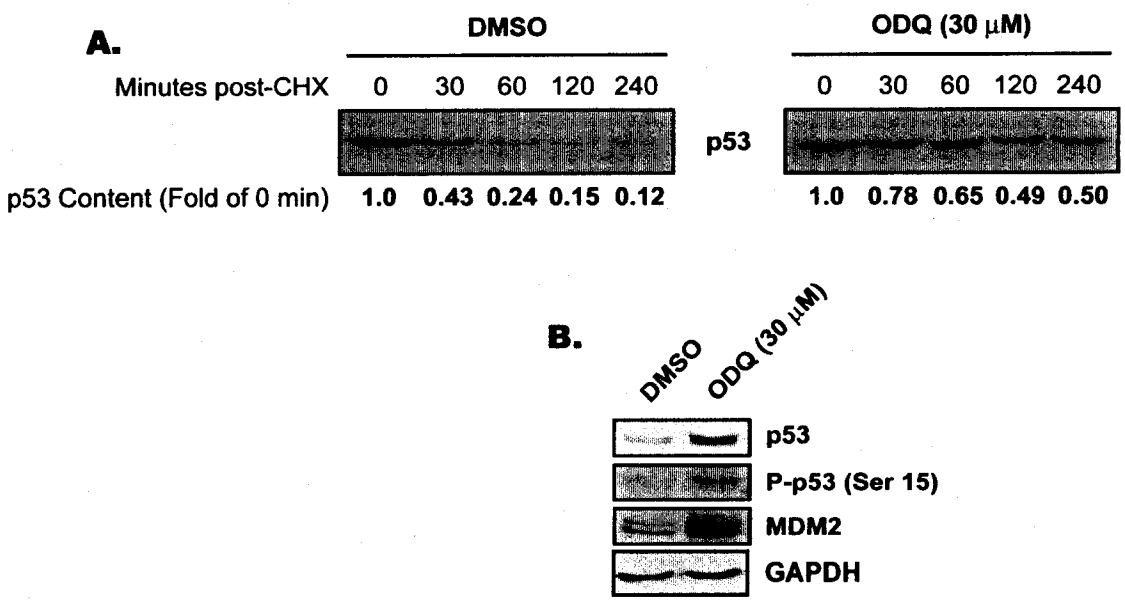

A.
DMSO
ODQ (30 μM)
Minutes post-CHX
0 30 60 120 240
0 30 60 120 240
p53
p53 Content (Fold of 0 min)
1.0 0.43 0.24 0.15 0.12
1.0 0.78 0.65 0.49 0.50
B.
DMSO
ODQ (30 μM)
p53
P-p53 (Ser 15)
MDM2
GAPDH

Figure 5

Discussion

The present study demonstrates that the basal activity of the soluble guanylyl cyclase/cGMP pathway plays an important role in the regulation of human cancer cell viability. Furthermore, our data represents, to our knowledge, the first report that directly demonstrates that this pathway influences mammalian cell viability by regulating the stability and activation of p53 and p53-mediated apoptosis.

sGC is activated by the binding of nitric oxide (NO) to its heme moiety (Waldman & Murad, 1987). sGC also has basal activity and contributes to most of the basal cGMP levels found in mammalian cells (Fiscus, 2002; Garthwaite et al., 1995; Waldman & Murad, 1987). While activation of sGC and elevation of cGMP levels inhibits apoptosis in many cell types (Fiscus, 2002; Fiscus et al., 2001; Fiscus et al., 2002; Kim et al., 1999), inhibition of basal sGC activity with specific inhibitors (e.g. ODQ) lowers basal cGMP levels and induces apoptosis (Fiscus, 2002; Flamigni et al., 2001; Garthwaite et al., 1995). For example, ODQ reduces cGMP, markedly increases caspase activity and results in a loss of cell viability (Flamigni et al., 2001). Furthermore, ODQ induces the processing of caspase-3 and -9, mitochondrial cytochrome c release, reduction of Bcl-2 content and dephosphorylation of Bad at Ser 112. Indeed, sGC is required for the basal viability of both uterine epithelial cells (Chan & Fiscus, 2003) and neural cells (Fiscus, 2002). The present study demonstrates that basal sGC activity/cGMP content is required for viability in cultured human ovarian cancer cells, and, as such, provides new insight into the mechanisms by which these cells may circumvent the normal induction of apoptosis. Moreover, the current study suggests that p53 may be intimately involved in the apoptosis resulting from decreased sGC activity/cGMP availability.

While ODQ-induced apoptosis was inhibited by both Atrial Natriuretic Factor, an activator of the particulate form of guanylyl cyclase (pGC), and the stable cGMP analog 8-Br-cGMP, neither agent completely blocked ODQ-induced apoptosis, although both reduced the percentage of apoptotic cells by >50%. It is possible that these agents were not able to fully restore basal cGMP levels, and thus could not completely restore cGMP-dependent cell survival. Moreover, 8-Br-cGMP is relatively, but not completely, resistant to phosphodiesterase-mediated degradation. Thus, the effects of this analog may be limited by degradation. Furthermore, ANF receptor abundance in ovarian cancer cells has not been examined, and may limit the effectiveness of this activator.

Over-expression of Xiap significantly attenuated ODQ-induced apoptosis. This, along with the observation that ODQ-induced apoptosis is associated with caspase-3 cleavage, suggests that ODQ-induced apoptosis likely proceeds through a caspase-mediated mechanism. This is consistent with previous data (Flamigni et al., 2001), which showed that ODQ markedly activates caspase-3 and -9, although a direct involvement of these caspases in ODQ-induced apoptosis was not demonstrated. By contrast, our data suggests a functional role for these caspases in ODQ-induced apoptosis, and provides further evidence that Xiap is a key regulator of apoptosis in human ovarian cancer cells.

p53 is a transcription factor that regulates the expression of genes involved in DNA repair, cell cycle arrest, and apoptosis. p53 is up-regulated during cell stress via a phosphorylation-dependent process by which p53 escapes degradation induced by its negative regulator, MDM2. Since MDM2 is a p53-responsive gene product, this system functions as a negative feedback loop, which prevents inappropriate induction of p53-mediated cell cycle arrest or apoptosis, yet permits a rapid response to cell stress.

Moreover, the MDM2-p53 interaction is promoted by a number of cell survival mediators (Mayo & Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001). p53 also contributes to apoptosis by a transcription-independent mechanism, likely via translocation to the mitochondria, where it binds to and inhibits the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-$_{XL}$, (Erster et al., 2004; Mihara et al., 2003).

ODQ up-regulated p53 in three ovarian cancer cell lines, and this was attenuated by co-treatment with 8-Br-cGMP, suggesting that maintenance of low basal p53 levels is promoted by tonic sGC activation and cGMP production. Addition of 8-Br-cGMP had no effect on the basal levels of p53. This is consistent with the hypothesis that basal p53 levels are already tightly regulated, and cannot be lowered further. However, removal of basal cGMP by inhibition of sGC eliminates its tonic inhibitory effect, and p53 levels are allowed to increase. Intriguingly, ODQ up-regulated p53 in the cisplatin-resistant C13* cell line. We previously showed that treatment of these cells with cisplatin does not markedly up-regulate p53 (Fraser et al., 2003), suggesting that the mechanism of up-regulation of p53 by ODQ is distinct from that of cisplatin. In addition, ODQ-induced apoptosis was attenuated by the specific p53 inhibitor pifithrin-alpha hydrobromide (PFT), by p53 RNAi, and in p53 mutant cells. All of these mechanisms of p53 inhibition attenuated ODQ-induced apoptosis by ~50%, suggesting that while p53 contributes to ODQ-induced apoptosis, p53-independent mechanism(s) are likely involved. Treatment with ODQ also down-regulated Xiap, and this may represent a p53-independent mechanism of apoptosis induction. This hypothesis is currently under investigation.

Thus, our study provides the first direct evidence that the sGC/cGMP pathway can regulate apoptosis, in part, via regulation of p53 content/function. While Heinloth et

al previously demonstrated that 8-Br-cGMP down-regulates p53 following treatment of macrophages with oxidized LDL (OxLDL) (Heinloth et al., 2002), they did not suggest a mechanism by which this occurs. Moreover, Heinloth et al did not address the question of whether down-regulation of p53 was involved in cGMP-mediated cell survival. In the current study, we demonstrate that inactivation of sGC/cGMP signalling by ODQ treatment up-regulates p53 and leads to a marked increase in p53 stability and p53-dependent apoptosis. Interestingly, ODQ also induced the phosphorylation of p53 on Ser15, a residue that lies adjacent to the MDM2 binding site, which is required for p53 stabilization and activation following cell stress, and is an important determinant of p53-mediated apoptosis (Ashcroft et al., 1999; Persons et al., 2000; Shieh et al., 1997; Siliciano et al., 1997; Unger et al., 1999b). Since ODQ also up-regulated MDM2, it is likely that depletion of sGC-derived cGMP results in phosphorylation-dependent attenuation of MDM2-mediated p53 degradation, and thus to stabilization and activation of p53. It is also possible that sGC-mediated cell survival results from a cGMP- or PKG-dependent inhibition of one or more kinases that target p53. Future studies will help to clarify the precise mechanism by which the sGC/cGMP pathway targets p53 stability.

Panahian et al demonstrated that over-expression of heme oxygenase-1 (HO-1) facilitates neuroprotection in mice, an effect that was associated with an up-regulation of cGMP levels as well as inhibition of nuclear localization of p53 (Panahian et al., 1999). Moreover, HO-1 co-localizes with p53 in the cytoplasm of motor neurons below the level of injury and with cGMP in the nucleus of injured cells (Panahian & Maines, 2001). However, these results do not directly implicate cGMP as a regulator of cell survival, or as a regulator of p53. Chen et al demonstrated that nitric oxide (NO)-induced apoptosis

was associated with an up-regulation of p53 and Bax. However, the authors demonstrated that these effects were cGMP-independent (Chen et al., 2001). Thus, while some correlative evidence exists to suggest a link between cGMP and p53, our report represents the first *direct* evidence demonstrating that the sGC/cGMP pathway facilitates cell survival by negatively regulating p53 content/function. Indeed, one previous study demonstrates that *activation* of the cGMP pathway up-regulates p53 and induces apoptosis in rat vascular endothelial cells (Suenobu et al., 1999). Thus, the role of the sGC/cGMP pathway as a cell fate determinant may be cell-type specific.

Interestingly, the Akt cell survival pathway plays an important role in regulating p53 in human ovarian cancer cells (Fraser et al., 2003). Akt phosphorylates and activates MDM2 (Mayo & Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001). Several reports have established that cGMP promotes Akt phosphorylation (Ciani et al., 2002; Falcone et al., 2002; Ha et al., 2003; Tejedo et al., 2004). Thus, it is possible that ODQ-mediated depletion of cGMP inactivates Akt, thereby attenuating the inhibitory effect of MDM2 on p53. However, we did not observe any effects of ODQ on phospho- or total Akt contents (data not shown), suggesting that the p53-dependent and/or -independent pathways of ODQ-induced apoptosis are not mediated via inactivation of Akt.

It is intriguing that ODQ-induced apoptosis is significantly, though incompletely, attenuated by 8-Br-cGMP. While we cannot completely exclude the possibility that ODQ may have non-specific actions which result in apoptosis, the finding that p53 content was returned to basal levels by 8-Br-cGMP suggests that this effect of ODQ, and the resulting *p53-mediated* apoptosis, is specific to the reduction in cGMP levels.

Interestingly, depletion of endogenous nitric oxide (NO) up-regulates p53 and sensitizes melanoma cells to cisplatin-induced apoptosis (Tang & Grimm, 2004). Since NO is known to function primarily, although not exclusively (Chen et al., 2001), through the activation of sGC and the production of cGMP, reductions in NO/sGC-derived cGMP levels may have been responsible for the up-regulation of p53, although this was not examined. Furthermore, NO promotes mammary tumour migration and invasion and Nitric Oxide Synthase (NOS) activity is associated with human breast cancer progression (Jadeski et al., 2003; Jadeski et al., 2000; Orucevic et al., 1999). Thus, activation of NO/cGMP may be an important mediator of tumorigenesis and cancer progression.

Moreover, the basal activity of sGC mediating the anti-apoptotic effects in human ovarian cancer cells may be dependent, at least in part, on the endogenous production of NO. Although the role of endogenous NO is not yet clear, recent data from our laboratory have shown that human ovarian cancer cells express all three forms of NOS, which likely results in elevated basal sGC activity (unpublished data).

It will be of interest to study the *in* vivo relationship between sGC/cGMP and p53 with respect to ovarian tumorigenesis. Very little is known regarding the precise factors that give rise to ovarian tumours, since most are not discovered until Stage III or IV, by which time it is difficult to decipher the molecular origins of the tumour. It will be critical to examine whether molecular abnormalities of sGC/cGMP are a common event in clinical ovarian cancer, where alterations in other apoptosis-related genes and gene products (e.g. PI3K, Akt, Bcl-2, p53) are frequently observed. Intriguingly, high levels of urinary cGMP correlate with poor prognosis in ovarian and other cancers (Luesley et al., 1986; Luesley et al., 1987; Turner et al., 1982a; Turner et al., 1982b), although

whether this cGMP is derived from the tumour itself, and whether cGMP plays a causative role in ovarian tumorigenesis and therapeutic response remains unclear.

Thus, these findings provide a new concept for the regulation of apoptosis in ovarian cancer cells and of ovarian tumour growth, showing a potential link between the anti-apoptotic effects of the sGC/cGMP signalling pathway and the regulation of p53, which has been identified as an important regulator of tumorigenesis and chemotherapeutic response.

Materials and Methods

Reagents

Hoechst 33258, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), aprotinin and cycloheximide were purchased from Sigma (St. Louis, MO, USA). Mouse monoclonal anti-p53 antibodies, clone 80 and clone DO-1, were purchased from Transduction Laboratories, Inc. (San Diego, CA, USA) and Santa Cruz Biotechnologies (Santa Cruz, CA, USA), respectively. Mouse monoclonal Bax antibody (clone 2D2) was from Santa Cruz Biotechnologies. Mouse monoclonal anti-p21 (clone DCS60), rabbit anti-caspase-3 (cleaved fragment), mouse anti-phospho-p53 (Ser15) and p53 siRNA were from from Cell Signaling Technology, Inc (Beverly, MA, USA). Rabbit polyclonal anti-Xiap antibody was from Trevigen, Inc (Gaithersberg, MD, USA). Mouse monoclonal anti-GAPDH antibody (ab8245) was from Abcam (Cambridge, UK). Pre-stained SDS-PAGE standards were from Bio-Rad (Hercules, CA, USA). ODQ and 8-Bromo-cGMP were from Calbiochem (La Jolla, CA, USA). Scrambled control siRNA was from Dharmacon Inc (Lafayette, CO, USA). RiboJuice siRNA transfection reagent

was purchased from Novagen, Inc (San Diego, CA, USA). Pifithrin-alpha hydrobromide was purchased from Tocris, Inc (Ellisville, MO, USA). Xiap-s and LacZ adenoviruses were synthesized in the laboratory of Dr. Ruth Slack, Adenovirus Core Facility, University of Ottawa. Atrial Natriuretic Factor (ANF) was a generous gift from Dr. Adolfo de Bold, University of Ottawa Heart Institute.

Cell lines and culture

All cell lines were maintained at 37°C and in an atmosphere of 5% CO_2/ 95% air. Wild-type p53, cisplatin-sensitive A2780s human ovarian cancer cells and the mutant p53, cisplatin-resistant counterpart cell line A2780cp were maintained in DMEM/F12 medium. OV2008 and C13* human ovarian cancer cells were maintained in RPMI 1640 medium. All media were supplemented with fetal bovine serum (10%), streptomycin (50 g/ml), penicillin (50 units/ml), fungizone (0.625 g/ml; Life Technologies, Inc. BRL, Carlsbad, CA, USA) and non-essential amino acids (1%), as previously reported (Fraser et al., 2003). Cells were plated at a density of 5 x 10^4 cells/cm^2 in 6-well plates 18h prior to experimental treatments. At the time of treatment, the cells were ≤85% confluent.

Hoechst 33258 Staining

At the end of the culture period, cells attached to the growth surface were removed by trypsin treatment [trypsin (0.05%), EDTA (0.53mM); 37 °C, 1 min]. Attached and floating cells were pooled, pelleted by centrifugation, and re-suspended in phosphate buffered formalin (10%) containing Hoechst 33258 (12.5 ng/ml). Cells were spotted onto slides for microscopy. Nuclear staining was observed and photographed using a Zeiss fluorescence microscope (magnification 400X). Cells with typical

apoptotic nuclear morphology (nuclear shrinkage, condensation, and fragmentation) were identified and counted as previously reported (Asselin et al., 2001a; Fraser et al., 2003; Sasaki et al., 2002; Sasaki et al., 2000), using randomly selected fields. A minimum of 200 cells were counted in each treatment group. The counter was 'blinded' to sample identity to avoid experimental bias.

Determination of Apoptosis by TUNEL Assay

Apoptosis also was assessed by the TUNEL method using the In Situ Cell Death Detection Kit, TMR Red assay kit from Roche (Laval, QC, Canada) according to the manufacturer's instructions. Briefly, cells were plated as above and treated with ODQ (0, 30 μM). After 24h of treatment, floating and attached cells were pooled and pelleted by centrifugation (5 min; 300 x g) and washed 2x in PBS. The cells were fixed in 10% phosphate buffered formalin for 1h on ice. Fixed cells were washed in PBS and permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate for 5 minutes. Cells were re-suspended in 50 μl TUNEL reaction mixture (5 μl enzyme + 45 μl label solution) and incubated at 37°C in the dark for 1h. Hoescht 33258 (1:1000) was added during the last 5 minutes to allow visualization of all nuclei under florescent microscopy. Cells were washed 2x in PBS and then plated on microscope slides and visualized by florescence microscopy. The percentage of cells undergoing apoptosis was determined by counting the number TUNEL positive nuclei divided by the total number of nuclei as determined by Hoescht 33258 staining.

cGMP Assay

cGMP contents were determined using Enzyme Immunoassay Kits (Direct Cyclic GMP) from Assay Designs, Inc (Ann Arbor, MI, USA). The cells were plated onto 100 mm plates and after two days, experiments were performed to determine the cGMP depletion effects of ODQ. Each plate represented approximately 7.5 million cells. The cells were exposed to ODQ at 10, 30, 50 and 100 μM for 20 min, followed by rapid removal of media, rapid washing of the cells with PBS and addition of 1.5 ml of ice-cold 0.1 N HCl. The cells (with the HCl cell-lysis solution) were set on ice for 10 min, followed by scraping the cells from the plate and transferring the samples to microfuge tubes. The cell lysates were centrifuges at 600 x g at room temperature and the supernatant fractions collected for cGMP analysis. Both the samples (200 μl) and the standards (200 μl, also prepared in the same 0.1 N HCl solution) were acetylated (as described in the protocol of the kits) in order to increase the sensitivity of the assay for cGMP measurement. Two aliquots (100 μl each) of the samples and standards were pipeted into the wells of a 96-well tray (provided in the kits). The addition of the antibody (specific for cGMP) and other reagents were added as described in the protocol of the kits. To increase the accuracy of the measurements of cGMP in samples with low cGMP content (i.e. ODQ-treated cells), we added two additional low-concentration standards (4 and 2 fmoles/well). The number of cells in the 100 mm dish was determined by counting cells with a hemocytometer. cGMP contents are reported as fmol/million cells.

Protein Extraction and Western Blot Analysis

Cells were pelleted and lysed in ice-cold lysis buffer (pH 7.4) containing 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM NaPPi, 10 %

Glycerol and 1% Triton X-100. Protease inhibitors PMSF (1 mM) and aprotonin (10 g/l), as well as 1 mM Na_3VO_4 were added to the lysis buffer freshly. Cell lysates were sonicated briefly (5 s/cycle, 3 cycles; 0 °C). The sonicates were incubated on ice for 1 h and pelleted by centrifugation (15, 000 x g; 20 min). The supernatant was taken as whole-cell lysate and stored at -20 °C for subsequent analyses. Protein concentration was determined using Bio-Rad DC protein assay kit. Equal amounts of proteins (30-70 μg) were loaded and resolved by 10% SDS-PAGE and electro-transferred (30V, 16 h) onto nitrocellulose membranes (Bio-Rad). Membranes were blocked (room temperature, 1 h) with 5% Blotto [Tris-HCl (10 mM; pH 8.0), NaCl (150 mM), Tween 20 (0.05%, v/v; TBS-Tween 20) containing skim milk (5%; w/v)], then incubated overnight with primary antibodies [p53 (1:1,000, Clones 80 and DO-1), p21 (1:1000), Xiap (1:2000), cleaved caspase-3 (1:1000), or GAPDH (1:30,000)], and subsequently with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody [1:2,000 in 5% Blotto; room temperature, 1 h; 1:20,000 for GAPDH]. Peroxidase activity was visualized with an ECL kit (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) after three washes (15 min/wash) with TBS-Tween 20. Signal intensity was determined densitometrically using Scion Image software, version 4.02 from Scion Corporation (Frederick, MD, USA). Even loading between lanes was determined using Ponceau-S stain and by densitometry of the anti-GAPDH blots. All blots were exposed to film for an appropriate duration to avoid saturation of the film.

p53 Half-Life Determination

After 18h of plating, culture medium was removed and replaced with serum-free medium containing ODQ (30 μM) or DMSO (1:2000) and then cells were returned to the

incubator for 6h. The medium was then removed, the cells were washed 2 times with serum-free medium and fresh medium was added containing 10 μg/ml cycloheximide. Cells were harvested at various time points following cycloheximide treatment (0, 30, 60, 120, 240 min). Whole-cell lysates were obtained and p53 content was analysed by Western blot using Alexa Fluor 680 goat anti-mouse secondary antibodies (Invitrogen) and analysed using a Typhoon Phosphorimager to ensure that all experimental groups were analysed within their 'linear', non-saturated range, and to avoid the confounding effects of ODQ-induced up-regulation of p53 during the 'pulse' period.

Adenoviral Infection

After 18 h of plating (10^6 cells/60-mm culture dish), cells were infected with adenoviral sense Xiap (Xiap-s) or LacZ cDNA at various multiplicities of infection (MOI) as indicated in the text as previously described (Li et al., 2001). Adenovirus infection efficiency at MOI of 5, as determined by an X-gal staining assay against LacZ construct infected cells, was >90%. Xiap over-expression was confirmed by Western blot analysis.

RNA Interference

18h after plating, 6 μl of transfection reagent was added to 244 μl of DMEM/F12 without serum. The mixture was vortexed and incubated for 5 minutes at room temperature. Following incubation, 7.5 μl of 10 μM stock siRNA construct was added. The mixture was incubated at room temperature for a further 15 minutes. During this period, the culture media was removed from the cells and the cells were washed once with PBS. The siRNA mixture was added in single drops to each well to ensure maximal

coverage, and an additional 1250 µl of complete (10% FBS) media was added. The cells were returned to the incubator and the media was removed 6 hours later and replaced with fresh, complete media for the duration of the culture (24h). Down-regulation was confirmed by Western blot analysis.

Statistical Analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was carried out by one- or two-way ANOVA or by Student's t-test (where appropriate) using PRISM software (Version 3.0; GraphPad, San Diego, CA, USA). Differences between multiple experimental groups were determined by the Bonferroni post-hoc test. Statistical significance was inferred at $p<0.05$.

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References

Ashcroft M, Kubbutat MH and Vousden KH. (1999). *Mol Cell Biol*, 19, 1751-8.
Asselin E, Mills GB and Tsang BK. (2001). *Cancer Res*, 61, 1862-8.
Chan SL and Fiscus RR. (2003). *Mol Hum Reprod*, 9, 775-83.
Chen HW, Jiang WS and Tzeng CR. (2001). *Fertil Steril*, 75, 1163-71.
Ciani E, Virgili M and Contestabile A. (2002). *J Neurochem*, 81, 218-28.
Erster S, Mihara M, Kim RH, Petrenko O and Moll UM. (2004). *Mol Cell Biol*, 24, 6728-41.
Estevez AG, Spear N, Manuel SM, Barbeito L, Radi R and Beckman JS. (1998). *Prog Brain Res*, 118, 269-80.
Falcone S, Mauro L, de Rose G, Paolucci C, Sciorati C, Ando S and Clementi E. (2002). *Biochem J*, 366, 165-73.
Fiscus RR. (2002). *Neurosignals*, 11, 175-90.
Fiscus RR, Tu AW and Chew SB. (2001). *Neuroreport*, 12, 185-9.
Fiscus RR, Yuen JP, Chan SL, Kwong JH and Chew SB. (2002). *J Card Surg*, 17, 336-9.
Flamigni F, Facchini A, Stanic I, Tantini B, Bonavita F and Stefanelli C. (2001). *Biochem Pharmacol*, 62, 319-28.
Fraser M, Leung BM, Yan X, Dan HC, Cheng JQ and Tsang BK. (2003). *Cancer Res*, 63, 7081-8.
Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K and Mayer B. (1995). *Mol Pharmacol*, 48, 184-8.
Ha KS, Kim KM, Kwon YG, Bai SK, Nam WD, Yoo YM, Kim PK, Chung HT, Billiar TR and Kim YM. (2003). *Faseb J*, 17, 1036-47.
Havrilesky L, Darcy M, Hamdan H, Priore RL, Leon J, Bell J and Berchuck A. (2003). *J Clin Oncol*, 21, 3814-25.
Heinloth A, Brune B, Fischer B and Galle J. (2002). *Atherosclerosis*, 162, 93-101.
Jadeski LC, Chakraborty C and Lala PK. (2003). *Int J Cancer*, 106, 496-504.
Jadeski LC, Hum KO, Chakraborty C and Lala PK. (2000). *Int J Cancer*, 86, 30-9.
Kerr JF, Winterford CM and Harmon BV. (1994). *Cancer*, 73, 2013-26.
Kim YM, Chung HT, Kim SS, Han JA, Yoo YM, Kim KM, Lee GH, Yun HY, Green A, Li J, Simmons RL and Billiar TR. (1999). *J Neurosci*, 19, 6740-7.
Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV and Gudkov AV. (1999). *Science*, 285, 1733-7.
LaCasse EC, Baird S, Korneluk RG and MacKenzie AE. (1998). *Oncogene*, 17, 3247-59.
Leitao MM, Soslow RA, Baergen RN, Olvera N, Arroyo C and Boyd J. (2004). *Gynecol Oncol*, 93, 301-6.
Levine EL, Renehan A, Gossiel R, Davidson SE, Roberts SA, Chadwick C, Wilks DP, Potten CS, Hendry JH, Hunter RD and et al. (1995). *Radiother Oncol*, 37, 1-9.
Li J, Feng Q, Kim JM, Schneiderman D, Liston P, Li M, Vanderhyden B, Faught W, Fung MF, Senterman M, Korneluk RG and Tsang BK. (2001). *Endocrinology*, 142, 370-80.
Liebermann DA, Hoffman B and Steinman RA. (1995). *Oncogene*, 11, 199-210.
Luesley DM, Blackledge GR, Chan KK and Newton JR. (1986). *Br J Obstet Gynaecol*, 93, 380-5.

Luesley DM, Chan KK, Newton JR and Blackledge GR. (1987). *Br J Obstet Gynaecol*, 94, 461-6.
Mayo LD and Donner DB. (2001). *Proc Natl Acad Sci U S A*, 98, 11598-603.
Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P and Moll UM. (2003). *Mol Cell*, 11, 577-90.
Moro MA, Russel RJ, Cellek S, Lizasoain I, Su Y, Darley-Usmar VM, Radomski MW and Moncada S. (1996). *Proc Natl Acad Sci U S A*, 93, 1480-5.
Mulsch A, Bauersachs J, Schafer A, Stasch JP, Kast R and Busse R. (1997). *Br J Pharmacol*, 120, 681-9.
Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, Masuyama N and Gotoh Y. (2002). *J Biol Chem*, 277, 21843-50.
Olesen SP, Drejer J, Axelsson O, Moldt P, Bang L, Nielsen-Kudsk JE, Busse R and Mulsch A. (1998). *Br J Pharmacol*, 123, 299-309.
Olivier M, Eeles R, Hollstein M, Khan MA, Harris CC and Hainaut P. (2002). *Hum Mutat*, 19, 607-14.
Orucevic A, Bechberger J, Green AM, Shapiro RA, Billiar TR and Lala PK. (1999). *Int J Cancer*, 81, 889-96.
Panahian N and Maines MD. (2001). *J Neurochem*, 76, 539-54.
Panahian N, Yoshiura M and Maines MD. (1999). *J Neurochem*, 72, 1187-203.
Persons DL, Yazlovitskaya EM and Pelling JC. (2000). *J Biol Chem*, 275, 35778-85.
Reap EA, Leslie D, Abrahams M, Eisenberg RA and Cohen PL. (1995). *J Immunol*, 154, 936-43.
Sasaki H, Kotsuji F and Tsang BK. (2002). *Gynecol Oncol*, 85, 339-50.
Sasaki H, Sheng Y, Kotsuji F and Tsang BK. (2000). *Cancer Res*, 60, 5659-66.
Sheets EE and Yeh J. (1997). *Ann Med*, 29, 121-6.
Shieh SY, Ikeda M, Taya Y and Prives C. (1997). *Cell*, 91, 325-34.
Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E and Kastan MB. (1997). *Genes Dev*, 11, 3471-81.
Suenobu N, Shichiri M, Iwashina M, Marumo F and Hirata Y. (1999). *Arterioscler Thromb Vasc Biol*, 19, 140-6.
Tang CH and Grimm EA. (2004). *J Biol Chem*, 279, 288-98.
Tejedo JR, Cahuana GM, Ramirez R, Esbert M, Jimenez J, Sobrino F and Bedoya FJ. (2004). *Endocrinology*, 145, 2319-27.
Turner GA, Ellis RD, Guthrie D, Latner AL, Monaghan JM, Ross WM, Skillen AW and Wilson RG. (1982a). *J Clin Pathol*, 35, 800-6.
Turner GA, Ellis RD, Guthrie D, Latner AL, Ross WM and Skillen AW. (1982b). *Br J Obstet Gynaecol*, 89, 760-4.
Unger T, Sionov RV, Moallem E, Yee CL, Howley PM, Oren M and Haupt Y. (1999). *Oncogene*, 18, 3205-12.
Waldman SA and Murad F. (1987). *Pharmacol Rev*, 39, 163-96.
Xiao CW, Yan X, Li Y, Reddy SA and Tsang BK. (2003). *Endocrinology*, 144, 623-30.
Zhou BP, Liao Y, Xia W, Zou Y, Spohn B and Hung MC. (2001). *Nat Cell Biol*, 3, 973-82.

Chapter 7 – Unpublished Data

Regulation of Integrin-Linked Kinase by XIAP

Our laboratory previously demonstrated that over-expression of XIAP increases Akt phosphorylation and confers resistance to CDDP-induced apoptosis (Asselin et al, 2001). Furthermore, data presented in this thesis suggest that the Akt pathway is an important mediator of XIAP-induced chemoresistance (Fraser et al., 2003) (Chapter 4; **Figure 2**). However, the molecular mechanisms by which XIAP influences Akt phosphorylation are not known.

As described in detail in chapter 2, Akt phosphorylation is regulated by PI3K through phosphorylation of the 3' OH group of the inositol ring of the membrane phospholipid PIP2, producing PIP3. Akt is recruited to the cell membrane through interaction of its PH domain with PIP3, and is phosphorylated on Thr308 and Ser473 by the PH domain-containing proteins PDK1 and ILK, respectively (Alessi et al., 1996).

To that end, we hypothesized that the XIAP-induced up-regulation of phospho-Akt was due to an increase in the content/activity of the upstream kinases ILK and/or PDK1. To test this hypothesis, we over-expressed XIAP in chemosensitive A2780s cells using our adenoviral gene delivery system (MOI = 0-10; 24h), as described in Chapter 4. Adeno-LacZ was included as a control such that all treatment groups received equivalent concentrations of adenovirus. Following infection, the cells were harvested as detailed above, and phospho-Akt (Ser473), ILK, and PDK1 protein contents were analyzed by Western blot.

As previously demonstrated (Asselin et al, 2001), over-expression of XIAP increased phospho-Akt (Ser473) content (**Figure 1A**). Interestingly, over-expression of

XIAP also significantly increased ILK content ($P<0.05$), but had no effect upon PDK1 content (**Figure 1A**).

The anti-ILK western blots showed three immunoreactive species around the predicted ILK size (~55kDa) (**Figure 1B**). Since *ILK* gene expression is responsive to TGF-β, we treated A2780s cells with TGF-β (10 ng/ml; DMSO control) following 18h of serum starvation to ensure minimal residual TGF-β receptor activation. The middle band showed an increased intensity in the presence of TGF-β, relative to either serum-starved control (lane 1 vs. lane 2) or DMSO-treated cells (lane 2 vs. lanes 3-5), while the top and bottom bands were insensitive to TGF-β (**Figure 1B**). This suggests that ILK is represented by the middle band, and this was the species that was quantified in **figure 1B**.

To further assess the relationship between XIAP and ILK, we next evaluated ILK mRNA content in response to XIAP over-expression by endpoint RT-PCR. Total RNA from A2780s cells infected with adenoviral XIAP cDNA (as above) was obtained and reverse transcribed as described in chapter 5. ILK and β-actin mRNA content was assessed by endpoint RT-PCR (94C, 30 sec; 54C, 30 sec; 72C, 1 min; 40 cycles ILK, 25 cycles, β-actin). To ensure amplification in the linear range, reactions were first optimized for cycle number by successive amplifications between 15-40 cycles. As shown in **figure 1C**, XIAP over-expression did not significantly up-regulate ILK mRNA content, although there was a trend towards increased ILK mRNA content at higher adenoviral XIAP concentrations.

The mechanism by which XIAP regulates ILK content is unclear. However, it does not appear to be mediated by changes in ILK mRNA content, suggesting a post-

translational mechanism. It is possible that XIAP enhances the stability of ILK, thereby up-regulating its steady-state levels. This could be mediated through XIAP-mediated ubiquitination (and subsequent proteasomal degradation) of molecules involved in promoting ILK stability. However, this hypothesis remains untested, and should be evaluated in follow-up experiments.

We next extended these data by determining the effects of XIAP over-expression on ILK kinase activity by *in vitro* kinase assay. Briefly, ILK was immunoprecipitated from 500 μg (as measured by protein assay; see Chapter 4) of whole-cell lysates of A2780s cells infected with adenoviral XIAP or LacZ (MOI = 10). Serum-starved (18h), EGF-treated (40 ng/ml; 15 minutes) A2780s cells were used as a positive control, while serum-starved (18h) A2780s cells were used as a negative control. Immunoprecipitates were incubated with recombinant Glycogen Synthase Kinase-3β (GSK-3β; Cell Signaling), in the presence of 1x kinase buffer (50 mM HEPES, 2mM $MgCl_2$, 2mM, 2mM $MnCl_2$, 5 mM NaF, 1 mM Na_3VO_4, pH 7.0) plus 200 mM ATP. After 25 minutes of incubation at 30C, the reactions were stopped by the addition of 2x SDS-PAGE sample buffer (see Chapter 4). The samples were resolved on 12% SDS-PAGE gels, transferred to nitrocellulose membranes (80V; 2h), and ILK kinase activity was assessed using an anti-phospho-GSK-3β antibody (Cell Signaling). As shown in **figure 2**, EGF induced the activation of ILK, relative to serum-starved cells (lane 2 vs. lane 1). However, XIAP over-expression failed to increase the kinase activity of ILK relative to the LacZ-infected cells, suggesting that activation of ILK is not the mechanism by which XIAP up-regulates phospho-Akt. However, since this experiment was conducted only once, follow-up studies should be performed to replicate this result and confirm the lack

of effect of XIAP over-expression on ILK activity. Moreover, PDK1 activity has not been similarly evaluated, and this should also be performed in follow-up experiments.

It is possible that XIAP may activate other known Ser473 kinases, including MAPKAPK2 (Rane et al., 2001), thus up-regulating phospho-Akt. Additionally, we have not yet evaluated PDK1 kinase activity, and as such cannot exclude the possibility that XIAP activates PDK1, thereby increasing PDK1-mediated Akt phosphorylation on Ser473. Alternatively, it is possible that XIAP over-expression may activate PI3K itself, or may interact with specific receptors, such as the EGF receptor (EGFR) known to participate in growth factor-mediated PI3K activation, thereby increasing PI3K-induced Akt phosphorylation. Indeed, the interaction of XIAP with cell surface receptors of the TGF-β family has been documented, suggesting that XIAP may interact with other receptor types. Additionally, we have observed that XIAP over-expression-induced Akt phosphorylation is dependent upon the presence of serum (unpublished observations), suggesting that XIAP may act as a co-activator of Akt phosphorylation. It is also possible that XIAP may inhibit the function of inhibitory phosphatases, including PTEN and/or SHIP, thereby increasing PIP3-dependent Akt phosphorylation. To that end, our laboratory recently demonstrated that down-regulation of PTEN sensitizes chemoresistant cells to CDDP (Yan et al., 2006).

Regulation of the CDDP-Induced Mitochondrial Accumulation of p53

While p53 mediates the transcriptional up- and down-regulation of genes implicated in apoptosis (see Chapter 2), evidence now suggests that transcription-independent mechanisms of p53-mediated apoptosis exist, and may play an important role in the mediation of p53-induced apoptosis. Recent evidence suggests that following

an apoptotic stimuli, a portion of total p53 rapidly translocates to the mitochondria and induces apoptosis by directly interacting with anti-apoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-XL. This accumulation preceeds the activation of p53-mediated gene transcription and targeted expression of p53 to the mitochondria is sufficient to induce apoptosis. These data suggest that mitochondrial p53 accumulation may contribute to the apoptotic response to cellular stress.

Figure 1 - Regulation of Phospho-Akt, ILK and PDK1 by XIAP

(A) A2780s cells were infected with adenoviral XIAP cDNA (MOI = 0-10 μM; LacZ control) and XIAP, phospho-Akt, ILK (upper panel), and PDK1 (lower panel) protein contents were assessed by Western blot. N=3. **(B)** A2780s cells were serum-starved for 18h and treated with TGF-β (10 ng/ml; lane 2) or DMSO (CTL 1-3; lanes 3-5) or left untreated (lane 1) and whole-cell lysates were analysed for ILK content by Western blot. Middle band is affected by TGF-β, suggesting that this is ILK. **(C)** A2780s cells were infected with adenoviral XIAP cDNA (as above), and total RNA was extracted and reverse transcribed as detailed in chapter 6. ILK and β-actin mRNA contents were assessed by endpoint semi-quantitative RT-PCR. N=3.

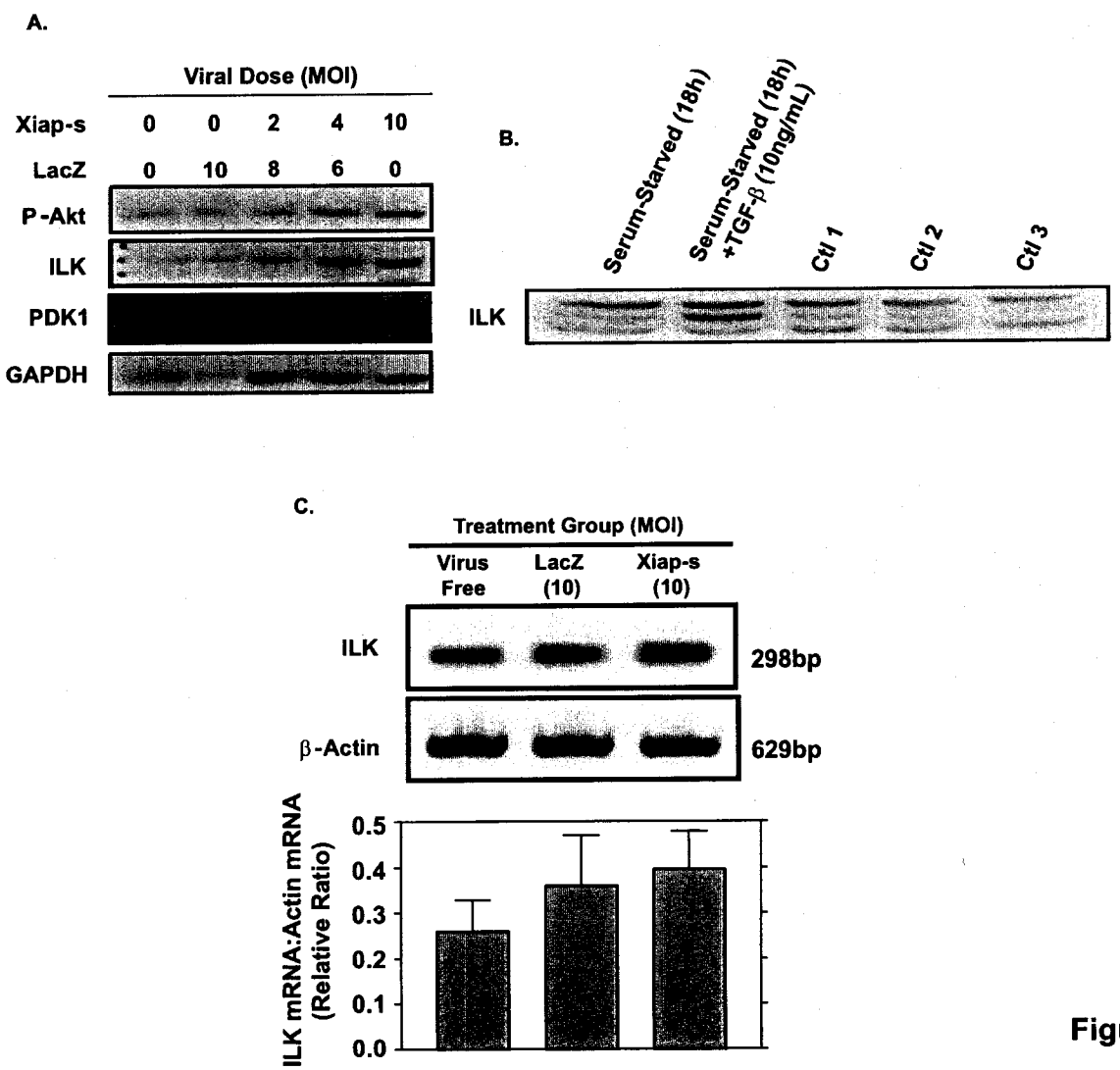

A.
Viral Dose (MOI)
Xiap-s 0 0 2 4 10
LacZ 0 10 8 6 0
P-Akt
ILK
PDK1
GAPDH
B.
Serum-Starved (18h)
Serum-Starved (18h) +TGF-β (10ng/mL)
Ctl 1
Ctl 2
Ctl 3
ILK
C.
Treatment Group (MOI)
Virus Free
LacZ (10)
Xiap-s (10)
ILK
298bp
β-Actin
629bp
ILK mRNA:Actin mRNA (Relative Ratio)
0.5
0.4
0.3
0.2
0.1
0.0

Figure 1

Figure 2 – Regulation of ILK Kinase Activity by XIAP Over-Expression

A2780s cells were infected with adenoviral XIAP cDNA (as above) or serum-starved for 18h and then treated with Epidermal Growth Factor (EGF; 40ng/ml) for 15 minutes prior to harvest. ILK was immunoprecipitated using a mouse monoclonal anti-ILK antibody (anti-FLAG control), and immunoprecipitates were incubated with recombinant Glycogen Synthase Kinase-3β in 1x kinase buffer (as indicated) in the presence of ATP for 30 min at 37C, followed by the addition of 30 μl of 2x SDS buffer. (see Chapter 4) Reaction products were separated by SDS-PAGE, and ILK activity was assessed by Western blot using an anti-phospho-GSK-3β antibody. N=1.

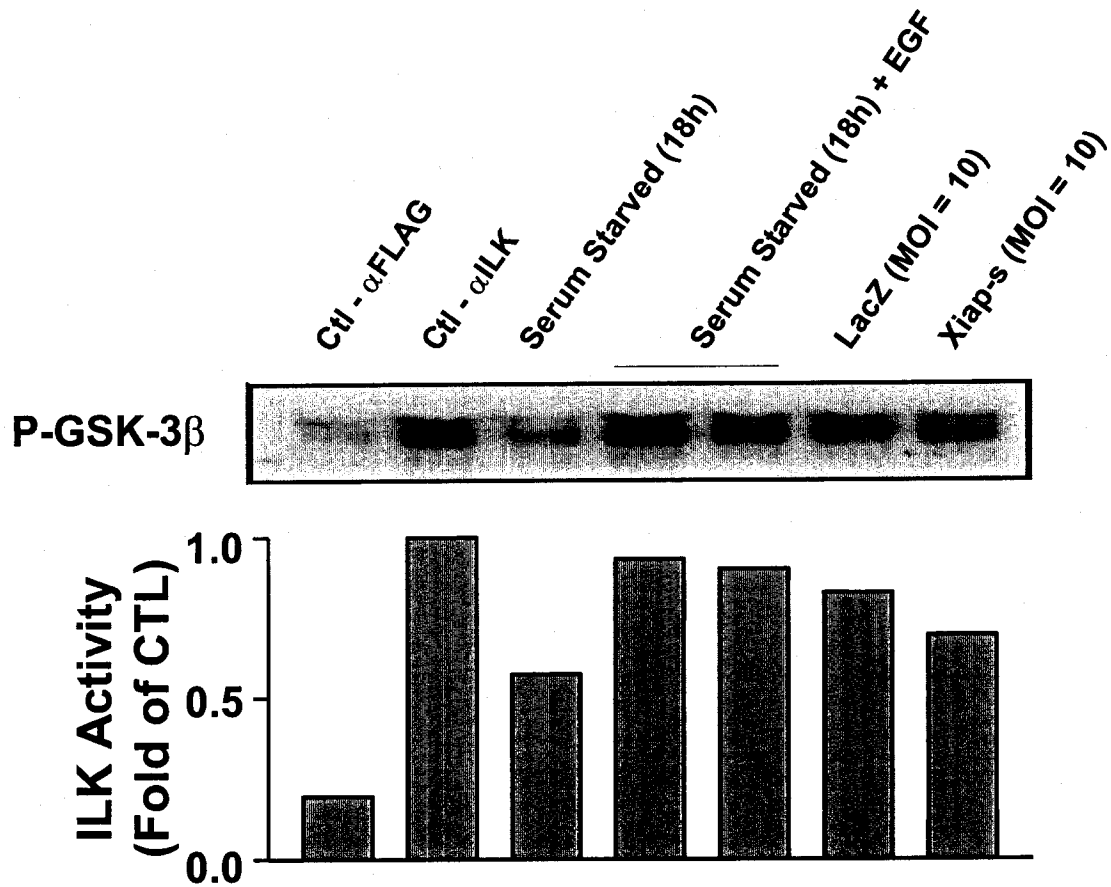

Ctl - αFLAG
Ctl - αILK
Serum Starved (18h)
Serum Starved (18h) + EGF
LacZ (MOI = 10)
Xiap-s (MOI = 10)
P-GSK-3β
ILK Activity
(Fold of CTL)
1.0
0.5
0.0

Figure 2

To that end, we evaluated the effects of CDDP on the mitochondrial accumulation of p53 in chemosensitive ovarian cancer cells (OV2008), and their chemoresistant variants (C13*). These cells were treated with CDDP (0-10 μM; 24h), and whole-cell and mitochondrial lysates were isolated as above. To obtain mitochondrial lysates, cell pellets were incubated with digitonin buffer (75 mM NaCl, 1mM NaH_2PO_4, 8mM Na_2HPO4_4, 250 mM sucrose, 190 μg/ml digitonin) to allow permeabilization of the cell membrane and leakage of the cytoplasm. These lysates were centrifuged at 14000 x g for 5 min, and the supernatents collected as cytoplasmic lysate. The pellets were washed 3x in digitonin buffer, incubated in mitochondrial lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP-40, 100 μM PMSF, 2 μg/ml aprotinin, pH 7.4) on ice for 1h, and then centrifiged at 14000 x g for 15 min. The supernatent was collected as the mitochondrial lysate. Protein contents for both whole cell and mitochondrial lysates were determined by assaying total protein content (see Chapter 4), and equivalent amounts were loaded onto SDS-PAGE gels, transferred to nitrocellulose membranes, and p53 content in both fractions was detected by Western blot using a mouse monoclonal anti-p53 antibody (clone DO-1; Santa Cruz). As shown in **figure 3A (left panel)**, CDDP induced the mitochondrial accumulation of p53 in the chemosensitive OV2008 cells, but not in the chemoresistant C13* cells, despite the presence of similar levels of p53 in whole-cell lysates in both cell lines. This suggests that CDDP-induced mitochondrial p53 accumulation is attenuated in chemoresistant cells, relative to their sensitive counterparts.

Since Akt-mediated chemoresistance involves the attenuation of p53-mediated apoptosis (Fraser et al., 2003) (Chapter 4 and 5), we next evaluated whether Akt

activation affects CDDP-induced mitochondrial p53 accumulation. A2780s-PHM6 and A2780-AAKT2 cells were treated with CDDP (0-10 μM; 24h), and mitochondrial and whole-cell lysates were evaluated as above. As shown in **figure 3A (right panel)**, p53 accumulated in the mitochondria of the control-transfected cells, but failed to do so in the mitochondria of cells expressing activated Akt despite the presence of p53 in whole-cell lysates. These results suggest that Akt inhibits p53 mitochondrial accumulation, and suggest that this may be one way in which Akt-mediated chemoresistance is mediated.

Since p53 is primarily a nuclear protein, we next evaluated the extent of nuclear contamination of the mitochondrial lysates by extracting nuclear and cytoplasmic fractions of CDDP-treated OV2008 and C13* cells using the NE-PER kit from Pierce (as in Chapter 5), and evaluating C23 (nucleolin) content in equivalent amounts (30 μg) of these fractions, as well as the mitochondrial lysates, by Western blot using a monoclonal anti-C23 antibody (Chapter 5). As shown in **figure 3B**, C23 was restricted to the nuclear fraction, with only small amounts of cytoplasmic contamination. However, C23 was undetectable in the mitochondrial fractions, suggesting that these lysates are free of nuclear contamination.

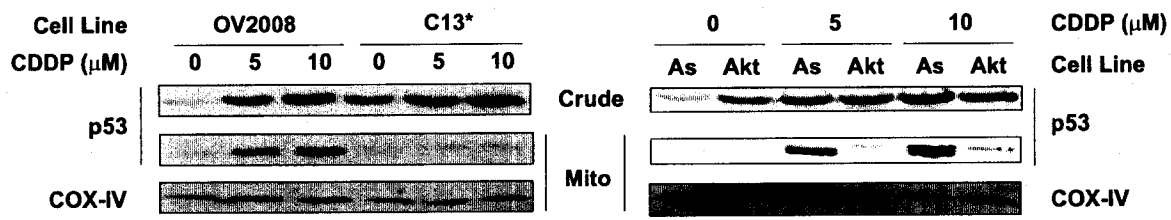
These results are consistent with those recently published in our laboratory (Yang et al., 2006). We demonstrated that the differential accumulation of p53 at the mitochondria of chemoresistant cells relative to their sensitive counterparts is not due to intrinsic differences in the mitochondria from these cells, that suppression of Akt in chemoresistant cells facilitates the mitochondrial accumulation of p53, and that targeted expression of mitochondrial p53 induces mitochondrial activation and apoptosis in *both* chemosensitive and chemoresistant cells, and that this occurs before the activation of

p53-mediated gene transcription. Taken together, these data suggest that Akt may contribute to a pathway which inhibits p53 mitochondrial accumulation, and that this represents a novel mechanism of Akt-mediated chemoresistance.

Figure 3 - Regulation of Mitochondrial p53 Accumulation

(A) A2780-CTL and A2780-AAkt2 (left panel) and OV2008 or C13* (right panel) cells were treated with CDDP (0-10 μM) for 24h, and mitochondrial lysates were obtained as indicated. Mitochondrial p53 contents were assessed by Western blot, using cytochrome c oxidase IV (COX IV) content as a loading control. **(B)** To determine the extent of nuclear contamination of the mitochondrial preparations C23 (nucleolin) content was assessed by Western blot on 30 μg (each) of nuclear, mitochondrial, and cytoplasmic lysates of OV2008 and C13* cells.

A.


Cell Line
OV2008
C13*
CDDP (μM)
0 5 10 0 5 10
p53
COX-IV
Crude
Mito
0 5 10
CDDP (μM)
As Akt As Akt As Akt
Cell Line
p53
COX-IV

B.

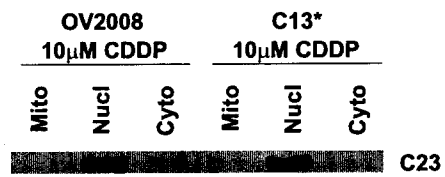

OV2008
10μM CDDP
C13*
10μM CDDP
Mito
Nucl
Cyto
Mito
Nucl
Cyto
C23

Figure 3

Confirmation of the Inhibitory Effects of Dominant-Negative Akt

Throughout this thesis, we have employed a dominant-negative form of Akt as a tool to ascertain the physiological role of Akt with respect to the regulation of cell fate and CDDP sensitivity. This construct consists of an Akt1 cDNA mutated to alanine at Thr308 and Ser473 (Kitamura et al., 1998), the phosphorylation of which is required for Akt activation (Alessi et al., 1996; Meier et al., 1997). In addition, this dominant-negative is mutated to alanine at Lys179, which lies within the kinase domain and is required for phosphate transfer (Krieg et al., 2003). The HA-tagged DN-Akt cDNA was cloned into an adenoviral expression vector, which also expresses Green Fluorescent Protein (GFP) on a separate Open Reading Frame (ORF). This system allows simple confirmation of DN-Akt expression by Western blot against the HA epitope, and of infection efficiency, by fluorescence microscopy with a FITC filter. In the current study, infection efficiency at an MOI of 80 was greater than 95% (**Figure 4**). The dominant-negative effects of both Ala308/Ala473-Akt ('double-A'-Akt) and Ala308/Ala473/Ala179-Akt ('triple-A'-Akt) have been extensively characterized (Kitamura et al., 1998; Krieg et al., 2003; Suhara et al., 2002). Additionally, we demonstrate here that this construct reduces both basal and EGF-induced Akt S473 phosphorylation, but had no effect upon basal or EGF-induced ERK1/2 phosphorylation (**Figure 5**).

Figure 4 - Confirmation of Adenoviral Infection Efficiency

C13* cells were infected with DN-Akt (MOI = 80; LacZ control) for 48h, and harvested by trypsinization as in Chapters 4, 5, and 6. Cells were fixed in 10% phosphate-buffered formalin and visualized under fluorescence (FITC) or bright-field microscopy. LacZ-infected cells were used to set the fluorescence background, and several fields of DN-Akt-infected cells were captured using a Q-Imaging digital camera into Q-Capture. Infection efficiency was determined as follows: number of GFP-positive cells (GFP) divided by total cell number (TOTAL) multiplied by 100 [100 x (GFP/TOTAL)].

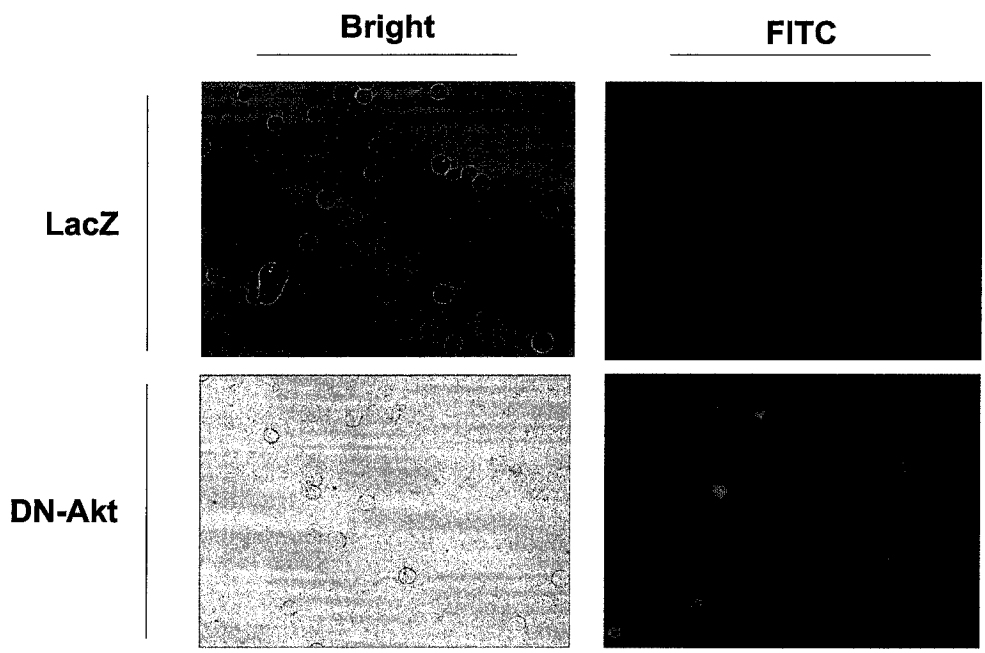


Figure 4

Figure 5 – Dominant-Negative Effects of Adenoviral DN-Akt

C13* cells were infected with DN-Akt as above, and then treated with or without Epidermal Growth Factor (EGF; 40 ng/ml) for 15 minutes. Cells were harvested by direct lysis, sonicated briefly, and HA-DN-Akt, phospho-Akt, and phospho-ERK contents were assessed by Western blot.

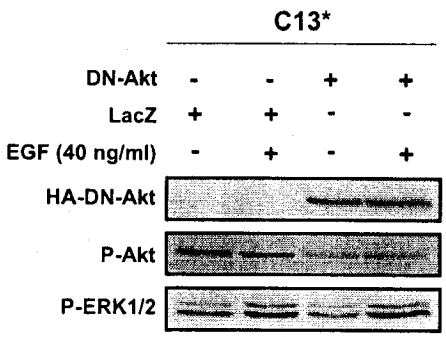


Figure 5

Phosphorylation does not Regulate p53 Mitochondrial Accumulation

Our data suggests that Akt attenuates CDDP-induced p53 phosphorylation and mitochondrial accumulation and that these effects are attenuated in chemoresistant cells relative to their sensitive counterparts. Furthermore, we demonstrated that phosphorylation of p53 on Ser15 and Ser20 is required for CDDP sensitivity (Chapter 5; **Figure 6**). Despite these correlations, however, it remains unclear whether phosphorylation of p53 is required for its mitochondrial accumulation in response to CDDP.

To that end, we evaluated the effects of p53 phosphorylation on p53 mitochondrial accumulation by transfecting chemoresistant, p53 mutant OVCAR-3 ovarian cancer cells with a wild-type or S15A/S20A HA-tagged p53 construct (2 μg; 24h; empty pcDNA3.1 as a control). Mitochondrial lysates (see above) were analysed for HA-p53 content by Western blot using a rat monoclonal anti-HA antibody. As shown in **figure 6**, HA-p53 accumulated in the mitochondria of OVCAR-3 cells, and this was not affected by mutation of Ser15 and Ser20 to alanine. This suggests that phosphorylation of these residues is not the mechanism by which p53 mitochondrial accumulation is regulated. Furthermore, since mutation of these sites significantly attenuated the ability of wild-type p53 to sensitize p53 mutant cells to CDDP-induced apoptosis, this result suggests that the pro-apoptotic effects of Ser15 and Ser20 phosphorylation is not mediated through changes in p53 mitochondrial accumulation.

Figure 6 - Effects of Altered p53 Phosphorylation on Mitochondrial p53 Accumulation

OVCAR-3 cells were transfected with control vector (pcDNA3.1), wild-type HA-p53, or HA-S15/20A-p53 (as described in Chapter 6) for 24h and mitochondrial and cytoplasmic lysates were obtained and assessed for HA-p53 content by Western blot. The absence of cytoplasmic contamination of the mitochondrial preparations was determined by assessing the protein content of lactate dehydrogenase (LDH), a cytoplasmic protein, in each of the fractions by Western blot.

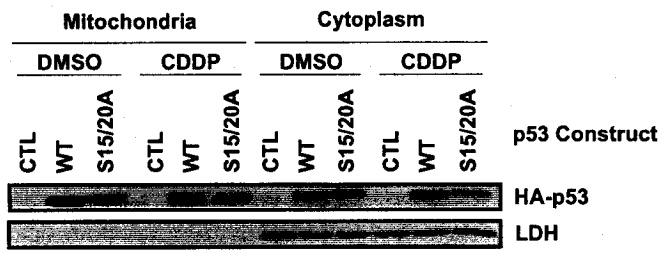

Mitochondria
Cytoplasm
DMSO
CDDP
DMSO
CDDP
CTL
WT
S15/20A
CTL
WT
S15/20A
CTL
WT
S15/20A
CTL
WT
S15/20A
p53 Construct
HA-p53
LDH

Figure 6

A2780cp is a p53 mutant, chemoresistant variant of the A2780s cell line. A2780cp is a heterozygous for *TP53*, possessing one wild-type allele, and one mutant allele. However, the identity of the mutant is unclear, since it was originally characterized as V173F (Skilling et al., 1996b), while subsequent studies showed a V172F mutant (Siddik et al., 1998). As described in Chapter 5, we evaluated the *TP53* sequence in these cells by direct sequencing of PCR amplicons of genomic DNA flanking codons 171-173. As shown in **figure 7**, our sequencing results suggest that A2780cp is heterozygous, with one wild-type GAG-GTT-GTG (Glu-Val-Val) allele and one mutant GAG-TTT-GTG (Glu-Phe-Val) allele.

Subsequent to this analysis, we sequenced exons 5-9 of *TP53* (encoding the DNA binding domain) for all of the ovarian cancer cells used in this study. Genomic DNA was extracted from the OVCA cell lines A2780s, A2780cp, OV2008, C13*, and OVCAR-3 using the DNeasy kit from Qiagen according to the manufacturer's instructions, and was subjected to exon-specific PCR amplification using the SNPCapture p53 Mutation Screening Kit from Panomics, Inc. PCR products, corresponding to TP53 genomic DNA from exons 5-9 (which encode the DNA binding domain of p53) were visualized by 2% agarose gel electrophoresis, and then cloned into the pCR4-TOPO sequencing vector from Invitrogen, according to the manufacturer's instructions. Following transformation and growth of chemically-competent E.Coli, plasmid DNA was purified by the Plasmid Miniprep Kit from Qiagen. Isolated plasmid DNA was sequenced by the Dye Terminator method at the Ottawa Genome Centre. OVCA cell line sequences were compared with the published human TP53 sequence using NCBI Blast2. The findings are summarized in Table 1, and show that A2780s, OV2008, and C13* cells possess wild-type *TP53*, while

A2780cp and OVCAR-3 are both mutant *TP53*. Interestingly, while A2780cp cells did show a V172F mutation, they additionally possess an Arg to Ser mutation at codon 260 (exon 8). By contrast, OVCAR-3 cells possess a Gln to Arg mutation at codon 317 (exon 9).

Figure 7 – *TP53* Mutational Status in Ovarian Cancer Cell Lines

Genomic DNA was extracted from A2780cp cells and the region flanking *TP53* codons 171-173 was amplified by PCR as described in chapter 5. PCR amplicons were directly sequenced. Automated base calls were verified by examining the generated chromatograms.

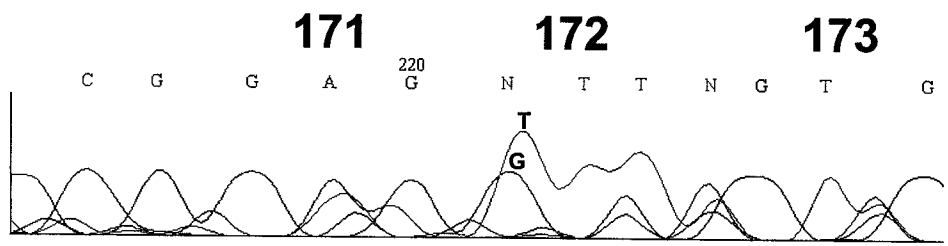


Figure 7

Table 1 – Sequence of *TP53* Exon 5-9 from Five OVCA Cell Lines

Cell Line	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9
A2780s	WT	WT	WT	WT	WT
A2780cp	V172F	WT	WT	R260S	WT
OV2008	WT	WT	WT	WT	WT
C13*	WT	WT	WT	WT	WT
OVCAR-3	WT	WT	WT	WT	Q317R

WT – wild-type

Chapter 8 – General Discussion

General Discussion

Overview and Significance

Ovarian cancer is the most lethal gynecological malignancy, and this is due in large part to the development of resistance to CDDP-based chemotherapy. While chemoresistance is a multi-factorial phenomenon, the aberrant regulation of components of apoptosis signaling pathways is commonly observed in chemoresistant tumours and cells, and is frequently associated with chemoresistance and poor prognosis. For this reason, a more complete understanding of the precise molecular mechanisms that govern the induction of apoptosis in ovarian cancer cells may ultimately improve our understanding of the clinical phenomenon of chemoresistance.

This thesis addresses the mechanisms of cell fate determination in human ovarian cancer cells. We have investigated if and how regulators such as p53, XIAP, Akt, and the sGC/cGMP pathway contributed to the modulation of apoptosis in ovarian cancer cells. Specifically, we have evaluated the contribution of apoptosis to the determination of CDDP sensitivity, and determined if and how these cell fate regulators interact to govern sensitivity to CDDP-induced apoptosis in ovarian cancer cells. The elucidation of the cellular and molecular mechanisms of chemoresistance in ovarian cancer cells may ultimately result in the development of treatment modalities for human ovarian cancer, thereby improving clinical outcomes for ovarian cancer patients.

Apoptosis as a Determinant of CDDP Sensitivity

Apoptosis is a key response of human ovarian tumours and cancer cells to chemotherapeutic agents, such as CDDP and is closely correlated to the *in vitro*

sensitivity of ovarian cancer cells to CDDP (Anderson et al., 2004; Gibb et al., 1997; Sato et al., 1999). Moreover, the aberrant regulation of components of apoptosis signaling pathways is frequently associated with poor clinical, *in vivo*, and *in vitro* response to these drugs (Kupryjanczyk et al., 2003; Murata et al., 2004; Sato et al., 1999; Skirnisdottir et al., 2002). This suggests that reduced apoptotic capacity may be an important determinant of chemoresistance in human ovarian cancer. As such, it is important to understand the precise molecular mechanisms by which ovarian cancer cell apoptosis is regulated.

Our laboratory has been particularly interested in examining the effects of CDDP on key apoptotic mediators in cultured ovarian cancer cells (Asselin et al., 2001a; Li et al., 2000; Sasaki et al., 2000). We have found that CDDP induces apoptosis in chemosensitive ovarian cancer cells, but not in their chemoresistant variants (Chapters 4 and 5). These findings suggest that the induction of apoptosis is a critical determinant of CDDP sensitivity in ovarian cancer cells.

Experimental Value of Matched Pair Ovarian Cancer Cell Lines

The elucidation of the molecular mechanisms of chemoresistance in ovarian cancer cells has been greatly facilitated by the use of matched pair cell lines, which consist of a chemosensitive parental cell line, and a resistant variant line, which has been created by selecting for CDDP resistance in the parental cell line through prolonged culture in the presence of increasing concentrations of CDDP. This results in a chemoresistant cell line that is, in theory, genotypically identical to the parental line with the exception of those factors that were selected by CDDP (i.e. factors that may determine chemosensitivity). As such, subsequent manipulations of these cells take place

on identical genetic backgrounds. This scenario is greatly preferable to the selection of essentially random chemosensitive and chemoresistant cell lines that, despite their phenotypes with respect to CDDP sensitivity, may have any number of other differences that may influence their response to the drug. As such, unmatched cell line pairs do not allow one to make firm conclusions about whether observed differences between chemosensitive and chemoresistant cells are related to this particular difference, or simply the result of random differences between these cell types. Moreover, this method of producing resistant sub-clones has important similarities to that of the clinical scenario, in which most ovarian tumours are sensitive to initial rounds of CDDP-based chemotherapy, but are resistant following disease recurrence. This suggests that selection of chemoresistant cells has occurred, and that these cells have repopulated the tumour.

However, it is important to recognize that the fact that parental cell lines *can be* selected for CDDP resistance suggests that the parental line is genotypically heterogenous. Individual cells within a completely homogenous cell line would be expected to respond in unison to stressors such as CDDP. As such, we must temper our conclusions with the knowledge that even these matched pair cell lines may have pre-existing genetic differences that affect their response to CDDP. Thus, it is critical to look for phenomena which exist across multiple, genetically unreleated cell line pairs, in order to establish whether specific molecular aberrances are merely chance occurrences, or are related to the differential cellular response to CDDP. To that end, the data presented in this thesis has been obtained, wherever possible, using multiple unrelated cell lines, in order to maximize its applicability and relevance.

Additionally, all cell lines used in this study were matched with respect to the number of post-thaw passages, in order to minimize any confounding effects associated with prolonged culture. It should, however, be noted that due to the mechanism of selection of resistant sub-clones (i.e. continuous passage in the presence of CDDP), these lines may be of higher passage, relative to their respective sensitive parental lines.

XIAP as a Determinant of Chemoresistance in Ovarian Cancer Cells

Data from our laboratory implicates the aberrant regulation of XIAP in the development of CDDP resistance in ovarian cancer (Asselin et al., 2001a; Li et al., 2000; Sasaki et al., 2000). Indeed, we demonstrated that over-expression of XIAP confers resistance to CDDP-induced apoptosis, while XIAP down-regulation sensitized chemoresistant cells to CDDP. Similar observations have been reported by other groups (Mansouri et al., 2003).

As such, modulation of XIAP represents a potential site for therapeutic intervention in the treatment of chemoresistant ovarian cancers. In this regard, there are two potential strategies that stand out. Recent work has identified at least three endogenous inhibitors of XIAP: Second Mitochondrial-Derived Activator of Caspases (Smac)/DIABLO, XIAP-Associated Factor-1 (XAF-1), and HtrA2/Omi (Du et al., 2000; Susin et al., 1996; Suzuki et al., 2001). A small peptide analogue of Smac has been developed, and this analogue potently sensitizes human cancer cells to TNF-α- and TRAIL-induced apoptosis (Li et al., 2004). We have also recently demonstrated that the Smac analogue peptide sensitizes ovarian cancer cells to CDDP-induced apoptosis (Yang et al., 2006). This peptide is currently in development for the treatment of human cancer (Li et al., 2004), and it is conceivable that targeted delivery of the Smac peptide to cancer

cells may provide a novel approach to treating chemoresistant ovarian cancer via suppression of XIAP.

Additionally, a XIAP antisense oligonucleotide (AEG35156) is currently in phase I clinical trials for the treatment of human cancer (Lacasse et al., 2005). Evaluation of this agent in combination with currently employed chemotherapeutics is currently underway, although the clinical effects of this agent have not yet been established.

One potential pitfall to the use of molecular manipulation of XIAP or XIAP-related proteins such as Smac is the lack of specificity of these agents. Our laboratory has reported that down-regulation of XIAP alone induces apoptosis in chemoresistant ovarian cancer cells (Sasaki et al., 2000), suggesting that selectivity of these XIAP attenuators will be required in order to avoid significant deleterious effects. This may require the development of novel drug delivery systems, such as the use of transgenes driven by ovarian epithelial cell-specific promoters (Connolly et al., 2003; Garson et al., 2003), thereby allowing specific expression of XIAP antisense oligonucleotides in the affected cell type.

Interaction between XIAP and AKT in the Determination of CDDP Sensitivity

The first evidence that XIAP-mediated chemoresistance may involve the PI3K/Akt pathway came with the observation that over-expression of XIAP confers resistance to CDDP and increases phospho-Akt content in chemosensitive ovarian cancer cells (Asselin et al., 2001a). Additionally, our laboratory has reported that XIAP-induced chemoresistance could be partially attenuated using the PI3K inhibitor LY294002, supporting the hypothesis that activation of the PI3K/Akt pathway may be an important mechanism by which XIAP elicits its anti-apoptotic effects. However, the biological

effects of LY294002 cannot be ascribed with certainty to an inactivation of Akt, since this agent may affect the activity of other kinases (McLaughlin & Demple, 2005). As such, we have extended this work by showing that while over-expression of XIAP confers resistance to CDDP-induced apoptosis in two chemosensitive ovarian cancer cell lines, this is significantly, albeit incompletely reversed by the concomitant expression of dominant-negative Akt (see below) (Fraser et al., 2003) (Chapter 4; **Figure 2**). This provides the first direct evidence suggesting that XIAP-mediated chemoresistance requires the Akt pathway, and contributes to the growing body of evidence suggesting that the anti-apoptotic effects of XIAP may not be restricted to the prototypical direct inhibition of the caspase cascade (Asselin et al., 2001a; Chai et al., 2001; Dan et al., 2004).

The dominant-negative Akt construct used in the current study consists of an Akt1 cDNA mutated to alanine at Thr308 and Ser473 (Kitamura et al., 1998), the phosphorylation of which is required for Akt activation (Alessi et al., 1996; Meier et al., 1997). In addition, this construct is mutated to alanine at Lys179, which lies within the kinase domain and is required for phosphate transfer (Krieg et al., 2003). This construct, which is HA-tagged at its carboxy terminus, was cloned into an adenoviral expression vector, which also expresses Green Fluorescent Protein (GFP) on a separate Open Reading Frame (ORF). This system allows simple confirmation of DN-Akt expression by Western blot against the HA epitope and of infection efficiency by fluorescence microscopy with a FITC filter. In the current study, infection efficiency at an MOI of 80 was greater than 95% (Chapter 7; **Figure 4**). The dominant-negative effects of both Ala308/Ala473-Akt ('double-A'-Akt) and Ala308/Ala473/Ala179-Akt ('triple-A'-Akt)

have been extensively characterized (Kitamura et al., 1998; Krieg et al., 2003; Suhara et al., 2002). Additionally, we demonstrate here that this construct reduces both basal and EGF-induced Akt S473 phosphorylation, but had no effect upon basal or EGF-induced ERK1/2 phosphorylation (Chapter 7; **Figure 3**).

Mechanism of XIAP-Induced Phospho-AKT Up-Regulation

While XIAP over-expression activates the Akt pathway, and while maximal XIAP-mediated chemoresistance requires Akt, the mechanism by which XIAP influences Akt phosphorylation/activation remain elusive. To that end, we hypothesized that XIAP up-regulates/activates the upstream PI3K-dependent kinases PDK1 and/or ILK, thereby leading to an increase in their ability to phosphorylate Akt. While we observed a statistically significant increase in ILK protein (but not mRNA) content in response to over-expression of XIAP, this did not translate into a measurable increase in ILK kinase activity, as measured by *in vitro* kinase assay (Chapter 7). This suggests that activation of ILK may not be implicated in XIAP-mediated Akt activation. Similarly, we did not observe changes in PDK1 protein content in response to XIAP over-expression, although we did not assay PDK1 kinase activity under these conditions.

It is possible that XIAP may directly activate PI3K, or may interact with and co-activate specific receptors, as has been observed for specific members of the TGF-β family of receptors (Birkey Reffey et al., 2001; Yamaguchi et al., 1999), thereby leading to PI3K activation. Either of these possibilites would lead to increased Akt phosphorylation through activation of PIP3-dependent upstream kinases. The observation that XIAP over-expression appears to activate Akt in a serum-dependent manner (unpublished observations from our laboratory) suggests that XIAP may act in

concert with other serum-derived factors to promote Akt activation. While we have not specifically examined the effects of serum on CDDP sensitivity (all CDDP treatments were done in the absence of serum), it is important to note that all adenoviral infections were performed in the presence of 2% serum, thus providing an appropriate environment for the observed effects of XIAP-mediated Akt activation.

It is also possible that XIAP may inhibit the expression and/or activity of the lipid phosphatases PTEN and/or SHIP1, which would result in the dephosphorylation of PIP3, thereby attenuating the phosphorylation of Akt. However, these hypotheses have not yet been evaluated, and are the focus of ongoing research in our laboratory.

Akt as an Independent Determinant of CDDP Resistance

While the PI3K/Akt pathway has received a great deal of attention with respect to its ability to suppress apoptosis in a wide-variety of cell types, only limited data has emerged to suggest a functional role for activation of this pathway in the determination of drug sensitivity. For example, stable transfection with a constitutively active form of the p110 (catalytic) subunit of PI3K conferred resistance to taxol in DOV-13 human ovarian cancer cells (Hu et al., 2002). Moreover, xenograft tumours derived from these cells in nude mice were resistant to taxol, relative to tumours derived from control-transfected cells while co-treatment with taxol and the PI3K inhibitor LY294002 sensitized the tumours to taxol and prolonged the median survival time of the activated PI3K-transfected cells.

We demonstrated that stable transfection of activated Akt2 in the chemosensitive A2780s ovarian cancer cell line rendered the cells resistant to CDDP-induced apoptosis (Fraser et al., 2003) (Chapter 4; **Figure 3A**). Furthermore, we demonstrated that

expression of dominant-negative Akt sensitized chemoresistant cells to CDDP-induced apoptosis (Chapter 4; **Figure 3B**). These results suggest that Akt activation, which is a frequent occurrence in human ovarian cancer (Bellacosa et al., 1995; Cheng et al., 1992; Sun et al., 2001b; Yuan et al., 2000), confers resistance to CDDP and that suppression of Akt is an effective means of overcoming CDDP resistance.

In the current study, Akt was activated by the presence of an amino terminus myristoylation tag, which targets Akt to the cell membrane, thus bypassing the physiological requirement for PIP3-dependent, PH-domain mediated Akt recruitment (Andjelkovic et al., 1997). Activation of Akt by this technique has been extensively characterized (Dan et al., 2004; Mende et al., 2001; Suhara et al., 2002; Sun et al., 2001b; Wang et al., 2006). Indeed, the A2780-AAkt2 cell line used in the current study displays markedly up-regulated Akt kinase activity, relative to the vector-transfected control cells (Dan et al., 2004; Yuan et al., 2003b).

Akt as a Therapeutic Target

The recent focus on specific kinases (e.g. PDGF, ErbB2, Bcl-Abl) as therapeutic targets (Baselga et al., 1998; Buchdunger et al., 1996; Druker et al., 1996; Pegram et al., 1998; Shaheen et al., 1999; Traxler et al., 1996) suggest that modulation of Akt or components of the Akt pathway may be a viable therapeutic intervention.

Trastuzamab, a monoclonal anti-ErbB2 antibody, has shown promise as an anti-breast cancer agent, particularly in those tumours which display ErbB2 elevation (reviewed in (Plosker & Keam, 2006)). This demonstrates that monoclonal antibody-based therapy is clinical applicable, and suggests that other targets, particularly those that

are amplified/activated in human tumours, may be amenable to similar treatment modalities.

To that end, recombinant monoclonal anti-Akt antibodies have recently been developed and engineered to ensure their cell permeability by fusion to a Kaposi fibroblast growth factor membrane translocation sequence (Shin et al., 2005). This antibody inhibits the activation of all three Akt family members and attenuates the activity of constitutively active Akt. Furthermore this anti-Akt antibody inhibits the development of xenograft tumours (Shin et al., 2005). As such, the use of monoclonal anti-Akt antibodies may be of clinical value for the treatment of human cancer, particularly of those cancer types, including ovarian cancer, which often depend on Akt activation for their growth.

It was originally believed that competitive inhibitors based upon the structure of ATP could not be made specific to individual kinases, since all kinases require the γ phosphate of ATP as a phosphate donor. However, several ATP analogue compounds, including Iressa and Gleevec, which antagonize the EGF receptor and the Bcl-Abl fusion protein, respectively, are currenly used in the treatment of human cancer (Goldman, 2001; West et al., 2006). This suggests that Akt may be amenable to similar agents, although to date, no such agent has been tested in human cancer cells. However, a recent report described the synthesis of three Akt-selective inhibitors (Breitenlechner et al., 2005; Breitenlechner et al., 2004), which could be used as lead compounds for the development of novel chemotherapeutics for the treatment of Akt-dependent human tumours.

Perhaps the most promising anti-Akt agent in current use is API-2, which was identified from a large compund library as a specific Akt inhibitor (Yang et al., 2004). API-2 is highly selective for Akt, induces apoptosis in cultured human cancer cells, and inhibits tumour growth in nude mice with xenografts derived from cells bearing constituitve Akt activation. Importantly, xenografts derived from cells without Akt activation were insensitive to API-2, suggesting that this molecule is highly specific for Akt. In ovarian cancer, where Akt activation occurs in nearly 30% of cases, API-2 or a derivative compound could be used to supplement existing chemotherapeutics. Indeed, in phase I and phase II clinical trials, an API-2-derived compound, TCN-P, showed anti-tumour activity, although its effects were limited by significant hepatotoxicity. However, these findings suggest that the Akt pathway may be amenable to novel chemotherapeutics, and that this may represent an important novel treatment modality for cancers in which Akt activation plays an important role.

Regulation of CDDP-Induced XIAP Down-Regulation by AKT

While our data implicates Akt as an important downstream mediator of the anti-apoptotic effects of XIAP, a reciprocal regulation has also been suggested. XIAP is an NF-κB-responsive gene product, and activation of NF-κB by FSH increases XIAP mRNA and protein content (Wang et al., 2002). This is related to the activation of the PI3K/Akt pathway, since FSH-mediated XIAP mRNA and protein up-regulation is inhibited by two PI3K inhibitors. As such, there may exist a feed-forward loop in some cells, whereby XIAP activates Akt by increasing its phosphorylation, while activated Akt induces NF-κB-mediated transcription of the *BIRC4* gene, thereby leading to up-regulation of XIAP mRNA and protein content.

We found that Akt activation did not increase basal XIAP protein levels in ovarian cancer cells (Chapter 4; **Figure 6**). However, while CDDP down-regulated XIAP protein content in control transfected chemosensitive ovarian cancer cells, this was inhibited by stable transfection of activated Akt (Fraser et al., 2003) (Chapter 4; **Figure 6**). Moreover, expression of dominant-negative Akt down-regulated XIAP in the presence of CDDP in chemoresistant cells. Previous results from our laboratory have demonstrated that CDDP does not affect XIAP mRNA content (Asselin et al., 2001a), suggesting CDDP regulates XIAP protein content at the post-translational level. As such, these data suggest that Akt influences XIAP at the protein level, and that the CDDP-induced down-regulation of XIAP is sensitive to Akt activation. Thus, the maintenance of high levels of XIAP in the presence of CDDP may be one mechanism by which Akt confers chemoresistance to ovarian cancer cells.

XIAP catalyzes its autoubiquitination following apoptotic stress, and this is mediated through its RING-Zn Finger domain, which possesses E3 ubiquitin ligase activity. While targeted deletion of this domain does not affect the anti-apoptotic effects of XIAP over-expression (Sasaki et al., 2002), it is possible that the regulation of XIAP protein content by CDDP may be mediated through this process.

Indeed, CDDP-induced XIAP down-regulation in ovarian cancer cells is blocked by inhibitors of the 26S proteasome, MG132 and lactacystin (Dan et al., 2004). Furthermore, neither a pan-caspase inhibitor nor a specific caspase-3 inhibitor blocked CDDP-induced XIAP down-regulation, suggesting that caspase-mediated processing of XIAP, which has been demonstrated during Fas-induced apoptosis (Johnson et al., 2000), is not the mechanism by which CDDP reduces XIAP content. By contrast, these results

suggest that the ubiqutin-proteasome pathway is required for this process. Additionally, and as we first demonstrated (Fraser et al., 2003) (Chapter 4), Akt activation blocks CDDP-induced XIAP down-regulation and this is associated with diminished XIAP autoubiquitination (Dan et al., 2004). Furthermore, Akt phosphorylates XIAP on Ser87, which attenuates its E3 ubiquitin ligase activity.

Taken togther, these data suggest that CDDP induces XIAP autoubiquitination and proteasomal degradation, and that Akt attenuates this process via direct phosphorylation of XIAP. As such, XIAP and Akt are reciprocal regulators of each other's biological function, thereby providing a tightly integrated anti-apoptotic signal.

Involvement of p53 in Chemoresistance

p53 is an important regulator of apoptosis, cell cycle progression, and the overall response to cell stress in mammalian cells, and *TP53* is the most frequently mutated gene in all human cancer. Importantly, mutation of *TP53* is often associated with chemoresistance and poor prognosis in human ovarian cancer, suggesting that p53 is required for chemosensitivity. However, the mechanisms by which wild-type p53 promotes chemosensitivity in ovarian cancer cells remain largely unknown. Additionally, the paradoxical finding that loss of p53 in cultured ovarian cancer cells sometimes results in *increased* sensitivity to CDDP (see Chapter 2) adds a further level of complexity to our understanding of the contribution of p53 to ovarian cancer cell fate and to CDDP sensitivity.

Functional Interaction Between AKT and p53

An involvement of altered p53 function in the determination of CDDP resistance in ovarian cancer has been suggested for some time, and numerous clinical, *in vivo*, and

in vitro studies have supported the hypothesis that p53 is required for CDDP sensitivity, as detailed above.

Our laboratory previously demonstrated that down-regulation of XIAP induces apoptosis in p53 wild-type, chemoresistant ovarian cancer cells, but that this is attenuated in chemoresistant cells possessing mutant p53 or that are p53 null (Sasaki et al., 2000). This suggests that XIAP-mediated chemoresistance may involve the inhibition of a p53-dependent apoptosis pathway.

Likewise, we observed that supression of Akt function sensitized p53 wild-type, chemoresistant cells to CDDP-induced apoptosis, but this was not seen in a mutant p53, chemoresistant cell line (Fraser et al., 2003) unless wild-type p53 was reconstituted, suggesting that p53 may be required for the pro-apoptotic effects of Akt inhibition. We also observed that pharmacologic inhibition of p53 attenuated the up-regulation of the p53-responsive gene product $p21^{WAF1/CIP1}$ and attenuated the chemosensitizing actions of dominant-negative Akt (Fraser et al., 2003). These data suggest that the ability to sensitize ovarian cancer cells to CDDP is dependent upon p53 function.

Interestingly, expression of DN-Akt up-regulated p53 in chemoresistant cells (Chapter 4; **Figure 6**), suggesting that basal Akt activity supresses p53 content. These data suggest that Akt may confer resistance by suppresing p53 content/function, although the precise mechanisms underlying this phenomenon are unclear.

Numerous reports have suggested that Akt can influence p53 content by phosphorylating MDM2, thereby promoting its nuclear translocation and its ability to ubiquitinate p53 (Gottlieb et al., 2002; Mayo & Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001). As such, expression of DN-Akt may up-regulate p53 content by

inhibiting MDM2-mediated p53 ubiquitination, although this has not been specifically addressed. Intriguingly, Yamaguchi et al showed that Akt can influence p53 function independently of changes in its content (Yamaguchi et al., 2001), suggesting that other mechanisms may be involved in the observed relationship between Akt and p53 with respect to chemosensitivity in ovarian cancer cells (discussed below).

Involvement of p53 in CDDP-Induced Apoptosis

Introduction of wild-type p53 into p53 mutant ovarian cancer cells *in vitro* results in enhanced sensitivity to CDDP, while xenograft tumours derived from p53 mutant cells are sensitized to CDDP by expression of wild-type p53 delivered by adenovirus (Song et al., 1999; Song et al., 1997), and clinical chemotherapy outcomes are significantly improved in patients with wild-type p53 tumours, relative to those with mutant p53 tumours (Righetti et al., 1996).

The bulk of the studies showing no *in vitro* role of p53 in determining CDDP sensitivity have relied upon non-specific methods to draw this conclusion. For instance, stable expression in the A2780 ovarian cancer cell line of the HPV-E6 gene, which encodes an E3 ubiquitin ligase known to target p53, down-regulates p53 and increases resistance to CDDP (Pestell et al., 2000). However, HPV-E6 is known to catalyze the ubiquitination and proteasomal degradation of other cellular proteins, including Rb (Wu et al., 2000), and thus the specific contribution of p53 down-regulation to the observed CDDP resistance is unclear. Similarly, stable over-expression of MDM2 results in enhanced chemosensitivity of cultured ovarian cancer cells (Mi & Ni, 2003), although it remains unclear whether this is strictly due to modulation of p53, or MDM2 target

proteins, such as Rb or $p21^{WAF1/Cip1}$ (Jin et al., 2003; Miwa et al., 2006; Uchida et al., 2005).

Paradoxical Findings Regarding the Involvement of p53 in Chemoresistance

Alternatively, studies showing enhanced *in vitro* CDDP sensitivity in the absence of p53 do not disinguish between changes in cell viability and sensitivity to CDDP-induced apoptosis, which, as discussed above, correlates with clinical CDDP sensitivity. Indeed, HPV-E6 expression, which down-regulates p53 and sensitizes A2780 cells to CDDP, actually reduces the percentage of cells undergoing apoptosis in response to CDDP (Pestell et al., 2000). This suggests that the loss of p53 may sensitize the cells to some other form of CDDP-induced cell death, possibly necrosis. This phenomenon is supported by evidence showing that fibroblasts from p53 knockout mice are insensitive to cyclophosphamide-induced apoptosis, relative to cells from wild-type littermates (Moallem & Hales, 1998). By contrast, these cells instead undergo necrosis, suggesting that p53 may protect cells against necrotic cell death. Thus, it is important to distinguish between these phenomenon, particularly since the induction of apoptosis appears to be an important clinical parameter with respect to CDDP sensitivity and overall patient survival rates. For this reason, we have used an assay for apoptosis (Hoescht 33258 staining) which is based on detecting the presence of typical apoptotic morphology, rather than other phenomenon (e.g. DNA fragmentation) which may occur during both apoptosis and necrosis.

However, the somewhat variable results obtained by different groups under similar experimental conditions (i.e. cell line, sensitivity assay) with respect to the role of p53 in this process cannot be ignored. One important consideration is that 'identical' cell

lines may, in fact, have developed key differences over time, and that these differences may affect the cellular response to CDDP. The A2780 cell line, for instance, which is a commonly employed tool for the study of ovarian cancer cell biology *in vitro*, has numerous variants, although the nomenclature distinguishing many of these lines does not permit an accurate reflection of their origin or their characteristics. For example, it has been demonstrated that CDDP does not up-regulate the protein content of the multi-domain, pro-apoptotic Bcl-2 family member Bax in A2780 cells (DeHaan et al., 2001). However, in the current study, we observed a clear up-regulation of Bax in these cells in response to CDDP, suggesting that these two cell lines may not be genetically identical. For this reason, it is important to examine these critical end-points in multiple cell lines, and to properly define the experimental conditions (i.e. p53 mutational status, CDDP concentration, cellular origin, passage number), to more accurately asses the role of p53 in the determination of CDDP sensitivity.

For these reasons, the precise role of p53 in the determination of CDDP-induced *apoptosis* remains largely un-examined. We show here that down-regulation of p53 by RNAi significantly reduces the percentage of cells undergoing CDDP-induced apoptosis (Chapter 5), while pharmacologic inhibition of p53 attenuates the pro-apoptotic effects of DN-Akt in the presence of CDDP (Chapter 4). This finding is particularly important, given the aforementioned controversy with respect to the role of p53 in the determination of chemosensitivity in ovarian cancer cells. Indeed, these data support the hypothesis that p53 may influence CDDP sensitivity by altering the apoptotic capacity of ovarian cancer cells, and provide some explanation as to a potential mechanism by which mutant p53 status confers chemoresistance to ovarian tumours.

Involvement of PUMA in CDDP-Induced, p53-Mediated Apoptosis

While p53 can induce apoptosis through up-regulation of gene products implicated in the induction of apoptosis, to date no single gene product has been shown to account for this phenomenon. However, recent evidence suggests that the BH3-only Bcl-2 family member PUMA is a critical downstream component of p53 in the propagation of apoptosis (Villunger et al., 2003). Indeed, PUMA knockout mice display almost identical phenotypes with respect to the induction of p53-dependent apoptosis as p53 knockout mice, suggesting that PUMA is a critical regulator of this process. This contrasts with Bax knockout mice, which display only partial resemblance to p53 knockouts with respect to the induction of apoptosis (Yu et al., 2005; Zhang et al., 2000).

We showed that CDDP up-regulates PUMA and that this is inhibited by down-regulation of p53 by RNAi, suggesting that p53 is required for the CDDP-induced up-regulation of PUMA. Additionally, down-regulation of PUMA by RNAi significantly attenuates CDDP-induced apoptosis, supporting the hypothesis that PUMA is a critical p53-dependent mediator of CDDP-induced apoptosis. This finding provides further evidence of the role of p53 in CDDP sensitivity in ovarian cancer cells.

However, it should be noted that while PUMA is clearly implicated in CDDP-induced, p53-mediated apoptosis, we cannot exclude the involvement of other p53-responsive gene products in this process. For example, Bax is also up-regulated by CDDP in chemosensitive A2780s cells, and is p53-responsive (Chapter 2). However, we did not observe Bax up-regulation in chemosensitive OV2008 cells, suggesting that Bax up-regulation may not be universally required for CDDP-induced apoptosis. Other potential downstream regulators of p53-mediated apoptosis include APAF-1, PTEN,

p53AIP1, and Fas, as discussed in chapter 2. While a complete characterization of the p53-mediated response to CDDP in ovarian cancer cells is beyond the scope of the current thesis, it is important to recognize that numerous p53-responsive genes may be implicated in the propagation of CDDP-induced apoptosis, although PUMA appears to play a critical role in this regard.

Regulation of p53 Nuclear Function

While our data clearly supports an important role for p53 in the propagation of CDDP-induced apoptosis, many chemoresistant ovarian cancer cell lines possess wild-type p53, yet fail to undergo apoptosis in response to CDDP. Additionally, while wild-type p53 status is generally associated with chemosensitivity and improved prognosis in ovarian cancer, many chemoresistant tumours show wild-type p53 status. This implies that additional factors, independent of p53 mutational status, may influence CDDP sensitivity.

In support of this hypothesis, we showed that CDDP up-regulates and activates p53, up-regulates PUMA, and induces apoptosis in chemosensitive OV2008 cells. However, both p53 and PUMA were abundant in the chemoresistant C13* cells, and these cells showed high levels of p53-DNA binding activity in the absence *and* presence of CDDP, although CDDP did not induce apoptosis in these cells (Chapter 5; **Figure 2B**). This observation strongly supports the hypothesis that both p53 and PUMA are necessary, but not sufficient, for CDDP-induced apoptosis.

Although we originally reported that C13* cells did not express measurable p53 content in the absence or presence of CDDP (Fraser et al., 2003) (Chapter 4), our subsequent data demonstrates that this original finding was not entirely correct, although

it is consistent with previous data from our laboratory (Sasaki et al., 2000). While C13* cells *do* express high basal p53 levels, in contradiction with our original findings, CDDP has a greater effect upon both p53 content and p53-DNA binding capacity in OV2008 cells than the resistant C13* cells, relative to the absence of CDDP in each cell line. Since these two assays both detected p53 content/function in C13* cells, it is likely that the original finding is flawed, possibily due to a failure of the previously used anti-p53 monoclonal antibody (Clone 80) to detect total p53 content in the C13* cells. We can only speculate as to the cause of this failure, but it is possible that this monoclonal antibody detects p53 only in the presence of specific post-translational modifications, such as phosphorylation or acetylation. This antibody detects an epitope in the C-terminal portion of the p53 molecule (amino acids 195-393; manufacturer's literature), which contain several sites of post-translational modification. However, it remains unclear whether this is the cause of the disparate results using different anti-p53 antibodies. However, the latter results, showing high levels of p53 protein content and activity, use two different anti-p53 antibodies, and are congruent to one another, suggesting that they are an accurate representation of these phenomena in C13* cells.

These results support the hypothesis that the presence of wild-type p53 is not sufficient to confer a chemosensitive phenotype. Interestingly, p53 levels are elevated in chemoresistant cells (Yazlovitskaya et al., 2001), as we have also observed. This phenomenon may result from a CDDP-induced selection during the development of chemoresistance, and this may provide a survival advantage to the chemoresistant cell since a tolerance of high levels of p53 may result in an inability to further activate this pathway, and thus to induce p53-dependent apoptosis. Our data supports this hypothesis

since both p53 activity and PUMA content were only marginally up-regulated by CDDP in the resistant C13* cells, whereas both of these parameters were markedly up-regulated in the chemosensitive OV2008 cells (Chapter 5; **Figures 2B and C**).

Additionally, we recently reported that CDDP induces the translocation of p53 to the mitochondria of chemosensitive ovarian cancer cells, but not their resistant variants (Yang et al., 2006) (Chapter 7). This does not appear to be due to intrinsic differences in the mitochondria from these cells, since isolated mitochondria from both chemosensitive and chemoresistant cells *in vitro* accumulate recombinant p53 and subsequently release Smac. Moreover, targeted mitochondrial p53 expression rapidly induces apoptosis in both cell types, suggesting that mitchondrial p53 accumulation is an important component of p53-mediated apoptosis, and that there is likely a defect in the subcellular shuttling of p53 in chemoresistant cells (Yang et al., 2006). These data, along with the data reported in this thesis, are consistent with the hypothesis that while p53 nuclear function is required for CDDP-induced apoptosis, other functions of p53, including its mitochondrial and cytosolic functions, may play additional supporting roles.

Implication of Altered p53 Phosphorylation in Chemoresistance

As such, while p53 is required for CDDP-induced apoptosis, p53-DNA binding activity, which is representative of p53 nuclear function, does not correlate with sensitivity to CDDP. These data suggest that p53 is necessary, but not sufficient, for CDDP sensitivity, and that other p53-dependent factors may influence CDDP sensitivity in ovarian cancer cells.

To that end, we showed that CDDP-induced phosphorylation of p53 on numerous residues, including Ser15, Ser20, Ser33, and Ser37, is attenuated in chemoresistant cells

in response to CDDP, relative to their chemosensitive counterparts (Chapter 5; **Figures 4A and B**). This was not explainable by the lack of total p53 in the chemoresistant cells, suggesting that this represents a specific reduction in phosphorylation.

p53 phosphorylation has been implicated in p53-induced apoptosis. In particular, Ser15 and Ser20 are critical for p53-induced apoptosis (Unger et al., 1999b), although, as discussed above, Ser46 also appears to be involved in this phenomenon in some cell types. However, if and how this process contributes to the regulation of CDDP sensitivity has not been previously evaluated. As such, our results suggest that reduced p53 phosphorylation may be an important determinant of CDDP resistance in ovarian cancer cells. This is further supported by our observation that mutation of Ser15 and/or Ser20, but not Ser37 to alanine, which prevent their phosphorylation, significantly attenuated the ability of wild-type p53 to sensitize chemoresistant, p53 mutant cells to CDDP-induced apoptosis (Chapter 6).

However, the mechanism by which phosphorylation of Ser15 and/or Ser20 contributes to CDDP sensitivity is unclear. Mutation of these sites did not reduce p53 content relative to wild-type, suggesting they are not required for steady-state p53 levels in these cells, as has been suggested (Unger et al., 1999b). PUMA was up-regulated by all p53 constructs to a similar extent, suggesting that PUMA up-regulation, and possibly p53 transactivation activity, is not dependent upon p53 phosphorylation. It should be noted that this is indirectly supported by the observation of high levels of PUMA protein content and p53-DNA binding activity in C13* cells in the absence of p53 phosphorylation (Chapter 5). However, while the results regarding PUMA regulation in C13* cells are clear, it is possible that our generic DNA binding assay, which uses a

consensus p53 oligonucleotide to assess p53-DNA binding, does not accurately reflect binding of p53 to individual specific gene promoters, and that inhibition of p53 phosphorylation in chemoresistant cells results in reduced expression of specific genes, which in turn contribute to p53-mediated apoptosis. Such a process has been observed with respect to Ser46, the phosphorylation of which is required for the up-regulation of p53AIP1. Thus, we cannot exclude the possible involvement of other p53-responsive gene products in the phosphorylation-dependent regulation of CDDP-induced, p53-mediated apoptosis. This should be examined in follow-up studies by evaluating the induction of other p53-responsive gene products by expression of wild-type and phosphorylation-deficient p53, and by evaluating the CDDP-mediated effects on these endpoints in chemosensitive ovarian cancer cells and their resistant variants.

However, others have similarly observed that in certain cell systems, p53 phosphorylation does not affect p53-mediated gene expression. As such, a much larger study of the precise effects of CDDP on overall p53-mediated gene expression in chemosensitive cells and their resistant variants is warranted, and may help to elucidate the contribution of p53 phosphorylation on this process.

Additionally, we found that mutation of p53 on Ser15 or Ser20, which markedly attenuated the pro-apoptotic capacity of p53, did not alter the accumulation of p53 at the mitochondria (Chapter 7; **Figure 6**). This suggests that phosphorylation of p53 is not required for the direct pro-apoptotic effects of p53 at the mitochondria. However, it is also possible that our forced expression system disrupts the normal regulation of p53 mitochondrial accumulation, and the results shown in **figure 6** represent an experimental

artifact. Additional experiments using shorter duration cultures should be performed to assess this hypothesis.

Regulation of Nuclear p53 Function by Akt

The observation that suppression of Akt function sensitized chemoresistant cells to CDDP-induced apoptosis in a p53-dependent manner suggests a functional relationship between Akt and p53. To that end, we showed that expression of dominant-negative Akt up-regulates p53 protein content. We also demonstrated that activation of Akt attenuates the CDDP-induced up-regulation of PUMA mRNA and protein content and reduces p53-DNA binding activity (Chapter 5; **Figure 3**). These effects were independent of changes in total p53 content, that Akt specifically inhibits p53 function. These results are consistent with those demonstrated by Yamaguchi et al (Yamaguchi et al., 2001), and suggest that Akt may confer resistance to CDDP by suppressing p53 function. Akt activation also reduced the protein content of the p53-responsive gene products Bax, MDM2, and $p21^{WAF1/CIP1}$, further supporting the hypothesis that Akt activation attenuates p53 nuclear function.

Akt phosphorylates MDM2 on Ser166 and Ser186, and this promotes the nuclear translocation of MDM2, thereby inducing the ubiquitination of p53 and facilitating its proteasomal degradation (Gottlieb et al., 2002; Mayo & Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001). However, we did not observe any decrease in p53 levels in the presence of Akt activation. By contrast, we observed *elevated* p53 levels in untreated cells expressing activated Akt. This apparent paradox may be explained, in part, by the observation that basal MDM2 levels were reduced in these cells, relative to the control transfected cells (Chapter 5; **Figure 3B**). This may result in diminished MDM2-induced

p53 degradation, thereby increasing basal p53 levels. Nevertheless, this basal p53 content appears to be inactive, since it did not display DNA binding activity.

Regulation of p53 Phosphorylation by Akt

While Akt attenuates the CDDP-induced up-regulation of PUMA, which is critical for CDDP-induced apoptosis, our data also supports a p53-dependent, but PUMA-*independent* mechanism of CDDP-induced apoptosis. As mentioned above, we also observed that CDDP-induced p53 phosphorylation is attenuated in chemoresistant cells, relative to their sensitive counterparts.

To date, there have been no reports of the regulation of basal or stress-induced p53 phosphorylation by Akt. Here we provide evidence that the CDDP-induced phosphorylation of numerous residues on p53, including Ser15, Ser20, Ser33, and Ser37, are attenuated by activation of Akt, and induced by DN-Akt (Chapter 5; **Figure 5**). This demonstrates, for the first time, that Akt inhibits CDDP-induced p53 phosphorylation, and suggests a further mechanism by which Akt-mediated chemoresistance may be conferred.

Additionally, we observed that while DN-Akt up-regulated total and phospho-p53, this was not further increased by CDDP. However, DN-Akt *does* sensitize chemoresistant cells to CDDP-induced apoptosis. This suggests that CDDP-induced apoptosis involves additional, p53-independent events, although p53 is clearly required. Furthemore, these results suggest that DN-Akt and CDDP have similar physiological effects upon p53 phosphorylation. One possible mechanism to explain this phenomenon is that CDDP may inhibit Akt content or activation in chemosensitive cells, whereas this effect is absent in chemoresistant cells. Our laboratory has previously demonstrated that

CDDP induces the caspase-3-mediated cleavage of Akt in A2780s cells (Asselin et al., 2001a), although it remains unclear whether this process is attenuated in chemoresistant cells, and this will be an important endpoint to evaluate in follow-up studies. Thus, while our results clearly implicate Akt in the regulation of CDDP-induced p53 phosphorylation and activation, it will be important to study the precise regulation of Akt content and activity by CDDP.

The mechanism by which Akt regulates p53 phosphorylation is unclear. CDDP-induced phosphorylation of Ser6 was not affected by Akt activation (Chapter 5; **Figure 5**). This suggests that the actions of Akt in this respect may be distal to DNA damage itself, since a reduction in DNA-CDDP adducts would be expected to lower Ser6 phosphorylation as well. However, the phosphorylations attenuated by Akt activation are regulated by diverse kinases, suggesting that Akt must act at a key regulatory step in the process, likely a proximal step implicated in the propagation of the DNA damage response to p53.

Possible Implication of ATR in the Akt-Dependent Regulation of p53 Phosphorylation

One candidate molecule for the Akt-mediated down-regulation of CDDP-induced p53 phosphorylation is ATR, which is activated by CDDP, and which is known to directly and indirectly facilitate the phosphorylation of p53 on numerous residues, including Ser15, Ser20, and Ser37. Intriguingly, ATR contains a putative Akt phosphorylation site at Ser436 (RRRLSS^{436}). As such, it is possible that Akt may phosphorylate ATR, thereby attenuating its activation, and reducing ATR-dependent p53 phosphorylation. Evaluation of this hypothesis will require an assessment of whether Akt

can direcly phosphorylate ATR, and whether ATR is required for p53 phosphorylation in response to (a) CDDP and (b) inhibition of Akt. While the role of ATR in CDDP-induced p53 phosphorylation and apoptosis has not been evaluated, it has been suggested that inhibition of ATR results in increased CDDP sensitivity (Yazlovitskaya & Persons, 2003). This paradoxical observation may be explained by the fact that apoptosis was not specifically addressed in this study and that the role of ATR was assessed using caffeine, a highly non-specific ATM/ATR inhibitor (Sarkaria et al., 1999). In addition to inhibiting ATM/ATR, caffeine inhibits other PI3K family members (Cheng et al., 2005), which may have opposing effects upon apoptosis regulation. As such, it will be important to study the specific role of ATR, in order to more fully understand the precise molecular mechanisms by which Akt affects p53 phosphorylation.

The Soluble Guanylyl Cyclase/cGMP Pathway as a Cell Fate Determinant

As detailed in chapter 2, recent data have suggested that the basal activity of the sGC/cGMP pathway is critical for the survival of numerous mammallian cell types. However, the precise mechanisms by this pathway elicits its pro-survival effects are unclear. We show here, for the first time, that this pathway is a critical determinant of ovarian cancer cell fate (Chapter 6). Depletion of basal cGMP content using the specific sGC inhbitor ODQ up-regulated total and phospho-p53, Bax, and $p21^{WAF1/CIP1}$ contents, increased p53 stability, and induced apoptosis that was partially sensitive to pharmacologic inhibition or down-regulation of p53, and was attenuated in p53 mutant cells relative to their wild-type counterparts. This suggests that cGMP may contribute to a pro-apoptotic pathway that involves suppression of p53-mediated apoptosis.

Interestingly, we found that following treatment with ODQ, up to a 2-fold greater percentage of cells were TUNEL positive than were apoptotic, as defined by Hoescht 33258 staining, although this was not consistent across multiple experiments. This is consistent with the hypothesis that the TUNEL assay does not adequately distinguish between apoptosis and necrosis, as has been previously suggested (Baima & Sticherling, 2002; Grasl-Kraupp et al., 1995), and suggests that the sGC/cGMP pathway may also protect cells against necrotic cell death. However, these hypotheses have not been evaluated.

The precise mechanisms by which sGC/cGMP affects p53 content, phosphorylation, and function remain unclear. PKG, which is the main effector of cGMP, phosphorylates substrates at a $(R/K)_{2-3}$-X_{1-2}-S/T (Kwan et al., 2004) consensus sequence. This sequence is found twice in MDM2 at Ser166 (RRAIS) and Ser186 (KRHKS), suggesting that PKG-mediated activation of MDM2 may help to suppress basal p53 content, and that reduction in basal cGMP levels may result in elevated p53 content via disruption of this pathway.

While our data supports a role for the sGC/cGMP pathway in the regulation of cell fate in ovarian cancer cells, it remains unclear whether this pathway is implicated in CDDP resistance, and if so, how this is mediated. Exposure to NO, which may enhance cGMP levels through activation of sGC, protects cells from CDDP-induced cytotoxicity (Xu et al., 2000), suggesting that this system can modulate CDDP sensitivity. Importantly, depletion of nitric oxide sensitizes melanoma cells to CDDP-induced apoptosis, suggesting that endogenous nitric oxide attenuates CDDP sensitivity (Tang & Grimm, 2004). The precise mechanism by which NO affects changes in CDDP

sensitivity is not known, nor is it clear whether the observed pro-survival effects of NO are mediated through changes in cGMP. Finally, it will be of great interest to evaluate the effects of CDDP on the expression and activity of the nitric oxide synthase enzymes (iNOS, eNOS, nNOS; see Chapter 2) in chemosensitive and chemoresistant ovarian cancer cells, to evaluate whether the regulation of these enzymes is implicated in CDDP-induced apoptosis, and whether any alterations in this pathway may be implicated in the pathogenesis of chemoresistance.

Future Directions

The current studies provide significant evidence that XIAP and Akt are important determinants of CDDP resistance in human ovarian cancer cells, that Akt-mediated chemoresistance may proceed through suppression of p53-mediated apoptosis, and that this may involve down-regulation of p53 nuclear function and its CDDP-induced phosphorylation. Additionally, the current studies provide evidence that the sGC/cGMP pathway is implicated in cell survival in these cells, and this may also be related to the down-regulation of p53-mediated apoptosis.

As such, in addition to the future experiments for this project proposed in the above sections, the following approaches should be considered as future directions in this regard.

Xenograft Models

However, the use of cultured cell lines, while convenient and informative, may not represent the behaviour of cancer cells *in vivo*. For this reason, the above studies should be extended to include an evaluation of pertinent endpoints using xenograft models of ovarian cancer. With the exception of C13*, all of the cell lines used in the

above studies form tumours following subcutaneous injection. Xenografts of CDDP-sensitive and -resistant ovarian cancer cell lines should be established in Swiss nude mice, and experiments conducted as indicated below.

To assess the regulation of Akt and its influence on CDDP sensitivity, A2780s and A2780-AAkt2 cells will be inoculated in nude mice as described. When the tumour has developed to 0.5cm in diameter, CDDP (1.5 mg/kg, i.p., clinical dose equivalent) will be administered and changes in tumour volume will be determined on alternate days and apoptotic (TUNEL and DNA ladder) and mitotic activity assessed. This tumour size is selected since the tumours can be readily measured, are unlikely to have developed necrosis in their core, and show similar extent of vascularization (to avoid potential differences in vascularization which may affect cell survival/outcome of studies). We expect that CDDP will induce apoptosis, suppress mitotic activity and growth of tumours developed from the A2780s, but not A2780-AAkt2 cells, suggesting that Akt activation leads to CDDP resistance, as shown *in vitro*. Interestingly, expression of active PI3K produced a similar response in another OVCA cell line (DOV13) treated with taxol *in vivo* (Hu et al., 2002).

For additional molecular manipulation of appropriate endpoints, two primary approaches may be employed. First, direct injection of adenoviral constructs (e.g. Adeno-DN-Akt, XIAP, and p53) into tumours is an established protocol our laboratory, and results in gene expression in the established tumour (Song et al., 1999). As such, this method may be employed. However, *in vivo* adenoviral gene delivery may result in expression in non-target tissues, thus complicating the interpretation of the results. Furthermore, adenovirus-based systems do not provide long-term expression of the gene

of interest, and as such their effects may diminish with time. For these reasons, the Tet-On (BD Biosciences) system can be used. This system involves the *in vitro* generation of cell lines stably expressing the reverse tetracycline-controlled transactivator (rtTA) under control of the CMV promoter. Clones of these cells, selected with puromycin, can then be subjected to a second stable transfection (G418 or hygromycin) with a vector containing the recombinant gene of interest, under the control of the Tet-responsive element. The gene of interest can be induced by the addition of doxycyline. Under this system, gene expression can be induced *only* in the appropriate cells, and can be maintained over time through the administration of doxycyline.

To demonstrate the functional role of p53 in Akt-mediated CDDP resistance *in vivo*, the p53 mutant cell lines A2780cp and A2780cp-DN-Akt (A2780cp stably expressing DN-Akt via G418 resistance) will be engineered using the above system to control the expression of wild-type p53. Xenografts of these cell lines will be established as above, and wild-type p53 expression induced through the addition of doxycycline in the drinking water. CDDP will be administered and endpoints evaluated as above. These experiments can be extended to include time-course and dose-response effects of doxycycline. If down-regulation of Akt function is effective in overcoming resistance, and if this depends on p53, we expect that DN-Akt will have no effect on CDDP-induced apoptosis/tumour regression in the p53 mutant cells unless wt-p53 is reconstituted.

Examination of Clinical Samples

Established cell lines provide a convenient and informative approach for the study of mechanisms of tumour progression and chemoresistance. However, their applicability

to the *in vivo* human situation should be established, since continuous passage of cell lines may result in a loss of characteristics of the original tumours.

To that end, the above studies should be extended to include primary cultured human ovarian cancer cells from ascites fluids pre- and post-chemotherapy and from solid tumours from patients with stage III/IV ovarian cancer undergoing surgical debulking. These cells can be treated with CDDP *in vitro*, and total and phospho- p53 content evaluated. If these factors are implicated in chemoresistance in human ovarian cancer, we expect that phospho-p53 levels will be low prior to CDDP treatment in cells from both the pre- and post-chemotherapy period, but increased by CDDP in cells from the chemosensitive pre-chemotherapy period, but not in the chemoresistant post-chemotherapy period. We expect a similar pattern for CDDP-induced apoptosis in these cells. Additionally, the effects of adenoviral DN-Akt expression on CDDP-induced apoptosis in primary ovarian cancer cells will be examined (as above). We expect that DN-Akt expression will sensitize chemoresistant primary human ovarian cancer cells to CDDP-induced apoptosis. Finally, chemoresistant cells that are not sensitized to CDDP by DN-Akt should be evaluate for p53-status by direct sequencing, to further establish the possible relationship between p53 mutational status and Akt-mediated chemoresistance.

Since ovarian tumours are characterized by cellular heterogeneity, in situ hybridization and IHC should be performed to localize mRNA and/or protein expression (e.g. PUMA, total/phospho-p53, total/phospho-Akt) in the context of cell proliferation and death (PCNA-IHC and TUNEL, respectively) (Li et al., 2001; Schneiderman et al., 1999). Intensity of IHC signal intensity will be semi-quantified using a scale system. It is unclear if specific OVCA cell types (i.e. serous, mucinous, endometrioid, clear cell,

transitional or undifferentiated) show the same expression patterns in the context of *in vivo* responsiveness to chemotherapy. If differences exist, this may account for the variance in disease distribution within the peritoneal cavity and time course of disease spread observed clinically and pathologically. Sections from ovarian tumors and paraffin-embedded tissues can be obtained from the Ovarian Tumor Bank, University of Ottawa. We expect that PUMA and phospho-p53 contents will be correlated with chemosensitivity and good prognosis, while phospho-Akt content will be correlated with chemoresistance and poor prognosis. In agreement with previous observations, we expect that high p53 content will be associated with chemoresistance and poor prognosis, since p53 over-expression is often a surrogate marker of p53 mutation. The above studies will demonstrate if the observations on OVCA cell lines are clinically relevant, and the regulation of p53 and Akt activation in ovarian tumour chemosensitivity. While these studies will be correlative, they will provide important clinical relevance for the above findings.

Conclusions

This thesis provides significant insights into the cellular and molecular mechanism of chemoresistance in human ovarian cancer cells. Specifically, we have demonstrated that XIAP and Akt are critical mediators of chemoresistance, and that thsese molecules confer chemoresistance, in part, through a reciprocal regulation whereby XIAP activates Akt by facilitating its phosphorylation, and Akt attenuates CDDP-induced down-regulation of XIAP, which has been shown to involve direct phosphorylation of XIAP by Akt, thereby protecting XIAP from autoubiquitination.

Additionally, we provide evidence that Akt is an independent determiant of chemoresistance, and that this is related to the regulation of p53. Specifically, Akt attenuates p53 nuclear activity and its CDDP-induced phosphorylation, while down-regulation of Akt up-regulates p53 content and phosphorylation, and sensitizes ovarian cancer cells to CDDP in a p53-dependent manner. Likewise, p53 is required for CDDP-induced apoptosis, and this involves the up-regulation of PUMA and its phosphorylation on Ser15 and Ser20. We also demonstrated that p53 phosphorylation is attenuated in chemoresistant cells, and is required for CDDP sensitivity.

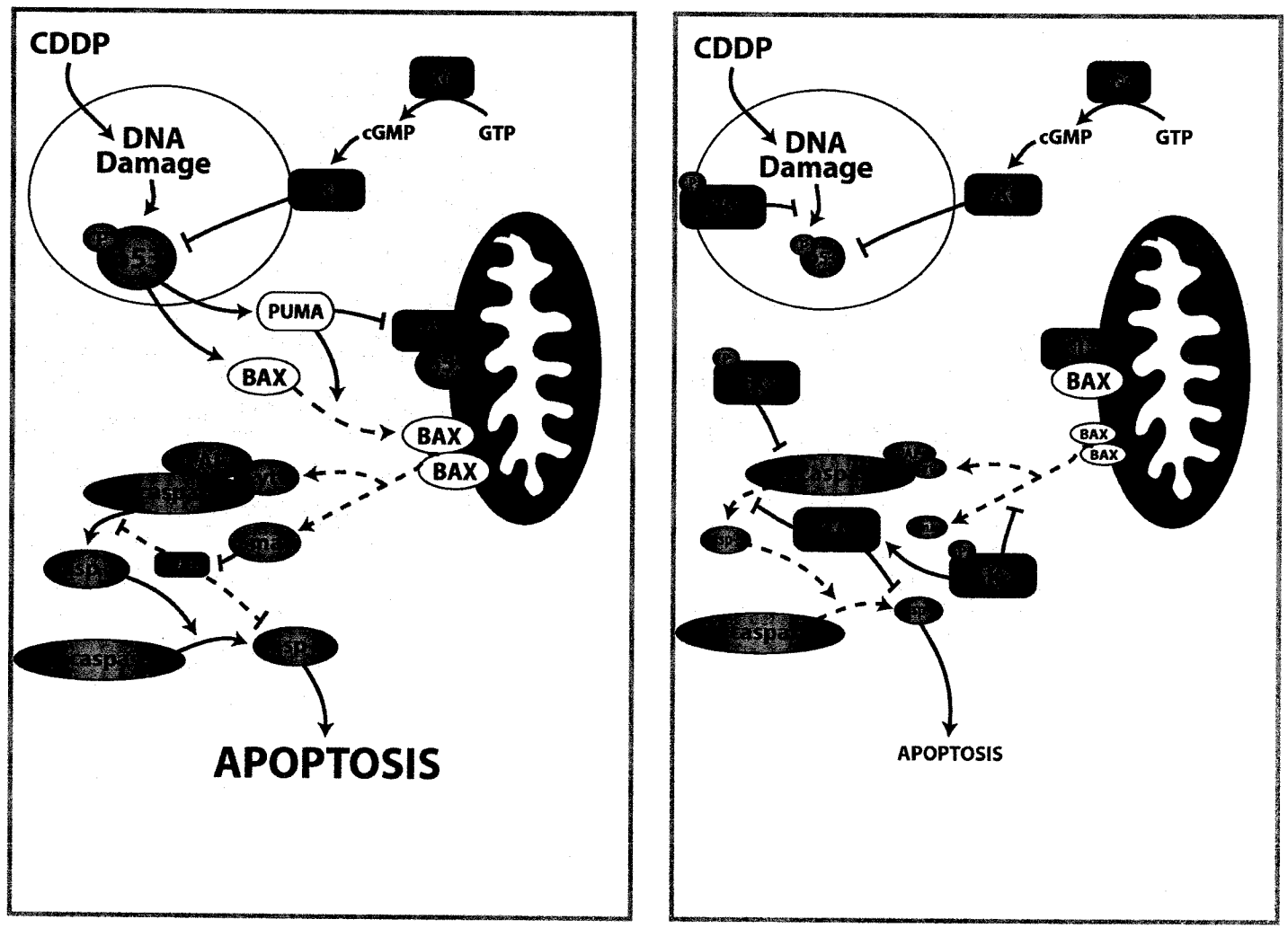
Finally, we provide evidence that the sGC/cGMP pathway is required for survival of ovarian cancer cells, and this is related to the inhibition of p53-mediated apoptosis.

Taken together, these data significantly extend our understanding of cell fate regulation in ovarian cancer cells, and provide evidence as to the mechanisms of chemoresistance in these cells (**Figure 1**). An improved understanding of the molecular and cellular mechanisms of chemoresistance may ultimately lead to improved treatment modalities for this disease.

Figure 1 – Hypothetical Model of Apoptosis Regulation and Chemoresistance in Human Ovarian Cancer Cells

While chemoresistance is a multi-factorial phenomenon, our model suggests that in chemosensitive cells, CDDP activates p53-mediated activation of PUMA and p53 phosphorylation, and these effects result in the activation of the mitochondria through inhibition of Bcl-2-mediated suppression of Bax. In chemoresistant cells, by contrast, PUMA levels and p53 phosphorylation do not respond to CDDP, thus preventing the release of pro-apoptotic proteins, such as cytochrome c and Smac from the mitochondria. Additionally, CDDP down-regulates XIAP in chemosensitive, but not resistant cells, resulting in increased activation of the caspase-9/caspase-3 cascade. Akt activation confers resistance by attenuating CDDP-induced p53 phosphorylation and by stabilizing XIAP. The sGC/cGMP pathway contributes to the inhibition of apoptosis through inhibition of p53-mediated apoptosis, although whether this pathway contributes to chemoresistance is not clear.

Pathways shown represent the key disruptions of apoptotic signaling in ovarian cancer cells, as examined in this thesis. To that end, a number of relevant pathways with defined roles in regulating CDDP sensitivity have been omitted.



CHEMOSENSITIVE CELL

CHEMORESISTANT CELL

Figure 1

Chapter 9 – References

List of References

Abedini MR, Qiu Q, Yan X and Tsang BK. (2004). *Oncogene.*

Abraham RT. (2001). *Genes Dev*, **15,** 2177-96.

Ackerman RC and Murdoch WJ. (1993). *Prostaglandins*, **45,** 475-85.

Adler V, Pincus MR, Minamoto T, Fuchs SY, Bluth MJ, Brandt-Rauf PW, Friedman FK, Robinson RC, Chen JM, Wang XW, Harris CC and Ronai Z. (1997). *Proc Natl Acad Sci U S A*, **94,** 1686-91.

Aghmesheh M, Nesland JM, Kaern J, Dorum A, Edwards L, Byth K, Friedlander M, Jackson P, Tucker KM and Russell PJ. (2004). *Gynecol Oncol*, **95,** 430-6.

Ahmed NN, Grimes HL, Bellacosa A, Chan TO and Tsichlis PN. (1997). *Proc Natl Acad Sci U S A*, **94,** 3627-32.

Akeshima R, Kigawa J, Takahashi M, Oishi T, Kanamori Y, Itamochi H, Shimada M, Kamazawa S, Sato S and Terakawa N. (2001). *Br J Cancer*, **84,** 1551-5.

Akiyama T, Ohuchi T, Sumida S, Matsumoto K and Toyoshima K. (1992). *Proc Natl Acad Sci U S A*, **89,** 7900-4.

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P and Hemmings BA. (1996). *Embo J*, **15,** 6541-51.

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB and Cohen P. (1997). *Curr Biol*, **7,** 261-9.

Altomare DA, Guo K, Cheng JQ, Sonoda G, Walsh K and Testa JR. (1995). *Oncogene*, **11,** 1055-60.

Aman MJ, Lamkin TD, Okada H, Kurosaki T and Ravichandran KS. (1998). *J Biol Chem*, **273,** 33922-8.

Ambrosini G, Adida C and Altieri DC. (1997). *Nat Med*, **3,** 917-21.

Anderson K, Lawson KA, Simmons-Menchaca M, Sun L, Sanders BG and Kline K. (2004). *Exp Biol Med (Maywood)*, **229,** 1169-76.

Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM and Hemmings BA. (1997). *J Biol Chem*, **272,** 31515-24.

Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K, Gambale F, Sadoul R and Martinou JC. (1997). *Science*, **277,** 370-2.

Antonsson B, Montessuit S, Sanchez B and Martinou JC. (2001). *J Biol Chem*, **276,** 11615-23.

Arango D, Corner GA, Wadler S, Catalano PJ and Augenlicht LH. (2001). *Cancer Res*, **61,** 4910-5.

Ashcroft M, Kubbutat MH and Vousden KH. (1999). *Mol Cell Biol*, **19,** 1751-8.

Ashcroft M, Taya Y and Vousden KH. (2000). *Mol Cell Biol*, **20,** 3224-33.

Asselin E, Mills GB and Tsang BK. (2001a). *Cancer Res*, **61,** 1862-8.

Asselin E, Wang Y and Tsang BK. (2001b). *Endocrinology*, **142,** 2451-7.

Atalay A, Crook T, Ozturk M and Yulug IG. (2002). *Biochem Biophys Res Commun*, **299,** 839-46.

Aunoble B, Sanches R, Didier E and Bignon YJ. (2000). *Int J Oncol*, **16,** 567-76.

Baima B and Sticherling M. (2002). *Am J Dermatopathol*, **24,** 130-4.

Bakkenist CJ and Kastan MB. (2003). *Nature*, **421,** 499-506.
Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y and Ziv Y. (1998). *Science*, **281,** 1674-7.
Barak Y, Juven T, Haffner R and Oren M. (1993). *Embo J*, **12,** 461-8.
Baselga J, Norton L, Albanell J, Kim YM and Mendelsohn J. (1998). *Cancer Res*, **58,** 2825-31.
Basu S, Bayoumy S, Zhang Y, Lozano J and Kolesnick R. (1998). *J Biol Chem*, **273,** 30419-26.
Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V and et al. (1995). *Int J Cancer*, **64,** 280-5.
Bellacosa A, Testa JR, Staal SP and Tsichlis PN. (1991). *Science*, **254,** 274-7.
Beltman J, Becker DE, Butt E, Jensen GS, Rybalkin SD, Jastorff B and Beavo JA. (1995). *Mol Pharmacol*, **47,** 330-9.
Bernstein C, Bernstein H, Payne CM and Garewal H. (2002). *Mutat Res*, **511,** 145-78.
Biggs WH, 3rd, Meisenhelder J, Hunter T, Cavenee WK and Arden KC. (1999). *Proc Natl Acad Sci U S A*, **96,** 7421-6.
Birkey Reffey S, Wurthner JU, Parks WT, Roberts AB and Duckett CS. (2001). *J Biol Chem*, **276,** 26542-9.
Bissonnette N, Wasylyk B and Hunting DJ. (1997). *Biochem Cell Biol*, **75,** 351-8.
Boolell M, Allen MJ, Ballard SA, Gepi-Attee S, Muirhead GJ, Naylor AM, Osterloh IH and Gingell C. (1996). *Int J Impot Res*, **8,** 47-52.
Borges HL, Chao C, Xu Y, Linden R and Wang JY. (2004). *Cell Death Differ*, **11,** 494-502.
Bowcock AM. (1993). *Breast Cancer Res Treat*, **28,** 121-35.
Breitenlechner CB, Friebe WG, Brunet E, Werner G, Graul K, Thomas U, Kunkele KP, Schafer W, Gassel M, Bossemeyer D, Huber R, Engh RA and Masjost B. (2005). *J Med Chem*, **48,** 163-70.
Breitenlechner CB, Wegge T, Berillon L, Graul K, Marzenell K, Friebe WG, Thomas U, Schumacher R, Huber R, Engh RA and Masjost B. (2004). *J Med Chem*, **47,** 1375-90.
Brown EJ and Baltimore D. (2000). *Genes Dev*, **14,** 397-402.
Brown R, Clugston C, Burns P, Edlin A, Vasey P, Vojtesek B and Kaye SB. (1993). *Int J Cancer*, **55,** 678-84.
Bruening W, Prowse AH, Schultz DC, Holgado-Madruga M, Wong A and Godwin AK. (1999). *Cancer Res*, **59,** 4973-83.
Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J and Greenberg ME. (1999). *Cell*, **96,** 857-68.
Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ and Lydon NB. (1996). *Cancer Res*, **56,** 100-4.
Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E and Fornace AJ, Jr. (1999). *Embo J*, **18,** 6845-54.
Burgering BM and Coffer PJ. (1995). *Nature*, **376,** 599-602.
Buschmann T, Potapova O, Bar-Shira A, Ivanov VN, Fuchs SY, Henderson S, Fried VA, Minamoto T, Alarcon-Vargas D, Pincus MR, Gaarde WA, Holbrook NJ, Shiloh Y and Ronai Z. (2001). *Mol Cell Biol*, **21,** 2743-54.
Butler MG, Hall BD, Maclean RN and Lozzio CB. (1987). *Am J Med Genet*, **27,** 645-9.

Caelles C, Helmberg A and Karin M. (1994). *Nature*, **370,** 220-3.
Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB and Siliciano JD. (1998). *Science*, **281,** 1677-9.
Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S and Reed JC. (1998). *Science*, **282,** 1318-21.
Carver DJ, Aman MJ and Ravichandran KS. (2000). *Blood*, **96,** 1449-56.
Casey G, Lopez ME, Ramos JC, Plummer SJ, Arboleda MJ, Shaughnessy M, Karlan B and Slamon DJ. (1996). *Oncogene*, **13,** 1971-81.
Cassinelli G, Supino R, Perego P, Polizzi D, Lanzi C, Pratesi G and Zunino F. (2001). *Int J Cancer*, **92,** 738-47.
Castoria G, Migliaccio A, Bilancio A, Di Domenico M, de Falco A, Lombardi M, Fiorentino R, Varricchio L, Barone MV and Auricchio F. (2001). *Embo J*, **20,** 6050-9.
Chai J, Shiozaki E, Srinivasula SM, Wu Q, Datta P, Alnemri ES and Shi Y. (2001). *Cell*, **104,** 769-80.
Chan SL and Fiscus RR. (2003). *Mol Hum Reprod*, **9,** 775-83.
Chao C, Herr D, Chun J and Xu Y. (2006). *Embo J*, **25,** 2615-22.
Chehab NH, Malikzay A, Appel M and Halazonetis TD. (2000). *Genes Dev*, **14,** 278-88.
Chehab NH, Malikzay A, Stavridi ES and Halazonetis TD. (1999). *Proc Natl Acad Sci U S A*, **96,** 13777-82.
Chen HW, Jiang WS and Tzeng CR. (2001). *Fertil Steril*, **75,** 1163-71.
Chen J, Halappanavar SS, St-Germain JR, Tsang BK and Li Q. (2004). *Cell Mol Life Sci*, **61,** 1675-83.
Chen J, Lin J and Levine AJ. (1995). *Mol Med*, **1,** 142-52.
Chen J, Marechal V and Levine AJ. (1993). *Mol Cell Biol*, **13,** 4107-14.
Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN and Testa JR. (1992). *Proc Natl Acad Sci U S A*, **89,** 9267-71.
Cheng JQ, Jiang X, Fraser M, Li M, Dan HC, Sun M and Tsang BK. (2002). *Drug Resist Updat*, **5,** 131-46.
Cheng JQ, Lindsley CW, Cheng GZ, Yang H and Nicosia SV. (2005). *Oncogene*, **24,** 7482-92.
Cheng JQ, Ruggeri B, Klein WM, Sonoda G, Altomare DA, Watson DK and Testa JR. (1996). *Proc Natl Acad Sci U S A*, **93,** 3636-41.
Chinnaiyan AM, O'Rourke K, Tewari M and Dixit VM. (1995). *Cell*, **81,** 505-12.
Chinnaiyan AM, Tepper CG, Seldin MF, O'Rourke K, Kischkel FC, Hellbardt S, Krammer PH, Peter ME and Dixit VM. (1996). *J Biol Chem*, **271,** 4961-5.
Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD and Green DR. (2005). *Science*, **309,** 1732-5.
Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, 3rd, Kaestner KH, Bartolomei MS, Shulman GI and Birnbaum MJ. (2001a). *Science*, **292,** 1728-31.
Cho H, Thorvaldsen JL, Chu Q, Feng F and Birnbaum MJ. (2001b). *J Biol Chem*, **276,** 38349-52.
Ciani E, Virgili M and Contestabile A. (2002). *J Neurochem*, **81,** 218-28.
Ciechomska I, Pyrzynska B, Kazmierczak P and Kaminska B. (2003). *Oncogene*, **22,** 7617-27.

Cimprich KA, Shin TB, Keith CT and Schreiber SL. (1996). *Proc Natl Acad Sci U S A*, **93,** 2850-5.
Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML and Wyllie AH. (1993). *Nature*, **362,** 849-52.
Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, Hua X, Harris SS, Vanderhyden BC and Hamilton TC. (2003). *Cancer Res*, **63,** 1389-97.
Cortez D, Guntuku S, Qin J and Elledge SJ. (2001). *Science*, **294,** 1713-6.
Crawford LV, Pim DC, Gurney EG, Goodfellow P and Taylor-Papadimitriou J. (1981). *Proc Natl Acad Sci U S A*, **78,** 41-5.
Cregan SP, Arbour NA, Maclaurin JG, Callaghan SM, Fortin A, Cheung EC, Guberman DS, Park DS and Slack RS. (2004). *J Neurosci*, **24,** 10003-12.
Crook NE, Clem RJ and Miller LK. (1993). *J Virol*, **67,** 2168-74.
Cui Y, Konig J, Buchholz JK, Spring H, Leier I and Keppler D. (1999). *Mol Pharmacol*, **55,** 929-37.
Currie RA, Walker KS, Gray A, Deak M, Casamayor A, Downes CP, Cohen P, Alessi DR and Lucocq J. (1999). *Biochem J*, **337 (Pt 3),** 575-83.
Dabholkar M, Bostick-Bruton F, Weber C, Bohr VA, Egwuagu C and Reed E. (1992). *J Natl Cancer Inst*, **84,** 1512-7.
Dabholkar M, Vionnet J, Bostick-Bruton F, Yu JJ and Reed E. (1994). *J Clin Invest*, **94,** 703-8.
Damia G, Filiberti L, Vikhanskaya F, Carrassa L, Taya Y, D'Incalci M and Broggini M. (2001). *Neoplasia*, **3,** 10-6.
Dan HC, Sun M, Kaneko S, Feldman RI, Nicosia SV, Wang HG, Tsang BK and Cheng JQ. (2004). *J Biol Chem*, **279,** 5405-12.
Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y and Greenberg ME. (1997). *Cell*, **91,** 231-41.
de Murcia G and Menissier de Murcia J. (1994). *Trends Biochem Sci*, **19,** 172-6.
Deckwerth TL, Elliott JL, Knudson CM, Johnson EM, Jr., Snider WD and Korsmeyer SJ. (1996). *Neuron*, **17,** 401-11.
Deguchi A, Thompson WJ and Weinstein IB. (2004). *Cancer Res*, **64,** 3966-73.
DeHaan RD, Yazlovitskaya EM and Persons DL. (2001). *Cancer Chemother Pharmacol*, **48,** 383-8.
del Peso L, Gonzalez-Garcia M, Page C, Herrera R and Nunez G. (1997). *Science*, **278,** 687-9.
Delcommenne M, Tan C, Gray V, Rue L, Woodgett J and Dedhar S. (1998). *Proc Natl Acad Sci U S A*, **95,** 11211-6.
Delia D, Mizutani S, Panigone S, Tagliabue E, Fontanella E, Asada M, Yamada T, Taya Y, Prudente S, Saviozzi S, Frati L, Pierotti MA and Chessa L. (2000). *Br J Cancer*, **82,** 1938-45.
Deligdisch L and Gil J. (1989). *Cancer*, **63,** 748-55.
Dellaire G and Bazett-Jones DP. (2004). *Bioessays*, **26,** 963-77.
Deng Y, Lin Y and Wu X. (2002). *Genes Dev*, **16,** 33-45.
Deora AA, Win T, Vanhaesebroeck B and Lander HM. (1998). *J Biol Chem*, **273,** 29923-8.
Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS and Reed JC. (1999). *Embo J*, **18,** 5242-51.

Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS and Reed JC. (1998). *Embo J*, **17,** 2215-23.
Deveraux QL, Takahashi R, Salvesen GS and Reed JC. (1997). *Nature*, **388,** 300-4.
Di Stefano V, Rinaldo C, Sacchi A, Soddu S and D'Orazi G. (2004). *Exp Cell Res*, **293,** 311-20.
Dokianakis DN, Varras MN, Papaefthimiou M, Apostolopoulou J, Simiakaki H, Diakomanolis E and Spandidos DA. (1999). *Clin Exp Metastasis*, **17,** 293-7.
Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr., Butel JS and Bradley A. (1992). *Nature*, **356,** 215-21.
Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J and Lydon NB. (1996). *Nat Med*, **2,** 561-6.
Du C, Fang M, Li Y, Li L and Wang X. (2000). *Cell*, **102,** 33-42.
Duckett CS, Nava VE, Gedrich RW, Clem RJ, Van Dongen JL, Gilfillan MC, Shiels H, Hardwick JM and Thompson CB. (1996). *Embo J*, **15,** 2685-94.
Dumaz N and Meek DW. (1999). *Embo J*, **18,** 7002-10.
Dupont J and Le Roith D. (2001). *Mol Pathol*, **54,** 149-54.
el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW and Vogelstein B. (1992). *Nat Genet*, **1,** 45-9.
Eliopoulos AG, Kerr DJ, Herod J, Hodgkins L, Krajewski S, Reed JC and Young LS. (1995). *Oncogene*, **11,** 1217-28.
Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A and Nagata S. (1998). *Nature*, **391,** 43-50.
Enomoto T, Inoue M, Perantoni AO, Terakawa N, Tanizawa O and Rice JM. (1990). *Cancer Res*, **50,** 6139-45.
Erster S, Mihara M, Kim RH, Petrenko O and Moll UM. (2004). *Mol Cell Biol*, **24,** 6728-41.
Espada J, Perez-Moreno M, Braga VM, Rodriguez-Viciana P and Cano A. (1999). *J Cell Biol*, **146,** 967-80.
Essmann F, Pohlmann S, Gillissen B, Daniel PT, Schulze-Osthoff K and Janicke RU. (2005). *J Biol Chem*, **280,** 37169-77.
Estevez AG, Spear N, Manuel SM, Barbeito L, Radi R and Beckman JS. (1998a). *Prog Brain Res*, **118,** 269-80.
Estevez AG, Spear N, Thompson JA, Cornwell TL, Radi R, Barbeito L and Beckman JS. (1998b). *J Neurosci*, **18,** 3708-14.
Falcone S, Mauro L, de Rose G, Paolucci C, Sciorati C, Ando S and Clementi E. (2002). *Biochem J*, **366,** 165-73.
Fan S, Chang JK, Smith ML, Duba D, Fornace AJ, Jr. and O'Connor PM. (1997). *Oncogene*, **14,** 2127-36.
Fathalla MF. (1971). *Lancet*, **2,** 163.
Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz LC, Trapani JA, Tomaselli KJ, Litwack G and Alnemri ES. (1996). *Proc Natl Acad Sci U S A*, **93,** 7464-9.
Filmus JE and Buick RN. (1985). *Cancer Res*, **45,** 4468-72.
Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG and Green DR. (1999). *J Biol Chem*, **274,** 2225-33.

Fiscella M, Ullrich SJ, Zambrano N, Shields MT, Lin D, Lees-Miller SP, Anderson CW, Mercer WE and Appella E. (1993). *Oncogene*, **8,** 1519-28.
Fiscus RR. (2002). *Neurosignals*, **11,** 175-90.
Fiscus RR, Rapoport RM and Murad F. (1983). *J Cyclic Nucleotide Protein Phosphor Res*, **9,** 415-25.
Fiscus RR, Tu AW and Chew SB. (2001). *Neuroreport*, **12,** 185-9.
Fiscus RR, Yuen JP, Chan SL, Kwong JH and Chew SB. (2002). *J Card Surg*, **17,** 336-9.
Flamigni F, Facchini A, Stanic I, Tantini B, Bonavita F and Stefanelli C. (2001). *Biochem Pharmacol*, **62,** 319-28.
Flesken-Nikitin A, Choi KC, Eng JP, Shmidt EN and Nikitin AY. (2003). *Cancer Res*, **63,** 3459-63.
Foray N, Marot D, Gabriel A, Randrianarison V, Carr AM, Perricaudet M, Ashworth A and Jeggo P. (2003). *Embo J*, **22,** 2860-71.
Ford D and Easton DF. (1995). *Br J Cancer*, **72,** 805-12.
Ford D, Easton DF and Peto J. (1995). *Am J Hum Genet*, **57,** 1457-62.
Fortin A, Cregan SP, MacLaurin JG, Kushwaha N, Hickman ES, Thompson CS, Hakim A, Albert PR, Cecconi F, Helin K, Park DS and Slack RS. (2001). *J Cell Biol*, **155,** 207-16.
Foster KA, Harrington P, Kerr J, Russell P, DiCioccio RA, Scott IV, Jacobs I, Chenevix-Trench G, Ponder BA and Gayther SA. (1996). *Cancer Res*, **56,** 3622-5.
Franceschi S, La Vecchia C, Booth M, Tzonou A, Negri E, Parazzini F, Trichopoulos D and Beral V. (1991). *Int J Cancer*, **49,** 57-60.
Fraser M, Chan SL, Chan SS, Fiscus RR and Tsang BK. (2005). *Oncogene*.
Fraser M, Leung BM, Yan X, Dan HC, Cheng JQ and Tsang BK. (2003). *Cancer Res*, **63,** 7081-8.
Fuchs B, O'Connor D, Fallis L, Scheidtmann KH and Lu X. (1995). *Oncogene*, **10,** 789-93.
Fuchs SY, Adler V, Pincus MR and Ronai Z. (1998). *Proc Natl Acad Sci U S A*, **95,** 10541-6.
Fujita E, Jinbo A, Matuzaki H, Konishi H, Kikkawa U and Momoi T. (1999). *Biochem Biophys Res Commun*, **264,** 550-5.
Gadducci A, Cosio S, Muraca S and Genazzani AR. (2002). *Eur J Gynaecol Oncol*, **23,** 390-6.
Gallion HH, Pieretti M, DePriest PD and van Nagell JR, Jr. (1995). *Cancer*, **76,** 1992-7.
Garcia A, Bussaglia E, Machin P, Matias-Guiu X and Prat J. (2000). *Int J Gynecol Pathol*, **19,** 152-7.
Garson K, Macdonald E, Dube M, Bao R, Hamilton TC and Vanderhyden BC. (2003). *J Soc Gynecol Investig*, **10,** 244-50.
Garthwaite J, Charles SL and Chess-Williams R. (1988). *Nature*, **336,** 385-8.
Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K and Mayer B. (1995). *Mol Pharmacol*, **48,** 184-8.
Gibb RK, Taylor DD, Wan T, O'Connor DM, Doering DL and Gercel-Taylor C. (1997). *Gynecol Oncol*, **65,** 13-22.
Goldman J. (2001). *Semin Hematol*, **38,** 28-34.
Gomes CP and Andrade LA. (2006). *Int J Gynecol Cancer*, **16 Suppl 1,** 254-8.
Gonzalez S, Prives C and Cordon-Cardo C. (2003). *Mol Cell Biol*, **23,** 8161-71.

Gottlieb TM, Leal JF, Seger R, Taya Y and Oren M. (2002). *Oncogene*, **21,** 1299-303.
Gras E, Pons C, Machin P, Matias-Guiu X and Prat J. (2001). *Int J Gynecol Pathol*, **20,** 335-40.
Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W and Schulte-Hermann R. (1995). *Hepatology*, **21,** 1465-8.
Green H, Soderkvist P, Rosenberg P, Horvath G and Peterson C. (2006). *Clin Cancer Res*, **12,** 854-9.
Griffith TM, Edwards DH, Lewis MJ and Henderson AH. (1985). *Eur J Pharmacol*, **112,** 195-202.
Gross A, Jockel J, Wei MC and Korsmeyer SJ. (1998). *Embo J*, **17,** 3878-85.
Gross TP and Schlesselman JJ. (1994). *Obstet Gynecol*, **83,** 419-24.
Gu W and Roeder RG. (1997). *Cell*, **90,** 595-606.
Gunz D, Hess MT and Naegeli H. (1996). *J Biol Chem*, **271,** 25089-98.
Ha KS, Kim KM, Kwon YG, Bai SK, Nam WD, Yoo YM, Kim PK, Chung HT, Billiar TR and Kim YM. (2003). *Faseb J*, **17,** 1036-47.
Hadari YR, Tzahar E, Nadiv O, Rothenberg P, Roberts CT, Jr., LeRoith D, Yarden Y and Zick Y. (1992). *J Biol Chem*, **267,** 17483-6.
Haldar S, Negrini M, Monne M, Sabbioni S and Croce CM. (1994). *Cancer Res*, **54,** 2095-7.
Hamaguchi K, Godwin AK, Yakushiji M, O'Dwyer PJ, Ozols RF and Hamilton TC. (1993). *Cancer Res*, **53,** 5225-32.
Hankinson SE, Colditz GA, Hunter DJ, Willett WC, Stampfer MJ, Rosner B, Hennekens CH and Speizer FE. (1995). *Cancer*, **76,** 284-90.
Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, Bell JC and Dedhar S. (1996). *Nature*, **379,** 91-6.
Hart WR. (2005). *Int J Gynecol Pathol*, **24,** 4-25.
Harvey M, McArthur MJ, Montgomery CA, Jr., Butel JS, Bradley A and Donehower LA. (1993). *Nat Genet*, **5,** 225-9.
Haslam RJ, Koide HB and Hemmings BA. (1993). *Nature*, **363,** 309-10.
Havrilesky L, Darcy M, Hamdan H, Priore RL, Leon J, Bell J and Berchuck A. (2003). *J Clin Oncol*, **21,** 3814-25.
Hayani A, Suarez CR, Molnar Z, LeBeau M and Godwin J. (1994). *J Med Genet*, **31,** 148-9.
Hedgecock EM, Sulston JE and Thomson JN. (1983). *Science*, **220,** 1277-9.
Heinloth A, Brune B, Fischer B and Galle J. (2002). *Atherosclerosis*, **162,** 93-101.
Heise C, Ganly I, Kim YT, Sampson-Johannes A, Brown R and Kirn D. (2000). *Gene Ther*, **7,** 1925-9.
Hengartner MO and Horvitz HR. (1994). *Cell*, **76,** 665-76.
Herod JJ, Eliopoulos AG, Warwick J, Niedobitek G, Young LS and Kerr DJ. (1996). *Cancer Res*, **56,** 2178-84.
Herrmann CP, Kraiss S and Montenarh M. (1991). *Oncogene*, **6,** 877-84.
Higashimoto Y, Saito S, Tong XH, Hong A, Sakaguchi K, Appella E and Anderson CW. (2000). *J Biol Chem*, **275,** 23199-203.
Hirao A, Cheung A, Duncan G, Girard PM, Elia AJ, Wakeham A, Okada H, Sarkissian T, Wong JA, Sakai T, De Stanchina E, Bristow RG, Suda T, Lowe SW, Jeggo PA, Elledge SJ and Mak TW. (2002). *Mol Cell Biol*, **22,** 6521-32.

Hoffman WH, Biade S, Zilfou JT, Chen J and Murphy M. (2002). *J Biol Chem*, **277,** 3247-57.
Hogdall EV, Kjaer SK, Blaakaer J, Christensen L, Glud E, Vuust J and Hogdall CK. (2006). *Gynecol Oncol*, **100,** 76-82.
Holschneider CH and Berek JS. (2000). *Semin Surg Oncol*, **19,** 3-10.
Honda R, Tanaka H and Yasuda H. (1997). *FEBS Lett*, **420,** 25-7.
Hu L, Hofmann J, Lu Y, Mills GB and Jaffe RB. (2002). *Cancer Res*, **62,** 1087-92.
Ignarro LJ, Buga GM, Wood KS, Byrns RE and Chaudhuri G. (1987). *Proc Natl Acad Sci U S A*, **84,** 9265-9.
Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE and Tschopp J. (1997). *Nature*, **388,** 190-5.
Ise T, Shimizu T, Lee EL, Inoue H, Kohno K and Okada Y. (2005). *J Membr Biol*, **205,** 139-45.
Ishikawa T and Ali-Osman F. (1993). *J Biol Chem*, **268,** 20116-25.
Isobe M, Emanuel BS, Givol D, Oren M and Croce CM. (1986). *Nature*, **320,** 84-5.
Iyer NG, Chin SF, Ozdag H, Daigo Y, Hu DE, Cariati M, Brindle K, Aparicio S and Caldas C. (2004). *Proc Natl Acad Sci U S A*, **101,** 7386-91.
Jabbur JR, Huang P and Zhang W. (2001). *Int J Mol Med*, **7,** 163-8.
Jadeski LC, Chakraborty C and Lala PK. (2003). *Int J Cancer*, **106,** 496-504.
Jadeski LC, Hum KO, Chakraborty C and Lala PK. (2000). *Int J Cancer*, **86,** 30-9.
Janiak F, Leber B and Andrews DW. (1994). *J Biol Chem*, **269,** 9842-9.
Janulis M, Silberman S, Ambegaokar A, Gutkind JS and Schultz RM. (1999). *J Biol Chem*, **274,** 801-13.
Jay G, Khoury G, DeLeo AB, Dippold WG and Old LJ. (1981). *Proc Natl Acad Sci U S A*, **78,** 2932-6.
Jiang K, Coppola D, Crespo NC, Nicosia SV, Hamilton AD, Sebti SM and Cheng JQ. (2000). *Mol Cell Biol*, **20,** 139-48.
Jiang M, Wei Q, Wang J, Du Q, Yu J, Zhang L and Dong Z. (2006). *Oncogene*.
Jin Y, Lee H, Zeng SX, Dai MS and Lu H. (2003). *Embo J*, **22,** 6365-77.
Johnson DE, Gastman BR, Wieckowski E, Wang GQ, Amoscato A, Delach SM and Rabinowich H. (2000). *Cancer Res*, **60,** 1818-23.
Judson PL, Watson JM, Gehrig PA, Fowler WC, Jr. and Haskill JS. (1999). *Cancer Res*, **59,** 2425-32.
Juvekar AS, Adwankar MK and Tongaonkar HB. (2000). *Cancer Biother Radiopharm*, **15,** 295-300.
Juven T, Barak Y, Zauberman A, George DL and Oren M. (1993). *Oncogene*, **8,** 3411-6.
Kaku T, Ogawa S, Kawano Y, Ohishi Y, Kobayashi H, Hirakawa T and Nakano H. (2003). *Med Electron Microsc*, **36,** 9-17.
Kamarajan P, Sun NK and Chao CC. (2003). *Biochem J*, **376,** 253-60.
Kane LP, Shapiro VS, Stokoe D and Weiss A. (1999). *Curr Biol*, **9,** 601-4.
Kapp LN, Painter RB, Yu LC, van Loon N, Richard CW, 3rd, James MR, Cox DR and Murnane JP. (1992). *Am J Hum Genet*, **51,** 45-54.
Kasof GM and Gomes BC. (2001). *J Biol Chem*, **276,** 3238-46.
Keri RA, Lozada KL, Abdul-Karim FW, Nadeau JH and Nilson JH. (2000). *Proc Natl Acad Sci U S A*, **97,** 383-7.

Kerr JF, Winterford CM and Harmon BV. (1994). *Cancer*, **73,** 2013-26.
Kerr JF, Wyllie AH and Currie AR. (1972). *Br J Cancer*, **26,** 239-57.
Kigawa J, Sato S, Shimada M, Takahashi M, Itamochi H, Kanamori Y and Terakawa N. (2001). *Hum Cell*, **14,** 165-71.
Kim SB, Kim JS, Lee JH, Yoon WJ, Lee DS, Ko MS, Kwon BS, Choi DH, Cho HR, Lee BJ, Chung DK, Lee HW and Park JW. (2006). *FEBS Lett*, **580,** 311-8.
Kim YM, Chung HT, Kim SS, Han JA, Yoo YM, Kim KM, Lee GH, Yun HY, Green A, Li J, Simmons RL and Billiar TR. (1999). *J Neurosci*, **19,** 6740-7.
Kimble J and Hirsh D. (1979). *Dev Biol*, **70,** 396-417.
Kinoshita H, Yoshikawa H, Shiiki K, Hamada Y, Nakajima Y and Tasaka K. (2000). *Int J Cancer*, **88,** 986-91.
Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH and Peter ME. (1995). *Embo J*, **14,** 5579-88.
Kiss Z and Crilly KS. (1995). *FEBS Lett*, **357,** 279-82.
Kitamura T, Ogawa W, Sakaue H, Hino Y, Kuroda S, Takata M, Matsumoto M, Maeda T, Konishi H, Kikkawa U and Kasuga M. (1998). *Mol Cell Biol*, **18,** 3708-17.
Kluck RM, Esposti MD, Perkins G, Renken C, Kuwana T, Bossy-Wetzel E, Goldberg M, Allen T, Barber MJ, Green DR and Newmeyer DD. (1999). *J Cell Biol*, **147,** 809-22.
Kmet LM, Cook LS and Magliocco AM. (2003). *Cancer*, **97,** 389-404.
Knudson CM, Tung KS, Tourtellotte WG, Brown GA and Korsmeyer SJ. (1995). *Science*, **270,** 96-9.
Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono M and Kuwano M. (1997). *Cancer Res*, **57,** 5475-9.
Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV and Gudkov AV. (1999). *Science*, **285,** 1733-7.
Konishi H, Kuroda S and Kikkawa U. (1994). *Biochem Biophys Res Commun*, **205,** 1770-5.
Konishi I, Kuroda H and Mandai M. (1999). *Oncology*, **57 Suppl 2,** 45-8.
Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL and Burgering BM. (1999). *Nature*, **398,** 630-4.
Krieg T, Landsberger M, Alexeyev MF, Felix SB, Cohen MV and Downey JM. (2003). *Cardiovasc Res*, **58,** 196-202.
Kubbutat MH, Jones SN and Vousden KH. (1997). *Nature*, **387,** 299-303.
Kupryjanczyk J, Szymanska T, Madry R, Timorek A, Stelmachow J, Karpinska G, Rembiszewska A, Ziolkowska I, Kraszewska E, Debniak J, Emerich J, Ulanska M, Pluzanska A, Jedryka M, Goluda M, Chudecka-Glaz A, Rzepka-Gorska I, Klimek M, Urbanski K, Breborowicz J, Zielinski J and Markowska J. (2003). *Br J Cancer*, **88,** 848-54.
Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ and Pavletich NP. (1996). *Science*, **274,** 948-53.
Kwan HY, Huang Y and Yao X. (2004). *Proc Natl Acad Sci U S A*, **101,** 2625-30.
LaCasse EC, Baird S, Korneluk RG and MacKenzie AE. (1998). *Oncogene*, **17,** 3247-59.
Lacasse EC, Kandimalla ER, Winocour P, Sullivan T, Agrawal S, Gillard JW and Durkin J. (2005). *Ann N Y Acad Sci*, **1058,** 215-34.
Laframboise S, Chapman W, McLaughlin J and Andrulis IL. (2000). *Cancer J*, **6,** 302-8.

Lambert PF, Kashanchi F, Radonovich MF, Shiekhattar R and Brady JN. (1998). *J Biol Chem*, **273,** 33048-53.
Lane DP. (1992). *Nature*, **358,** 15-6.
Lane DP and Crawford LV. (1979). *Nature*, **278,** 261-3.
Lasky T and Silbergeld E. (1996). *Environ Health Perspect*, **104,** 1324-31.
Lautier D, Canitrot Y, Deeley RG and Cole SP. (1996). *Biochem Pharmacol*, **52,** 967-77.
Lavarino C, Pilotti S, Oggionni M, Gatti L, Perego P, Bresciani G, Pierotti MA, Scambia G, Ferrandina G, Fagotti A, Mangioni C, Lucchini V, Vecchione F, Bolis G, Scarfone G and Zunino F. (2000). *J Clin Oncol*, **18,** 3936-45.
Lee CW, Sorensen TS, Shikama N and La Thangue NB. (1998). *Oncogene*, **16,** 2695-710.
Lee KB, Parker RJ, Bohr V, Cornelison T and Reed E. (1993). *Carcinogenesis*, **14,** 2177-80.
Lees-Miller SP, Sakaguchi K, Ullrich SJ, Appella E and Anderson CW. (1992). *Mol Cell Biol*, **12,** 5041-9.
Leitao MM, Soslow RA, Baergen RN, Olvera N, Arroyo C and Boyd J. (2004). *Gynecol Oncol*, **93,** 301-6.
Levine EL, Renehan A, Gossiel R, Davidson SE, Roberts SA, Chadwick C, Wilks DP, Potten CS, Hendry JH, Hunter RD and et al. (1995). *Radiother Oncol*, **37,** 1-9.
Lewis J, Burstein E, Reffey SB, Bratton SB, Roberts AB and Duckett CS. (2004). *J Biol Chem*, **279,** 9023-9.
Li DW, Liu JP, Schmid PC, Schlosser R, Feng H, Liu WB, Yan Q, Gong L, Sun SM, Deng M and Liu Y. (2006). *Oncogene*.
Li J, Feng Q, Kim JM, Schneiderman D, Liston P, Li M, Vanderhyden B, Faught W, Fung MF, Senterman M, Korneluk RG and Tsang BK. (2001). *Endocrinology*, **142,** 370-80.
Li J, Sasaki H, Sheng YL, Schneiderman D, Xiao CW, Kotsuji F and Tsang BK. (2000). *Biol Signals Recept*, **9,** 122-30.
Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X and Harran PG. (2004). *Science*, **305,** 1471-4.
Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X. (1997). *Cell*, **91,** 479-89.
Li SB, Schwartz PE, Lee WH and Yang-Feng TL. (1991). *J Natl Cancer Inst*, **83,** 637-40.
Liebermann DA, Hoffman B and Steinman RA. (1995). *Oncogene*, **11,** 199-210.
Lincoln TM, Dey N and Sellak H. (2001). *J Appl Physiol*, **91,** 1421-30.
Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, Farahani R, McLean M, Ikeda JE, MacKenzie A and Korneluk RG. (1996). *Nature*, **379,** 349-53.
Liu FT, Newland AC and Jia L. (2003). *Biochem Biophys Res Commun*, **310,** 956-62.
Liu L, Li H, Underwood T, Lloyd M, David M, Sperl G, Pamukcu R and Thompson WJ. (2001). *J Pharmacol Exp Ther*, **299,** 583-92.
Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA and Elledge SJ. (2000). *Genes Dev*, **14,** 1448-59.
Liu Y, Heyman M, Wang Y, Falkmer U, Hising C, Szekely L and Einhorn S. (1994). *Int J Cancer*, **58,** 663-7.
Lockshin RA and Williams CM. (1965). *J Insect Physiol*, **11,** 123-33.

Lu YS, Yeh PY, Chuang SE, Gao M, Kuo ML and Cheng AL. (2006). *J Endocrinol*, **188,** 311-9.
Ludwick C, Gilks CB, Miller D, Yaziji H and Clement PB. (2005). *Int J Gynecol Pathol*, **24,** 205-17.
Luesley DM, Blackledge GR, Chan KK and Newton JR. (1986). *Br J Obstet Gynaecol*, **93,** 380-5.
Luesley DM, Chan KK, Newton JR and Blackledge GR. (1987). *Br J Obstet Gynaecol*, **94,** 461-6.
Luo X, Budihardjo I, Zou H, Slaughter C and Wang X. (1998). *Cell*, **94,** 481-90.
Lynch HT, Krush AJ, Larsen AL and Magnuson CW. (1966a). *Am J Med Sci*, **252,** 381-90.
Lynch HT, Shaw MW, Magnuson CW, Larsen AL and Krush AJ. (1966b). *Arch Intern Med*, **117,** 206-12.
Lynch HT, Shaw TG and Lynch JF. (2004). *Am J Med Genet C Semin Med Genet*, **129,** 5-22.
MacFarlane M, Ahmad M, Srinivasula SM, Fernandes-Alnemri T, Cohen GM and Alnemri ES. (1997). *J Biol Chem*, **272,** 25417-20.
MacFarlane M, Merrison W, Bratton SB and Cohen GM. (2002). *J Biol Chem*, **277,** 36611-6.
Mano Y, Kikuchi Y, Yamamoto K, Kita T, Hirata J, Tode T, Ishii K and Nagata I. (1999). *Eur J Cancer*, **35,** 1214-9.
Mansouri A, Zhang Q, Ridgway LD, Tian L and Claret FX. (2003). *Oncol Res*, **13,** 399-404.
Marchenko ND, Zaika A and Moll UM. (2000). *J Biol Chem*, **275,** 16202-12.
Matzuk MM, Finegold MJ, Su JG, Hsueh AJ and Bradley A. (1992). *Nature*, **360,** 313-9.
Mayer BJ, Ren R, Clark KL and Baltimore D. (1993). *Cell*, **73,** 629-30.
Mayo LD and Donner DB. (2001). *Proc Natl Acad Sci U S A*, **98,** 11598-603.
Mazoyer S, Puget N, Perrin-Vidoz L, Lynch HT, Serova-Sinilnikova OM and Lenoir GM. (1998). *Am J Hum Genet*, **62,** 713-5.
McCormick F. (1999). *Trends Cell Biol*, **9,** M53-6.
McGowan CH and Russell P. (2004). *Curr Opin Cell Biol*, **16,** 629-33.
McGuire WP, 3rd. (2003). *J Clin Oncol*, **21,** 133-5.
McGuire WP, 3rd and Markman M. (2003). *Br J Cancer*, **89 Suppl 3,** S3-8.
McGuire WP, Hoskins WJ, Brady MF, Kucera PR, Partridge EE, Look KY, Clarke-Pearson DL and Davidson M. (1996a). *N Engl J Med*, **334,** 1-6.
McGuire WP, Hoskins WJ, Brady MF, Kucera PR, Partridge EE, Look KY, Clarke-Pearson DL and Davidson M. (1996b). *Semin Oncol*, **23,** 40-7.
McLaughlin LM and Demple B. (2005). *Cancer Res*, **65,** 6097-104.
Mechetner EB and Roninson IB. (1992). *Proc Natl Acad Sci U S A*, **89,** 5824-8.
Meek DW and Eckhart W. (1988). *Mol Cell Biol*, **8,** 461-5.
Meggio F, Marin O and Pinna LA. (1994). *Cell Mol Biol Res*, **40,** 401-9.
Meier R, Alessi DR, Cron P, Andjelkovic M and Hemmings BA. (1997). *J Biol Chem*, **272,** 30491-7.
Mende I, Malstrom S, Tsichlis PN, Vogt PK and Aoki M. (2001). *Oncogene*, **20,** 4419-23.
Metzinger DS, Taylor DD and Gercel-Taylor C. (2005). *Cancer Lett.*
Mi RR and Ni H. (2003). *Gynecol Oncol*, **90,** 238-44.

Micheau O, Lens S, Gaide O, Alevizopoulos K and Tschopp J. (2001). *Mol Cell Biol*, **21,** 5299-305.
Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P and Moll UM. (2003). *Mol Cell*, **11,** 577-90.
Mikhailov V, Mikhailova M, Pulkrabek DJ, Dong Z, Venkatachalam MA and Saikumar P. (2001). *J Biol Chem*, **276,** 18361-74.
Miki Y, Swensen JJ, Hobbs MR, DeHoff BS, Rosteck PR, Skolnick MH and Neuhausen SL. (1995). *Genomics*, **25,** 295-7.
Mirza A, McGuirk M, Hockenberry TN, Wu Q, Ashar H, Black S, Wen SF, Wang L, Kirschmeier P, Bishop WR, Nielsen LL, Pickett CB and Liu S. (2002). *Oncogene*, **21,** 2613-22.
Miwa S, Uchida C, Kitagawa K, Hattori T, Oda T, Sugimura H, Yasuda H, Nakamura H, Chida K and Kitagawa M. (2006). *Biochem Biophys Res Commun*, **340,** 54-61.
Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B and Reed JC. (1994). *Oncogene*, **9,** 1799-805.
Moallem SA and Hales BF. (1998). *Development*, **125,** 3225-34.
Momand J, Zambetti GP, Olson DC, George D and Levine AJ. (1992). *Cell*, **69,** 1237-45.
Moncada S, Palmer RM and Higgs EA. (1988). *Hypertension*, **12,** 365-72.
Montagna M, Dalla Palma M, Menin C, Agata S, De Nicolo A, Chieco-Bianchi L and D'Andrea E. (2003). *Hum Mol Genet*, **12,** 1055-61.
Morita K, Ono Y, Fukui H, Tomita S, Ueda Y, Terano A and Fujimori T. (2000). *Pathol Int*, **50,** 219-23.
Moro MA, Russel RJ, Cellek S, Lizasoain I, Su Y, Darley-Usmar VM, Radomski MW and Moncada S. (1996). *Proc Natl Acad Sci U S A*, **93,** 1480-5.
Muller E and Scheidtmann KH. (1995). *Oncogene*, **10,** 1175-85.
Mulsch A, Bauersachs J, Schafer A, Stasch JP, Kast R and Busse R. (1997). *Br J Pharmacol*, **120,** 681-9.
Munchausen LL and Rahn RO. (1975). *Biochim Biophys Acta*, **414,** 242-55.
Murad F, Waldman S, Molina C, Bennett B and Leitman D. (1987). *Prog Clin Biol Res*, **249,** 65-76.
Murad F, Waldman SA, Fiscus RR and Rapoport RM. (1986). *J Cardiovasc Pharmacol*, **8 Suppl 8,** S57-60.
Murata T, Haisa M, Uetsuka H, Nobuhisa T, Ookawa T, Tabuchi Y, Shirakawa Y, Yamatsuji T, Matsuoka J, Nishiyama M, Tanaka N and Naomoto Y. (2004). *Int J Mol Med*, **13,** 865-8.
Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME and Dixit VM. (1996). *Cell*, **85,** 817-27.
Nakajima M, Fujiki Y, Kyo S, Kanaya T, Nakamura M, Maida Y, Tanaka M, Inoue M and Yokoi T. (2005). *J Clin Pharmacol*, **45,** 674-82.
Nakano K and Vousden KH. (2001). *Mol Cell*, **7,** 683-94.
Negri E, Franceschi S, Tzonou A, Booth M, La Vecchia C, Parazzini F, Beral V, Boyle P and Trichopoulos D. (1991). *Int J Cancer*, **49,** 50-6.
Nemajerova A, Erster S and Moll UM. (2005). *Cell Death Differ*, **12,** 197-200.
Nguyen HN, Averette HE, Hoskins W, Sevin BU, Penalver M and Steren A. (1993). *Cancer*, **72,** 3007-11.

Nicosia SV. (1987). *Med Clin North Am*, **71,** 1-9.
Nilson JH, Abbud RA, Keri RA and Quirk CC. (2000). *Recent Prog Horm Res*, **55,** 69-89; discussion 89-91.
O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S and Huang DC. (1998). *Embo J*, **17,** 384-95.
O'Driscoll M, Ruiz-Perez VL, Woods CG, Jeggo PA and Goodship JA. (2003). *Nat Genet*, **33,** 497-501.
Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T and Tanaka N. (2000a). *Science*, **288,** 1053-8.
Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y and Taya Y. (2000b). *Cell*, **102,** 849-62.
Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, Masuyama N and Gotoh Y. (2002). *J Biol Chem*, **277,** 21843-50.
Ogryzko VV, Schiltz RL, Russanova V, Howard BH and Nakatani Y. (1996). *Cell*, **87,** 953-9.
Ohtani K, DeGregori J and Nevins JR. (1995). *Proc Natl Acad Sci U S A*, **92,** 12146-50.
Okamura S, Arakawa H, Tanaka T, Nakanishi H, Ng CC, Taya Y, Monden M and Nakamura Y. (2001). *Mol Cell*, **8,** 85-94.
Oldham SM, Clark GJ, Gangarosa LM, Coffey RJ, Jr. and Der CJ. (1996). *Proc Natl Acad Sci U S A*, **93,** 6924-8.
Olesen SP, Drejer J, Axelsson O, Moldt P, Bang L, Nielsen-Kudsk JE, Busse R and Mulsch A. (1998). *Br J Pharmacol*, **123,** 299-309.
Olivier M, Eeles R, Hollstein M, Khan MA, Harris CC and Hainaut P. (2002). *Hum Mutat*, **19,** 607-14.
Oren M. (2003). *Cell Death Differ*, **10,** 431-42.
Oren M and Levine AJ. (1983). *Proc Natl Acad Sci U S A*, **80,** 56-9.
Orucevic A, Bechberger J, Green AM, Shapiro RA, Billiar TR and Lala PK. (1999). *Int J Cancer*, **81,** 889-96.
Ouchi T, Monteiro AN, August A, Aaronson SA and Hanafusa H. (1998). *Proc Natl Acad Sci U S A*, **95,** 2302-6.
Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB, Zhang WW, Kruzel E and et al. (1995). *Mol Cell Biol*, **15,** 3032-40.
Ozalp SS, Yalcin OT, Tanir M, Kabukcuoglu S and Etiz E. (2002). *Eur J Gynaecol Oncol*, **23,** 337-40.
Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM and Donner DB. (1999). *Nature*, **401,** 82-5.
Palmer RM, Ashton DS and Moncada S. (1988a). *Nature*, **333,** 664-6.
Palmer RM, Rees DD, Ashton DS and Moncada S. (1988b). *Biochem Biophys Res Commun*, **153,** 1251-6.
Panahian N and Maines MD. (2001). *J Neurochem*, **76,** 539-54.
Panahian N, Yoshiura M and Maines MD. (1999). *J Neurochem*, **72,** 1187-203.
Park JH and Levitt L. (1993). *Blood*, **82,** 2470-7.
Park SJ, Kim YY, Ju JW, Han BG, Park SI and Park BJ. (2001). *Biochem Biophys Res Commun*, **289,** 1205-10.
Parrilla-Castellar ER, Arlander SJ and Karnitz L. (2004). *DNA Repair (Amst)*, **3,** 1009-14.

Patschinsky T, Knippschild U and Deppert W. (1992). *J Virol*, **66,** 3846-59.
Pectasides D, Fountzilas G, Aravantinos G, Kalofonos HP, Efstathiou E, Salamalekis E, Farmakis D, Skarlos D, Briasoulis E, Economopoulos T and Dimopoulos MA. (2005). *Gynecol Oncol*, **97,** 436-41.
Pegoraro L, Palumbo A, Erikson J, Falda M, Giovanazzo B, Emanuel BS, Rovera G, Nowell PC and Croce CM. (1984). *Proc Natl Acad Sci U S A*, **81,** 7166-70.
Pegram MD, Lipton A, Hayes DF, Weber BL, Baselga JM, Tripathy D, Baly D, Baughman SA, Twaddell T, Glaspy JA and Slamon DJ. (1998). *J Clin Oncol*, **16,** 2659-71.
Perego P, Giarola M, Righetti SC, Supino R, Caserini C, Delia D, Pierotti MA, Miyashita T, Reed JC and Zunino F. (1996). *Cancer Res*, **56,** 556-62.
Perez RP. (1998). *Eur J Cancer*, **34,** 1535-42.
Perkins C, Kim CN, Fang G and Bhalla KN. (1998). *Cancer Res*, **58,** 4561-6.
Persons DL, Yazlovitskaya EM and Pelling JC. (2000). *J Biol Chem*, **275,** 35778-85.
Pestell KE, Hobbs SM, Titley JC, Kelland LR and Walton MI. (2000). *Mol Pharmacol*, **57,** 503-11.
Pestell KE, Medlow CJ, Titley JC, Kelland LR and Walton MI. (1998). *Int J Cancer*, **77,** 913-8.
Peters M, DeLuca C, Hirao A, Stambolic V, Potter J, Zhou L, Liepa J, Snow B, Arya S, Wong J, Bouchard D, Binari R, Manoukian AS and Mak TW. (2002). *Proc Natl Acad Sci U S A*, **99,** 11305-10.
Petty R, Evans A, Duncan I, Kurbacher C and Cree I. (1998). *Pathol Oncol Res*, **4,** 97-102.
Phillips NJ, Ziegler MR, Radford DM, Fair KL, Steinbrueck T, Xynos FP and Donis-Keller H. (1996). *Cancer Res*, **56,** 606-11.
Picksley SM, Vojtesek B, Sparks A and Lane DP. (1994). *Oncogene*, **9,** 2523-9.
Pieretti M, Hopenhayn-Rich C, Khattar NH, Cao Y, Huang B and Tucker TC. (2002). *Cancer Invest*, **20,** 11-23.
Plooy AC, van Dijk M and Lohman PH. (1984). *Cancer Res*, **44,** 2043-51.
Plosker GL and Keam SJ. (2006). *Drugs*, **66,** 449-75.
Prat J, Ribe A and Gallardo A. (2005). *Hum Pathol*, **36,** 861-70.
Puget N, Torchard D, Serova-Sinilnikova OM, Lynch HT, Feunteun J, Lenoir GM and Mazoyer S. (1997). *Cancer Res*, **57,** 828-31.
Purdie D, Green A, Bain C, Siskind V, Ward B, Hacker N, Quinn M, Wright G, Russell P and Susil B. (1995). *Int J Cancer*, **62,** 678-84.
Qin LF and Ng IO. (2002). *Cancer Lett*, **175,** 27-38.
Rak J, Mitsuhashi Y, Bayko L, Filmus J, Shirasawa S, Sasazuki T and Kerbel RS. (1995). *Cancer Res*, **55,** 4575-80.
Ramus SJ, Bobrow LG, Pharoah PD, Finnigan DS, Fishman A, Altaras M, Harrington PA, Gayther SA, Ponder BA and Friedman LS. (1999). *Genes Chromosomes Cancer*, **25,** 91-6.
Rane MJ, Coxon PY, Powell DW, Webster R, Klein JB, Pierce W, Ping P and McLeish KR. (2001). *J Biol Chem*, **276,** 3517-23.
Reap EA, Leslie D, Abrahams M, Eisenberg RA and Cohen PL. (1995). *J Immunol*, **154,** 936-43.

Reles A, Wen WH, Schmider A, Gee C, Runnebaum IB, Kilian U, Jones LA, El-Naggar A, Minguillon C, Schonborn I, Reich O, Kreienberg R, Lichtenegger W and Press MF. (2001). *Clin Cancer Res*, **7,** 2984-97.

Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW, Liddington RC and Salvesen GS. (2001). *Cell*, **104,** 791-800.

Righetti SC, Della Torre G, Pilotti S, Menard S, Ottone F, Colnaghi MI, Pierotti MA, Lavarino C, Cornarotti M, Oriana S, Bohm S, Bresciani GL, Spatti G and Zunino F. (1996). *Cancer Res*, **56,** 689-93.

Risch HA, Marrett LD and Howe GR. (1994). *Am J Epidemiol*, **140,** 585-97.

Rosell R, Taron M, Barnadas A, Scagliotti G, Sarries C and Roig B. (2003). *Cancer Control*, **10,** 297-305.

Rosenberg B, VanCamp L, Trosko JE and Mansour VH. (1969). *Nature*, **222,** 385-6.

Rossig L, Jadidi AS, Urbich C, Badorff C, Zeiher AM and Dimmeler S. (2001). *Mol Cell Biol*, **21,** 5644-57.

Roy N, Mahadevan MS, McLean M, Shutler G, Yaraghi Z, Farahani R, Baird S, Besner-Johnston A, Lefebvre C, Kang X and et al. (1995). *Cell*, **80,** 167-78.

Sakahira H, Enari M and Nagata S. (1998). *Nature*, **391,** 96-9.

Sakakura C, Sweeney EA, Shirahama T, Igarashi Y, Hakomori S, Nakatani H, Tsujimoto H, Imanishi T, Ohgaki M, Ohyama T, Yamazaki J, Hagiwara A, Yamaguchi T, Sawai K and Takahashi T. (1996). *Int J Cancer*, **67,** 101-5.

Salazar H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, Boente MP, Lynch HT and Hamilton TC. (1996). *J Natl Cancer Inst*, **88,** 1810-20.

Sanchez-Prieto R, Rojas JM, Taya Y and Gutkind JS. (2000). *Cancer Res*, **60,** 2464-72.

Sanna MG, da Silva Correia J, Ducrey O, Lee J, Nomoto K, Schrantz N, Deveraux QL and Ulevitch RJ. (2002a). *Mol Cell Biol*, **22,** 1754-66.

Sanna MG, da Silva Correia J, Luo Y, Chuang B, Paulson LM, Nguyen B, Deveraux QL and Ulevitch RJ. (2002b). *J Biol Chem*, **277,** 30454-62.

Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM and Abraham RT. (1999). *Cancer Res*, **59,** 4375-82.

Sasaki H, Kotsuji F and Tsang BK. (2002). *Gynecol Oncol*, **85,** 339-50.

Sasaki H, Sheng Y, Kotsuji F and Tsang BK. (2000). *Cancer Res*, **60,** 5659-66.

Sato N, Tsunoda H, Nishida M, Morishita Y, Takimoto Y, Kubo T and Noguchi M. (2000). *Cancer Res*, **60,** 7052-6.

Sato S, Kigawa J, Minagawa Y, Okada M, Shimada M, Takahashi M, Kamazawa S and Terakawa N. (1999). *Cancer*, **86,** 1307-13.

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S and et al. (1995a). *Science*, **268,** 1749-53.

Savitsky K, Sfez S, Tagle DA, Ziv Y, Sartiel A, Collins FS, Shiloh Y and Rotman G. (1995b). *Hum Mol Genet*, **4,** 2025-32.

Scambia G, Masciullo V, Benedetti Panici P, Marone M, Ferrandina G, Todaro N, Bellacosa A, Jain SK, Neri G, Piffanelli A and Mancuso S. (1997). *Br J Cancer*, **75,** 1547-53.

Scheid MP, Schubert KM and Duronio V. (1999). *J Biol Chem*, **274,** 31108-13.

Schlesselman JJ. (1995). *Obstet Gynecol*, **85,** 793-801.

Schlossmann J, Ammendola A, Ashman K, Zong X, Huber A, Neubauer G, Wang GX, Allescher HD, Korth M, Wilm M, Hofmann F and Ruth P. (2000). *Nature*, **404,** 197-201.
Schneiderman D, Kim J-M, Senterman M and Tsang BK. (1999). *Apoptosis*, **4,** 271-82.
Schultz DC, Vanderveer L, Berman DB, Hamilton TC, Wong AJ and Godwin AK. (1996). *Cancer Res*, **56,** 1997-2002.
Schuyer M and Berns EM. (1999). *Mol Cell Endocrinol*, **155,** 143-52.
Scolnick DM, Chehab NH, Stavridi ES, Lien MC, Caruso L, Moran E, Berger SL and Halazonetis TD. (1997). *Cancer Res*, **57,** 3693-6.
Scorrano L and Korsmeyer SJ. (2003). *Biochem Biophys Res Commun*, **304,** 437-44.
Scully R, Puget N and Vlasakova K. (2000). *Oncogene*, **19,** 6176-83.
Scully RE. (1995). *J Cell Biochem Suppl*, **23,** 208-18.
Selvakumaran M, Lin HK, Miyashita T, Wang HG, Krajewski S, Reed JC, Hoffman B and Liebermann D. (1994). *Oncogene*, **9,** 1791-8.
Selvakumaran M, Pisarcik DA, Bao R, Yeung AT and Hamilton TC. (2003). *Cancer Res*, **63,** 1311-6.
Shaheen RM, Davis DW, Liu W, Zebrowski BK, Wilson MR, Bucana CD, McConkey DJ, McMahon G and Ellis LM. (1999). *Cancer Res*, **59,** 5412-6.
Shayesteh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, Collins C, Pinkel D, Powell B, Mills GB and Gray JW. (1999). *Nat Genet*, **21,** 99-102.
Sheets EE and Yeh J. (1997). *Ann Med*, **29,** 121-6.
Shelling AN, Cooke IE and Ganesan TS. (1995). *Br J Cancer*, **72,** 521-7.
Shen DW, Goldenberg S, Pastan I and Gottesman MM. (2000). *J Cell Physiol*, **183,** 108-16.
Shieh SY, Ahn J, Tamai K, Taya Y and Prives C. (2000). *Genes Dev*, **14,** 289-300.
Shieh SY, Ikeda M, Taya Y and Prives C. (1997). *Cell*, **91,** 325-34.
Shin I, Edl J, Biswas S, Lin PC, Mernaugh R and Arteaga CL. (2005). *Cancer Res*, **65,** 2815-24.
Shono T, Tofilon PJ, Schaefer TS, Parikh D, Liu TJ and Lang FF. (2002). *Cancer Res*, **62,** 1069-76.
Siddik ZH, Mims B, Lozano G and Thai G. (1998). *Cancer Res*, **58,** 698-703.
Silberman S, Janulis M and Schultz RM. (1997). *J Biol Chem*, **272,** 5927-35.
Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E and Kastan MB. (1997). *Genes Dev*, **11,** 3471-81.
Silke J, Ekert PG, Day CL, Hawkins CJ, Baca M, Chew J, Pakusch M, Verhagen AM and Vaux DL. (2001). *Embo J*, **20,** 3114-23.
Silva EG, Tornos C, Bailey MA and Morris M. (1991). *Arch Pathol Lab Med*, **115,** 377-81.
Skilling JS, Sood A, Niemann T, Lager DJ and Buller RE. (1996a). *Oncogene*, **13,** 117-23.
Skilling JS, Squatrito RC, Connor JP, Niemann T and Buller RE. (1996b). *Gynecol Oncol*, **60,** 72-80.
Skirnisdottir I, Seidal T, Gerdin E and Sorbe B. (2002). *Int J Gynecol Cancer*, **12,** 265-76.
Song K, Cowan KH and Sinha BK. (1999). *Oncol Res*, **11,** 153-9.
Song K, Li Z, Seth P, Cowan KH and Sinha BK. (1997). *Oncol Res*, **9,** 603-9.
Song Z, Yao X and Wu M. (2003). *J Biol Chem*, **278,** 23130-40.

Stambolic V, MacPherson D, Sas D, Lin Y, Snow B, Jang Y, Benchimol S and Mak TW. (2001). *Mol Cell*, **8,** 317-25.
Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP and Mak TW. (1998). *Cell*, **95,** 29-39.
States JC and Reed E. (1996). *Cancer Lett*, **108,** 233-7.
Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR and Lipp J. (1998). *J Exp Med*, **188,** 211-6.
Stevens EV, Raffeld M, Espina V, Kristensen GB, Trope CG, Kohn EC and Davidson B. (2005). *Cancer*, **103,** 2313-9.
Struewing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, Timmerman MM, Brody LC and Tucker MA. (1997). *N Engl J Med*, **336,** 1401-8.
Suenobu N, Shichiri M, Iwashina M, Marumo F and Hirata Y. (1999). *Arterioscler Thromb Vasc Biol*, **19,** 140-6.
Suhara T, Kim HS, Kirshenbaum LA and Walsh K. (2002). *Mol Cell Biol*, **22,** 680-91.
Sulston JE. (1976). *Philos Trans R Soc Lond B Biol Sci*, **275,** 287-97.
Sulston JE and Horvitz HR. (1977). *Dev Biol*, **56,** 110-56.
Sulston JE, Schierenberg E, White JG and Thomson JN. (1983). *Dev Biol*, **100,** 64-119.
Sun M, Paciga JE, Feldman RI, Yuan Z, Coppola D, Lu YY, Shelley SA, Nicosia SV and Cheng JQ. (2001a). *Cancer Res*, **61,** 5985-91.
Sun M, Wang G, Paciga JE, Feldman RI, Yuan ZQ, Ma XL, Shelley SA, Jove R, Tsichlis PN, Nicosia SV and Cheng JQ. (2001b). *Am J Pathol*, **159,** 431-7.
Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geuskens M and Kroemer G. (1996). *J Exp Med*, **184,** 1331-41.
Suzuki M, Saito S, Saga Y, Ohwada M and Sato I. (2000). *Cancer Genet Cytogenet*, **118,** 132-5.
Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K and Takahashi R. (2001). *Mol Cell*, **8,** 613-21.
Szegezdi E, Fitzgerald U and Samali A. (2003). *Ann N Y Acad Sci*, **1010,** 186-94.
Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS and Reed JC. (1998). *J Biol Chem*, **273,** 7787-90.
Takai H, Tominaga K, Motoyama N, Minamishima YA, Nagahama H, Tsukiyama T, Ikeda K, Nakayama K, Nakanishi M and Nakayama K. (2000). *Genes Dev*, **14,** 1439-47.
Tang CH and Grimm EA. (2004). *J Biol Chem*, **279,** 288-98.
Tang Y, Zhou H, Chen A, Pittman RN and Field J. (2000). *J Biol Chem*, **275,** 9106-9.
Tejedo JR, Cahuana GM, Ramirez R, Esbert M, Jimenez J, Sobrino F and Bedoya FJ. (2004). *Endocrinology*, **145,** 2319-27.
Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS and Dixit VM. (1995). *Cell*, **81,** 801-9.
Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M, Scaffidi C, Krammer PH, Peter ME and Tschopp J. (1997). *Nature*, **386,** 517-21.
Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh SY, Taya Y, Prives C and Abraham RT. (1999). *Genes Dev*, **13,** 152-7.
Traxler PM, Furet P, Mett H, Buchdunger E, Meyer T and Lydon N. (1996). *J Med Chem*, **39,** 2285-92.

Tsou TC, Tsai FY, Wu MC and Chang LW. (2003). *Toxicol Appl Pharmacol*, **191,** 177-87.
Tsujimoto Y, Cossman J, Jaffe E and Croce CM. (1985). *Science*, **228,** 1440-3.
Tsujimoto Y, Finger LR, Yunis J, Nowell PC and Croce CM. (1984). *Science*, **226,** 1097-9.
Tsuruta Y, Mandai M, Konishi I, Kuroda H, Kusakari T, Yura Y, Hamid AA, Tamura I, Kariya M and Fujii S. (2001). *Eur J Cancer*, **37,** 531-41.
Turner GA, Ellis RD, Guthrie D, Latner AL, Monaghan JM, Ross WM, Skillen AW and Wilson RG. (1982a). *J Clin Pathol*, **35,** 800-6.
Turner GA, Ellis RD, Guthrie D, Latner AL, Ross WM and Skillen AW. (1982b). *Br J Obstet Gynaecol*, **89,** 760-4.
Uchida C, Miwa S, Kitagawa K, Hattori T, Isobe T, Otani S, Oda T, Sugimura H, Kamijo T, Ookawa K, Yasuda H and Kitagawa M. (2005). *Embo J*, **24,** 160-9.
Unger MA, Nathanson KL, Calzone K, Antin-Ozerkis D, Shih HA, Martin AM, Lenoir GM, Mazoyer S and Weber BL. (2000). *Am J Hum Genet*, **67,** 841-50.
Unger T, Juven-Gershon T, Moallem E, Berger M, Vogt Sionov R, Lozano G, Oren M and Haupt Y. (1999a). *Embo J*, **18,** 1805-14.
Unger T, Sionov RV, Moallem E, Yee CL, Howley PM, Oren M and Haupt Y. (1999b). *Oncogene*, **18,** 3205-12.
Uren AG, Pakusch M, Hawkins CJ, Puls KL and Vaux DL. (1996). *Proc Natl Acad Sci U S A*, **93,** 4974-8.
van Riper DA, McDaniel NL and Rembold CM. (1997). *Biochim Biophys Acta*, **1355,** 323-30.
van Steeg H. (2001). *Toxicol Lett*, **120,** 209-19.
Vanderhyden BC, Shaw TJ and Ethier JF. (2003). *Reprod Biol Endocrinol*, **1,** 67.
Vasey PA, Jones NA, Jenkins S, Dive C and Brown R. (1996). *Mol Pharmacol*, **50,** 1536-40.
Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ, Adams JM and Strasser A. (2003). *Science*, **302,** 1036-8.
Wajant H. (2003). *Mol Interv*, **3,** 124-7.
Waldman SA and Murad F. (1987). *Pharmacol Rev*, **39,** 163-96.
Wang G, Barrett JW, Stanford M, Werden SJ, Johnston JB, Gao X, Sun M, Cheng JQ and McFadden G. (2006). *Proc Natl Acad Sci U S A*, **103,** 4640-5.
Wang J, Chun HJ, Wong W, Spencer DM and Lenardo MJ. (2001). *Proc Natl Acad Sci U S A*, **98,** 13884-8.
Wang Y, Chan S and Tsang BK. (2002). *Endocrinology*, **143,** 2732-40.
Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB and Korsmeyer SJ. (2000). *Genes Dev*, **14,** 2060-71.
Weill CL and Greene DP. (1990). *Brain Res Dev Brain Res*, **55,** 143-6.
Weiss NS, Homonchuk T and Young JL, Jr. (1977). *Gynecol Oncol*, **5,** 161-7.
Weiss RS, Matsuoka S, Elledge SJ and Leder P. (2002). *Curr Biol*, **12,** 73-7.
Wen SF, Mahavni V, Quijano E, Shinoda J, Grace M, Musco-Hobkinson ML, Yang TY, Chen Y, Runnenbaum I, Horowitz J, Maneval D, Hutchins B and Buller R. (2003). *Cancer Gene Ther*, **10,** 224-38.
West HL, Franklin WA, McCoy J, Gumerlock PH, Vance R, Lau DH, Chansky K, Crowley JJ and Gandara DR. (2006). *J Clin Oncol*, **24,** 1807-13.

Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA and et al. (1995). *Immunity*, **3,** 673-82.
Wolf CR, Hayward IP, Lawrie SS, Buckton K, McIntyre MA, Adams DJ, Lewis AD, Scott AR and Smyth JF. (1987). *Int J Cancer*, **39,** 695-702.
Wong HK, Fricker M, Wyttenbach A, Villunger A, Michalak EM, Strasser A and Tolkovsky AM. (2005). *Mol Cell Biol*, **25,** 8732-47.
Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C and Micklem G. (1995). *Nature*, **378,** 789-92.
Wu L, Goodwin EC, Naeger LK, Vigo E, Galaktionov K, Helin K and DiMaio D. (2000). *Mol Cell Biol*, **20,** 7059-67.
Wu Xiao C, Asselin E and Tsang BK. (2002). *Biol Reprod*, **67,** 436-41.
Wu Z, Earle J, Saito S, Anderson CW, Appella E and Xu Y. (2002). *Mol Cell Biol*, **22,** 2441-9.
Wyllie AH, Morris RG, Smith AL and Dunlop D. (1984). *J Pathol*, **142,** 67-77.
Xiao CW, Yan X, Li Y, Reddy SA and Tsang BK. (2003). *Endocrinology*, **144,** 623-30.
Xu J, Xin S and Du W. (2001). *FEBS Lett*, **508,** 394-8.
Xu W, Liu L, Smith GC and Charles G. (2000). *Nat Cell Biol*, **2,** 339-45.
Xu W, Liu LZ, Loizidou M, Ahmed M and Charles IG. (2002). *Cell Res*, **12,** 311-20.
Xue D, Shaham S and Horvitz HR. (1996). *Genes Dev*, **10,** 1073-83.
Yaginuma Y, Hayashi H, Kawai K, Kurakane T, Saitoh Y, Kitamura S, Sengoku K and Ishikawa M. (1997). *Exp Cell Res*, **233,** 233-9.
Yaginuma Y and Westphal H. (1992). *Cancer Res*, **52,** 4196-9.
Yamaguchi A, Tamatani M, Matsuzaki H, Namikawa K, Kiyama H, Vitek MP, Mitsuda N and Tohyama M. (2001). *J Biol Chem*, **276,** 5256-64.
Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H and Matsumoto K. (1999). *Embo J*, **18,** 179-87.
Yan X, Fraser M, Qiu Q and Tsang BK. (2006). *Gynecol Oncol*.
Yanez L, Groffen J and Valenzuela DM. (1987). *Oncogene*, **1,** 315-8.
Yang BF, Xiao C, Roa WH, Krammer PH and Hao C. (2003). *J Biol Chem*, **278,** 7043-50.
Yang L, Dan HC, Sun M, Liu Q, Sun XM, Feldman RI, Hamilton AD, Polokoff M, Nicosia SV, Herlyn M, Sebti SM and Cheng JQ. (2004). *Cancer Res*, **64,** 4394-9.
Yang S, Kuo C, Bisi JE and Kim MK. (2002). *Nat Cell Biol*, **4,** 865-70.
Yang X, Fraser M, Moll UM, Basak A and Tsang BK. (2006). *Cancer Res*, **66,** 3126-36.
Yang Y, Fang S, Jensen JP, Weissman AM and Ashwell JD. (2000). *Science*, **288,** 874-7.
Yazlovitskaya EM, DeHaan RD and Persons DL. (2001). *Biochem Biophys Res Commun*, **283,** 732-7.
Yazlovitskaya EM and Persons DL. (2003). *Anticancer Res*, **23,** 2275-9.
Yoshida K, Liu H and Miki Y. (2006). *J Biol Chem*, **281,** 5734-40.
Yoshida K and Miki Y. (2004). *Cancer Sci*, **95,** 866-71.
Yu J, Zhang L, Hwang PM, Kinzler KW and Vogelstein B. (2001). *Mol Cell*, **7,** 673-82.
Yu JH, Lim JW, Kim KH, Morio T and Kim H. (2005). *Free Radic Biol Med*, **39,** 590-602.
Yu JJ, Bicher A, Ma YK, Bostick-Bruton F and Reed E. (2000). *Cancer Lett*, **151,** 127-32.
Yuan ZQ, Feldman RI, Sussman GE, Coppola D, Nicosia SV and Cheng JQ. (2003a). *J Biol Chem*.

Yuan ZQ, Feldman RI, Sussman GE, Coppola D, Nicosia SV and Cheng JQ. (2003b). *J Biol Chem*, **278,** 23432-40.
Yuan ZQ, Sun M, Feldman RI, Wang G, Ma X, Jiang C, Coppola D, Nicosia SV and Cheng JQ. (2000). *Oncogene*, **19,** 2324-30.
Zaffaroni N, Pennati M, Colella G, Perego P, Supino R, Gatti L, Pilotti S, Zunino F and Daidone MG. (2002). *Cell Mol Life Sci*, **59,** 1406-12.
Zhang H, Somasundaram K, Peng Y, Tian H, Zhang H, Bi D, Weber BL and El-Deiry WS. (1998). *Oncogene*, **16,** 1713-21.
Zhang HS, Postigo AA and Dean DC. (1999). *Cell*, **97,** 53-61.
Zhang K, Chew M, Yang EB, Wong KP and Mack P. (2001). *Mol Pharmacol*, **59,** 837-43.
Zhang L, Yu J, Park BH, Kinzler KW and Vogelstein B. (2000). *Science*, **290,** 989-92.
Zhang M, Liu W, Ding D and Salvi R. (2003). *Neuroscience*, **120,** 191-205.
Zhang T, Guan M, Jin HY and Lu Y. (2005). *Gynecol Oncol*, **97,** 501-7.
Zheng W, Magid MS, Kramer EE and Chen YT. (1996). *Am J Pathol*, **148,** 47-53.
Zhou BP, Liao Y, Xia W, Zou Y, Spohn B and Hung MC. (2001). *Nat Cell Biol*, **3,** 973-82.
Zou H, Henzel WJ, Liu X, Lutschg A and Wang X. (1997). *Cell*, **90,** 405-13.
Zou L and Elledge SJ. (2003). *Science*, **300,** 1542-8.
Zou L, Liu D and Elledge SJ. (2003). *Proc Natl Acad Sci U S A*, **100,** 13827-32.

ACADEMIC MEMBERSHIPS AND OTHER RELEVANT ASSOCIATIONS

2005-2006	Member, Canadian Science Writer's Association
2003-2004	Associate Member, American Association for Cancer Research
2003	Member, Ottawa Hospital Family Campaign Organizing Committee
2002-2003	Member, Canadian Fertility and Andrology Society

AWARDS AND DISTINCTIONS

• Gerry Taichman Award for Best Research Achievement by a Graduate Student (Doctoral Program)	Department of Cellular and Molecular Medicine, University of Ottawa	2004
• Outstanding Student Seminar (Ph.D.)	Department of Cellular and Molecular Medicine, University of Ottawa	2004
• 3rd Place Poster Presentation – Ph.D.	Canadian Conference on Ovarian Cancer Research	2004
• 2nd Place Poster Presentation – Ph.D.	Canadian Reproductive Biology Workshop	2004
• Canada Graduate Scholarship – Doctoral Research Award	Canadian Institutes of Health Research (CIHR)	2004-2006
• Canada Graduate Scholarship (declined)	Natural Science and Engineering Research Council (NSERC)	2004-2006
• Ontario Graduate Scholarship (declined)	Ontario Ministry of Education	2004-2006
• National Excellence Scholarship	University of Ottawa	2004-2006
• 1st Place – Oral Presentation	2003 Ottawa Health Research Institute Annual Research Day	2003
• Graduate (Doctoral) Admission Scholarship	Faculty of Graduate and Postdoctoral Studies, University of Ottawa	2003-2007
• Ontario Graduate Scholarship in Science and Technology	Government of Ontario – Ministry of Education and Ottawa Health Research Institute	2001-2003
• Entrance Award	Department of Cellular and Molecular Medicine, University of Ottawa	2001
• Samuel Lunenfeld Summer Studentship	The Hospital for Sick Children	2001
• Canadian Genetic Diseases Network Summer Studentship	Montreal Genome Centre	2000
• Entrance Award	McGill University	1997-1998

PUBLICATIONS

1. Yang X., **Fraser M.**, Moll U.M., Basak A., and Tsang B.K. (2006) Akt-mediated Cisplatin resistance in Ovarian Cancer: Modulation of p53 Action on Caspase-dependent Mitochondrial Death Pathway. *Cancer Research.* Mar 15;66(6):3126-36.

2. Yan X, **Fraser M.**, Qiu Q., and Tsang B.K. (2006) Over-Expression of PTEN Sensitizes Human Ovarian Cancer Cells to Cisplatin-Induced Apoptosis in a p53-Dependent Manner. *Gynecologic Oncology* [epub ahead of print].

3. **Fraser M.**, Chan S.L., Chan S., Fiscus R.R., and Tsang B.K. (2006) Regulation of p53 and suppression of apoptosis by the soluble guanylyl cyclase pathway in human ovarian cancer cells. *Oncogene* Apr 6;25(15):2203-12.

4. **Fraser M.**, and Tsang B.K. (2004) The Role of X-Linked Inhibitor of Apoptosis Protein in Chemoresistant Ovarian Cancer. *The Ovary, Second Edition*, Elsevier Science. Ch. 37, pp 613-624.

5. **Fraser M.**, Leung B., Yan X., Dan H.C., Cheng J.Q., and Tsang B.K. (2003) p53 is a Determinant of Xiap/Akt-Mediated Chemoresistance in Human Ovarian Cancer Cells. *Cancer Research.* 63: 7081-7088.

6. **Fraser M.**, Leung B, Jahani-Asl A., Yan X., Thompson W.E., and Tsang B.K. (2003) Chemoresistance in human ovarian cancer: the role of apoptotic regulators. *Reproductive Biology and Endocrinology.* 1:66.

7. Cheng J.Q, Jiang X., **Fraser M.**, Li M., Dan H.C, Sun M., and Tsang B.K. (2002) Role of X-linked inhibitor of apoptosis protein in chemoresistance in ovarian cancer: possible involvement of the phosphoinositide-3 kinase/Akt pathway. *Drug Resistance Updates.* 5(3-4):131-46.

8. Heon E., Paterson A.D, **Fraser M.**, Billingsley G., Priston M., Balmer A., Schorderet D.F, Verner A., Hudson T.J., and Munier F.L. (2001) A progressive autosomal recessive cataract locus maps to chromosome 9q13-q22. *American Journal of Human Genetics.* 68(3):772-7.

MANUSCRIPTS SUBMITTED

1. **Fraser M.**, and Tsang B.K. (2006) Akt-Mediated Chemoresistance in Human Ovarian Cancer Cells is Mediated Through Inhibition of p53 Function and Phosphorylation. *Oncogene.*

MANUSCRIPTS IN PREPARATION

1. Boulay H.M., Qiu Q., **Fraser M.**, Senterman M., Basak A., and Tsang B.K. (2005) Proprotein Convertase 4 is a Cell Survival Mediator and Determinant of Chemoresistance in Human Ovarian Cancer Cells. *Cancer Research.*

2. Yang X., **Fraser M.**, and Tsang B.K. (2006) Akt inhibits the mitochondrial release of Apoptosis Inducing Factor during Cisplatin-Induced Apoptosis in Human Ovarian Cancer Cells. *Gynecologic Oncology.*

3. Leung L.H., **Fraser M.**, Fiscus R.R., and Tsang B.K. (2006) Nitric Oxide Sensitizes Human Ovarian Cancer Cells to Cisplatin-Induced Apoptosis Via p53 Up-Regulation. *Gynecologic Oncology.*

INVITED TALKS

1. **Fraser M.** and Tsang B.K. (2005) Regulation of p53-Mediated Apoptosis in Human Ovarian Cancer Cells. 2005 Ottawa Reproductive Biology Workshop, Ottawa, ON.

2. **Fraser M.**, Leung B., Yan X., Cheng J.Q., and Tsang B.K. (2003) p53 Status is a Determinant of Xiap-/Akt-Mediated Chemoresistance in Human Ovarian Cancer. 2003 Ottawa Health Research Institute Research Day, Ottawa, ON.

3. **Fraser M.**, Leung B., Yan X., Cheng J.Q., and Tsang B.K. (2003) p53 Status is a Determinant of Akt-Mediated Chemoresistance in Human Ovarian Cancer. 2003 Ottawa Reproductive Biology Workshop, Ottawa, ON.

4. **Fraser M.**, and Tsang B.K. (2001) Xiap and Chemoresistance in Human Ovarian Cancer. 1st Annual Ottawa Health Research Day Retreat, Ottawa, ON.

PUBLISHED ABSTRACTS

1. **Fraser M.**, Yang X., Abedini M., Yan X., Jahani-asl A., Boulay H., Wang H., and Tsang B.K. Program on Ovarian Cancer Biology: Mechanisms of Chemoresistance. 3rd Canada-Japan Bilateral Workshop on Human Reproduction & Reproductive Biology, Ottawa, ON, 2004.

2. **Fraser M**, Chan S.L., Fiscus R.R., and Tsang B.K. (2004) Down-regulation of XIAP and induction of apoptosis by the soluble guanylate cyclase inhibitor ODQ in human ovarian cancer cells. Canadian Conference on Ovarian Cancer, Ottawa, ON, 2004.

3. **Fraser M**, Chan S.L., Fiscus R.R., and Tsang B.K. (2004) Down-regulation of XIAP and induction of apoptosis by the soluble guanylate cyclase inhibitor ODQ

in human ovarian cancer cells. American Association for Cancer Research, 2004 Annual Meeting, Orlando, FL.

4. **Fraser M.**, Leung B., Yan X., Cheng J.Q., and Tsang B.K. (2003) p53 Status is a Determinant of Akt-Mediated Chemoresistance in Human Ovarian Cancer. American Association for Cancer Research, 2003 Annual Meeting, Toronto, ON.

5. **Fraser M.**, and Tsang B.K. (2002) Overexpression of X-linked Inhibitor of Apoptosis Protein Increases Integrin-Linked Kinase Content in Human Ovarian Cancer Cells. Canadian Fertility and Andrology Society, 2002 Annual Meeting, La Malbaie, QC.

ASSIGNED MENTORSHIPS

01/2005-present	Shadia Al-Bahlani, Ph.D. student
04/2005-11/2005	Elaine Leung, Ph.D. student, visiting from Chinese University of Hong Kong
05/2004-08/2004	Sarah Chan, Undergraduate Summer Student
05/2003-12/2003	Siu Lan Chan, M.Sc. student, visiting from Chinese University of Hong Kong