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The Effect of Cisplatin on the Role of Proprotein Convertases (PCs) in Human Ovarian Cancer Cells

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The Effect of Cisplatin on the Role of Proprotein Convertases (PCs) in Human Ovarian Cancer Cells

By

Hillary Michelle Boulay

This thesis is submitted as a partial fulfillment of the Masters in Science program in Cellular and Molecular Medicine

Faculty of Medicine, University of Ottawa

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ABSTRACT

Proprotein convertases (PCs) have been implicated in cancer progression as well as cell survival, although their role in ovarian cancer and drug sensitivity is largely unknown. Of all the PCs, PC4 has the most restricted expression; present only in reproductive tissues (testis, ovary and placenta). The expression and regulation of PC4 were investigated using a pair of cisplatin-sensitive and –resistant cell lines as an *in vitro* model of ovarian cancer. PC4 is expressed in the ovarian cancer cell lines as well as in tumours from patients diagnosed with the disease. Over-expression of PC4 in chemosensitive cells attenuated cisplatin (CDDP)-induced apoptosis, while inhibition of PC4 activity or down-regulation of PC4 by siRNA sensitized chemoresistant cells to the apoptotic effects of CDDP. Furthermore, current data suggests that the role of PC4 in chemoresistance may be mediated through the processing and activation of IGF-II. These data demonstrate for the first time that PC4 is anti-apoptotic and imply its involvement in the chemoresistance of human ovarian cancer cells.

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LIST OF ABBREVIATIONS

A	Alanine
aa	Amino acid
ADAM	Disintegrin-type metalloproteinase
AIF	Apoptotic inducing factor
AMC	7-amino-4-methylcoumarin
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine triphosphate
BMP	Bone morphogenic protein
bp	Base pairs
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BSA	Bovine serum albumin
CDDP	Cisplatin (cis diamminedichloroplatinum)
cDNA	Complementary DNA
CMK	Chloromethylketone
CRE	cAMP responsive element
D	Aspartic acid
DAB	3,3'-diaminobenzidine
DISC	Death inducing signaling complex
DMEM-F12	Dulbecco's Modified Eagle Medium-F12
DMSO	Dimethylsulphoxide
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor

ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FAK	Focal adhesion kinase
Fas-L	Fas ligand
GAPDH	Glyceraldehyde 3' phosphate dehydrogenase
H	Histidine
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis protein
ICC	Immunocytochemistry
IGF	Insulin-like growth factor
IGF-II	Insulin-like growth factor II
IGF-1R	Insulin-like growth factor 1 receptor
IGF-IIR	Insulin-like growth factor 2 receptor
IGFBP	Insulin-like growth factor binding proteins
IHC	Immunohistochemistry
IR-A	Insulin receptor A
IRS-1	Insulin receptor substrate-1
K	Lysine
Kb	kilo bases
kDa	kilodaltons
KO	Knock-out
L	Leucine
LB	Luria-Bertani
LOI	Loss of imprinting
LRP	Lung-related resistance protein
M6PR	Mannose-6-phosphate receptor
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2
MDR1	Multidrug resistance 1
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid

MRP1	Multidrug resistance protein 1
MRP2	Multidrug resistance protein 2
MT-MMP	Membrane-type matrix metalloproteinase
NARC	Neural-apoptosis regulated-convertase
NER	Nuclear excision repair
NFκB	Nuclear factor kappa B
OSE	Ovarian surface epithelium
OVCA	Ovarian cancer
PACAP	Pituitary adenylate cyclase-activating polypeptide
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PC	Proprotein convertase
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PI3K	Phosphotidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
Q	Glutamine
R	Arginine
RNA	Ribonucleic acid
RNAi	RNA interference
RPMI 1640	Roswell Park Memorial Institute 1640
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
S	Serine
siRNA	Small inhibitory RNA
T	Threonine
TBS-T	Tris buffered saline Tween-20
TGF-β	Transforming growth factor beta

TNF	Tumour necrosis factor
TNF R1	Tumour necrosis factor receptor 1
TNF R2	Tumour necrosis factor receptor 2
UV	Ultraviolet
V	Valine
VEGF	Vascular endothelial growth factor
WB	Western blot
WT	Wild-type
X	Any amino acid
XIAP	X-linked inhibitor of apoptosis protein
Y	Tryptophan

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CHAPTER 1: INTRODUCTION

1. Human Epithelial Ovarian Cancer

i. Etiology and Epidemiology

Human ovarian cancer is the most common and lethal gynecological cancer and represents the leading cause of death from gynecologic malignancy in the western world. It arises from the simple epithelium, a single layer of flat-to cuboidal epithelial cells that covers the ovary and accounts for 90% of all ovarian malignancies (Auersperg *et al*, 1998). In 2005, it is expected that 2400 women in Canada will develop the disease while 1600 women will succumb to ovarian cancer (Canadian Cancer Statistics, 2005). If diagnosed early the disease is curable, however there are no reliable means for early detection except for genetic screening in a small subset of individuals, and to date there is no test that has been shown to reduce mortality (Auersperg *et al*, 1998). Furthermore, there is a lack of specific symptoms in the early stages of the disease and much of the failure to obtain better cure rates in ovarian cancer is a consequence of late diagnosis (Young *et al*, 2001). Reports suggest that only 25% of patients are diagnosed when the disease is still restricted to the ovary. As the disease advances and the cancer spreads beyond the ovary, treatment becomes largely ineffective due to inoperable conditions. Although most patients respond to current treatment regimens with combination chemotherapy, recurrent drug-resistant disease ultimately hinders successful treatment outcomes. The five year survival rate for patients with stage III or IV of the disease is approximately 20-40 % (Legros *et al*, 1997). Despite current knowledge and intensive research efforts these numbers have not changed in recent decades.

The etiology and early events in ovarian carcinogenesis are poorly understood accounting, in part for the low cure rates. In the past, a variety of environmental agents have been implicated but not proven to play a role in the development of ovarian cancer including diet, talc, industrial pollutants, smoking, asbestos and infectious agents (Herbst, 1994). Accumulating evidence suggests that nulliparity and hyperovulation treatment for infertility increase the risk of ovarian cancer, while use of oral contraceptives and pregnancy are protective against the disease (Auersperg *et al,* 1998). These observations are consistent with the early hypothesis of Fathalla, first proposed in 1971, that frequent ovulation contributes to increased risk because of the repeated rupture of the epithelium at the site of ovulation and that the ensuing wound repair and cell proliferation provides opportunity for genetic alterations that may lead to carcinogenesis. Additionally, a strong family history of ovarian cancer is a major known risk factor. Hereditary ovarian cancer accounts for 5-10% of cases (Auersperg *et al*, 1998) and has been associated with germline mutations in the BRCA1 and BRCA2 genes, with BRCA1 mutations playing a major role in ovarian cancer susceptibility (Xu *et al*, 1996). These genes regulate DNA damage responses and have been defined as tumour suppressors (Kote-Jarai *et al*, 1999). However, it is worth noting that not all carriers of these predisposing mutations develop ovarian cancer, which suggests a role for interactions with other genetic and epigenetic influences. Cases of epithelial ovarian cancer that are not associated with heredity, the majority of cases, arise as a result of the accumulation of genetic damage over the course of a lifetime and are termed sporadic cancers. Over the past several decades, researchers have identified numerous alterations or mutations in oncogenes and tumour suppressor genes which play important roles in the regulation of proliferation, cell survival,

programmed cell death and senescence, in the development of ovarian cancer, however there is still much to be understood regarding the early molecular events leading to ovarian cancer development.

ii. Pathology of Epithelial Ovarian Carcinomas

Histopathologically, ovarian carcinomas are among the most complex of all human malignancies (Van Niekerk *et al*, 1993). One of the most interesting aspects in ovarian carcinogenesis is the marked changes in cellular differentiation that accompanies neoplastic progression. As described earlier, ovarian surface epithelium (OSE) is a simple epithelium with some stromal features. However, as the malignancy progresses, it loses its stromal properties and acquires the characteristics of the Mullerian-duct epithelia of the female reproductive tract, *i.e.* the oviduct, endometrium, and uterine cervix (Auersperg *et al*, 1998). Tumourigenesis may be initiated in epithelial inclusion cysts or invaginations which are often formed in the process of ovulatory repair or changes in the ovarian surface with aging. Cyst formation results in the disruption of the connective tissues, exposing OSE cells to further proliferative cues from steroid producing cells (estrogens), gonadotropins, follicular fluids and cytokines within the ovary (Cramer & Welch, 1983). Ovarian carcinomas are divided into several classes based on the cell types of the tissues they resemble, including serous (fallopian tube-like), endometrioid (endometrium-like) and mucinous (endocervical-like) adenocarcinomas. Serous adenocarcinomas are the most common form of malignant epithelial ovarian cancer and account for approximately 80% of cases. Among the less common forms are clear cell

carcinomas and transitional cell or Brenners carcinomas which resemble the menosephros and urinary tract, respectively (Herbst *et al*, 1992). Histologically, the tumours form polarized epithelia, papillae, cysts and glandular structures and are thus more differentiated and complex than the simple epithelia from which they arose. Additionally, pathological diagnosis often reflects the characteristics of the major cell type within the tumour, however many epithelial-derived ovarian tumours are heterogeneous in appearance and contain cells with varying levels of differentiation.

iii. Treatment of Ovarian Cancer

Ovarian carcinomas are usually diagnosed by detection of an adnexal mass on pelvic examination. Patients with ovarian cancer frequently develop ascites, and a swollen abdomen may be the first symptom, either due to ascites or tumour spread (Herbst *et al*, 1992). The currently preferred treatment regimen for ovarian cancer is cytoreductive surgery to remove the bulk of the tumour followed by combination chemotherapy. However, these are largely ineffective in terms of long term treatment. Approximately 75% of patients present with advanced-stage disease, which are surgically incurable and thus chemotherapy is a critical component of treatment. Chemotherapy with platinum-based drugs such as cisplatin or carboplatin, in combination with paclitaxel (Taxol), are the first-line chemotherapeutic agents for treatment of ovarian cancer and have shown promising results in a large number of cases (Williams & Hamilton, 2001). Despite the initial clinical response rates of approximately 80% that is achieved with these drugs, the tumour ultimately recurs in most patients within 3 years (Eltabbakh and Awtrey, 2001). Therefore, the key underlying reason for the poor outcome of these

treatment regimens is the development of chemoresistance and/ or the expansion of resistant subpopulations of cells within the tumour. Overcoming drug resistance and gaining a better understanding of the molecular events associated with ovarian carcinogenesis is crucial to developing therapeutics that will lead to successful treatment outcomes for ovarian cancer patients.

2. Chemoresistance in Ovarian Cancer

The major goal of cancer chemotherapy is to commit tumour cells to apoptosis following exposure to the anti-tumour agents. Although cisplatin is a very potent inducer of apoptosis (Henkels & Turchi, 1997), resistance does develop and tumour cells fail to undergo apoptosis at clinically relevant drug concentrations. Chemoresistance has therefore been identified as a major hurdle in the effective treatment of ovarian cancer. However, the molecular mechanisms of chemoresistance are multi-factorial and poorly understood. Interestingly, when resistance is seen, it is not just to the primary agent used but to a diverse number of other drugs as well. Cross-resistance is seen both clinically as well as in drug-resistant models of ovarian cancer (Williams & Hamilton, 2001).

The success of chemotherapy is dependent on at least two cellular events. First the drug has to be taken up by the tumour cells, and remain there long enough to elicit its pharmacological effects and secondly the drug must reach its target site within the cell. Molecular mechanisms that may underlie chemoresistance therefore include alterations in the pharmacokinetic and pharmacodynamic properties of the tumour (Williams &

Hamilton, 2001). In general, changes in pharmacokinetic properties include alterations in drug metabolism at both cellular and organ levels, drug accumulation and modified drug targets. In contrast, pharmacodynamic changes revolve around the development of cellular adaptations such as increased DNA repair and DNA damage tolerance.

Increased drug efflux and/or decreased drug influx can directly reduce the accumulation of the chemotherapeutic agent in the tumour cells. Several genes, including *MDR1*, *MRP1*, *MRP2* and *LRP* play a key role in drug transport. Chemotherapy-resistant cell lines displayed many phenotypic differences compared with the parental lines from which they were derived, with reduced drug accumulation being commonly observed. Resistant cell lines were found to over-express a transmembrane P-glycoprotein, the gene product of *MDR1*, which functions as an energy-dependent drug efflux pump (Bradley *et al*, 1989). Over-expression of this protein may account for the cross-resistance phenomena observed in tumour cells as many chemotherapeutic agents, especially those derived from natural products such as vinca alkaloids and taxanes, are substrates for the protein pump (Williams & Hamilton, 2001). Consistent with this finding was the observation that transfection with the *MDR1* gene into sensitive cells was sufficient to produce the multi-drug resistance phenotype (Bourhis *et al*, 1989). *MRP2* has been found to be expressed in a number of cisplatin resistant cell lines (Taniguchi *et al*, 1996). Moreover, LRP expression in advanced ovarian carcinoma has been correlated to poor cisplatin response (Izquierdo *et al*, 1995). However, the literature continues to debate the involvement and relevance of the multi-drug resistant phenotype and P-glycoproteins in chemoresistance associated with ovarian cancer (Aalders *et al, 1999)*. Serial specimens

from 8 patients with ovarian cancer showed no change in the level of P-glycoprotein expression during the course of treatment (Rubin, 1990). Furthermore cisplatin is not a substrate for the P-glycoprotein efflux pump, however it could be a substratre for the so-called glutathione pump or canalicular multispecific organic anion transporter (cMOAT).

The primary cellular target for alkylating agents and platinum based drugs is DNA and studies have shown that the level and persistence of DNA adducts induced by these agents correlate directly with cytotoxicity (Siddik, 2003). Therefore, reducing the extent of DNA damage would increase resistance and can occur via an increased rate of DNA repair within the cell. Repair of platinum adducts occurs primarily through the nucleotide excision repair pathway (NER) (Reed, 1998) The involvement of this phenomenon in chemoresistance is highlighted by the finding that repair-deficient cells are hypersensitive to alkylating agents and cisplatin (Eastman, 1987). Furthermore, cisplatin-resistant cell lines display increased DNA repair, as evidenced by the loss of platinum adducts (Parker *et al*, 1991) and reactivation of cisplatin-damaged plasmid DNA (Sheibani *et al*, 1989).

Chemoresistance can be acquired through chronic drug exposure or can present as *de novo* or intrinsic forms. In addition to the mechanisms mentioned above, the phenomenon of chemoresistance has also been associated with altered expression of genes involved in transducing the signal of DNA damage to activate the apoptotic machinery (Reed *et al*, 1996). Accumulating evidence suggests that defects in intra- and extra-cellular apoptotic mechanisms are an important cause of resistance to cytotoxic

agents such as cisplatin (Kaufmann & Earnshaw, 2000; Arts *et al*, 2000) and this has been the focus of our research. It has been accepted that the apoptotic capacity of the cancer cell is pivotal in determining its response to chemotherapeutic agents (Fraser *et al*, 2003). Recent studies have shown that homeostasis of human ovarian surface epithelial cells is maintained by a delicate balance in the expression and actions of tumour suppressors (e.g. p53, Fas and Fas-L) and cell survival factors (e.g. inhibitor of apoptosis proteins (IAPs) and intermediates of the PI3K/Akt pathway) (Cheng *et al*, 2002). Inhibitors of apoptosis proteins (IAPs) represent a family of intracellular anti-apoptotic proteins that include X-linked Inhibitor of Apoptosis Protein (Xiap), Human Inhibitor of Apoptosis Protein –1 and –2 (Hiap-1 and-2), Neuronal Apoptosis Inhibitory Protein (Naip), survivin, livin and Kidney Inhibitor of Apoptosis (Kiap) (Ambrosini *et al*, 1997; Liston *et al*, 1996). These proteins play a key role in cell survival by modulating death-signaling pathways. It has been suggested that Xiap induces cell survival via inhibition of caspase-3 and caspase-7, two cell death proteases downstream of many pro-apoptotic signaling pathways (Deveraux *et al*, 1997). However, recent studies have shown that Xiap also interacts with a number of fundamental cell survival pathways, such as the PI3K/Akt pathway. Several studies from our lab have demonstrated the importance of this cell survival pathway with regards to chemosensitivity (Cheng *et al*, 2002; Fraser *et al*, 2003). Intermediates of this pathway are frequently altered in human ovarian cancer and elevated levels of PI3K and AKTs were shown to be associated with poor prognosis and chemoresistance (Sun *et al*, 2001; Philp *et al*, 2001). An increasing body of evidence suggests that chemoresistance in ovarian cancer is in part attributed to over-expression of cell survival molecules such as IAPs, PI3K and AKT with a corresponding down-

regulation of pro-apoptotic factors such as Fas and FasL, leading to increased activity of cell survival signaling pathways and ultimately suppressing apoptosis.

Taken together, it can be concluded that the high levels of resistance seen in ovarian cancer are likely the net effect of several complex but unrelated cellular mechanisms. This multi-factorial feature of chemoresistance compounds the difficulty of finding ways to circumvent cisplatin or overall chemotherapy resistance as a therapeutic strategy and remains the focus of many research efforts.

3. Apoptosis

i. Apoptotic *vs.* necrotic cell death

There are two major mechanisms of cell death; necrosis and apoptosis. Cells damaged by external injury undergo necrosis, a poorly regulated and passive process that involves cell swelling, rupture of plasma and organelle membranes leading to the lysis of the cell and ultimately elicits an immune response (Palumbo & Yeh, 1995). In contrast, cells that are induced to commit programmed suicide because of internal or external stimuli (physiological, pathological or cytotoxic) undergo apoptosis (Ghobrial *et al*, 2005). Apoptosis or "programmed cell death" is a highly regulated, genetically controlled mechanism of cellular self-destruction and represents a normal physiological process (Kerr *et al*, 1972; Wyllie *et al*, 1980). It occurs during embryonic development, normal cellular homeostasis, as well as drug-induced tumour cell death (Hickman, 1992) as a means to eliminate damaged, virally infected or otherwise harmful cells. A fundamental feature of apoptosis is that the process results from a decision made by the cell, based on

cues and stimuli from its environment, whether it will live or die. Upon making the decision to die, cellular material is dismantled, packaged and rapidly phagocytosed by adjacent cells, so that an immune response is avoided (Kerr *et al*, 1972). All cells possess the machinery to undergo apoptosis and although some cells require *de novo* synthesis of some of the signaling molecules, the process can be readily and rapidly activated, emphasizing the importance for tight regulation of this process. Deregulation of apoptosis has many pathophysiological consequences; excess activation of programmed cell death has been implicated in various autoimmune diseases and neurodegenerative disorders whereas an escape or inability of cells to undergo cell death is a common etiological factor of cancer (Vincent & Feldman, 2002).

Apoptosis is characterized by unique morphological and biochemical features including nuclear and cytoplasmic shrinkage, blebbing of the cell surface, chromatin condensation, nuclear pyknosis and fragmentation into membrane-enclosed vesicles (Earnshaw *et al*, 1999). Biochemically, there is an activation of endogenous endonucleases to elicit internucleosomal DNA fragmentation [180-190 base-pair (bp) multiples], reduction in mitochondrial membrane potential, translocation of phosphatidylserine to the outer plasma membrane, with a dependence on energy supplied by ATP as well as on active protein synthesis (Wyllie *et al*, 1980; Dive & Wyllie, 1993).

ii. Apoptotic signaling cascades

Apoptosis can take place through several cellular pathways including the receptor-, mitochondria- and endoplasmic reticulum (ER)-mediated cell death pathways which ultimately converge on the activation of caspases that cleave regulatory and structural molecules, culminating in irreversible cell death. Although each of these pathways is able to function independently, the apoptotic response of the cell is likely dictated by cross-talk between all 3 signaling cascades (Figure 1).

Extrinsic pathway

The extrinsic or cytoplasmic pathway of apoptosis is triggered by the activation of death receptors on the cell membrane. These death receptors are members of the tumour necrosis factor (TNF) superfamily and include Fas (APO-1/CD95), TNF R1, DR3, DR4 (TRAIL R1), DR5 (TRAIL R2) and DR6 (Zapata *et al*, 2001). When a death stimulus triggers the pathway, membrane bound Fas ligand (FasL) interacts with Fas to form the death-inducing signaling complex (DISC) containing the Fas-associated death domain protein (FADD) and the proforms of caspase-8 and -10. Formation of the signaling complex ultimately leads to the activation of caspase-8, which can then act on downstream effector caspases to initiate the apoptotic program. Although the ligand and adaptor molecules are different for the other death receptors, similar pathways leading to the activation of caspase-8 appear to be activated upon ligand binding (Ashkenazi *et al*, 1999). TNF-α, the prototype of the TNF superfamily, interacts with TNF R1 or TNF R2 to induce cell death signaling cascades however its actions are mediated through the activation of the transcription factors, AP-1 and NFkappaB (Nagata, 1995).

Figure 1: **Major apoptotic signaling cascades**

Death receptor (extrinsic), mitochondrial (intrinsic) and ER-mediated cell death pathways represent the major apoptotic pathways, all of which culminate in the activation of caspases. In all instances, irreversible cell death is induced via caspase-3 mediated proteolytic cleavage of important regulatory and structural molecules.

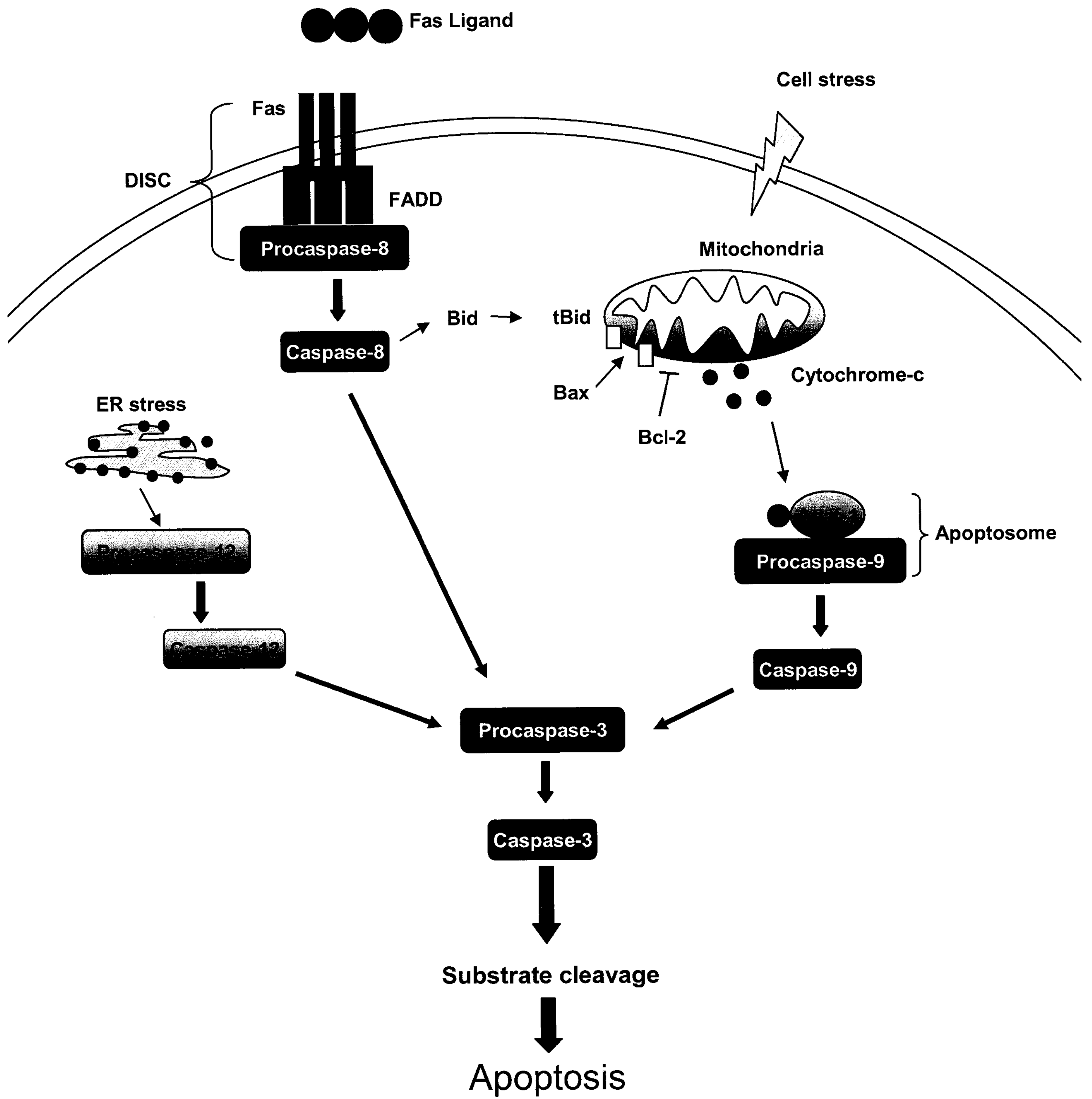


Figure 1

Intrinsic pathway

The role of mitochondria in the induction of apoptotic cascades has received a lot of interest in recent years. The mitochondria-dependent or intrinsic pathway is triggered by stress signals such as DNA damage (induced by radiation or chemotherapeutic agents), reactive oxygen or nitrogen species as well as growth-factor withdrawal (Henry-Mowatt *et al*, 2004). In response to apoptotic stimuli, the mitochondrial membrane becomes permeable and a number of soluble pro-apoptotic proteins are released from the intermembrane space into the cytosol (Henry-Mowatt *et al*, 2004; Hougardy *et al*, 2005) including cytochrome c (Yang *et al*, 1997), apoptotic inducing factor (AIF) (Susin *et al*, 1999), SMAC/Diablo (Du *et al*, 2000) and HtrA2/Omi (Suzuki *et al*, 2001). The actions of cytochrome c release have been extensively studied. Once released into the cytosol, it triggers the formation of the apoptosome, a multimeric molecule comprised of the apoptotic protease activating factor-1 (Apaf-1) and proform of caspase-9, ultimately leading to caspase-9 activation. Similar to the activation of caspase-8 by the death receptor pathway, caspase-9 can then activate downstream effector caspases such as caspase-3 to initiate the apoptotic cascade. The release of apoptogenic proteins from the mitochondria is regulated by the BcL-2 family of proteins of which there are pro-apoptotic members (Bax, Bak) and anti-apoptotic members (Bcl-2, Bcl-XL) (Henry-Mowatt *et al*, 2004). The relative ratio of pro- to anti-apoptotic Bcl-2 family members establishes the cellular sensitivity to apoptotic stimuli through the mitochondrial pathway (Green & Reed, 1998).

Apoptotic pathway induced by ER stress

Endoplasmic reticulum-induced apoptosis is a recently identified death pathway that requires caspase-12 as an initiator caspase (Nakagawa *et al*, 2000). In response to ER-specific stress-inducing stimuli such as brefeldin A, tunicamycin and thapsigargan, caspase-12 is activated, whereas treatment with non-ER stressors such as staurosporine, Fas and TNF has no effect on caspase-12 cleavage (Nakagawa *et al*, 2000). In addition, caspase-12 deficient murine cells are resistant to apoptosis induced by ER stressors, further establishing a role for caspase-12 in this process (Morishima *et al*, 2002).

iii. Executioners of apoptosis: Caspases

As mentioned above, all three of the apoptotic signaling pathways converge on the final pathway that leads to the execution of the death signal. The activation of cysteine-dependent aspartate-directed proteases or caspases is believed to be the point of no-return in the cell death cascade. Caspase-8, -9, and -10 are the major initiator caspases. Their activation occurs through death-receptor ligation or mitochondrial release of apoptogenic proteins, respectively, and allows them to initiate the caspase cascade and act on downstream substrates. The major effector caspases include caspase-3, -6 and -7, which, once activated, are responsible for the cleavage of many cellular targets during apoptosis.

iv. Cisplatin-induced apoptosis

The action of cisplatin (*cis*-diammine-dichloro-platinum; CDDP) is associated with its ability to form DNA-protein and DNA-DNA inter- and intra-strand cross-links. However, increasing evidence strongly favours intra-strand adducts as the lesions responsible for its cytotoxic action (Pinto and Lippard, 1985) and that apoptosis may be the cellular underpinning of cisplatin-induced cell death (Li *et al*, 2000; Sasaki *et al*, 1999; Arts *et al*, 2000). Cisplatin enters the cell by passive diffusion, where its chloride ions are replaced with water to form the reactive, positively charged species that interacts with DNA strands to form the adducts, ultimately resulting in cell cycle arrest at G_1, S, and G_2-M and induction of apoptosis (Vaisman *et al*, 1997; Sorenson *et al*, 1988). Although the platinum analogues are not cell cycle specific, highly proliferating cells in late G_1 and S phases are most susceptible to the effects of cisplatin. There are several families of proteins that may be involved in the pathway that links cisplatin-induced DNA damage to apoptosis, including those involved in nuclear excision repair and mismatch repair (Gonzalez *et al*, 2001). Recent studies have shown that the nuclear excision repair pathway is responsible for correcting cisplatin-DNA adducts. The DNA damaging effects of cisplatin are also associated with the expression of specific death genes (Fas and Fas-L) and down-regulation of "survival" counterparts (Li *et al,* 2000; Sasaki *et al,* 1999). Increased activities of kinases, phosphorylation of p53 and MDM2, stabilization of p53 and the subsequent G_1 cell cycle arrest are all important cellular events in cisplatin-induced apoptosis (Sasaki *et al*, 1999). Understanding the molecular mechanisms by which cisplatin induces cell death will provide a fundamental approach for increasing the sensitivity of cells to this anti-cancer agent.

4. Proprotein Convertases

i. Discovery of proprotein convertases

In order to regulate biological activity, a wide variety of proteins are synthesized as inactive precursors that subsequently undergo conversion to their mature active forms. Proteolytic processing is a post-translational modification by which the cell can diversify and regulate the products of its genes. This process is crucial for the activation of many proteins as well as their cellular localization. In mammalian species, proteolytic processing of biological substrates is carried out by a group of highly specific proteolytic enzymes known as proprotein convertases (PCs). The search for these enzymes began in 1967 with the discovery that insulin was produced by cleavage from a larger precursor (pro-insulin) (Steiner *et al*, 1967; Chretien & Li, 1967) and since the discovery of the mammalian prototype enzyme Furin in 1990 (van de Ven *et al*, 1990), proprotein convertases have been the subject of vast research. During these years, many attempts were made to biochemically purify and characterize these endoproteolytic, bioactivating enzymes. Genetic complementation studies in yeast resulted in the isolation of the gene encoding kexin, a subtilisin-like serine protease that activates the secreted yeast α-mating factor and killer toxin proproteins by cleavage at dibasic sites (Julius *et al*, 1984). It was soon shown that kexin could also cleave proprotein precursors in mammalian cells (Thomas *et al*, 1988), suggesting that this was the long-sought after eukaryotic proprotein processing enzyme and that mammalian homologs must therefore exist. Furin was discovered to be the mammalian ortholog of kexin (van de Ven *et al*, 1990) and since then seven mammalian members of the PC family have been identified including Furin, PCI/PC3, PC2, PC4, PACE4, PC5/PC6 and PC7/LPC/PC8 (Seidah & Chretien, 1997).

Two additional members, SKI-1 and NARC that belong to different subtypes, have most recently been classified into this family (Seidah *et al,* 1999; Seidah *et al,* 2003).

ii. Structure of proprotein convertases

PCs are multi-domain, calcium-dependent serine proteases and members of the family share a common domain structure consisting of a signal peptide followed by pro-, catalytic-, middle- or P- and carboxyl-terminal domains (Figure 2). Sequence homology is highest among the catalytic domains while there is distinct variability at the C-terminal domains of each family member. The N-terminal signal peptide is required for entry of the protein into the secretory pathway, and is cleaved following the translocation of the nascent peptide through the membrane of the endoplasmic reticulum (Bergeron *et al*, 2000). Like many other proteins, PCs are synthesized as inactive zymogens and require proteolytic processing for maturation and activation (Seidah *et al*, 1994). The pro-segment domain serves as an intramolecular chaperone as well as an endogenous inhibitor. Primary cleavage of the pro-segment domain occurs autocatalytically and is important for sorting the enzymes out of the ER (Creemers *et al*, 1995). Once cleaved the pro-segment remains bound to the active site of the catalytic domain where it is released through a secondary cleavage only once the enzyme has reached its appropriate intracellular target and pH and Ca^{2+} levels are optimal (Anderson *et al*, 1997). Thus enzyme activation is spatially and temporally regulated. This model of activation has been demonstrated for PC1 (Rouille *et al*, 1995), PC4 (Seidah *et al*, 1998), PC5 (De Bie *et al*, 1996), PC7 (Munzer *et al*, 1997) as well as Furin (Anderson *et al*, 1997).

Figure 2: **Structure of PC family members**

Structural comparison of members of the mammalian PCs and their related yeast kexin and bacterial subtilisin proteases. Protein is comprised of a signal peptide, as well as pro-, catalytic, P- and C'-terminal domains. Note that only Furin and PC7 contain transmembrane domains in their C-terminal regions. Downward arrow (↓) indicates the site of cleavage after the proform that yields the mature bioactive protein.

(Adapted from Bergeron et al, 2000)

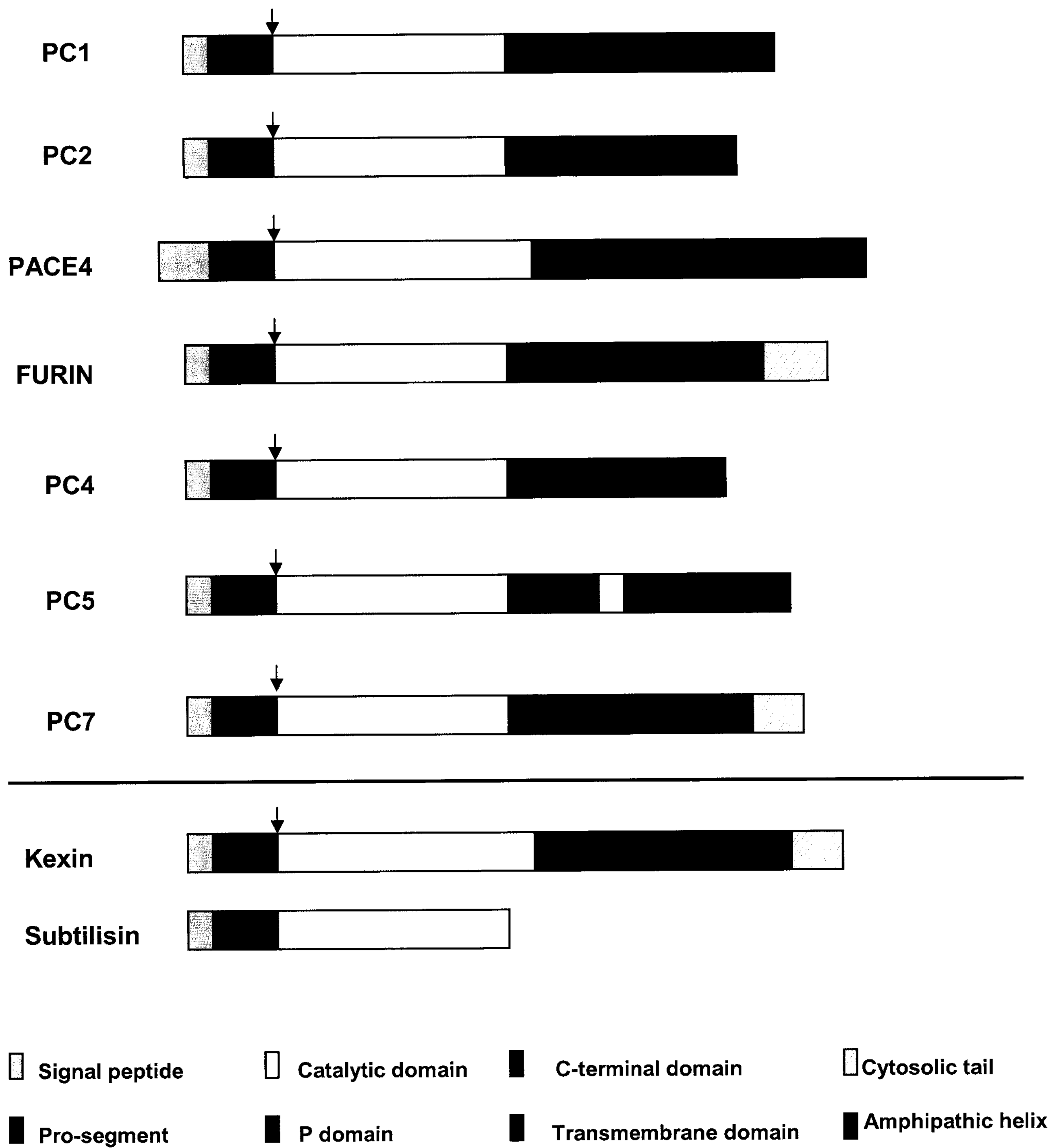


Figure 2

The catalytic domain contains the active site of the enzyme, with the typical catalytic triad of subtilisin-related serine proteases, amino acids D, H and S (Bergeron *et al*, 2000). Sites required for substrate recognition were identified by mutational analysis, confirming that negatively charged residues of the catalytic pocket interact with positively charged residues of the substrate (Roebroek *et al*, 1994). The P- or middle domain, located C-terminally to the catalytic region, is essential for the correct folding and stability of the enzyme and contains a highly conserved RGD motif. The highly variable C-terminal domains of each PC family member contain one or more Cys-rich regions, transmembrane and cytosolic domains as well as amphipathic helices (Bergeron *et al*, 2000).

iii. Function of PCs

There is high homology among the catalytic domains of this family of enzymes suggesting that they share a common evolutionary path. It is suggested that they arose from a single ancestral gene and evolved through duplications, translocations, insertions or deletions (Seidah *et al*, 1999). Except for Furin and PACE4 which are closely linked, all of the other PC genes are dispersed on various chromosomes. The multiplicity of PC genes suggests both redundancy and differentiation of functions among their products (Seidah & Chretien, 1999). Redundancy serves the survival of a biological system while differentiation affords complexity to it. Additionally some PC members exhibit multiple protein isoforms that are produced through alternative splicing of mRNA. However, the ongoing debate over the past several decades has been to delimit the degree of overlap and distinctiveness between the members of this family. The generation of biologically

active proteins requires two major components, the polypeptide precursor substrate and the proteolytic enzyme(s) responsible for the conversion of the precursor. Although many PCs could cleave the same precursor *in vitro,* it is the regulation of the level of their individual cellular expression and activities as well as their intraorganellar localization that is critical for each *in vivo* processing reaction.

Understanding the physiological functions of PC family members has been achieved by the use of knock-out or spontaneous mutations for specific PC members. Studies in mice revealed that PC1 knockouts are embryonic lethal, while the heterozygotes are viable, however reports of a double mutant PC1 human patient suggests that there may be key differences between mice and humans. Inactivation of PC2 in mice, revealed a small decrease in growth with an otherwise normal phenotype. Upon further observations decreased processing of glucagon, insulin and somatostatin in pancreatic islet cells were seen (Furuta *et al*, 1997). Mild phenotypic changes suggest that there may be some compensatory mechanism at work, and since PC1 is also co-expressed in many endocrine and neural cells it likely exerts the back up role (Seidah & Chretien, 1999). Furin is expressed very early in development, suggesting a critical involvement in embryogenesis since inactivation of the gene results in dimorphic embryos that die between e10.5 and e11.5 (Roebroek *et al*, 1998). PACE4 is also expressed in early development, playing a role in anterior patterning and PACE4 knockout mice display a similar phenotype to the furin knockouts with ~25% of the homozygote embryos dying between e13.5-15.5 (Constam & Robertson, 2000). Functional inactivation of PC5/6 also results in embryonic lethality, suggesting its importance in early development (Essalmani

et al, 2006). The availability of PC-null mice enriches the understanding of their biological functions and provides useful models for human pathologies, ultimately leading to identification of their physiological substrates.

iv. Tissue distribution

Proprotein convertases cleave their substrates at paired or single basic residues with the general motif (H/K/R)-X_n-(K/R)↓ or (H/K/R)-X_n-(K/R)-R↓ where n= 0, 2, 4, or 6 and X is usually not a cysteine (Hosaka *et al*, 1991; Seidah *et al*, 1994; Khatib *et al*, 2002). This recognition site is conserved in many protein precursors and is targeted by most of the PC family members. Studies demonstrate that PCs exhibit a remarkable temporal and spatial specificity of expression patterns, making them available in various combinations and proportions in different loci. Based on tissue distribution and intracellular localization, the mammalian PCs can be classified into several groups. Furin, PC7, PC5-B and SKI-1 are the only members of the PC family with a transmembrane domain and cycle between the trans-Golgi network and the cell surface. Therefore they are capable of processing proproteins secreted in both the biosynthetic (ER and Golgi apparatus; for extracellular secretion) and endocytic (ER; intracellular proteins) pathways (Molloy *et al*, 1999). PC1 and PC2 are primarily expressed in neuro-endocrine cells where they are localized in dense core secretory granules and process proteins secreted by the secretory pathway. PC5 and PACE4 are expressed in both endocrine and non-endocrine cells and process precursors in both the constitutive and regulated secretory pathways (Seidah & Chretien, 1999). Except for PC4 which has been shown to be

expressed only in reproductive tissues, the enzymes exhibit a wide tissue distribution and although there may be some overlap, each enzyme has its own unique expression pattern.

v. PCs and their substrates

Proprotein convertases have a wide variety of substrates involved in numerous cellular processes and various pathological conditions. The PCs are usually activating proteases and have not been reported to inactivate polypeptides (Khatib *et al*, 2002). Predicted substrates, based on the presence of a dibasic consensus sequence, include the precursors of neuropeptides (enkephalin, dynorphin), peptide hormones (insulin, somatostatin), growth and differentiation factors (BMP/TGF-β superfamily), receptors, enzymes (PCs, matrix metalloproteinases), adhesion molecules, blood coagulation factors, plasma proteins, viral coat proteins, and bacterial toxins. After proteolysis by the convertases, the mature peptides/proteins are usually subject to several other post-translational modifications necessary to achieve full bioactivity with the most common being the removal of carboxy-terminal basic residues by carboxypeptidase E or D (CPE, CPD) (Reznik *et al*, 2001).

vi. PCs and cancer

One of the hallmarks of malignancy is the presence of altered autocrine or paracrine regulatory mechanisms controlling cell growth, proliferation and cell survival (Khatib et al, 2005). The involvement of proprotein convertases in tumourigenesis and cancer has been extensively studied by several researchers and multiple approaches support the hypothesis that these enzymes have a role in onset and progression of the disease. Increased PC expression has been associated with enhancement of metastatic

spread and tumour cell proliferation. Early studies revealed high Furin expression in advanced lung tumours (Mbikay *et al*, 1997), and this association has also been confirmed in other cancers such as breast (Cheng *et al*, 1997), and head and neck carcinomas (Bassi *et al*, 2001). In addition, PACE4 expression was also significantly higher in breast and head and neck cancers, while its expression in squamous cell carcinomas was implicated with tumour progression and invasiveness (Hubbard *et al*, 1997). Another study reported a significant association between increased expression of PC1 and PC2 in neuroendocrine tumours, suggesting their involvement in the malignancy of tumours from a neural and/or endocrine origin (Jin *et al*, 1999).

It has been reported that proprotein convertases process a number of protein precursors involved in the genesis and progression of cancer. These include various growth factors (i.e. IGF-1, IGF-2, EGF, TGF-β1, VEGF and PDGF) and their receptors, matrix metalloproteinases involved in the degradation of the extracellular matrix, and cell adhesion molecules such as integrins (Table 1). Once active, these substrate proteins are crucial in the processes of cellular transformation, acquisition of the tumourigenic phenotype and metastases formation (Khatib *et al*, 2002). Due to their role in the activation of critical proteins implicated in neoplasia, PCs may be novel therapeutic targets for treatment of various cancers.

Table 1: Sequence of cleavages sites of proprotein convertase substrates linked to tumourigenesis and cancer

	Precursor Protein		Sites of processing	NCBI accession
Growth factors				
	TGF-β1		SSRHRR↓AL	XP_008912
	Insulin	1-	TPKTRR↓EA	
		2-	GSLQKR↓GI	XP028180
	IGF-1		PAKSAR↓SV	P01343
	IGF-2		PAKSER↓DV	XP028189
	EGF	1-	HHYSVR↓NS	
		2-	KWWELR↓HA	P01133
	PDGF-A		PIRRKR↓SI	NP002598
	PDGF-B		LARGRR↓SL	NP148937
	PDGF-C		FGRKSR↓VV	NP057289
	PDGF-D		HDRKSK↓VD	AAK56136
	VEGF-C		HSIIRR↓SL	P49767
	VEGF-D		YSIIRR↓RI	NP_004460
	HGF		KTKQLR↓VV	XP052260
	BDNF		SMRVRR↓HS	XP006027
	β-NGF		THRSKR↓SS	XP002122
	APRIL		RSRKRR↓HS	O75888
Growth factor receptors				
	Insulin receptor		PSRKRR↓SL	XP048347
	IGF-1 receptor		PERKRR↓DV	IGHUR1
	HGF receptor		EKRKKR↓ST	P08581
Integrins				
	Integrin α3		PQRRRR↓QL	XP008432
	Integrin α4		HVISKR↓ST	XP039011
	Integrin α5		HHQQKR↓EA	AAH08786
	Integrin α6		NSRKKR↓EI	NP000201
	Integrin α7		RDRRRR↓EL	Q13683
	Integrin α8		HLVRKR↓DV	AAA93514
	Integrin Av		HLITKR↓DL	XP002379
Matrix metalloproteinases				
	STR-3	1-	SLRPPR↓CG	
		2-	RNRQKR↓FV	P24347
	MT-1 MMP	1-	AMRRPR↓CG	
		2-	NVRRKR↓YA	P50281
	MT-2 MMP	1-	WMKRPR↓CG	
		2-	RRRRKR↓YA	P51511
	MMP-2		TMRKPR↓CG	P08253
	MMP-9		AMRTPR↓CG	XP006273
	MMP-13		VMKKPR↓CG	XP040746
	ADAM 1		PPRSRK↓PD	AAA74920
	ADAM 9		LLRRRR↓AV	NP003807

(This table was adapted from Khatib *et al*, 2002)

PCs control tumour cell proliferation by activating growth factors and their receptors. The availability of growth factors is critical for malignant transformation and metastasis and may ultimately result in a transformed phenotype. These molecules mediate cell entry and progression through the cell cycle, implicating their importance in growth and proliferation. Many of these proteins are synthesized as precursors and are processed and activated by PC-like enzymes. Transforming growth factor β (TGF-β) is proteolytically activated by Furin and represents an important regulatory mechanism for cell growth and proliferation. Similarly IGF-I mediated cell growth and proliferation requires PC processing of the IGF-I receptor (Khatib *et al*, 2001). Hwang and colleagues also demonstrated that cleavage of the insulin receptor by PCs is essential for the signal transduction of insulin (2000). Enhanced expression of both PCs and their substrates, as in the case of some cancers, would thus increase tumour cell proliferation and cell growth.

Metastatic spread and tumour cell invasion requires remodeling of the extracellular matrix (ECM), a reduction in cell adhesion and an increase in cell motility and PCs are involved in all of these processes. ECM degradation is a complex process involving a cascade of proteolytic events in which the primary step is likely mediated by PCs (Khatib *et al*, 2002). PCs activate several metalloproteinases through proteolytic cleavage such as MMPs, MT-MMPs, and ADAMs which are involved in the tightly controlled process of ECM degradation (Taylor *et al*, 2003). Furthermore, PCs modulate the cell adhesion and signaling capacity of integrins, transmembrane receptors that bind a variety of cell surface ligands and components of the ECM. This binding results in the

transfer of signal-to-cytoplasmic components that control cell migration, shape, growth and survival (Giancotti & Mainiero, 1994). A study by Bassi and colleagues demonstrated that overexpression of Furin caused a significant increase in the invasive potential of head and neck human tumour cell lines of low and moderate aggressive potential *in vitro* and *in vivo* (Bassi et al, 2001a). Furthermore, overexpression of PACE4 in non-tumorigenic skin keratinocytes resulted in their malignant conversion, increased invasive potential and significantly increased the processing of stromelysin-3, MT2-MMP, MMP-2 and MMP-9 (Mahloogi *et al*, 2002).

The potential clinical and pharmalogical role of PCs has fostered the development of peptide and protein-based inhibitors which have significantly highlighted the role for these proteolytic enzymes in the regulation of cancer cell behaviour. The anti-tumour effect of PC inhibition was first demonstrated by Khatib and colleagues. Treatment with the general PC inhibitor, α1-PDX, in colon carcinoma cells significantly delayed the appearance, indicidence and vascularization of palpable tumours in nude mice (Khatib et al, 2001). The effect of PC inhibition on *in vivo* invasion of tumour cells was further confirmed in a report by Bassi and colleagues where treatment of head and neck squamous carcinoma cells with α1-PDX lead to a 70-80% reduction in the ability of the tumour cells to invade tracheal wall xenotransplants which was shown to be associated with decreased processing and activity of the matrix metalloproteinase MMP-2 (Bassi et al, 2001b).

vii. PCs and cell survival

The role of PCs in cell survival is only beginning to unfold and can be attributed to their role in the maturation of various proteins that are known to be anti-apoptotic mediators participating in various autocrine/paracrine mechanisms. The most significant group of proteins implicated in this role is growth factors and their corresponding receptors including insulin-like growth factor (IGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) (Khatib *et al*, 2002). An important study validating the ability of proprotein convertases to determine cell fate was carried out by Khatib and colleagues, whereby expression of the general PC inhibitor α1-PDX in HT-29 colon carcinoma cells and Jurkat leukemia cells exaggerated serum deprivation-induced apoptosis (Khatib *et al*, 2001). This is key evidence that strongly suggests a pro-survival role for PCs. The protective effects of many proteins or growth factors processed by PCs are dependent on their ability to induce a cascade of events leading to the phosphorylation of downstream effector pathways, including FAK, PI-3K and IRS-1. Following phosphorylation, these molecules mediate their anti-apoptotic effects through activation of several negative death regulators such as Bcl-2 or inhibition of IL-converting enzyme like caspases (Jarpe *et al*, 1998). Our lab has previously demonstrated that the PI-3K/AKT pathway represents an important determinant of chemoresistance in ovarian cancer cells (Cheng *et al*, 2002; Fraser *et al*, 2004). Evidence of increased PC expression in various cancers as well as their involvement in cell survival through actions on this pathway has led to the hypothesis that proprotein convertases may be mediators of cell survival in human epithelium ovarian cancer cells.

viii. PC4: a unique PC family member

While extensive research has been done on other PC family members, fewer studies have focused on PC4. The enzyme was originally discovered in 1992, and was included in the PC family solely based on its cDNA sequence (Seidah *et al*, 1992; Nakayama *et al*, 1992). Five mRNA transcripts were identified in the rat, differing in their C- or N-terminal truncations (Seidah *et al*, 1992), while multiple isoforms were also seen in the mouse and it anticipated that multiple forms also exist in humans. It has been demonstrated that these isoforms arise from alternative splicing of a single PC4 gene (Mbikay *et al*, 1994). The gene contains 15 exons and extends over 9.5 kb. Putative transcriptional and regulatory elements in the mouse sequence include three CCAAT boxes (putative CTF binding sites), 11 GGGCGG motifs (putative Sp1 binding sites), two imperfect cAMP-responsive elements (CRE) and a glucocorticoid-responsive element (GRE) (Mbikay *et al*, 1994) however beyond sequence analysis the transcriptional regulation of PC4 is not clearly understood. Like other PC family members, PC4 consists of a signal peptide, a prodomain, a catalytic domain, a P-domain and a carboxy-terminal domain (Figure 3). Its locus (*Pcsk4*) has been mapped to chromosome 10 in the mouse and 19 in the human (Mbikay *et al*, 1995).

Unlike other members of the PC family which are expressed in a wide variety of tissues and cells, PC4 expression is highly restricted. A large number of tissues have been screened for PC4 expression including 1) the central nervous system: hippocampus, hypothalamus, olfactory bulb, cerebral cortex, brainstem and spinal cord 2) pituitary 3) peripheral tissues: heart, adrenal gland, small intestine, colon, trachea, esophagus, lung,

thyroid gland, kidney, bladder, spleen, liver, pancreas and muscle (Seidah et al, 1992). However, to date, abundant levels of PC4 mRNA have only been detected in testicular germ cells, specifically spermatocytes and round spermatids and, to a lesser extent in hormonally stimulated ovaries (Seidah *et al*, 1992; Tadros *et al*, 2001). The subfertility phenotype of female *Psck4* -/- mice suggests that PC4 is also expressed in female reproductive tissues. Most recently PC4 mRNA and protein were detected in trophoblast cells of the human placenta throughout different gestational ages (Qiu *et al*, 2005). The tissue distribution of PC4 would thus suggest a reproductive role for this particular PC family member.

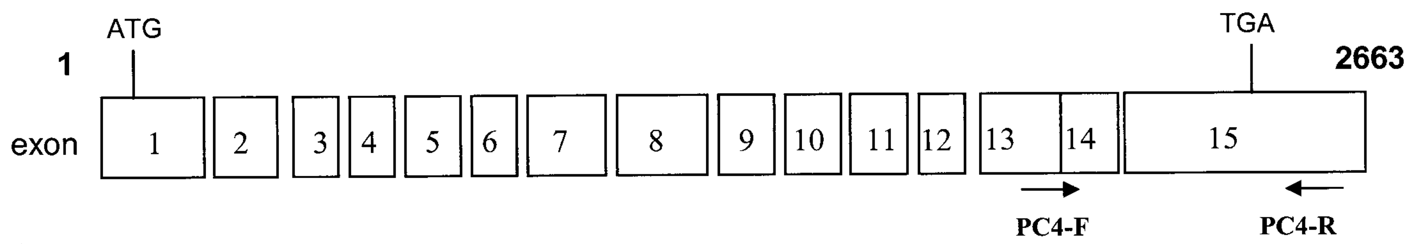
To elucidate the physiological role of PC4, its locus was disrupted with a LacZ insert through homologous recombination in embryonic stem cells and mice carrying the mutation were generated (Mbikay *et al*, 1997). The study revealed that PC4 null mice had compromised fertility. Consistent with the observed pattern of PC4 expression, male knock-out mice were infertile. Sperm from male mice were less able to fertilize eggs *in vitro*, and the eggs that were fertilized were not viable as they failed to develop to the blastocyst stage (Mbikay *et al*, 1997). In addition, female knockout mice had delayed folliculogenesis, which may also contribute to the reduced fertility of these animals. From these observations, the quest for PC4 substrates began. Co-expression of PC4 with several potential substrates, such as pro-enkephalins, pro-opiomelanocortin, 7B2, pro-renin, amyloid precursor protein, and pro-von Willebrand factor by several research groups has so far yielded negative results (De Strooper *et al*, 1995). Studies by Akimura and colleagues demonstrated that PC4 processes a key substrate that may explain the phenotype observed in the PC4 null-mice.

Figure 3: **PC4 structure**

Schematic diagram of mRNA and protein structure for human PC4. In the mRNA sequence, start (ATG) and stop (TGA) codons are indicated as well as primers used for PCR amplification. In the protein structure, S stands for signal peptide, PRO for prosegment and C' for C-terminal domain. Cleavage of the prosegment is required for enzymatic activation and is indicated by RVKR.

(Adapted from Mbikay et al, 1994)

mRNA



Protein

Figure 3

Pituitary adenylate cyclase activating polypeptide is expressed in many tissues with the highest levels expressed in the brain and testis (Arimura *et al*, 1991). High levels of PACAP are present in the cap and acrosome phases of spermatids but not in mature spermatozoa, Leydig or Sertoli cells, implying its involvement in fertility (Shioda *et al*, 1994). Furthermore its expression is consistent with that of PC4 in testicular germ cells. Mature PACAP consists of two peptides which are derived from proteolytic cleavage of a proform. Co-expression analysis revealed that the PACAP precursor was only processed when cells were co-transfected with PC1, PC2 or PC4 (Li *et al*, 1998a). PC1 and PC2 are expressed at high levels in the brain while they are not expressed in the gonads, suggesting that PC4 is the endoprotease responsible for the processing of PACAP in these tissues. Consistent with this finding, testis and ovaries from PC4 null mice do not contain the processed forms of PACAP, PACAP-38 and PACAP-27 (Li *et al*, 1998b).

Based on the localization in the testis and studies with recombinant enzymes, it has been postulated that pro-insulin-like growth factors (pro-IGFs) -I and –II are also substrates of PC4. Co-transfection studies demonstrated that pro-IGF-II could be processed at Arg-104 by Furin, PACE4, PC5, and PC7 however none of these PC family members could yield mature IGF-II(1-67) (Duguay *et al.*, 1998). Using synthetic peptides and intramolecularly quenched substrates Basak *et al* (1999, 2004) demonstrated that PC4 cleaves pro-IGF-1 and pro-IGF-II more efficiently than other PCs to generate mature forms of IGF-I and II, respectively. Furthermore, recent studies from our laboratory have demonstrated that PC4 is also expressed in the human placenta where it is involved in the proteolytic maturation of IGF-II, cleaving the precursor at Arg 104 to yield big IGF-II(1-

104) and subsequently at Arg 68 leading to activation of its biological function and increased ability to induce AKT phosphorylation in human trophoblast cells (Qiu *et al*, 2005).

5. Growth factors: Insulin-like Growth Factor Family

The insulin-like growth factor (IGF) system is comprised of the ubiquitously expressed IGF ligands (IGF-I and IGF-II), cell surface receptors that mediate the biological effects of IGFs, including the IGF-I receptor (IGF-IR), the IGF-II receptor (IGF-IIR) and the insulin receptor, as well as a family of binding proteins. The bioavailability and half lives of the IGF peptides in the circulation and extracellular fluid are regulated through association with the IGF binding proteins. This signaling system is essential for normal embryonic and postnatal growth, and plays an important role in the function of a healthy immune system, lymphopoiesis, myogenesis and bone growth among other physiological functions (Denley *et al*, 2005). The IGF system has also been implicated in various pathophysiological conditions and is thought to play a prominent role in tumourigenesis (LeRoith *et al*, 2003).

Like other PC substrates, IGF-I and IGF-II are synthesized as proproteins and undergo proteolytic cleavage to become active (Daughaday *et al*, 1989). In rodents IGF-II expression is high during embryonic development and diminishes after birth while IGF-I expression is low during the prenatal period and increases significantly during puberty and adulthood, with the liver being the major contributor to IGF-I in the circulation. In contrast, in humans both IGF-I and IGF-II are expressed in multiple tissues throughout

life. It is worth noting that the circulating levels of IGF-II are several-fold higher than that of IGF-I, which is consistent with the concept that these ligands have potentially divergent roles in human physiology.

The IGF ligands exert their biological effects through interactions with a variety of cell surface receptors present on target cells. Both IGF-I and IGF-II interact with the IGF-IR, a transmembrane tyrosine kinase receptor functionally related to the insulin receptor (Ulrich *et al*, 1986). Recently it has been shown that IGF-II can also interact with insulin receptor exon 11− (IR-A) to activate its intrinsic tyrosine kinase domain activities, leading to predominantly proliferative effects (Frasca *et al*, 1999). These activated receptors initiate signaling cascades that ultimately result in regulation of a number of biological responses. The type 2 IGF receptor (IGF-IIR) and a family of high affinity IGF binding proteins (IGFBPs) modulate the availability of IGF-I and IGF-II to bind the receptors. IGF-II binds to the type 2 receptor (IGF-IIR) which acts as an internalization and degradation pathway, clearing IGF-II from the circulation. However, this receptor has no signaling activity (LeRoith *et al*, 1995).

i. Role of IGF signaling in cell survival

The insulin-like growth factor system plays a pivotal role in processes controlling cell proliferation, survival under stress and maintenance of the transformed phenotype (LeRoith *et al*, 2003). Accumulating evidence has demonstrated that signaling through the IGF system also plays a critical role in cell survival and prevention of apoptosis. Activation of the IGF-IR is particularly important for the transduction of survival-

promoting signals. Levels of IGF-IR directly relate to the degree of apoptosis induction and levels of this receptor are generally lower in cells that are susceptible to developmental cell death than in resistant cells (Resnicoff *et al*, 1995). Interaction of the signaling ligand, IGF-I or IGF-II with the IGF-I receptor induces survival-promoting signaling cascades and increased phosphorylation of downstream protein pathways. Briefly, binding of the ligand to the IGF-IR induces a conformational change in the transmembrane β-subunit that fully activates the receptor tyrosine kinase through autophosphorylation of tyrosine residues. These residues can then act as docking sites for the adaptor proteins insulin-receptor substrate 1-4 (IRS 1-4) and Shc, ultimately leading to the activation of phosphatidylinositol-3 kinase (PI3K), the mitogen-activated protein kinase (MAPK) and 14-3-3 pathways (Baserga, 2000). The ultimate targets of these pathways include members of the Ets and forkhead transcription factor families (Figure 4). Thus IGF binding at the cell surface can elicit changes in gene expression that mediate the anti-apoptotic effects (LeRoith *et al*, 2003).

The best defined pathway for which IGF signaling via its type 1 receptor prevents apoptosis is mediated through PI3K/ AKT cell signaling. PI3K is activated by binding of the SH2 domain to IRS-1, ultimately leading to the phosphorylation of its downstream effector, AKT. Activated AKT has been shown to prevent apoptosis in a variety of cell types, namely through the phosphorylation and inactivation of pro-apoptotic proteins such as caspase-9 (Kermer *et al*, 2000), and Bad, an apoptogenic mitochondrial protein (Bai *et al*, 1999).

Figure 4: **IGF signaling pathways**

Control of cell survival by IGF signaling through the IGF-I receptor. Following activation of the receptor by ligand binding, several downstream signaling molecules are activated including those of the PI3K/AKT and MAPK pathways. These can modulate both effectors and inhibitors of apoptosis at several distinct points in the apoptotic cascade.

(Adapted from Vincent & Feldman, 2002)

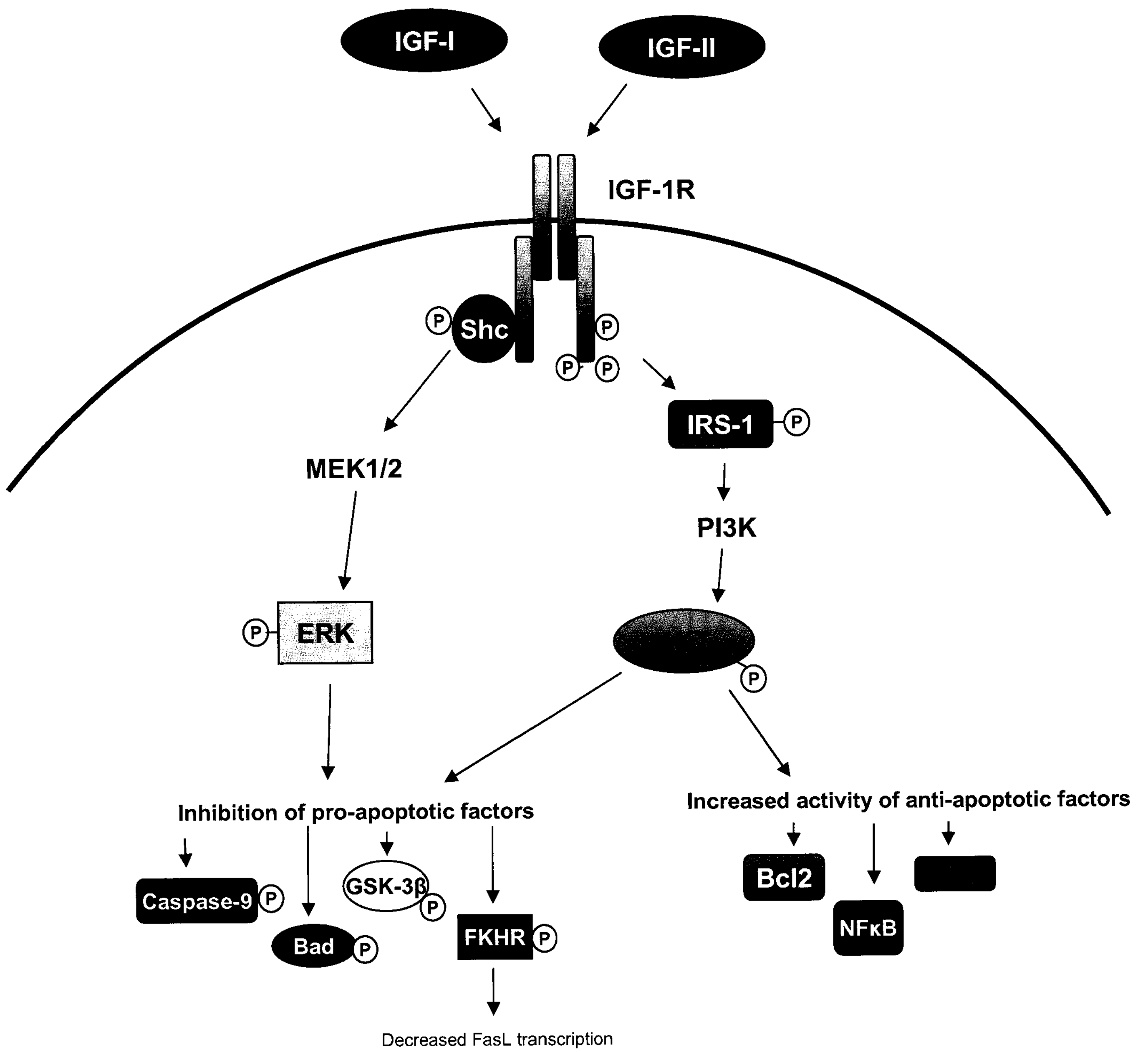


Figure 4

In addition, activated AKT either increases or sustains the levels of several anti-apoptotic proteins including Bcl-2 and BclX (Chrysis *et al*, 2001) as well as XIAP (Dan *et al*, 2004).An alternative pathway for transduction of survival signals from the IGF-IR is through the mitogen-activated protein kinase (MAPK) pathway which signals through the Shc proteins leading to Ras activation (Peruzzi *et al*, 1999). Peruzzi *et al*, proposed an additional pathway which depends on the integrity of a serine quartet at residues 1280-1283 of the human IGF-IR which binds isoforms of the 14-3-3 proteins, ultimately targeting Raf-1 to the mitochondria to inhibit apoptosis (Peruzzi *et al*, 1999). Interestingly all of these pathways lead to BAD phosphorylation, suggesting that the cell surface receptor has multiple pathways for which it can promote survival signals (Navarro & Baserga, 2001).

An additional mechanism by which IGF signaling may be involved in cell survival is through the regulation of calcium signaling. Studies have shown that the IGF-IR and other growth factor receptors can act as modulators of ion channels. Ligand binding to the IGF-IR leads to rapid activation of voltage-dependent calcium channels. Downstream effects of this calcium efflux include regulation of calcium-dependent transcription factors such as MEF2 and CREB which promote the expression of several anti-apoptotic proteins including Bcl-2 (Blair *et al*, 1997; 1999). Consistent with this are the findings of Linnerth and colleagues, whereby administration of IGF-II significantly induced CREB phosphorylation and cell survival in a human lung cancer cell line (Linnerth *et al*, 2005).

ii. IGF-II and ovarian cancer

Increased IGF-II expression has been associated with the development of several types of malignancies including those of the prostate (Tennant *et al*, 1996), colon (Tricoli *et al*, 1986) and breast (Osborne *et al*, 1989). Previous studies have implicated the IGF system in the etiology of human epithelial ovarian cancer, although its role in the progression of the disease remains unclear (Conover *et al*, 1998; Sayer *et al*, 2005). IGF-II gene expression has been shown to be elevated in ovarian cancers compared to normal ovarian tissue (Lancaster *et al*, 2004) while several ovarian cancer tumours and cell lines express IGF-I, IGF-II and the type-1 receptor (Yee *et al*, 1991; Conover *et al*, 1998). The involvement of the IGF system in ovarian cancer was demonstrated by the finding that primary ovarian epithelial cell lines expressed all major components of the IGF system and were able to functionally respond to exogenous IGFs (Conover *et al*, 1998). A recent study by Sayer and colleagues highlighted the importance of IGF-II in ovarian cancer, through examination of 109 epithelial ovarian cancers. It was demonstrated that total IGF-II expression was 300-fold higher in ovarian cancers compared to normal surface epithelium, and that IGF-II expression is associated with more aggressive ovarian cancer phenotypes, higher stage at diagnosis, and shorter overall survival (Sayer *et al*, 2005). Further elucidation of the role of the IGF system and IGF-II signaling pathway in ovarian cancer development and progression will increase our understanding of ovarian cancer biology and may yield novel opportunities for intervention and treatment.

6. Rationale for the proposed studies

Human ovarian epithelial cancer is the fifth most frequent cause of cancer death in women (Canadian Cancer Statistics, 2005). Poor prognosis and low survival rates are a consequence of a poor understanding of the early events leading to ovarian carcinogenesis, absence of specific symptoms and thus late diagnosis, and ultimately chemoresistance. The phenomenon of chemoresistance has been attributed to altered expression of genes involved in part in the regulation of apoptosis (Reed *et al*, 1996) and increasing evidence indicates that defects in intra- and extra-cellular apoptotic mechanisms are an important cause of resistance to cytotoxic agents such as cisplatin.

In the past decade, extensive research has been aimed at better understanding the function and role of proprotein convertases (PCs) in a variety of biological processes and disease states. Khatib and colleagues suggested an involvement of this family of proteolytic enzymes in cell survival through their ability to process protein precursors that act as anti-apoptotic mediators (Khatib *et al*, 2001). However, the precise role of PCs in the determination of cell fate has not been clearly established. Evidence of increased PC expression in various cancers as well as their putative involvement in cell survival has led to the proposed work which implicates proprotein convertases in the chemoresistance of human epithelial ovarian cancer cells. Little is known about the involvement of PCs in human ovarian cancer and, to our knowledge, the role of PCs in chemoresistance has not yet been investigated. The present studies represent novel work in this field and may reveal a new and interesting role for proprotein convertases in cancer cell physiology.

7. Hypothesis

Proprotein convertase 4 is an anti-apoptotic mediator and, through its processing of IGF-II which is involved in cell survival signaling, confers chemoresistance in human ovarian cancer cells.

8. Overall Objective and Specific Objectives

To examine the role and regulation of proprotein convertases in human ovarian epithelial cancer cells by cisplatin as well as their possible involvement in the regulation of chemosensitivity.

Specific Objectives:

1. To determine if proprotein convertases (PCs) are expressed in human ovarian cancer cell lines and if their expression is modulated by the chemotherapeutic drug cisplatin *in vitro;*

2. To determine whether proprotein convertases influence cisplatin sensitivity in ovarian cancer cells;

3. To elucidate the mechanisms by which these proteolytic enzymes may be involved in chemoresistance.

CHAPTER 2: MATERIALS AND METHODS

1. MATERIALS

Culture media (RPMI-1640, DMEM-F12), fetal bovine serum, penicillin-streptomycin, trypsin-EDTA, TEMED, M-MLV reverse transcriptase, Lipofectamine and Plus Reagents, Stealth RNAi for human PC4, anti-v5 mouse monoclonal IgG antibody and all primer sets used for RT-PCR were purchased from Invitrogen (Burlington, ON, Canada). Cisplatin, DMSO and Hoechst 33258 were obtained from Sigma (St. Louis, MO, USA). Goat anti-rabbit and goat anti-mouse IgG HRP conjugate, acrylamide (electrophoresis grade), *N, N'*-methylene bis-acrylamide, DC Protein Assay kit, sodium dodecyl sulphate (SDS), Tris, nitrocellulose membranes and SDS polyacrylamide gel electrophoresis (SDS-PAGE) prestained molecular weight standards (low range) were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). The pCI-neo mammalian expression vector was purchased from Promega (Madison, WI, USA). The anti-PC4 rabbit polyclonal antibody (PC4-606) was kindly provided by Dr. Majambu Mbikay (Diseases of Aging Program, Ottawa Health Research Institute). Primary antibody against PC2 (goat polyclonal IgG) and ImmunoCruz Staining System for rabbit primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PARP rabbit polyclonal IgG was purchased from Cell Signaling Technologies (Beverly, MA, USA) and anti-Furin polyclonal rabbit IgG from Zymed Laboratories (San Francisco, CA, USA). Mouse monoclonal anti-GAPDH antibody was purchased from Abcam (Cambridge, MA, USA). All Falcon culture wares were purchased from BD Biosciences (Mississauga, ON, Canada). Prestained wide range molecular weight protein standards and MassRuler™ DNA ladder were purchased from

MBI Fermentas (Newington, NH, USA). Enhanced chemiluminescence (ECL™) kit and Hyperfilm ECL™ high performance chemiluminescence film were purchased from Amersham Life Sciences (Oakville, ON, Canada). RNeasy Mini kit, Plasmid MiniPrep kit and HotStarTaq DNA polymerase were from QIAGEN (Mississauga, ON, Canada). The fluorogenic substrate Boc-RVRR-AMC was purchased from Bachem AG (Torrance, CA, USA). The PC4-specific inhibitors used in the present study were a kind gift from Dr. Ajoy Basak (Diseases of Aging Program, Ottawa Health Research Institute).

2. METHODS

Cell culture

The present research involves the use of 4 established human epithelial ovarian cancer cell lines. These include A2780s, A2780cp, OV2008 and C13*. Cisplatin-sensitive cell lines (A2780s and OV2008) were derived from ovarian serous cystadenocarcinomas of two separate patients without prior chemotherapy, and their respective cisplatin-resistant variants A2780cp and C13* respectively were established following *in vitro* cisplatin challenges (Hamilton *et al, 1985).* C13* and A2780cp cells carry wild-type p53 and mutant p53, respectively. Cell lines were kindly provided by Dr. R. Goel at the Ottawa Regional Cancer Center.

Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640; OV2008 and C13*) and Dulbecco's Modified Eagle Medium-F12 (DMEM-F12; A2780s and A2780cp) supplemented with 10% (vol/vol) fetal bovine serum, streptomycin (100 μg/ml), penicillin (100 U/ml), and fungizone (0.625 μg/ml) at 37°C under 5% CO_2 and 95% air. Prior to each experiment, cells were seeded on appropriate size multi-well plates

(12- or 6-well) or dishes (60 mm) in media with 10% fetal bovine serum for 12-18 h to allow for proper attachment before the initiation of treatment.

Transient transfection

A pCIneo vector for expression of rat PC4 was constructed by insertion of the full-length rat PC4 cDNA fragment with a V5 epitope at the carboxy terminal. The empty PCIneo vector was used as a negative control. At 60-80 % confluence, cells were transfected with 1μg of the expression vectors using Lipofectamine Plus Transfection reagents (Invitrogen, Burlington ON, Canada). At 24 h after transfection, cells were treated with cisplatin (2.5 and 5 μM) or DMSO (vehicle control) for an additional 24 h and then harvested for analysis.

PC4 small interfering RNA transfection

Stealth RNAi molecules, targeted to nucleotides 2239 to 2264 of the human PC4 mRNA coding sequence (Genbank Accession no. AY358963) were designed and supplied by Invitrogen Life Technologies (Burlington, ON, Canada). Scrambled sequences used as negative controls were purchased from Dharmacon RNA Technologies. C13* cells were seeded at 35% confluence in 6-well plates and transfected the following day with 100 nM of PC4 siRNA or scrambled sequence using Ribojuice siRNA transfection reagents (Novagen, San Diego, CA). At 24-48 h thereafter, cells were treated for 24 h with cisplatin (10 μM) or DMSO and harvested for analysis.

Site-directed Mutagenesis

Site-directed mutagenesis was carried out on the rat PC4 cDNA sequence (NM_133559) at the dibasic consensus site (RVK**R**↓; R110A) where the proform is autocatalytically cleaved *in vivo*. This site is conserved among rat, mouse and human PC4 cDNA sequences. The pCIneo rPC4(R110A) mutant was obtained by point mutation in wild-type pCIneo rPC4/v5 plasmid using QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Briefly, mutagenic primers were designed to convert the Arginine residue at position 110 of the rat PC4 sequence to an Alanine (F: 5' CTTTGAGGCGGCGGGTGAAGGCTTCCCTGGTGGTACCCACAG 3' and R: 5' CTGTGGGTACCACCAGGGAAGCCTTCACCCGCCGCCTCAAAG 3'). Wild-type rPC4 plasmid was used as the template and high fidelity *Pfu* DNA polymerase incorporated the mutagenic primers through extension during thermocycling (95°C-30s, 55°C-1min, 68°C-7min; 12 cycles). The resulting PCR product contained the mutated plasmid which was selected following *DpnI* digestion of the parental template. The *DpnI*-digested DNA was transformed into TOPO10 cells (Invitrogen) and cultured overnight on LB (+ ampicillin) agar plates. Colonies were picked the following day and grown overnight in LB broth with ampicillin. Plasmid DNA was extracted using the Qiagen MiniPrep kit as per the manufacturer's instructions. Successful mutation of the wild-type plasmid was confirmed through sequencing.

Chemosensitive OV2008 cells were transiently transfected with either the wild-type or mutant R110A rPC4 expression vectors (empty vector serving as a control) and subsequently treated with cisplatin (5 μM, 24 h).

Determination of Apoptosis

At the end of the culture period, cells attached to the growth surface were removed by trypsin treatment (Trypsin 0.05 % and 0.53 mM EDTA; 37°C for 3-5 min). Attached and detached cells were pooled, pelleted and resuspended in neutral-buffered formalin (10%) with Hoechst 33258 stain (12.5 ng/ml, Sigma) at 4°C. At 24 h thereafter, cells were spotted onto slides and assessed for typical apoptotic nuclear morphology (nuclear shrinkage, condensation, and fragmentation) under a fluorescent microscope with the appropriate filter combination. At least 200 cells/treatment group were counted and assessed under randomly selected fields. Slides were blinded to avoid experimental bias.

Semi-quantitative RT-PCR Analysis

i. RNA Extraction

Total RNA from cultured ovarian cancer cells was isolated using RNeasy Mini Kit (QIAGEN, Mississauga, ON), according to manufacturer's instructions. Briefly, cell pellets were lysed and homogenized using a buffer containing guanidine isothiocyanate and β-mercaptoethanol and spun through QIA shredder columns. Equal volume of 70% ethanol was added to the cell lysates to optimize binding of RNA to the silica-gel spin column. RNA remained bound to the spin column, while contaminants were removed in several washing steps. RNA was eluted in RNase-free water, and its concentration and purity were determined spectrophotometrically (absorbance at 260 nm). The relative purity was expressed as a ratio of the readings at 260 nm and 280 nm (1 unit of

absorbance = 40 μg/mL). Isolated RNA was treated with RNAase-free DNAase to eliminate any potential genomic DNA contamination.

ii. Reverse Transcription

Aliquots of total RNA were used for first strand cDNA synthesis. Two μg of total RNA were incubated with oligodT primers (0.2 μg, Ambion) and incubated at 70°C (5 min) to preferentially transcribe messenger RNA. A master mix containing 5x reaction buffer, 10 mM dNTPs, RNase inhibitor (20 IU) was added to the mixture, and incubated at 37°C (5 min). One μL of RevertAid Enzyme (H Minus M-MuLV RT; Fermentas) reverse transcriptase was added and reverse transcribed under the following conditions: 42°C (60 min) and 70°C (10 min).

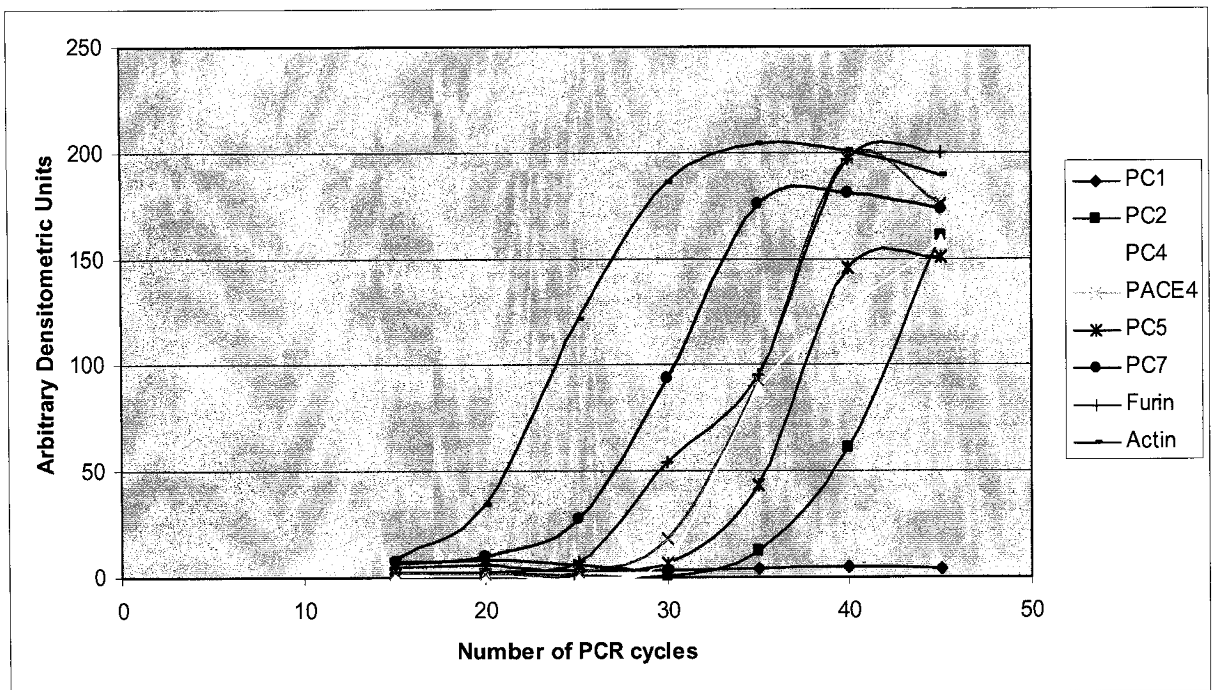
iii. Polymerase Chain Reaction (PCR)

The PCR amplification reaction was performed by mixing 0.2 units of HotStart Taq DNA polymerase (QIAGEN, Missassauga, ON), 2 μL 10X PCR buffer, 0.4 μL dNTPs [10 mM each of dATP, dCTP, dGTP, and dUTP], 1 μL of cDNA from the RT reaction and 1μM each forward and reverse primers in a final volume of 20 μL. Primers for PC1A, PC1B, PC2, PACE4, PC5, PC7 and Furin were chosen from unique sequences of human cDNA sequences (Cheng *et al*, 1997) and all primers were designed so that the forward and reverse primers were separated by at least one intron so that any genomic contamination could be detected if present. Primer pairs for cDNA amplification (in the 5'-3' direction), annealing temperatures, and expected product sizes are indicated in Table 2. Amplification conditions were optimized and the linear range of the PCR

reaction for each target gene was determined empirically prior to evaluation of experimental samples (Figure 5). HotStarTaq^{TM} DNA polymerase was activated at 95°C for 15 min, followed by an amplification cycle of 94°C for 1 min, 54-59°C (depending on primer annealing temperature) for 45 s, and 72°C for 90 s, which was repeated 20-40 times (depending on the gene sequence being amplified). A final extension cycle at 72°C for 10 min was carried out for each reaction. As a means to correct for quantity of cDNA used in each PCR reaction, the cytosolic protein β-actin was also amplified for each treatment group. PCR products [8μL, mixed with 6X loading buffer (Fermentas)] were resolved on 1.5% agarose gels containing ethidium bromide (100 V, 45 min), visualized under UV light and images were digitally captured (Molecular Analyst, BioRad). Quantification of relative mRNA levels was determined using densitometric analysis of PCR bands (Scion Image, Scion Inc).

Figure 5: **Optimization of PCR conditions**

Optimization of reaction conditions for PC primer sets. PCR saturation curves for each PC primer set and the housekeeping gene β-actin. The size of expected amplification product (bp) is indicated by arrows. Right-hand column indicates optimized annealing temperature and number of cycles for each primer set.



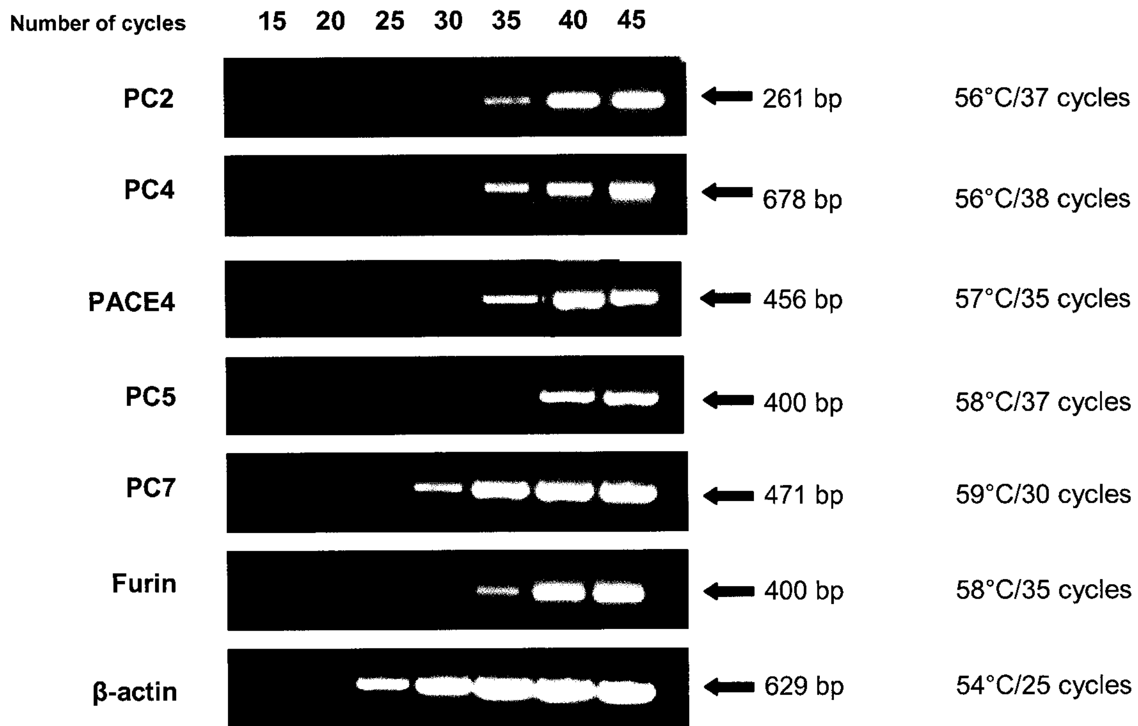


Figure 5

Table 2: Primers and conditions for PCR amplifications

Gene	Annealing Temp (°C)	No. of cycles	Expected Product size	Primer pair sequences
PC1	54	40	495 bp	F: TCT TTT GCG CTT GGT GTG R: TGG CAT AAA TGT CCG TGT G
PC2	56	40	261 bp	F: AAA GAA GGA TGA AGG GTG GTT R: GTG TAG GCT GCG TCT TCT CTT G
PC4	55	40	678 bp	F: TTC ATG TCC ACC CAC TTC TG R: TCG CTT TCT GAG CTG ACA AC
PACE4	57	35	456 bp	F: CTA TGG ATT TGG TTT GGT GGA C R: AGG CTC CAT TCT TTC AAC TTC C
PC5	58	35	510 bp	F: TGC GCT CCA TCT ACA AAG R: CAT TGC AGT GGT CTG GTC
PC7	59	35	471 bp	F: ATC ATT GTC TTC ACA GCC R: AAG CCT GTA GGT CCC TC
Furin	59	35	399 bp	F: TAT GGC TAC GGG CTT TTG G R: TTC GCT GGT GTT TTC AAT CTC T
β-actin	54	25	629 bp	F: GGA CTT CGA GCA AGA GAT GG R: CAC CTT CAC CGT TCC AGT TT
7B2	56	40	393 bp	F: GGT ACC CAG ACC CTC CAA AT R: TGT CAT TCA CAG GGA CTC CA
IGF-II	56	35	288 bp	F: CGT TGA GGA GTG CTG TTT CC R: GTC TTG GGT GGG TAG AGC AA
IGF-I	58	35	250 bp	F: TGG ATG CTC TTC AGT TCG TG R: CCT GCA CTC CCT CTA CTT GC
IGF-IR	58	35	575 bp	F: AAC CCC AAG ACT GAG GTG TG R: CGC TGA TCC TCA ACT TGT GA
IGF-IIR /M6PR	56	35	265 bp	F: CTC CGA TAT TCG GAT GGA GA R: CAG GTC ATA GCG CTT CTT CC
Insulin R	58	35	A: 600bp B: 636bp	F: AAC CAG AGT GCG TCT GAG GAT R: CCG TTC CAG AGC GAA GTG CTT

Immunohistochemistry and immunocytochemistry

The presence of PC4 protein was examined by immunohistochemistry in human ovarian cancer cell lines and human ovarian tumour specimens, using the polyclonal PC4 606 antibody (courtesy of Dr. M. Mbikay, OHRI). To validate the specificity of the PC4 polyclonal antibody, deparaffinized and rehydrated sections of ovarian tissue from PC4 wild-type and knockout mice were incubated with the PC4 antibody (1:50; overnight, 4°C) followed by incubation with a fluorescent conjugated secondary antibody (Alexa Fluor 594 1:100; Molecular Probes, 1 h, RT) and counterstained with Hoechst 33258. PC4 protein was visualized under fluorescent microscopy with the appropriate filter combinations. Positive immunofluorescent staining was observed in the corpus lutea and interstitial cells of the ovary in the PC4 WT ovaries but not in those of the knockouts (Figure 6). No positive signals were observed in the follicular cells or oocytes. These observations are consistent with previous findings from Tadros *et al* (2001) in which a strong immunoreaction for β-gal (used to represent PC4 expression since a LacZ insert in the *Psck4* gene is under the control of the PC4 promoter to generate knock out mice) was detected in ovarian theca, interstitium and corpora lutea of heterozygote (+/-) mice but not +/+ mice. Primary antibodies were substituted with normal rabbit serum as a negative control. Adjacent sections were stained using hematoxylin and eosin to demonstrate ovarian morphology.

i. ***Detection of PC4 protein in cultured ovarian cancer cells***

Cells were plated on 8-well chamber slides (Becton Dickinson Canada, Mississauga, ON), incubated overnight in RPMI supplemented with 10% serum, and fixed with 10% phosphate buffered formalin (30 min, RT). Cells were rinsed with PBS

Figure 6: **Validation of PC4 606 antibody in WT and KO mouse ovaries**

Immunohistochemistry of ovarian sections from wild-type (PC4 +/+; a-d, g) and PC4 knockout (PC4 -/-; e-f, h) mice confirmed the specificity of the PC4-606 antibody. Strong positive PC4 signal was detected in the interstitial cells of wild-type animals (b; 200x, d; 400x). Fluorescent signal was undetectable in the knockout ovaries (f; 200x). Hematoxylin and eosin (H&E) stained adjacent sections were used to indicate ovarian morphology. Negative controls where the primary antibody was omitted were performed for all sections (g, h).

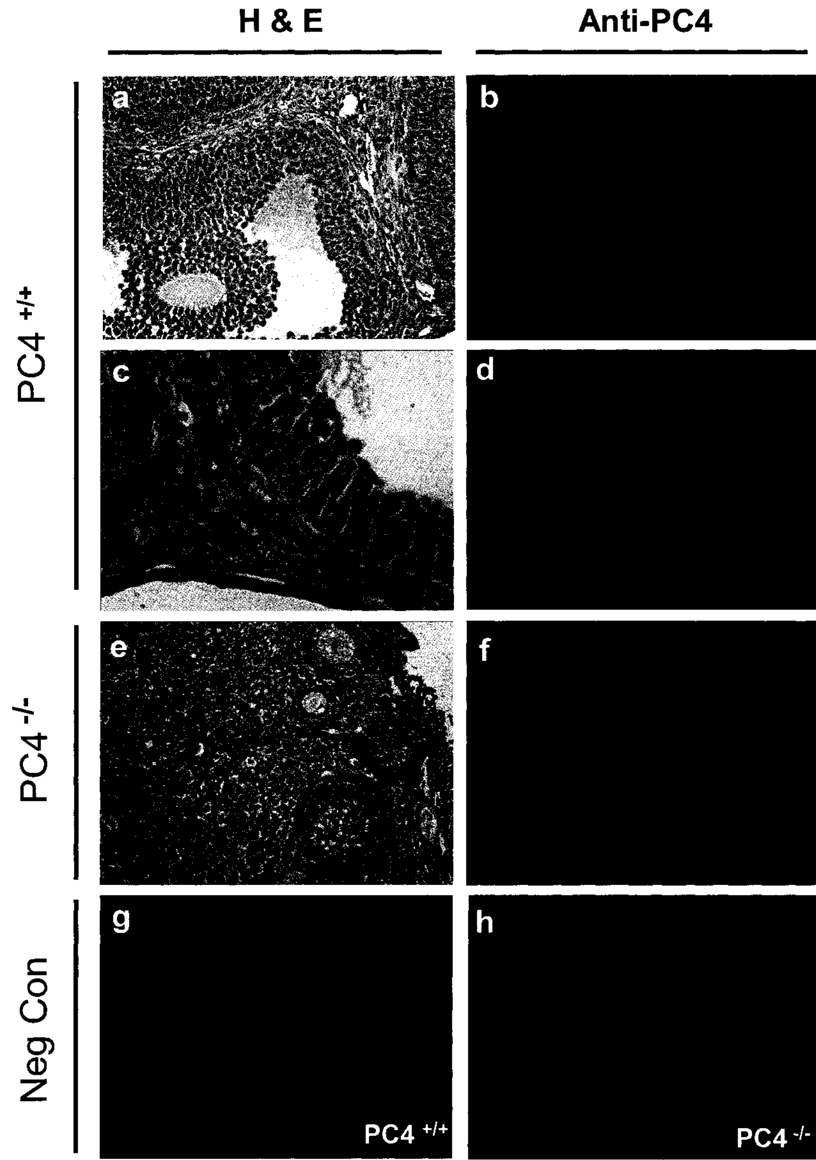


Figure 6

and endogenous peroxidase activity was blocked using 3% H_2O_2 (10 min, RT). Cells were permeabilized with 0.1% Triton X-100 in PBS (10 min, 4°C) and subsequently rinsed with PBS and incubated for 1hr with goat serum block buffer (ImmunoCruz Staining System, Santa Cruz Biotechnology, California, USA). Cells were then incubated overnight at 4°C with the anti-PC4 primary antibody (1:50 in PBS with 0.2% Triton-X100). After rinsing with PBS, cells were incubated with the secondary biotinylated goat anti-rabbit antibody (30 min, RT) (Santa Cruz Biotechnology) and subsequently with HRP-Streptavidin solution (30 min, RT). Enzyme reactivity of the horseradish peroxidase (HRP) was visualized using DAB (DakoCytomation, Mississauga, Ontario) as a substrate and sections were examined under light microscopy. Images of the sections were captured using Q Capture imaging software (Qimaging Corp., Burnaby, British Columbia). As a negative control, the primary antibody was omitted and all negative controls showed no immunoreactivity.

To examine the effects of cisplatin on PC4 protein levels, ovarian cancer (OVCA) cells were cultured for various times in the absence or presence of cisplatin (10μM) and harvested as described previously. Cell pellets were washed twice in ice cold PBS and resuspended to 1-5 x 10^6 cells /ml. Ten μL of the cell suspension were transferred to 0.25% gelatin coated reaction slides and allowed to air dry. Once dry, the cells were fixed with 10% formalin (30 min, RT) and excess formalin was removed by several washes in PBS. Slides were air dried and stored at -20°C until they were processed for PC4 protein content, using the immunocytochemistry techniques described above. Relative levels of PC4 immunostaining between experimental groups at different time

points were assessed by evaluation of immunosignal strength. Cells with weak or no immunostaining were counted in at least 300 cells. Data are presented as a percentage of PC4 positive staining cells in total. For each replicate, PC4 immunosignals were assessed at one sitting to ensure consistency in observation, and the evaluator was blinded to the experimental group to avoid bias.

ii. Detection of PC4 protein in human ovarian tumours

Human epithelial ovarian tumour specimens were obtained from the Ottawa Ovarian Tumour Bank. Slides were deparaffinized in xylene (3 x 10 min) and re-hydrated in decreasing grades of ethanol (100%, 95%, 80%, 70%, 50%; 5 min each). Endogenous peroxidase activity was inhibited by incubation of slides with 3% (v/v) hydrogen peroxide in water (10 min, RT). To reduce non-specific binding, sections were blocked in 10% normal goat serum (40 min, RT), and incubated with: 1) anti-PC4 (1:50) or 2) negative control rabbit IgG (1:200) overnight (18-24 h, 4°C). After incubation with the primary antibody, slides were washed in PBS and subjected to secondary antibody-linked biotinylated anti-rabbit IgG (40 min, RT) and subsequently with a streptavidin complex (40 min, 37°C). Immunostaining was completed by the addition of substrate-chromagen DAB (< 5 min) and the reaction was stopped by inserting the slides in PBS. Sections were dehydrated in ethanol and xylene, preserved in Cytoseal mounting medium (plus coverslip), and images were captured using the Q Capture software imaging system. Adjacent sections were stained with hematoxylin and eosin to demonstrate cell morphology.

Protein Extraction and Western Blot Analysis

At the end of the culture period, cells were harvested and cell pellets were resuspended in cell lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 10% (v/v) glycerol, 1% (v/v) Triton X-100) supplemented with freshly-added protease inhibitors (1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/μL aprotinin and 1 mM sodium orthovanadate) with sonication (30 X 1 s pulses). Proteins were allowed to solubilize for 1 h at 4°C. Insoluble material was removed by centrifugation (14000 *g*, 4°C, 20 min) and the supernatant was taken as whole cell lysate and stored at -20°C for further analysis. Protein concentrations were determined spectrophotometrically using the BioRad DC protein assay kit (Bradford method), and BSA as a standard (0.1875-3.0 μg/μL) (BioRad, Canada). One-third of the total volume of 4X-sample buffer (200 mM Tris-HCl, 400 mM DTT, 8% (w/v) SDS, 40% (v/v) glycerol and 0.4% (w/v) bromophenol blue; pH 6.8) was added and samples were boiled for 5 min prior to denaturing SDS-PAGE.

For examination of IGF-II protein levels, conditioned media (1 ml) were collected at the end of the culture period and concentrated with Microcon YM-3 columns (Millipore Corporation, Bedford, MA) to approximately 20 μL. The membranes were blocked for 1 h in 5% milk and then immunoblotted with monoclonal IGF-II(1-67) (clone S1F2 1:500, Upstate, Lake Placid, NY).

Equivalent amounts of total protein (40-80 μg) or concentrated spent media were loaded onto acrylamide gels (8-15 %), resolved by SDS-polyacrylamide gel electrophoresis and electrotransfered onto nitrocellulose membranes. Ponceau-S staining was used to confirm even protein loading between lanes. The membranes were blocked (1 h, room temperature) with 5% skim milk in Tris-buffered saline with 0.05 % Tween (TBS-T) and subsequently incubated overnight at 4°C with primary antibodies diluted in blotto. Primary antibodies were detected with horseradish peroxidase-conjugated goat IgG raised against the corresponding species (BioRad) diluted in blotto (1 h, RT). Horseradish peroxidase activity was visualized using an Enhanced Chemiluminescence Detection kit (Amersham Pharmacia Biotech), recorded on HyperFilm MP (Amersham Pharmacia Biotech) and developed in a Kodak X-Omat film developer. Results were scanned and densitometrically analyzed using Scion Image software (Scion Inc.).

To re-probe membranes for subsequent protein detection, membranes were incubated in stripping buffer (1 M Tris-HCl, 10 % SDS, 14.3 M β-ME) for 15-20 min at 56°C with occasional shaking, and washed in water (3 x 5 min) and TBS-T (5 min) before addition of the primary antibody (overnight, 4°C).

Table 3: Antibodies used for Western blotting (WB), immunohistochemistry (IHC) and immunocytochemistry (ICC).

Antigen	Species raised against	Species	Monoclonal or polyclonal	Working Dilution	Company	Application
PC2	Human	Goat	Polyclonal	1 :200	Santa Cruz Biotechnology, Santa Cruz, USA	WB
Furin	Human	Rabbit	Polyclonal	1 :500	Zymed Laboratories,	WB
PC4	Human	Rabbit	Polyclonal	1:50	Gift from Dr. Mbikay, OHRI	ICC, IHC
PARP	Human	Rabbit	Polyclonal	1:1000	Cell Signaling	WB
V5		Mouse	Monoclonal	1:2000	Invitrogen, Burlington, ON, Canada	WB
IGF-II	Human	Mouse	Monoclonal	1:500	Upstate, Lake Placid, NY	WB
GAPDH		Mouse	Monoclonal	1:20000	Abcam, Cambridge, MA, USA	WB

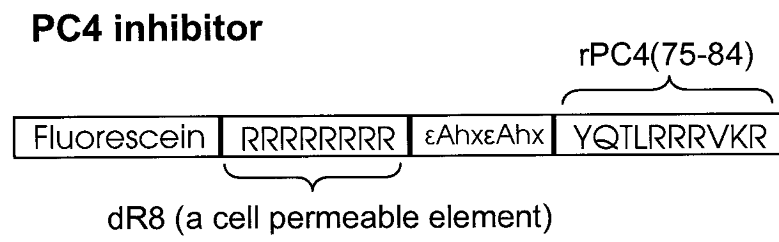
PC4 Inhibitor assays

The PC4-specific inhibitors used in the present study were developed from the PC4 prodomain. The inhibitor contains a 10 amino acid (YQTLRRRVKR) sequence for rat PC4 at the C-terminal of the pro-domain [rPC4(75-84)], upstream of the primary activation site with a cell permeable element, 8-dextro-Arginine (dR8), attached via a linker [2 units of epsilon amino hexanoic acid (εAhx)] (Figure 7A). A fluorescent moiety was also linked to its N-terminal to facilitate the detection of its cellular transport via fluorescence microscopy (Figure 7B). *In vitro* studies using small fluorogenic substrates indicate that the inhibitor has significantly less inhibitory effects on other PC family members (Basak *et al.* unpublished). Prior to cell culture experiments, the inhibition efficiency of the peptide was examined using a previously described cell-free system (Basak *et al*, 1999) at different concentrations (0, 1, 10, 50, 100 μM). These *in vitro* studies with the fluorogenic substrate (Boc-RVRR-MCA) indicated that the peptide had a potent inhibitory effect on recombinant PC4 with maximal inhibition being reached between 10-50 μM (Figure 7C). Cells were seeded overnight in 12-well plates and the following day were cultured with or without the PC4 inhibitor (0, 25, 50 μM) in 1 ml of serum-free medium. The PC4 inhibitor was dissolved in DMSO and each experimental group received an equal amount of DMSO as control (1:1000, vehicle). Following 1 h of pre-treatment with the PC4 inhibitor, cells were treated with or without cisplatin (10 μM) for 24 h and harvested for analysis. The permeability of the inhibitors was examined after 1h and following 24 h of cell culture using fluorescence microscopy with appropriate filters.

Figure 7: **PC4 inhibitor structure, permeability, function**

The PC4 inhibitor was designed from its own prodomain sequence (aa75-84) near the primary activation site (A). A cell permeable element (8-dextro-arginine; dR8) was attached via a linker to the PC4 sequence and a fluorescent moiety at the N-terminal facilitates the detection of its intra-cellular transport (B). *In vitro* studies with fluorogenic substrates indicate that the peptide has minimal inhibition activity towards other PC family members (Basak, unpublished) but has a potent inhibitory effect on recombinant PC4 (C).

A



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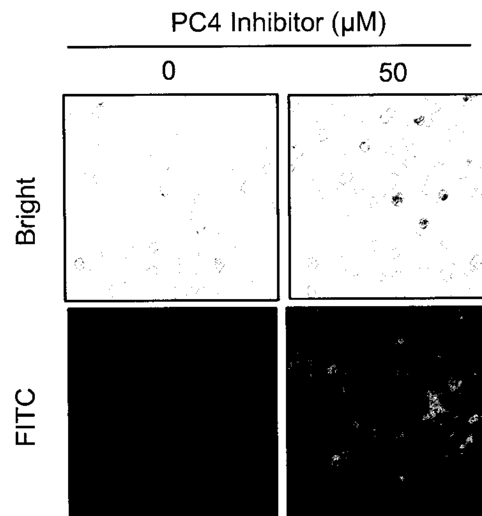

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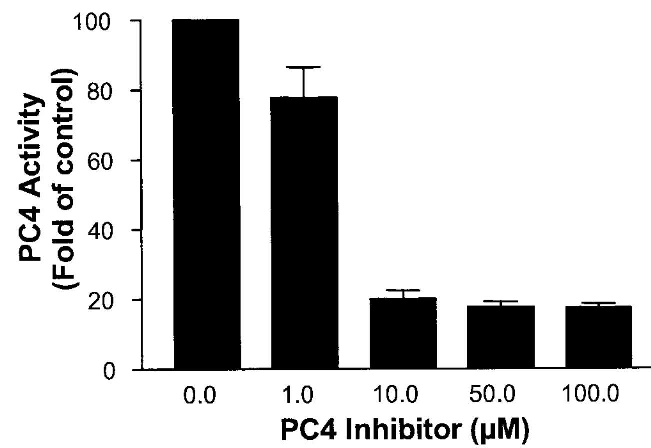


Figure 7

Statistical Analysis

Experimental results are expressed as the mean ± SEM of at least three independent experiments. Data were analyzed by analysis of variance (ANOVA) with Bonferonni post-tests to assess differences between experimental groups (PRISM 3.0; GraphPad Software Inc.) Statistical significance was inferred at $P<0.05$.

CHAPTER 3: RESULTS

1. Expression of Proprotein Convertases in Human Ovarian Cancer Cells

The first objective of the present study was to determine which PC family members are expressed in human ovarian cancer cell lines. It was hypothesized that all members of the PC family (Furin, PC4, PACE4, PC5, and PC7) would be expressed in ovarian cancer cells with the exception of PC1 and PC2, the expression of which is typically restricted to neuroendocrine-like tissues and cell types, and since the expression of proprotein convertases has been demonstrated in various types of tumours and cancer cell lines (Mbikay *et al*, 1997; Cheng *et al*, 1997; Bassi *et al*, 2001; Jin *et al*, 1999). RT-PCR analyses of chemosensitive (A2780s and OV2008) and chemoresistant (A2780cp and C13*) human ovarian cancer cells revealed the presence of mRNA for 6 of the 7 PC family members in all 4 of the cell lines examined including Furin, PC2, PC4, PACE4, PC5, and PC7 (Figure 8). PC1 was not detected in any of the ovarian cancer cell lines under the conditions examined. Contrary to the initial hypothesis, PC2 was detected in all of the OVCA cell lines. This was an interesting but unexpected finding since PC2 is typically only found within endocrine or neuroendocrine cells and the ovarian cancer cells examined are of epithelial origin. Unlike other PC family members, PC2 requires the presence of an accessory molecule, neuroendocrine protein 7B2 for its enzymatic activation and expression. To further support a functional presence of PC2 in ovarian cancer cells, PCR analyses for 7B2 was performed. Like PC2, this neuroendocrine marker was detected in all the OVCA cell lines examined (Figure 8B).

Figure 8: **PC expression in ovarian cancer cell lines**

A) PC4, PACE4, PC5, PC7 and Furin are expressed in both chemosensitive (A2780s and OV2008) and chemoresistant (A2780cp, C13*) ovarian cancer cells as confirmed by RT-PCR, B) PC2 and 7B2 expression in ovarian cancer cells, C) PC1 was not expressed in any of the cell lines examined. Normal brain tissue was used as a positive control for neuroendocrine specific expression.

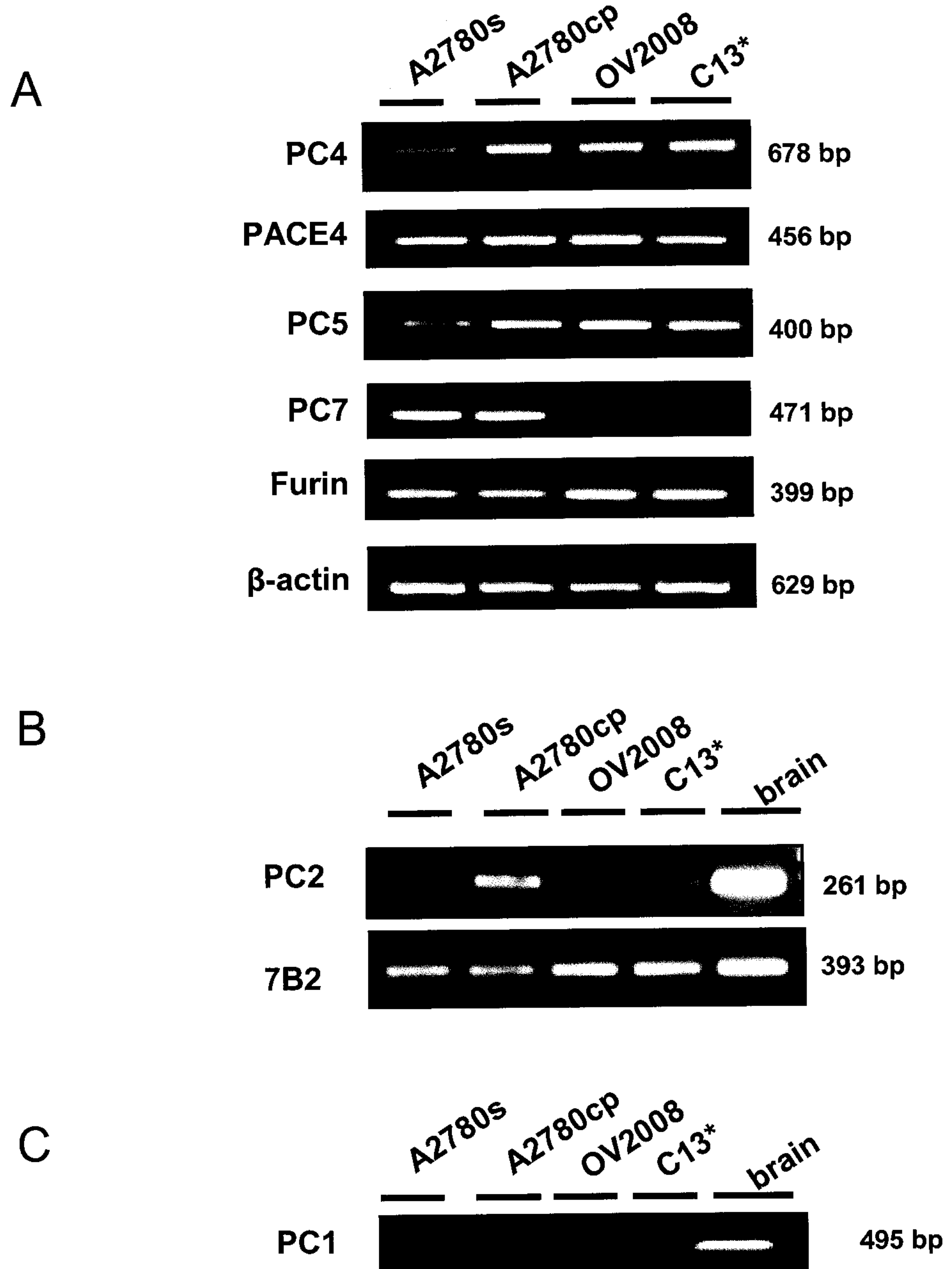


Figure 8

2. Effect of cisplatin treatment on PC mRNA content in OVCA cells

In order to determine whether changes in proprotein convertase mRNA levels may be a consequence of the chemotherapeutic action of cisplatin (CDDP), and whether there is a difference in the responsiveness between chemosensitive and -resistant ovarian cancer cells, OVCA cells were cultured in the absence or presence of CDDP and mRNA content of the PC family members was examined by RT-PCR. Initially, the concentration-dependent effects of CDDP on the mRNA levels of PC family members were examined using real-time semi-quantitative PCR. Optimization of the reaction conditions for several primer sets proved difficult and this technique was later abandoned for conventional thermocycler PCR. Following careful examination of the experimental design and technical procedures, it was determined that a 24 h treatment period with CDDP was inappropriate for detecting changes in mRNA levels since it is likely that any effect on PC mRNA levels would occur at time points prior to 24 h. To address this issue, the effect of CDDP on mRNA levels of PC family members in OVCA cells was examined over time (0, 2, 4, 8, 24 h).

Cisplatin treatment caused a significant induction of apoptosis in chemosensitive OV2008 cells but not in C13* cells after 24 h (Figure 9A). CDDP (5 μM) had no significant effect on the mRNA levels of any of the PC family members examined (Furin, PACE4, PC5, PC7, PC2) over a 24 h time period (Figure 9B-F, respectively).

Figure 9: **Effect of cisplatin on PC mRNA content in OVCA cells**

A) Effects of cisplatin (CDDP; 5 μM) on apoptosis in ovarian cancer cells over time; chemosensitive cells undergo an increased rate of apoptosis where as the chemoresistant counterparts do not respond. B-F) PC (Furin, PC2, PACE4, PC5, PC7) mRNA levels following (0, 2, 4, 8, 24 h) cisplatin treatment (- , +). Total RNA from ovarian cancer cells was isolated, reverse-transcribed (2μg) and analyzed using PCR. Data is corrected against corresponding β-actin levels and expressed as fold of 0 h non-treated OV2008 group. Data represent mean ± SEM of 4 independent experiments.

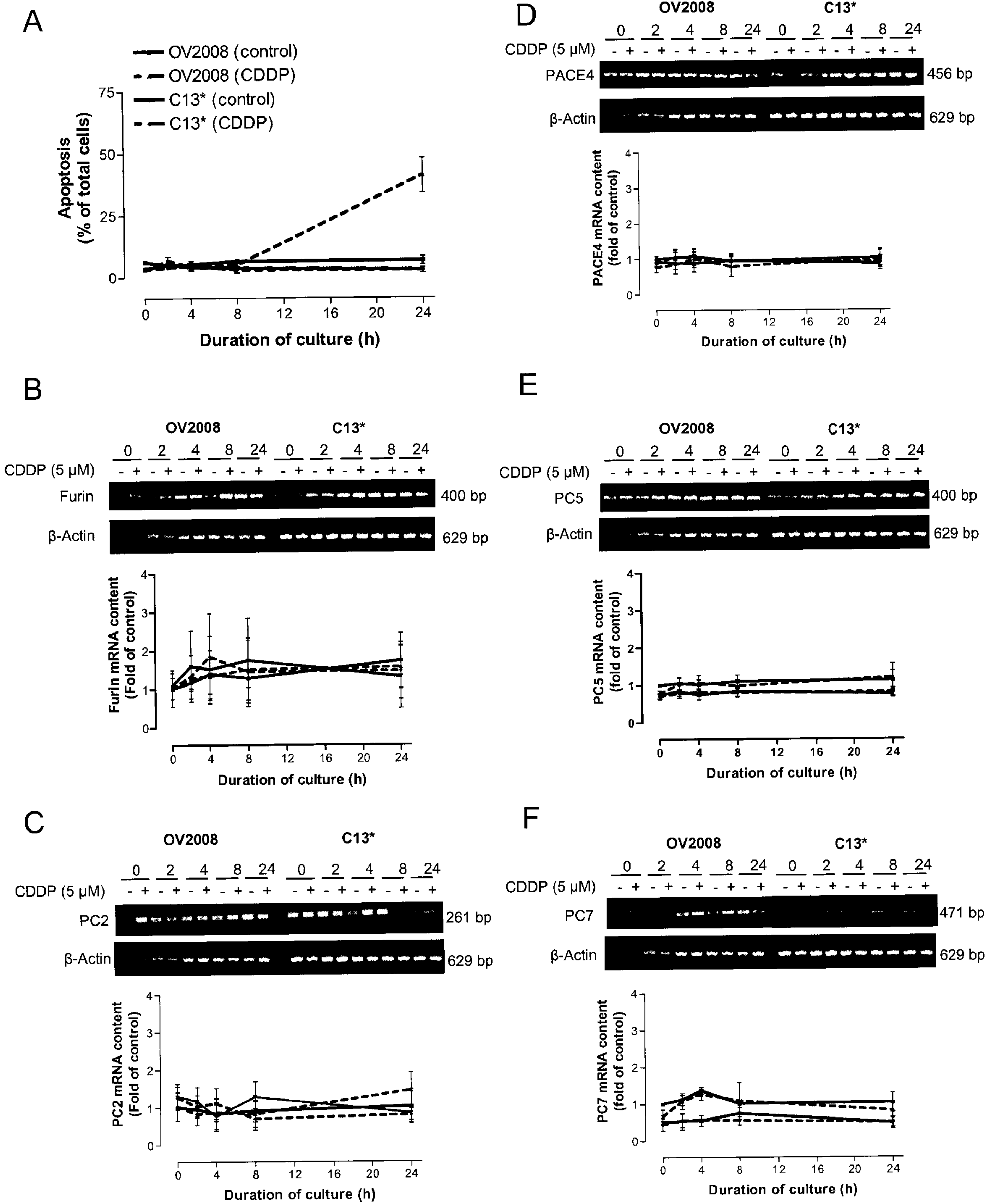


Figure 9

In addition, RT-PCR analysis revealed that CDDP treatment had no significant effect on PC4 mRNA content over various durations of culture (0, 2, 4, 8, 24 h; 5 μM; Figure 10A) and concentrations of CDDP (0, 5, 10 μM; 8 h; Figure 10B). Taken together, these data suggest that the potential involvement of PCs in chemoresistance of human ovarian cancer cells is not mediated through the regulation of proprotein convertase mRNA levels by CDDP.

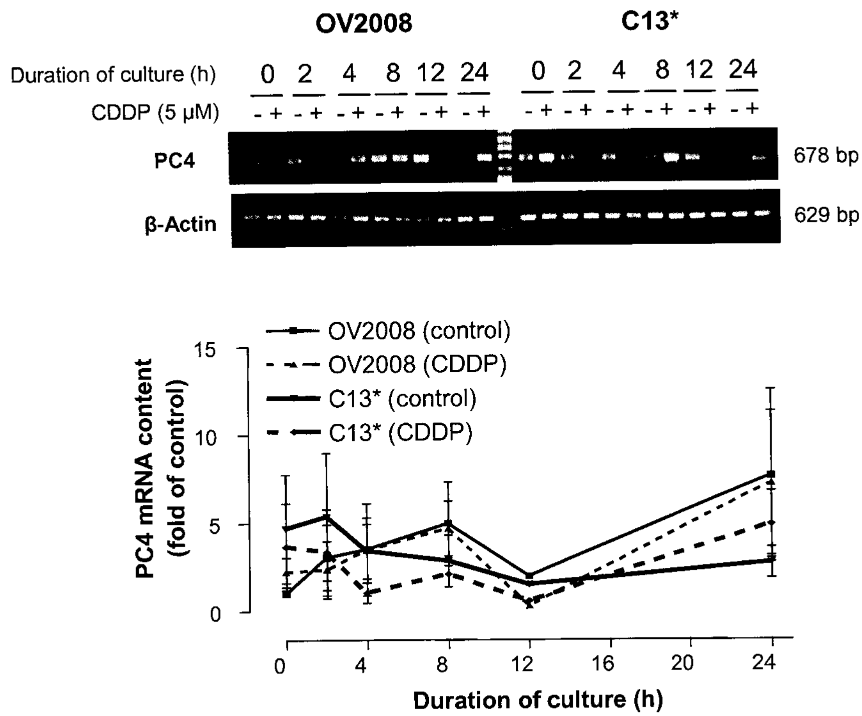
3. Effect of cisplatin treatment on PC protein content

A pitfall of the present research is that there are few proprotein convertase antibodies commercially available, making the examination of the effects of cisplatin on protein content of the different PC family members difficult. Antibodies against Furin and PC2 are available, have been used successfully and allowed us to examine the effects of CDDP on the protein content of these members. OVCA cells were cultured for 24 h with increasing concentrations of CDDP, harvested and the protein extracted for analysis. Treatment with the chemotherapeutic drug significantly induced apoptosis in a concentration-dependent manner in the chemosensitive cells but not in the chemoresistant cells ($P<0.001$; Figure 11A). Western blot analysis revealed that CDDP did not have any significant effect on Furin protein content at any of the concentrations examined (0, 5, 10 μM) in either the chemosensitive OV2008 cells nor in their resistant variant, C13* (Figure 11B). CDDP appeared to slightly increase PC2 protein content, but this failed to reach statistical significance. However, this observation was evident in both the chemo-

Figure 10: Effect of cisplatin on PC4 mRNA in OVCA cells

Effect of CDDP treatment on PC4 mRNA content following time-course (A; 0, 2, 4, 8, 24 h) and concentration-response studies (B; 0, 5, 10 μM), as determined by RT-PCR. PC4 mRNA content was corrected against corresponding β-actin levels and data expressed as fold of control (OV2008 at 0 h non-treated or 0 μM CDDP). Data are represented as the mean ± SEM of at least 3 independent experiments.

A



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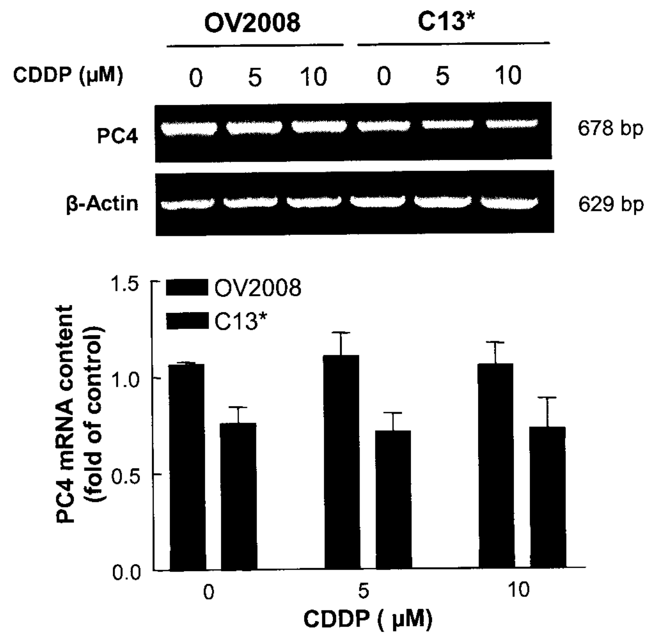


Figure 10

Figure 11: **Effect of CDDP on the protein content of Furin and PC2**

PC content in OVCA cell extracts (80 μg/lane) were analyzed by Western blot. A) CDDP treatment significantly induces apoptosis in OV2008 but not in C13* cells * $P<0.05$ *** $P<0.001$ (relative to C13* at corresponding CDDP concentration). Representative immunoblots of Furin (100 kDa) (B) and PC2 (68 kDa) (C) contents corrected to GAPDH (fold of control units). Cisplatin treatment did not cause any significant changes in Furin content in either cell line. PC2 content was increased with CDDP however this trend was observed in both sensitive and resistant cells. Data are represented as the mean ± SEM of 5 independent experiments.

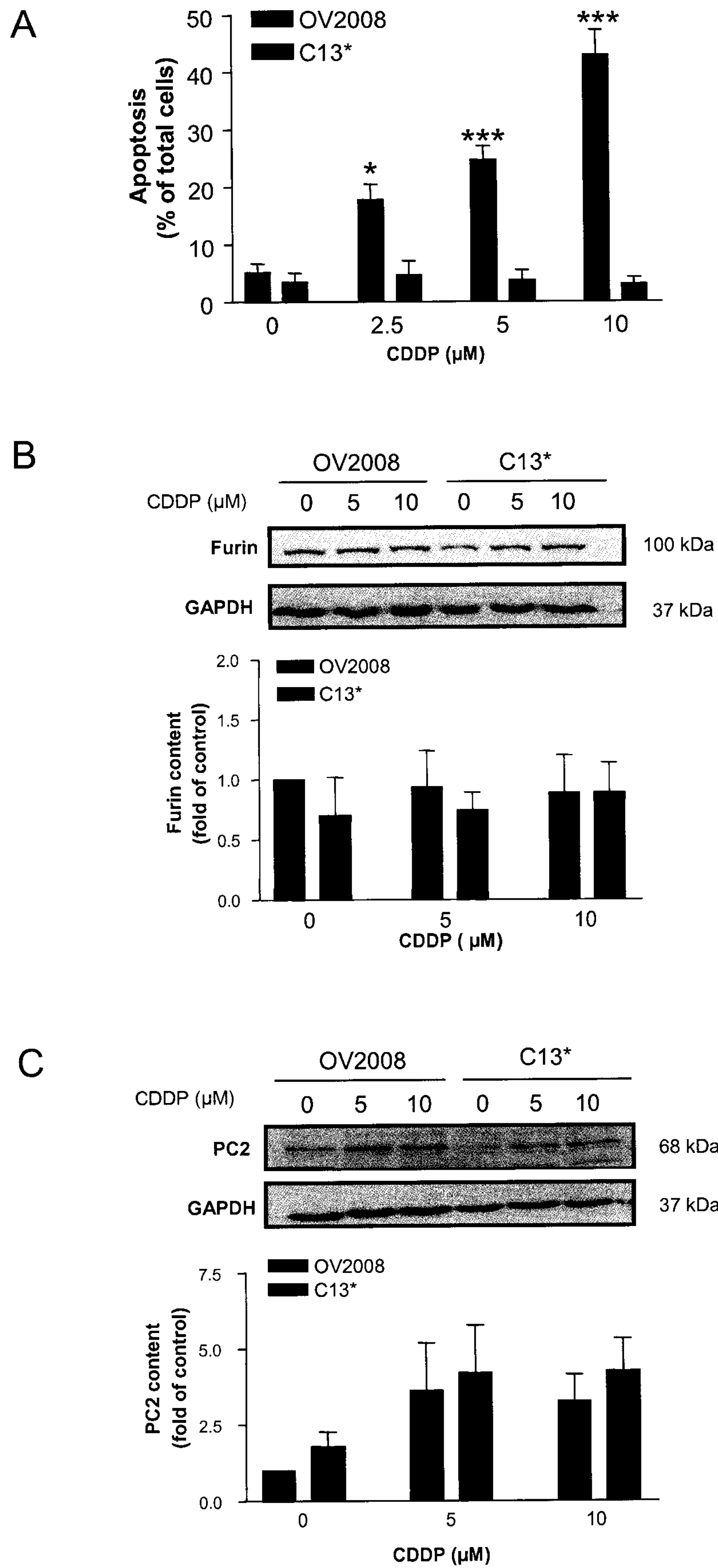


Figure 11

sensitive and resistant cells (Figure 11C), suggesting that the protein levels of these particular PC family members (Furin and PC2) are not differentially regulated by cisplatin in chemosensitive and -resisant ovarian cancer cells.

Although one anti-PC4 antibody is commercially available, it reacts with many proteins, making it difficult to assess which bands are specific to PC4. This was confirmed when sperm lysates from PC4 wild-type (WT) and knock-out (KO) mice were analyzed as positive and negative controls, respectively (Figure 12). A 54 kDa band, the expected size of mature PC4 was seen in all samples including the KO sperm lysate suggesting that this band was non-specific, while none of the other immunoreactive bands corresponded to the expected size of mature PC4. For these reasons, examination of PC4 protein levels is very difficult to assess by Western blot and this method for examining PC4 protein levels was discontinued. An additional polyclonal antibody (PC4-606), kindly provided by Dr. Majambu Mbikay (Diseases of Aging Program, OHRI), recognizes the native form of PC4 and can therefore be used for immunohistochemistry. Validation of this antibody was done using ovaries from PC4 WT and KO mice and confirmed the specificity of the antibody (See Materials and Methods). As an alternative method to confirm the presence of PC4 protein in OVCA cells, immunocytochemistry using this PC4 antibody was carried out on cultured cells. Examination by light microscopy demonstrated the presence of PC4 protein in both chemosensitive (OV2008) and chemoresistant (C13*) ovarian cancer cells (Figure 13). PC4 immunosignals were

Figure 12: Non-specificity of PC4 antibody for Western blotting

Specificity of a commercially available polyclonal PC4 antibody was examined by Western blot. Sperm lysates from wild-type and knock-out PC4 mice were used as positive and negative controls and compared against cell lysate from ovarian cancer cells (C13*) and human trophoblast cells (HTR). C13* cells transfected with full-length rat PC4 (C13* + rPC4) were also included as a positive control. The expected size of the proform of PC4 is 72 kDa and 54kDa for the mature enzymatically active form. A band ~54 kDa was seen in all lysates including the negative control, suggesting that this band is non-specific. A strong band at 72 kDa can be seen in the cells transfected with full-length PC4.

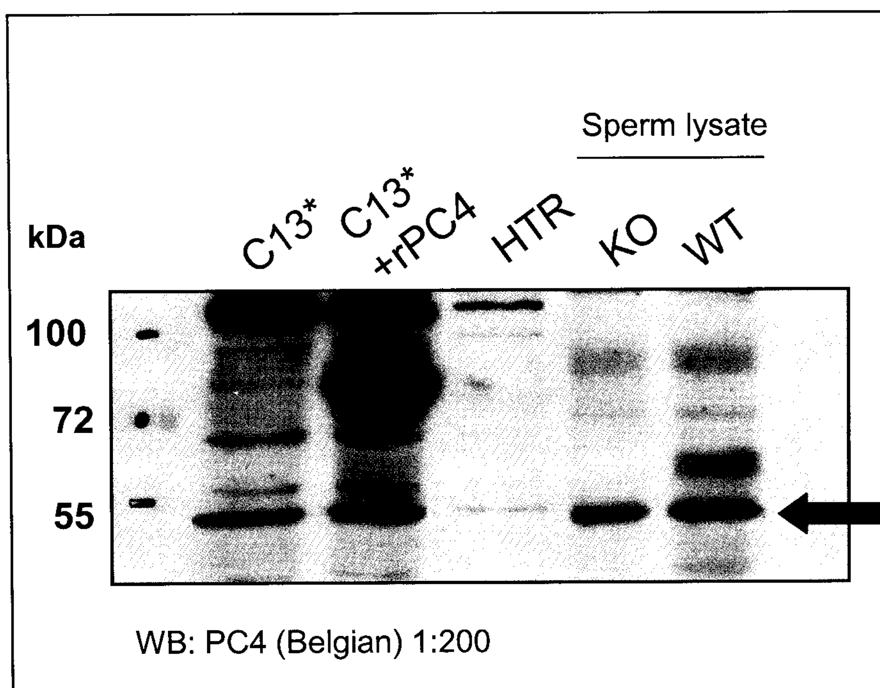


Figure 12

Figure 13: **PC4 protein in OV2008 and C13* cells**

Detection of PC4 protein in ovarian cancer cells (OV2008, C13*) by immunohistochemistry using the polyclonal PC4-606 antibody. Cells were cultured overnight on 8-well chamber slides in RPMI with 10% FBS. PC4 immunosignals are perinuclear in location as indicated by black arrows. Primary antibody was omitted as a negative control. Magnification, X200.

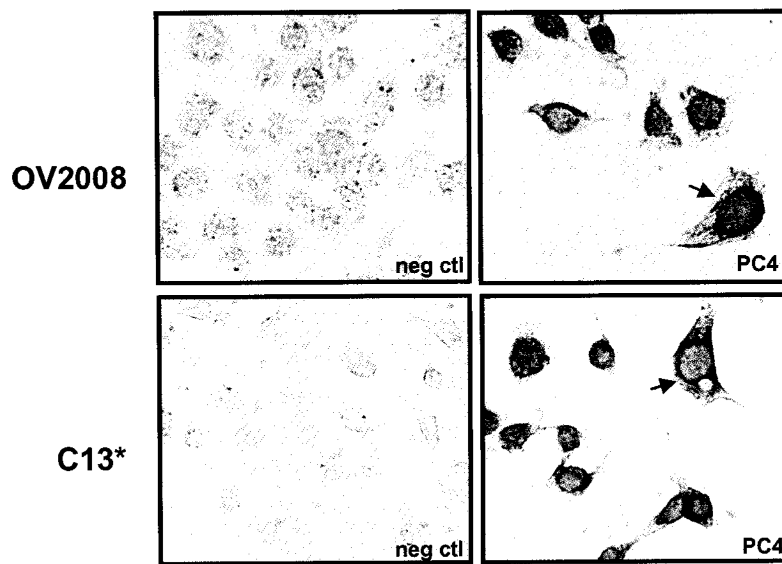


Figure 13

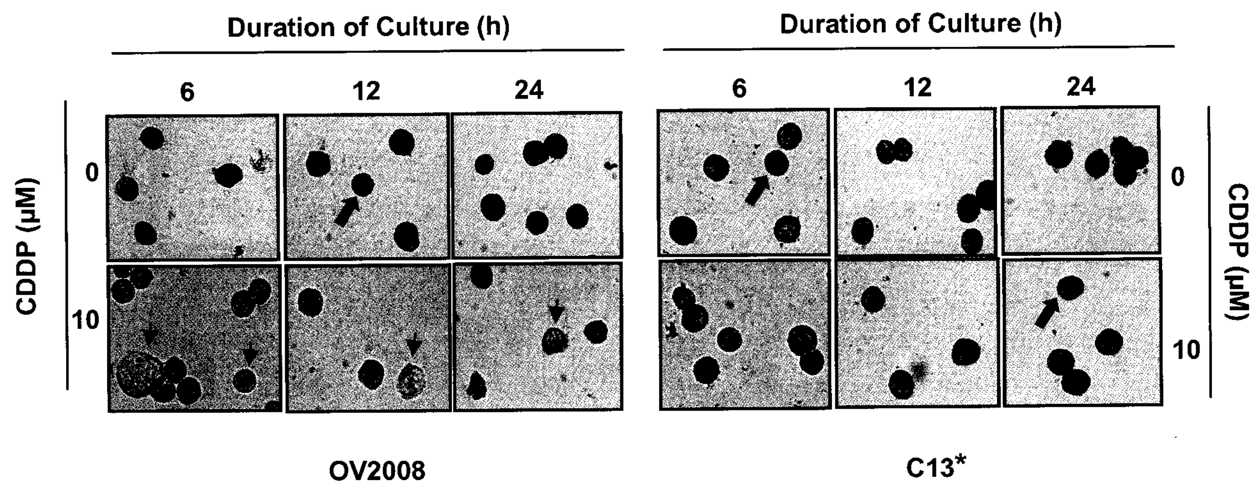
perinuclear in location (black arrows), which is consistent with the expected pattern of PC4 expression within the cell, namely endoplasmic reticulum and Golgi apparatus.

With the confirmation that PC4 protein is expressed in ovarian cancer cells, our next objective was to investigate the effects of CDDP on PC4 protein levels in chemosensitive and –resistant human ovarian cancer cells. OV2008 and C13* cells were cultured in the absence or presence of CDDP for various durations of culture (0, 6, 12, 24 h) and PC4 immunosignal intensity was qualitatively compared between chemosensitive and resistant cells at different time points after CDDP treatment. PC4 immunostaining remained relatively stable in both the chemosensitive and -resistant cells during 24 h of culture in the absence of CDDP. However, a drastic decrease in the number of cells staining positive for PC4 was observed in the cisplatin-sensitive cells following CDDP treatment as early as 6 h. This response was maintained through all other time points (Figure 14B). In contrast, CDDP failed to influence PC4 immunostaining in the chemoresistant C13* cells. To ensure that this effect of cisplatin was not cell line-specific, we examined the response in another pair of OVCA cell lines, A2780s/A2780cp. Consistent with the previous results, a decrease in PC4 immunostaining was observed in the chemosensitive cells as early as 6h and maintained through 24h of culture with no response seen in the resistant cells (Figure 14 C).

Figure 14: Effect of CDDP on PC4 protein

A) Influence of cisplatin on PC4 immunosignals over time in human ovarian cancer cells. Black arrows indicate cells with low or absent PC4 staining while red arrows indicate PC4 positive cells. B) OV2008 and C13* and C) A2780s and A2780cp cells were cultured in the absence or presence of CDDP (10 μM; DMSO as control) for up to 24 h and analyzed by ICC. After 6 h of CDDP treatment, the number of PC4 positive staining cells significantly decreased, and remained low in the chemosensitive cells but not in the resistant counterpart cells. Cell culture alone had no effect on PC4 immunostaining. Data are presented as the number of PC4 positive staining cells as a percentage of the total number of cells. Magnification, X400. The effect of cisplatin-induced apoptosis in each of these cell lines is also shown in the lower panels. Data represents mean ± SEM from three independent experiments.

A



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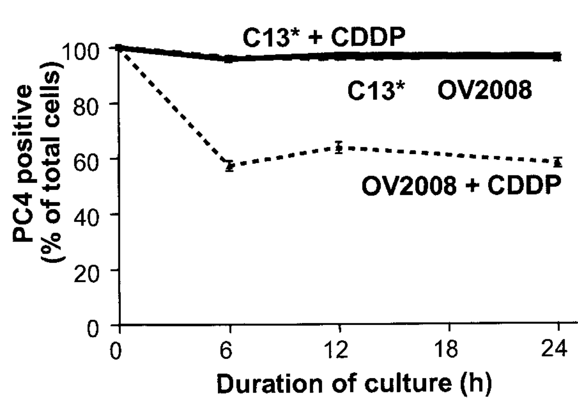

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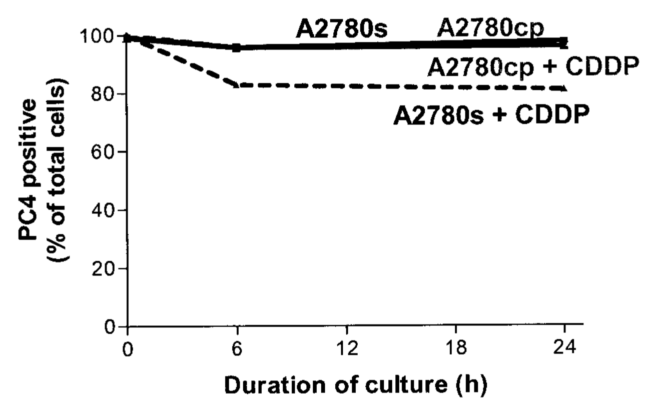
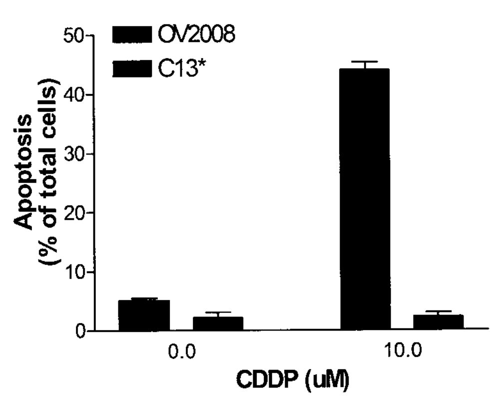




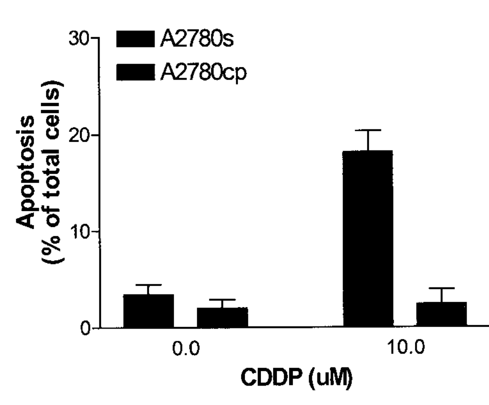


Figure 14

4. Over-expression of PC4 protects chemosensitive ovarian cancer cells from CDDP- induced apoptosis

To examine whether PC4 is indeed involved in the regulation of cisplatin sensitivity in human ovarian cancer cells, we investigated the effect of CDDP on apoptosis in chemosensitive ovarian cancer cells following PC4 over-expression. Chemosensitive OV2008 cells were transiently transfected with rat PC4 cDNA (1μg) or empty PCIneo vector (as a transfection control) for 24 h and treated with CDDP (2.5 and 5 μM) or dimethyl sulfoxide (DMSO; vehicle control) for an additional 24 h. CDDP significantly induced apoptosis in a concentration-dependent manner in the empty vector transfected-cells, however over-expression of rat PC4 (rPC4) cDNA attenuated cisplatin-induced apoptosis ($P<0.001$; Figure 15). Over-expression of PC4 was confirmed by immunoblotting with V5 antibody.

It is well established that PC4, like the other PC family members, requires proteolytic processing to become enzymatically active (Seidah *et al*, 1998). To further establish that the protective effects of PC4 over-expression were associated with its activation, site-directed mutagenesis was carried out on the wild-type rPC4 cDNA sequence (NM_133559) at the dibasic consensus site (RVK**R**↓; R110A) where the proform is autocatalytically cleaved *in vivo.* This site is conserved among rat, mouse and human PC4 cDNA sequences (Genbank accession no: Q78EH2, NP_032819, AAQ89322, respectively). Chemosensitive OV2008 cells were transiently transfected with either the wild-type or mutant R110A rPC4 expression vectors (empty vector serving as a control) and subsequently treated with CDDP (5 μM, 24 h). Consistent with the previous observations, cells over-expressing WT rPC4 exhibited suppressed levels of

cisplatin-induced apoptosis (*P*<0.001) compared to control, while cells transfected with the R110A rPC4 mutant had similar levels of cisplatin-induced apoptosis as the control cells, suggesting that the processing of PC4 at this site is required for the protective effect of PC4 (Figure 16). Western blot analysis confirmed wild-type and mutant PC4 over-expression.

Taken together, these findings suggest that PC4 is an anti-apoptotic mediator and may play an important role in regulating cisplatin sensitivity of ovarian cancer cells.

Figure 15: **Over-expression of wild-type PC4 protects chemosensitive (OV2008) cells from cisplatin-induced apoptosis.**

Apoptotic response of OV2008 cells transiently transfected with PCIneo vector (1 μg; 24 h) containing rPC4/v5 cDNA (empty vector as control) and treated with cisplatin (2.5 - 5 μM for 24 h; DMSO as control); ** $P<0.01$, *** $P<0.001$ (relative to empty vector at respective CDDP concentration). Data represent mean ± SEM of four independent experiments. PC4 over-expression was confirmed by V5 immunoblotting.

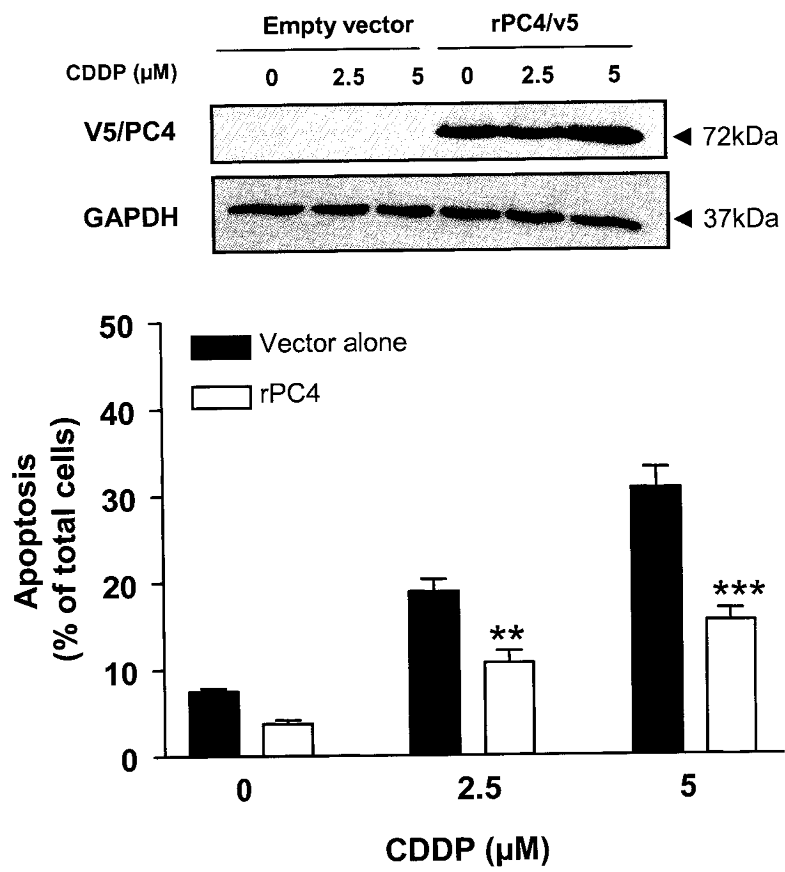


Figure 15

Figure 16: Over-expression of WT rPC4 vs. R110A mutant

OV2008 cells were transfected with control, wild-type, or proform mutant (R110A) PC4 expression vectors (1 μg; 24 h), cultured in the absence or presence of CDDP (5 μM, 24 h) and their apoptotic response was examined. *** $P<0.001$ (relative to control at same concentration) +++ $P<0.001$ (relative to WT rPC4 at same concentration). Over-expression was confirmed by western blotting with V5.

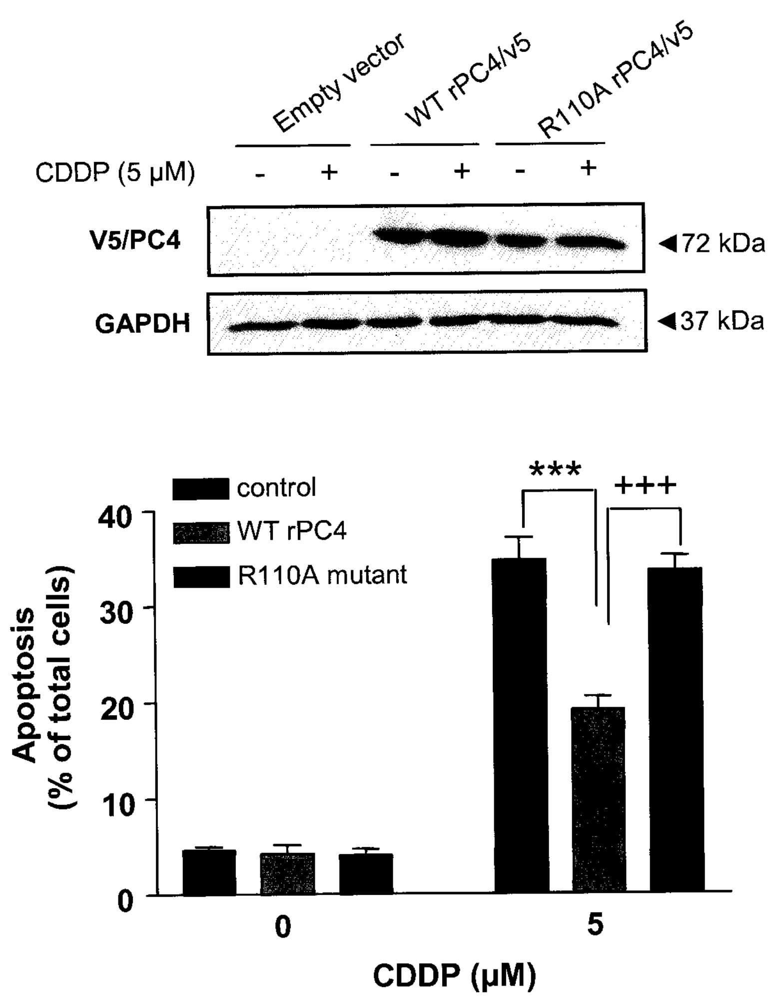


Figure 16

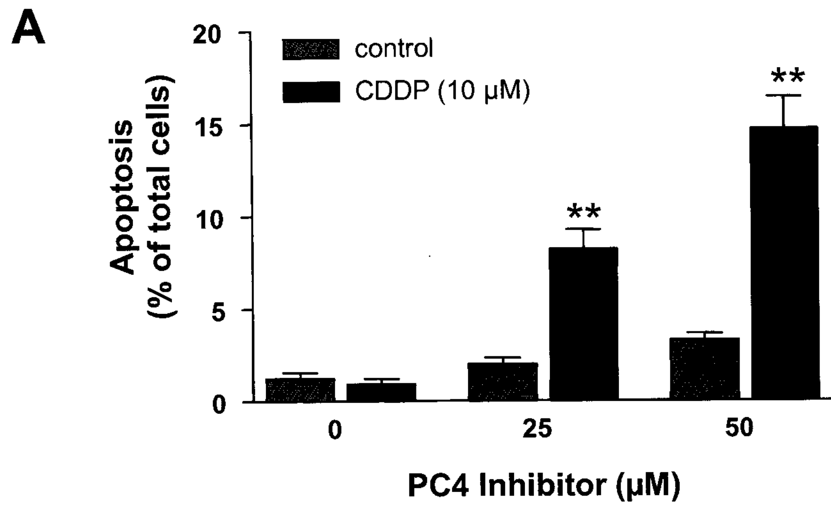
5. Inhibition of PC4 activity and down-regulation of PC4 sensitizes chemoresistant ovarian cancer cells to cisplatin-induced apoptosis

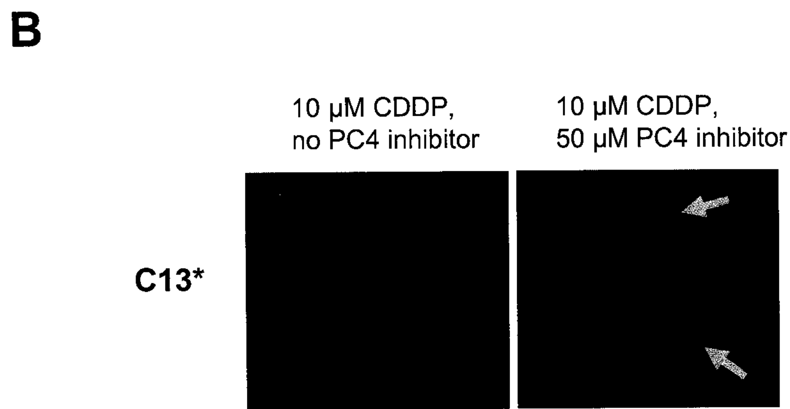
To further investigate the hypothesis that PC4 is a determinant of chemoresistance in ovarian cancer cells, we used a PC4-specific inhibitor to examine the effect of PC4 activity on cell survival following cisplatin challenge. The chemoresistant variant of OV2008 cells (C13*) were treated with the PC4-specific inhibitor in the absence and presence of CDDP (10 μM) and the effects on apoptosis examined. In cell culture, pre-treatment with the PC4-specific inhibitor (25 and 50 μM) sensitized chemoresistant C13* cells to cisplatin-induced apoptosis in a concentration-dependent manner ($P<0.001$) (Figure 17A). Apoptosis was determined by assessment of nuclear morphology using Hoechst staining (Figure 17B). Typical morphological features of apoptotic cells include chromatin condensation and nuclear fragmentation (as indicated by the yellow arrows).

Since changes in nuclear morphology occur at later stages of apoptosis, molecular markers of apoptosis were also examined. Activation of caspase-3 is critical for apoptotic execution and its activity can be evaluated by cleavage of its substrate poly(ADP-ribose) polymerase (PARP). Proteolytic cleavage of intact 116 kDa PARP into an 89 kDa fragment increased in C13* cells cultured in the presence of both the PC4 inhibitor and CDDP (Figure 17C). PARP cleavage was also detected in the inhibitor alone group suggesting that inhibition of PC4 activity may have implications on basal cell survival.

Figure 17: **Effect of PC4-specific inhibitor on OVCA cell survival**

Inhibition of PC4 sensitizes chemoresistant (C13*) cells to cisplatin. (A) Concentration-dependent sensitization of C13* cells to cisplatin-induced apoptosis by PC4 inhibitor (0, 25, 50 μM) during a 24 h culture period. *** $P<0.001$ (relative to control at respective CDDP concentration). Data represents mean ± SEM of 5 independent experiments. (B) Apoptotic nuclear morphology (Hoechst staining; magnification X400; yellow arrows indicate typical morphological features of apoptotic cells (DNA fragmentation and nuclear condensation) while the red arrow indicates a healthy cell. (C) Representative western blots showing increased PARP cleavage following co-treatment with PC4-inhibitor and CDDP.





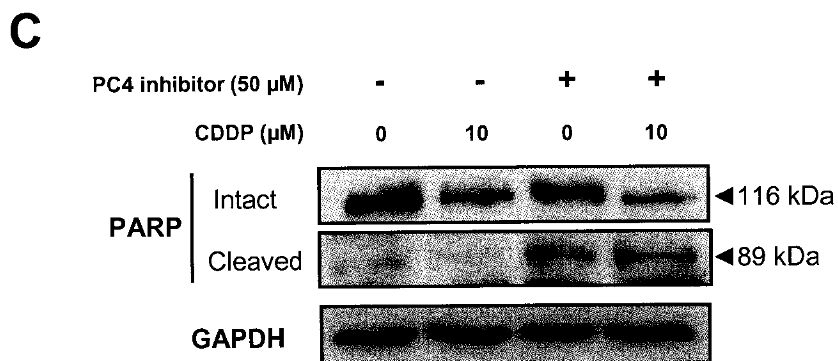


Figure 17

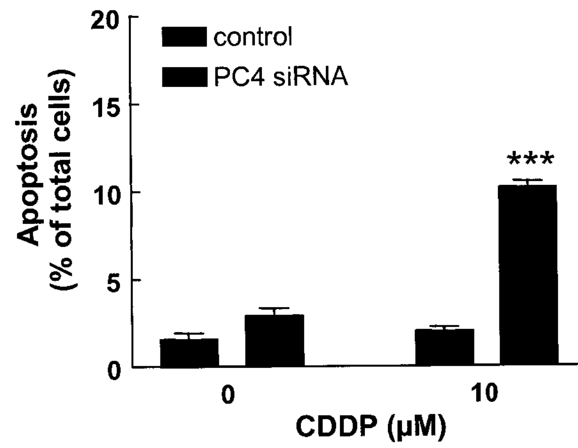
To further examine the effects of PC4 on cell survival, siRNA targeted to the C-terminal of human PC4 coding sequence was designed. Chemoresistant C13* cells were cultured and transfected with 100 nM of either control (scrambled sequence) or PC4 siRNA for 24 h and then treated with or without CDDP (10 μM) for an additional 24 h. Cells treated with both the PC4 siRNA and CDDP had significantly increased levels of apoptosis ($P<0.001$) compared to either the CDDP or PC4 siRNA alone groups (Figure 18A), suggesting that treatment with the targeted PC4 siRNA sensitized chemoresistant ovarian cancer cells to the apoptotic effects of cisplatin. To examine the efficiency of the PC4 siRNA, we examined the mRNA levels of PC4 following siRNA treatment. Chemoresistant cells were cultured and transfected with either the control or PC4 siRNA for 8 h, harvested and processed for PCR. RT-PCR analysis revealed that treatment with the PC4 siRNA significantly reduced PC4 mRNA content compared to the control siRNA group (Figure 18B). In addition, immunocytochemistry with the PC4 606 antibody was performed on the OVCA cells treated with the PC4 siRNA and confirmed the down-regulation of the PC4 gene product in the siRNA experimental group (Figure 18C).

Together with the results from the PC4 inhibitor experiments, these data support the hypothesis that PC4 is a pro-survival molecule and may be a determinant of chemoresistance in ovarian cancer cells.

Figure 18: **Effect of PC4 siRNA**

Down-regulation of PC4 sensitizes chemoresistant (C13*) cells to cisplatin-induced apoptosis. (A) Apoptotic response of C13* cells transfected with control or PC4 siRNA (100 nM, 24 h), and cultured in the absence or presence of CDDP (10 μM, 24 h) *** $P<0.001$ (relative to control siRNA at 10 μM CDDP) (B) RT-PCR confirmed the PC4 siRNA (100 nM, 8 h) efficiency as depicted by the changes in PC4 mRNA (corrected against β-actin mRNA) ** $P<0.01$ (relative to control siRNA). Data represents mean ± SEM of six independent experiments. (C) Immunocytochemistry with polyclonal PC4 antibody confirmed down-regulation of the PC4 gene product by the PC4 siRNA (100 nM, 24 h).

A



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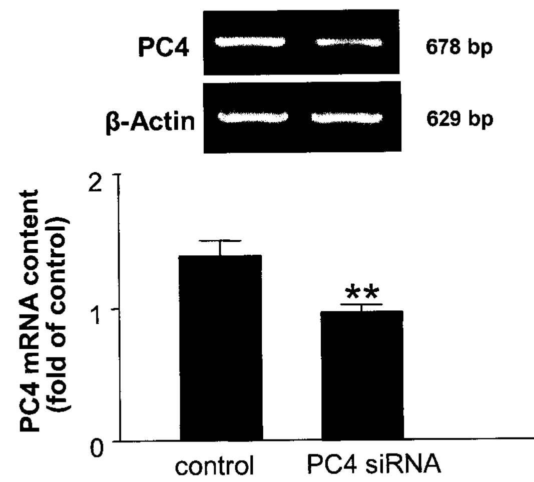

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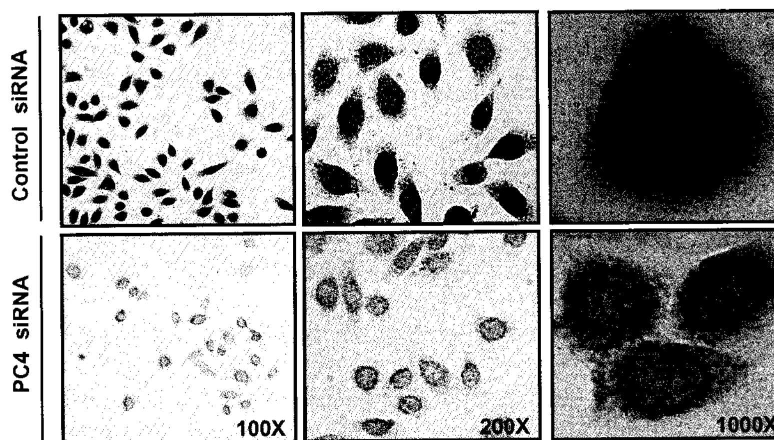


Figure 18

6. Expression of insulin-like growth factor-II signaling components in OVCA cells

Proprotein convertases process many substrates implicated in the onset and progression of cancer (reviewed in Khatib *et al*, 2002). Recent studies have demonstrated that PC4 plays a key role in the proteolytic processing of IGF-II (Qiu *et al*, 2005) while several studies have implicated members of the insulin-like growth factor (IGF) family in human ovarian cancer (Kalli & Conover, 2003; Sayer *et al*, 2005). To examine whether the ovarian cancer cells used in the present study express the components required for IGF-II signaling, RT-PCR analysis was used. Primers were designed from human cDNA sequences for each of the signaling members from Genbank. RT-PCR confirmed the expression of IGF signaling components in OVCA cells (Figure 19). IGF-I was expressed at very low levels in A2780s and A2780cp cells while levels were undetectable in OV2008 and C13* cells. IGF-II mRNA was detected in all four cell lines examined. All three of the receptors involved in IGF-II signaling, IGF-IR, IGF-II R and the insulin receptor, were detected in all of the cell lines examined. These data suggest that components required for IGF-II signaling, at least at the ligand/ receptor level, are expressed in human ovarian cancer cells.

Figure 19: **IGF signaling components in OVCA cells**

Components for IGF-II signaling are expressed in human ovarian cancer cells. RT-PCR detected transcripts for IGF-II ligand and its receptors, IGF-IR, IGF-IIR and the insulin receptor. cDNA from human trophoblast (HTR) cells was used as a positive control.

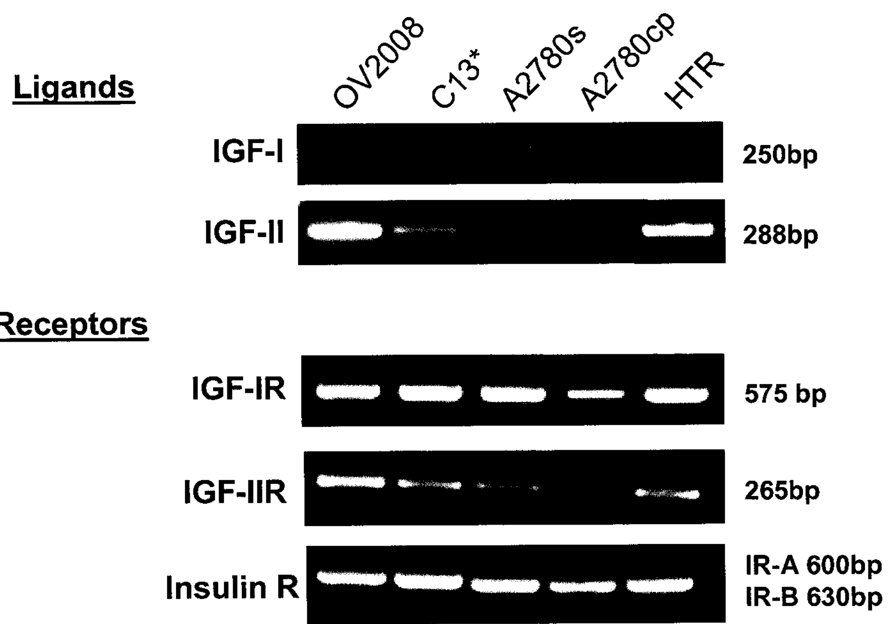


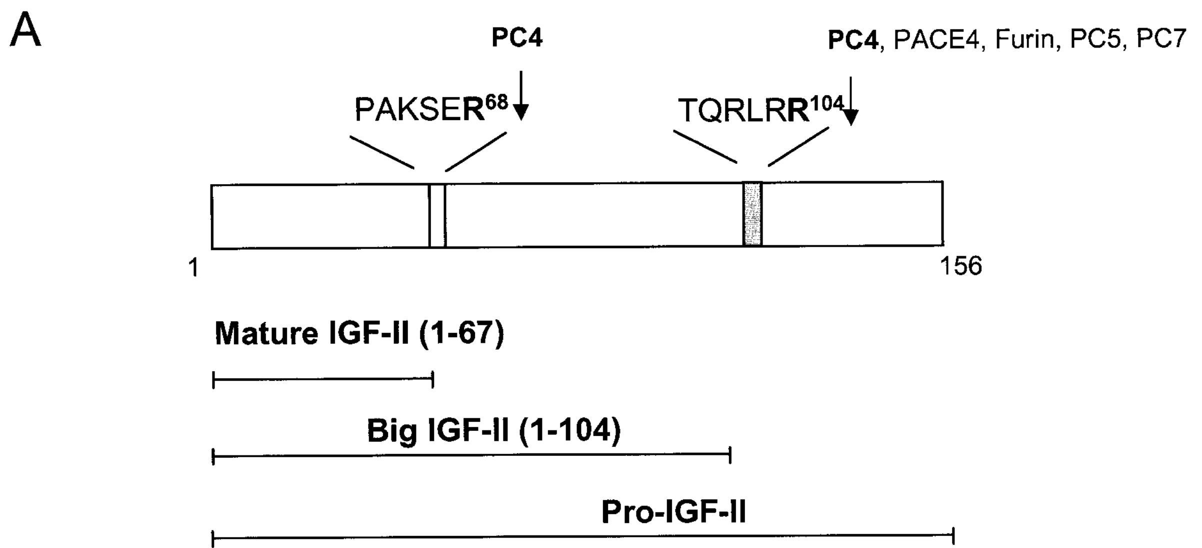
Figure 19

7. PC4 processes pro-IGF-II to its mature form in ovarian cancer cells

Previous studies from our laboratory have highlighted the role of PC4 in the processing of pro-IGF-II (Qiu *et al,* 2005), namely the final proteolytic cleavage of big IGF-II(1-104) to yield mature, bioactive IGF-II(1-67) (Figure 20A). Since this was demonstrated in an immortalized trophoblast cell line, we wanted to confirm that PC4 was involved in proIGF-II processing in the ovarian cancer cell lines used in the present study. To test this hypothesis, OVCA cells were cultured with increasing concentrations of the PC4 inhibitor (50, 100 μM) for various durations (48 or 96 h). If PC4 is indeed involved in the final maturation of IGF-II, it is expected that inhibition of PC4 activity would result in reduced production of mature IGF-II(1-67) with a corresponding accumulation of big IGF-II(1-104). After 48 h of incubation with the cell permeable inhibitor, an accumulation of big IGF-II and a slight decrease in mature IGF-II(1-67) could be detected in the spent media from C13* cells by Western blot (Figure 20B). Furthermore, after 96 h this effect was even more evident with mature IGF-II levels being drastically reduced in a concentration-dependent manner. These data provide key evidence that PC4 is responsible for the proteolytic processing of IGF-II, a known cell survival mediator, in ovarian cancer cell lines. These results lend further insight into the possible role of PC4 in chemoresistance.

Figure 20: Processing of IGF-II by PC4 in OVCA cells

PC4 processes IGF-II to its mature form in human ovarian cancer cells. (A) Schematic representation of pro-IGF-II structure. Sequences of PC4 cleavage sites are indicated by downward arrows. (B) Inhibition of PC4 drastically reduces processing of proIGF-II. C13* cells were treated with increasing concentrations of PC4 inhibitor (0, 50, 100 μM) for 48 and 96 h. Processed fragments of IGF-II (big IGF-II and mature IGF-II) are detected in the spent culture media of OVCA cells by western blotting with IGF-II antibody.



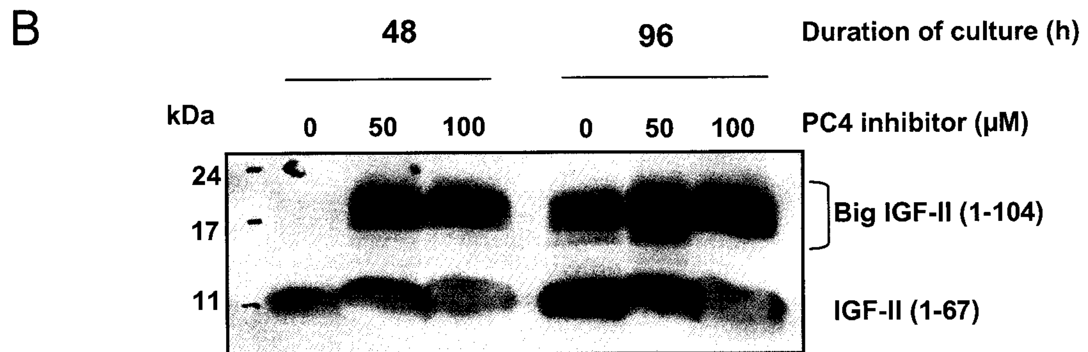


Figure 20

8. IGF-II attenuates the effects of the PC4 inhibitor

Previous results indicated that chemoresistant OVCA cells could be sensitized to CDDP by the PC4-specific inhibitor and that incubation of these cells with the same inhibitor resulted in a marked decrease in mature IGF-II secretion into the spent culture media. Taken together, these results suggested that perhaps the effect of the PC4 inhibitor on the chemoresistant cells was associated with decreasing mature IGF-II levels. To further test this hypothesis, C13* cells were pretreated (1 h) with the PC4 inhibitor, and then treated with cisplatin (10 μM). Recombinant mature IGF-II (10 nM) was added to the culture media 1 h after CDDP (depending on the experimental group) and cells were cultured for 24 h. Consistent with previous results, examination of nuclear morphology by Hoechst staining revealed that treatment with the PC4 inhibitor sensitized the resistant cells to CDDP, as evidenced by the increased number of apoptotic cells (Figure 21). Moreover, treatment with mature IGF-II abrogated the pro-apoptotic effects of the PC4 inhibitor, with cells in this treatment group having a 50% reduction in apoptosis compared to the inhibitor alone group ($6.1 \pm 0.11\%$ *vs*. $13 \pm 0.090\%$).

Since treatment with the PC4 inhibitor increased cisplatin-sensitivity of the resistant cells and this appeared to be manifested through IGF-II processing, our next objective was to examine whether CDDP directly influenced pro-IGF-II processing. Chemosensitive and –resistant cells were treated with increasing concentrations of CDDP for 24 h and IGF-II processing in the spent culture media was analyzed by Western blot. Because there is no house keeping marker in the spent media, we were unable to accurately control for protein loading and the Ponceau stained membranes

Figure 21: **IGF-II attenuates the effects of the PC4 inhibitor**

IGF-II attenuates the effects of the PC4 inhibitor. Chemoresistant (C13*) cells were treated with or without the PC4 inhibitor (50 μM), recombinant IGF-II (10 nM) was added to the culture media for 24 h and apoptotic response examined. *** $P<0.001$ (relative to control at same CDDP concentration) +++ $P<0.001$ (relative to PC4 inhibitor alone at same CDDP concentration). Data represents mean ± SEM of three independent experiments.

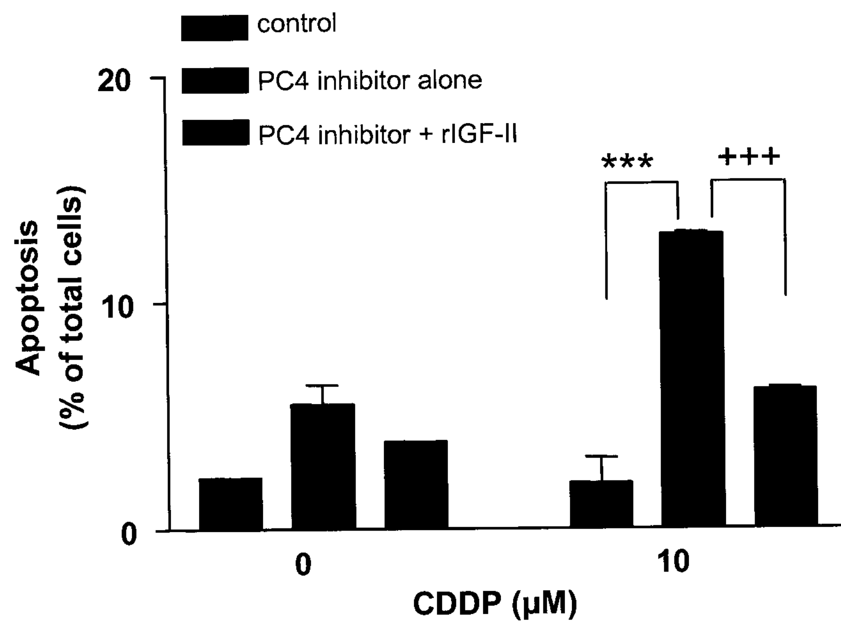
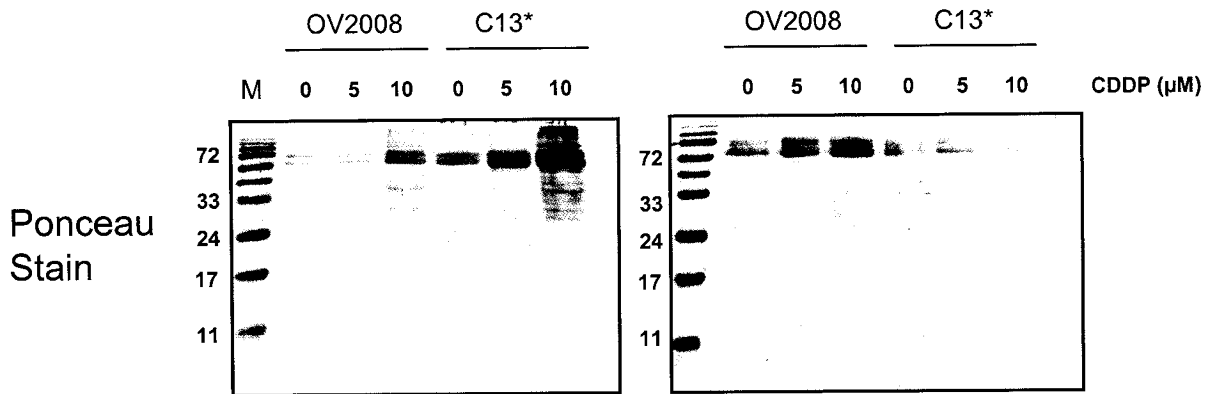


Figure 21

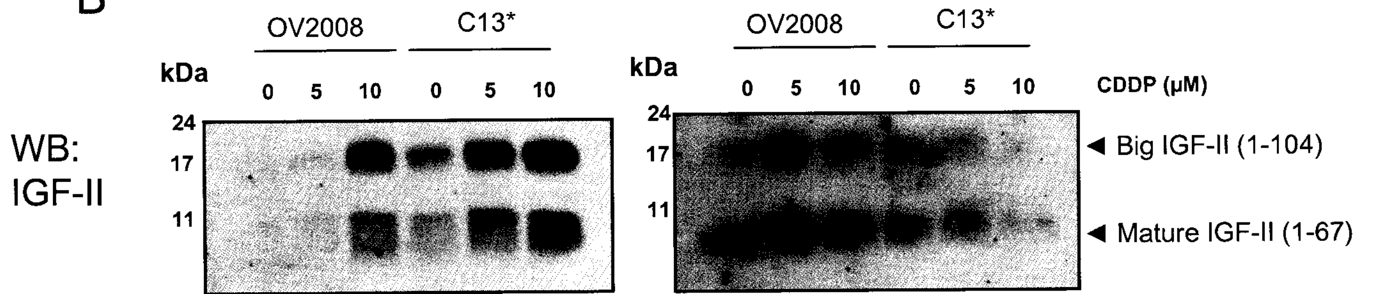
Figure 22: Lack of effect of CDDP on IGF-II processing

OV2008 and C13* cells were treated with CDDP (5, 10 µM) for 24 h and IGF-II in spent media was examined by WB. A) Representative Ponceau stained membranes while the lower panel B) shows the corresponding Western blot. Changes in mature IGF-II levels correlate with protein loading seen in upper panel. C) Effect of cisplatin on IGF-II processing. Data represented as ratio of big IGF-II to mature IGF-II in at least three independent experiments.

A



B



C

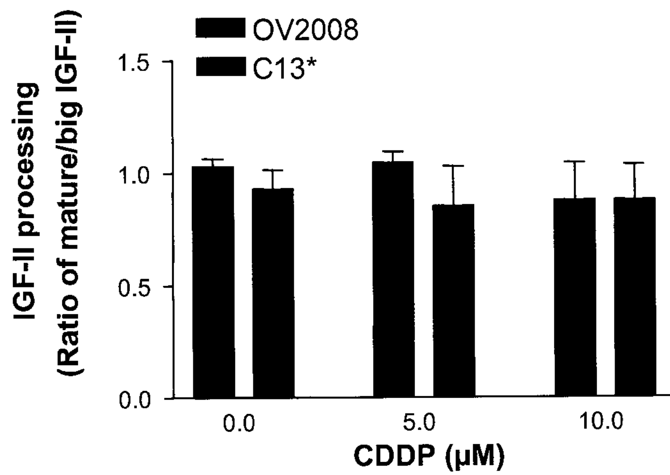


Figure 22

were the best indication of protein content in each lane. Unfortunately, no change in mature IGF-II levels could be detected (Figure 22).

9. IGF-II protects chemosensitive OVCA cells from cisplatin-induced apoptosis

Ligand binding of IGF-II to the IGF-I R provokes both mitogenic as well as anti-apoptotic effects and this phenomenon has been observed in multiple forms of cancer (LeRoith *et al,* 2003). The role of IGF-II in human ovarian cancer has not been extensively studied, however it has been reported that ovarian cancer cell lines may become dependent on the expression of IGF peptides and their autocrine/paracrine signaling (Kalli & Conover, 2003). In the present study, we investigated if IGF-II had an anti-apoptotic effect on chemosensitive cells. OV2008 cells were treated with or without CDDP (5 μM), and subsequently treated with recombinant IGF-II (10 nM; 1 h after CDDP) for 24 h to examine if addition of mature IGF-II could prevent the cells from undergoing cisplatin-induced apoptosis. Hoechst staining analysis indicated that addition of IGF-II to the culture media significantly reduced the apoptosis induced by the chemotherapeutic agent ($P<0.01$; Figure 23). Taken together, our results indicate that IGF-II can act as a cell survival factor to protect against cisplatin-induced apoptosis in human ovarian cancer cells *in vitro*.

Figure 23: IGF-II protects OV2008 cells from CDDP-induced apoptosis

IGF-II protects chemosensitive cells from cisplatin-induced apoptosis. OV2008 cells were treated with cisplatin (5 μM, 24 h) in the absence and presence of recombinant IGF-II (10 nM) ** $P<0.01$ (relative to control at same CDDP concentration). Data represents mean ± SEM of three independent experiments.

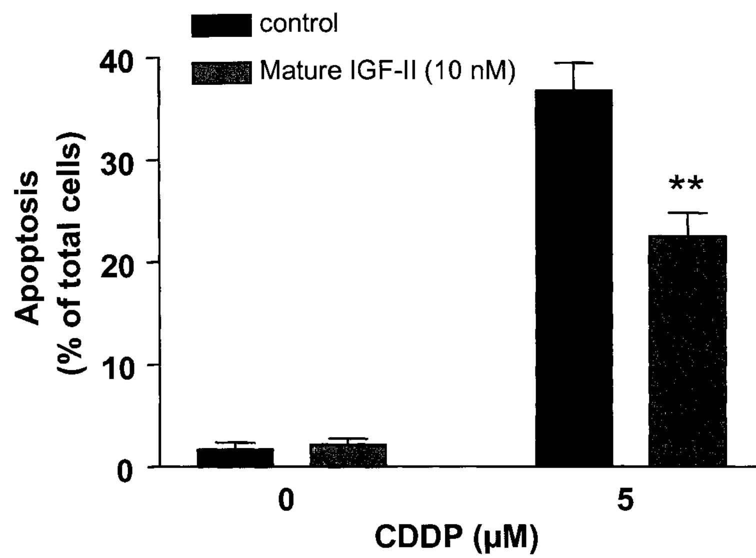


Figure 23

10. Human epithelial ovarian cancer tumours express PC4

To determine whether proprotein convertase 4 (PC4) is expressed in human ovarian carcinomas, immunohistochemistry was performed on ovarian tumour specimens obtained from patients during surgical debulking using the polyclonal PC4 antibody (PC4 606-06). Strong PC4 immunosignals were detected in the malignant epithelial cells of specimens from a stage III papillary serous carcinoma (B) as well as a clear cell carcinoma (D) with weaker or absent signals being seen in the stromal tissue (Figure 24). PC4 immunoreactivity was specifically localized to cytoplasm or perinuclear region of these cells.

Figure 24: **PC4 expression in human epithelial ovarian tumours**

Expression of PC4 in a papillary serous carcinoma (B) and clear cell carcinoma (D) was detected by immunohistochemistry with PC4 polyclonal antibody. Black arrows indicate PC4 immunosignals in tumour cells whereas the red triangles indicate stromal tissue. Adjacent sections were stained with hematoxylin and eosin (H & E) to indicate cellular morpholgy (A, C). Magnification X400. Images are representative of eight tumours examined (n=8).

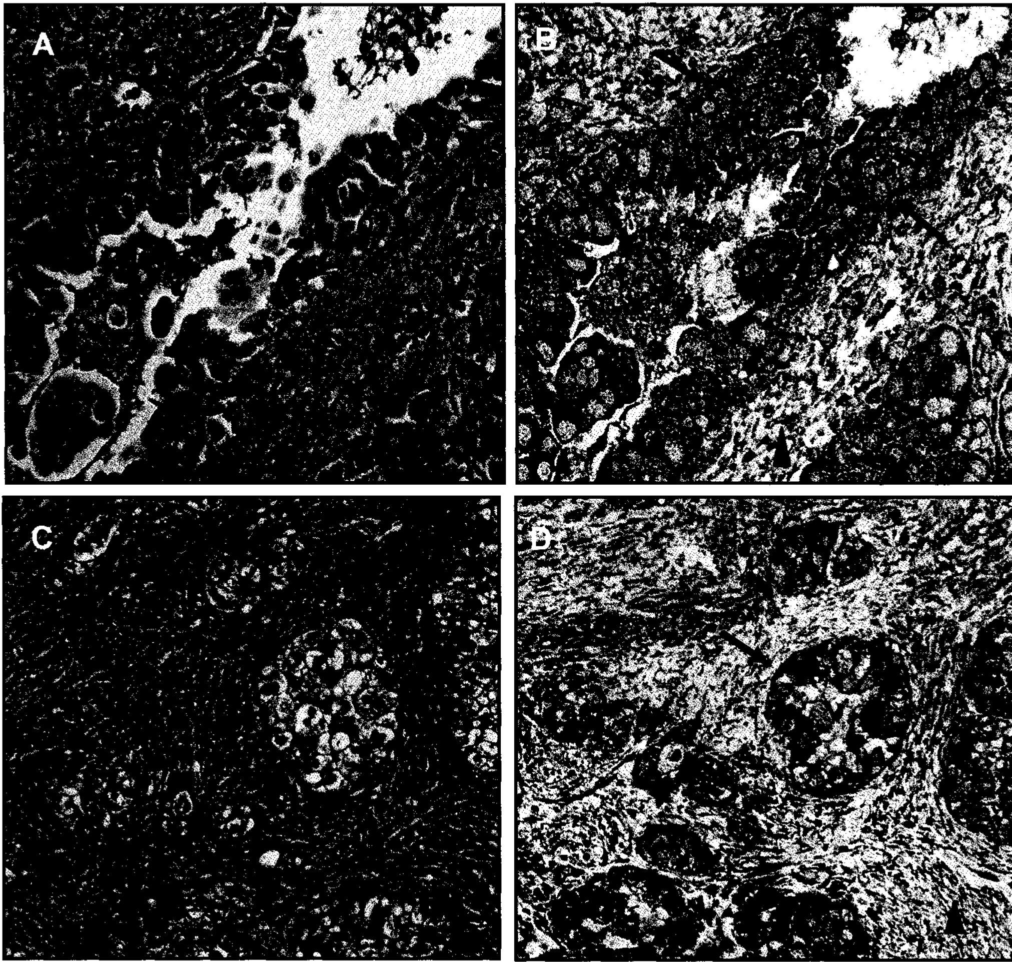


Figure 24

CHAPTER 4: DISCUSSION

i. Expression of PC family members in OVCA cells

The expression of proprotein convertase (PC) family members in many types and stages of cancers and in normal tissues have been shown at both the mRNA and protein level, although little has been reported on the expression of PCs in human ovarian cancer. In the present study, we have investigated which PCs are expressed in human ovarian cancer cells. Expression of Furin, PACE4, PC5 and PC7 in OVCA cells was expected since they are known to have a ubiquitous expression pattern (Seidah and Chretien, 1997). PC4 is expressed exclusively in gonadal tissues and has recently been shown to be expressed in trophoblast cells of the placenta, suggesting a reproductive function for this enzyme (Basak *et al*, 1999; Qiu *et al*, 2005). PC4 mRNA was detected in all four ovarian cancer cells examined and its presence is therefore consistent with its restricted localization in reproductive tissues. Interestingly, PACE4 was shown to be severely down-regulated in human ovarian cancer cells (Fu *et al*, 2003), but this was not the case in our study as PACE4 mRNA was easily detected by RT-PCR. The cell lines used in the present study were not examined by Fu *et al* and may be exceptions to their observation. PC1 and PC2 are restricted to dense-core secretory granules typically found in cells possessing a secretory pathway, including endocrine and neuroendocrine tissues. Contrary to the initial hypothesis, PC2 as well as its accessory protein 7B2, were expressed in the OVCA cell lines examined. Expression of the neuroendocrine marker 7B2 suggests the presence of secretory granules within this cell type. Overall these results concur with previous studies demonstrating the expression of proprotein convertases in

various tumours and established cancer cell lines (Mbikay *et al*, 1997; Cheng *et al*, 1997; Bassi *et al*, 2001; Jin *et al*, 1999). However, this study represents, to our knowledge, the first thorough evaluation of PC expression in human ovarian cancer cells.

Following the observation that PCs are expressed in cultured ovarian cancer cells, we next investigated whether the expression of any of the family members was regulated by the chemotherapeutic drug, cisplatin *in vitro*. As mentioned previously, chemoresistance is a key hindrance in the successful treatment of this disease and gaining a better understanding of the molecular mechanisms that underlie chemoresistance is critical for improving survival outcomes. Identifying and understanding the differences between chemosensitive and chemoresistant cell populations will provide important insight into the phenomenon of chemoresistance. We therefore used a pair of cisplatin-sensitive (OV2008) and –resistant (C13*) ovarian cancer cells, derived from the same genetic background as an *in vitro* model to examine the regulation of PCs in cisplatin-mediated apoptosis and their possible role in chemoresistance.

Since changes in mRNA levels is one mechanism by which protein content is regulated, we examined the effects of cisplatin on the mRNA levels of various PC family members by RT-PCR. Initially, the concentration-dependant effects of cisplatin were examined using real-time semi-quantitative RT-PCR. Optimization of the reaction conditions for several primer sets proved difficult and only 3 of 8 primer pairs yielded amplification of a single product (PC5, PC7 and the house-keeping gene β-actin). Successful PCR amplification heavily depends on the first few cycles to generate copies

of the template which are then exponentially amplified. One reason for difficulties with optimizing the reactions may be that the transcripts were low in abundance and therefore the short cycling times, inherent to the Real-Time method, were insufficient for the amplification reaction to get started. Additionally, each of the primer pairs being used were taken from the literature (Cheng *et al*, 1997) and designed to amplify products that were 400-700 bp in length so that the forward and reverse primers were separated by at least one intron to eliminate genomic contamination. Efficient amplification of products by Real-Time PCR typically uses primers that amplify products between 100-200 bp. Due to these difficulties, endpoint PCR was used successfully and the conditions were optimized accordingly (as described in Materials and Methods). Time-course studies revealed that the mRNA content for all of the PCs examined (Furin, PC2, PC4, PACE4, PC5, PC7) was not affected by CDDP. These results suggest that cisplatin does not regulate proprotein convertases at the mRNA level in ovarian cancer cells. Although cisplatin regulates various gene products at the transcriptional level through its DNA-damaging effects, in the present study there were no differences in cisplatin-induced changes in mRNA levels of any of the PCs between chemosensitive and -resistant ovarian cancer cells. This phenomenon has been observed with several important mediators of chemoresistance in ovarian cancer cells and is consistent with the observations of Asselin and colleagues, whereby cisplatin had no effect on mRNA abundance of XIAP, PI3-K and AKT (Asselin *et al*, 2001) in chemosensitive and resistant ovarian cancer cells.

ii. Effect of CDDP on PC protein content in ovarian cancer cells

Cisplatin-induced apoptosis in many cell types is associated with the down regulation of multiple cell survival factors (e.g. XIAP, intermediates of PI3K pathway) and a corresponding increase in pro-apoptotic mediators (e.g. p53, Fas, FasL,), ultimately increasing the apoptotic capacity of the cell (Asselin *et al.*, 2001; Fraser *et al.*, 2003a; Li *et al.*, 2001; Schneiderman *et al.*, 1999). However, whether changes in PC protein content are associated with cisplatin-induced apoptosis remains unknown and requires further investigation. In the present study, the effects of CDDP on Furin and PC2 protein content in OVCA cells were examined by Western blotting, and no significant changes were observed in both chemosensitive and -resistant cell lines, suggesting that neither of these PC family members are differentially regulated by cisplatin at the protein level.

A major challenge of the present study is the lack of specific antibodies for PC4. To date, there is only one antibody commercially available. However this polyclonal PC4 antibody immunoreacts with many proteins, making it difficult to assess which bands are specific to PC4 by Western analysis. In addition, this PC4 antibody immunoreacts with a non-specific band in the area of interest for PC4 (~54kDa; the putative molecular weight of the enzymatically active form). The non-specificity of this antibody may explain why no decrease in the putative PC4 54kDa band was observed following cisplatin or PC4 siRNA treatment in previous experiments. For these reasons, examination of PC4 protein levels by Western blot was abandoned and immunohistochemical methods using a second PC4 antibody were adapted.

A validated polyclonal PC4 antibody that recognizes the native form of the protein was used to examine the effects of CDDP on PC4 protein content by immunocytochemistry. CDDP caused a marked decrease in PC4 immunoreactivity in chemosensitive cells but not in their resistant counterpart while RT-PCR analysis suggested that this observation was likely the result of post-translational events since no differences in PC4 mRNA could be detected under the same experimental conditions (time-course or concentration response studies). Previous studies from our laboratory have shown that cisplatin-induced apoptosis in chemosensitive cells is associated with changes in nuclear morphology detectable by 12 h and significant by 24 h. Earlier events associated with apoptotic cell death, including caspase activation, FAK cleavage and XIAP down-regulation, can also be detected in these same cells as early as 6 h after cisplatin treatment (Sasaki *et al.*, 2002). The observed decrease in PC4 protein levels at 6 h in the chemosensitive cells is therefore temporally related to other earlier molecular events observed during cisplatin-induced cell death, suggesting that PC4 may play a role in the initiation of apoptosis.

To date, no putative regulators of PC4 have been identified that may explain the molecular mechanism by which CDDP down-regulates PC4. Within the cell, protein content is often regulated via proteosomal or lysosomal degradation. This may be one possibility for the cisplatin-induced changes in PC4 protein. Cisplatin has been shown to decrease the protein content of XIAP, an important determinant of chemoresistance through its inhibitory interactions with caspase-9 and-3, in chemosensitive ovarian cancer cells (Sasaki *et al*, 2000; Cheng *et al*, 2002). A recent study by Dan and colleagues

further demonstrated that the cisplatin-induced decrease in XIAP was associated with the ubiquitination and subsequent degradation via the proteasome pathway in ovarian cancer cells (Dan *et al*, 2004). Cisplatin may therefore target PC4 for degradation by increasing ubiquitination or sumoylation of the protein, however further research is needed to clarify which process is involved. Alternatively, it has been shown that cisplatin-resistant cell lines exhibit defects in lysosomal processing. Chauhan and colleagues demonstrated that the cisplatin-resistant counterpart of a human epidermoid carcinoma cell line exhibited slower uptake and degradation of radioisotope- tagged EGF compared to the sensitive cells, suggesting that the cisplatin-resistant cells may have defects in lysosomal acidification (Chauhan *et al*, 2003). It is possible that PC4 is degraded via the lysosome in the chemosensitive cells and that the lack of cisplatin-induced PC4 down-regulation in resistant cells is due to defects in lysosomal processing. However, further studies are required to confirm or refute these possibilities.

Additionally, the decrease in PC4 levels did not appear to be due to any general cytotoxic effects of cisplatin. Previous studies using the same cell lines under identical experimental conditions have shown that cisplatin treatment increases the expression of Fas and FasL (Schneiderman *et al.*, 1999), suggesting that protein synthesis is being maintained within the cells. Furthermore, expression of the housekeeping protein GAPDH is also maintained at steady levels following cisplatin treatment, even in cells undergoing apoptosis. Recognizing the limitations of the immunocytochemical method, these studies provided the rationale for further examination of the implications of modulating PC4 levels within the cell and the effects it may have on cisplatin sensitivity.

iii. Possible involvement of PC4 in cell survival

Proprotein convertases have been implicated in cell survival of cancer cells through the processing and activation of anti-apoptotic precursors, including growth factors, their receptors and integrins (Khatib *et al.*, 2002; Khatib *et al.*, 2001; Siegfried *et al.*, 2003). Khatib and colleagues demonstrated that expression of the general PC inhibitor α1-PDX in HT-29 colon carcinoma and Jurkat leukemia cells exaggerated serum deprivation-induced apoptosis, suggesting that proprotein convertases may play a role in the determination of cell fate (Khatib *et al.*, 2001). However, a general inhibitor was used in this study and it was unclear as to which PC family member was responsible for the observed phenotype. Using a PC4-specific inhibitor and siRNA in the present study, we have provided clear evidence for the involvement of PC4 in the direct regulation of cisplatin-induced apoptosis. This, to our knowledge, is the first evidence of an involvement of a specific PC family member in the determination of cell fate and cisplatin sensitivity. Our data supports the hypothesis that failure of cisplatin to decrease PC4 protein levels in chemoresistant cells may, in part, confer resistance to the drug. This observation is consistent with other cell survival determinants (e.g. XIAP, FLIP) in this system (Li *et al.*, 2001; Abedini *et al.*, 2004). This notion was further supported by the protective effects of PC4 observed in the over-expression studies and sensitization of chemoresistant cells to cisplatin through inhibition of PC4 activity and down-regulation of the gene product.

While increasing evidence has suggested that defects in intra- and extra-cellular apoptotic mechanisms are an important cause of resistance to cytotoxic agents, the precise action of cisplatin in human ovarian cancer cells, as well as the differences in response to the chemotherapeutic agent between sensitive and resistant cells, remains unclear. We have postulated that one mechanism by which cisplatin induces apoptosis in chemosensitive cells is through the down-regulation of the cell survival mediator PC4. Indeed, over-expression of PC4 significantly reduced cisplatin-induced apoptosis in the chemosensitive cells while down regulation of the gene product in the resistant cells mimicked the chemosensitive phenotype. Interestingly, molecular manipulation of PC4 through over expression, although significantly reducing the amount of cisplatin-induced apoptosis, did not completely protect the chemosensitive cells from the drug. This may be explained by the fact that cisplatin has multiple targets in the induction of cell death and the PC4 pathway may only account for one intracellular mechanism by which apoptosis is achieved. Alternatively, there may be species differences between rat and human PC4, and perhaps if the human PC4 cDNA had been available, more drastic effects would have been observed. The transfection efficiency of the expression vector is another factor to consider. Unfortunately this was not assessed in the present study since the control vector did not contain a LacZ or GFP insert, although it is possible that increasing the transfection efficiency might further decrease the level of apoptosis. In contrast, inhibition of PC4 activity or down-regulation of PC4 using siRNA, increased the incidence of CDDP-induced apoptosis to about 15% (compared to ~3%). These observations are consistent with many other studies carried out on proteins known to be key determinants of chemoresistance. Indeed, down-regulation of XIAP or AKT by anti-

sense or dominant-negative expression in chemoresistant cells sensitized the cells to cisplatin-induced apoptosis, although the level of apoptosis in both instances only reached 15% at the same concentration of cisplatin (10 μM; Sasaki *et al*, 2000; Fraser *et al*, 2003), suggesting that chemoresistance of human ovarian cancer cells is a result of cross-talk signaling through multiple pathways, and not one single molecule can fully explain the observed phenomenon.

Unlike the original study of Khatib *et al* (2001) which implicates a pro-survival role for PCs, the present studies employed a peptide inhibitor specific to PC4 to examine its involvement in ovarian cancer cell survival. Necessary experiments were carried out to ensure that the inhibitor was indeed specific for PC4. *In vitro* studies with various fluorogenic substrates indicated that the inhibition constant (Ki) for recombinant PC4 was in the nanomolar range, whereas those for other recombinant PCs were in the micromolar range, suggesting that this peptide has minimal inhibition activity towards other PC family members (Basak, unpublished). Qiu *et al* (2005) further confirmed the specificity of the inhibitor by demonstrating that the synthetic PC4 inhibitor had no effect on the processing of the IGF-I receptor, a known Furin substrate, whereas treatment with the Furin inhibitor (decanoyl-RVKR-CMK) prevented the maturation of pro-IGF-I receptor in HTR8/SVneo cells. The synthesis of most PCs as inactive precursors provides the cells with the means to regulate their enzymatic activities spatially and temporally, minimizing inappropriate protein activation. The PC4-specific inhibitor was designed to mimic the proform of the enzyme, which acts as a potent endogenous inhibitor by binding to the catalytic domain.

Accumulating evidence supports a role for PCs in the processing of molecules presumed to be involved in the pathophysiology of cancer, thus there is increasing demand for the development of specific inhibitors for therapeutic intervention. Many attempts to inhibit substrate processing by PCs *ex vivo* have been made, including the use of irreversible chloromethylketone inhibitors and reversible peptide inhibitors, but they are limited by their poor cellular permeability and cytotoxicity. Consistent with the present study, a recent investigation with a Furin inhibitor derived from the propeptide of the enzyme demonstrated effective suppression of the processing of pro-IGF-1R and pro-TGF-β1, two important Furin substrates, and significantly decreased cell proliferation, anchorage-independent growth and invasiveness potential of head and neck squamous carcinoma cells compared to the control treatment group (Lopez de Cicco *et al*, 2005). The specificity and ability of the PC4 peptide inhibitor to readily enter the cells offers one the opportunity to examine the role of PC4 in cell survival and chemosensitivity in human ovarian cancer.

iv. Role of PCs in chemoresistance of cancer cells

The role of proprotein convertases in the regulation of chemosensitivity is largely unknown and has not been documented. We speculate that the role of PCs in this process can be explained in part by their involvement in the processing of growth factors and their receptors. The interaction of several growth factors (e.g. PDGF, EGF, and IGF) with their receptors induces intracellular signaling cascades that promote cell survival. It is well documented that PDGF, EGF as well as IGF signaling (tyrosine phosphorylation of the receptor) can lead to AKT phosphorylation/activation (Langley *et al*, 2004;

Kaplan-Albuquerque *et al*, 2003; Zhang *et al*, 2004; Qiu *et al*, 2004). We and others have also established that AKT is an important determinant of chemoresistance in ovarian cancer cells and that over-expression of this protein kinase confers resistance to cisplatin-sensitive OVCA (Cheng *et al*, 2002; Fraser *et al*, 2003). Many growth factors are synthesized as precursor proteins and must be proteolytically processed to their mature forms to become biologically active (Khatib *et al*, 2001; Khatib *et al*, 2002; Siegfried *et al*, 2003). Recent studies have demonstrated that PC4 is responsible for the processing of pro-IGF-II into its active form in the human placental trophoblast cell line and that mature IGF-II can increase phospho-AKT content to a greater extent then the proform of the protein (Qiu *et al*, 2005). It is hypothesized that the processing of these growth factors by PCs leads to increased activation of their receptors and ultimately induction of downstream signaling cascades and promotion of cell survival.

v. Role of IGF-II in the regulation of apoptosis and chemosensitivity

The insulin-like growth factor (IGF) system plays a pivotal role in processes controlling cell proliferation, survival under stress and maintenance of the transformed phenotype (LeRoith & Roberts, 2003), although its possible role in ovarian malignancies requires further investigation. Direct evidence exists for the involvement of this growth factor system in human epithelial ovarian cancer (Kalli & Conover, 2003; Sayer *et al.*, 2005). IGF-II expression is 300-fold higher in ovarian cancers compared to normal surface epithelium, and IGF-II is associated with more aggressive phenotypes, higher stage at diagnosis, and shorter overall survival (Sayer *et al*, 2005). Tumour progression and metastasis associated with IGF-II over-expression may be manifested through

multiple mechanisms including loss of imprinting, loss of heterozygosity, excessive transcriptional activation, loss of transcriptional suppression, and alteration in IGF-binding proteins (Toretsky & Helman, 1996). In addition to dysregulation of IGF-II gene expression, abnormal processing of IGF-II may also alter its biological activity. Current data suggests that increased post-translational processing of IGF-II may also contribute to its anti-apoptotic actions. The ability of IGF-II to induce potent cell survival signaling cascades in various cell types (Linnerth *et al.*, 2005; Peruzzi *et al.*, 1999; Vincent & Feldman, 2002), led us to postulate that the involvement of PC4 in chemoresistance may be mediated through the processing and ultimately the signaling of the mature IGF-II. This hypothesis is consistent with findings that mature IGF-II suppresses apoptosis induced by chemotherapeutic agents (Alexia *et al.*, 2004; Leng *et al.*, 2001; Lund *et al.*, 2004). Lund and colleagues demonstrated that inhibition of IGF-II by a neutralizing antibody significantly increased both etoposide and cisplatin-induced apoptosis in human hepatocarcinoma cells. Furthermore, addition of recombinant mature IGF-II rendered human colon cancer cells resistant to butyrate-induced apoptosis (Leng *et al*, 2001). However, whether IGF-II signaling suppresses cisplatin-induced apoptosis in our cell type is unknown. The ovarian cancer cell lines used in the present study express the necessary components for IGF-II signaling. Moreover, addition of recombinant IGF-II(1-67) to the culture media significantly reduced cisplatin-induced apoptosis in the chemosensitive cells, suggesting that IGF-II signaling may in part, confer resistance. Although it is believed that the anti-apoptotic effects of IGF-II are mediated principally through the IGF-IR, other receptors responsive to IGF-II (IGF-II R, IR-B) are present in the cells and may play a role in the observed effects. Although we have postulated that

the anti-apoptotic effects of IGF-II are mediated through the P13K/AKT pathway, it should be noted that other cell survival pathways may be involved, including those of MAPK and 14-3-3 proteins (Peruzzi *et al*, 1999; Parizzas *et al*, 1997). Future experiments should focus on delineating which is the predominant signaling cascade involved in IGF-II-mediated chemoresistance in human ovarian cancer cells.

vi. PC4-mediated chemoresistance

Although there is much redundancy among PC substrates, PC4 has been shown to be the sole processor of precursors of several key cell survival mediators. Present in both the testis and ovary, pituitary adenylate cyclase-activating polypeptide (PACAP) is processed solely by PC4 in these tissues (Li *et al.*, 2000; Li *et al.*, 1998). Increasing evidence supports the role of PACAP in cell survival, highlighting its protective effects against apoptosis induced by various stressors, including serum deprivation, ceramide, and ethanol, although the majority of these studies involve neuronal cell types where PACAP is in high abundance (Gutierrez-Canas *et al.*, 2003; Vaudry *et al.*, 2003; Vaudry *et al.*, 2002). In a more recent study, PC4 has been shown to participate in the proteolytic processing and activation of pro-IGF-II (Qiu *et al.*, 2005). IGF-II is synthesized as a biologically inactive pro-IGF-II peptide (156 aa) and while IGF-II protein is found in various molecular weights; the most active form, with regard to binding to its receptors, is 7.5 kDa fragment, IGF-II(1-67) (Kiess *et al*, 1994). Accumulation of this functional isoform can be detected in the spent culture media of ovarian cancer cells and has been shown to be a result of proteolytic processing by PC4 as evidenced by its drastic reduction following treatment with the PC4 inhibitor.

In the present study, we put forth the idea that PC4-mediated chemoresistance is associated with processing of pro-IGF-II to its mature form, but the current evidence is only correlative. Unfortunately, we were unable to demonstrate a direct link between cisplatin and decreased IGF-II processing, only that cisplatin down-regulates PC4 protein content in chemosensitive cells and that manipulation of this cell survival factor influences cisplatin-sensitivity in human ovarian cancer cells. Although there appeared to be some cisplatin-induced changes in mature IGF-II, they were negligible when corrected for uneven protein loading and taken as a ratio of mature to big IGF-II protein contents. The ovarian cancer cells used in the present study express and secrete IGF-II, although 48 to 96 h of culture were required to detect sufficient IGF-II accumulation and changes in processing with the PC4 inhibitor. Following CDDP treatment, a significant number (~50%) of chemosensitive cells have undergone apoptosis by 24 h. Thus the window of opportunity to examine the effects of CDDP on IGF-II processing is difficult to attain as this duration is insufficient to detect changes by a gross measure such as Western blot. The IGF-II receptor regulates the levels of IGF-II in the microenvironment of the cell by binding, internalizing and targeting it for lysosomal degradation. It is possible that the IGF-II receptor in the ovarian cancer cells used in the present studies may be influencing the levels of IGF-II present in the media and prevents its detection during the first 24 h and that longer culture duration allowed IGF-II receptor saturation and detectable accumulation of the processed products in the media.

The IGF-II gene is maternally imprinted (Rainier *et al.*, 1993), meaning that normal cells only express IGF-II through the paternal allele. Interestingly, loss of imprinting (LOI) has been reported in approximately 17% of malignant ovarian epithelial tumours (Chen *et al.*, 2000; Kim *et al.*, 1998), although the LOI status of IGF-II in the cell lines used in the present study is not known and requires further investigation. If present, this condition could potentially increase IGF-II levels by allowing transcription from both alleles. Over-expression of various PC family members has been noted in many types of malignant tumours (Bassi *et al.*, 2001; Cheng *et al.*, 1997; Jin *et al.*, 1999; Mbikay *et al.*, 1997), while over-expression of PC4 has not been reported in ovarian cancer. High levels of PC4, as detected in the ovarian cancer cells used in the present study, in combination with increased levels of IGF-II could result in an established autocrine/paracrine loop critical for cell survival. Whether PC4 is in fact over-expressed in ovarian malignancies compared to normal surface epithelium has not been determined although this could be done by RT-PCR. The cell survival determinants PC4 and IGF-II provide novel targets for therapeutic intervention and a better understanding of their signaling pathway in ovarian carcinoma will hopefully increase successful treatment outcomes.

vii. Conclusions and Future Directions

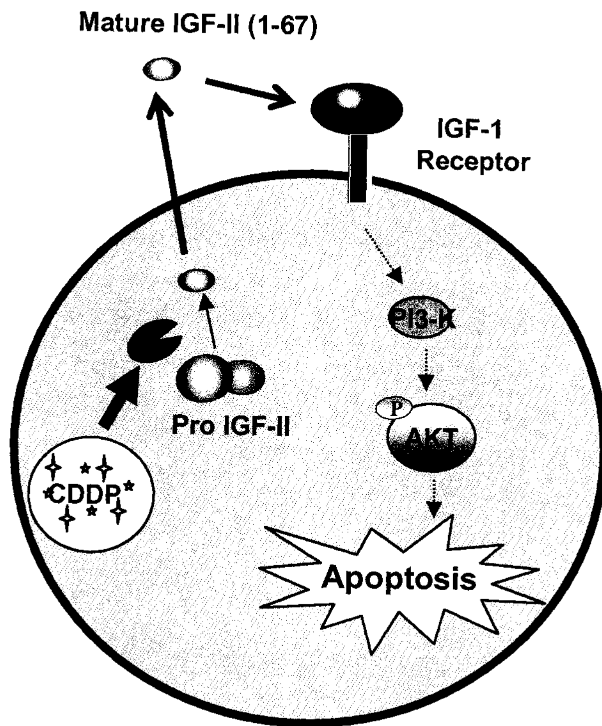
Using a pair of chemosensitive and –resistant cells as an *in vitro* model we have investigated in this thesis, the role and regulation of proprotein convertases in human ovarian cancer cells. We have tested the hypothesis that PCs are anti-apoptotic mediators and determinants of chemoresistance in human ovarian cancer cells and have demonstrated that one family member, PC4 exerts such a role. This study is, to our knowledge, the first to report the expression of PC4 in human ovarian cancer cells as well as to demonstrate its involvement in the regulation of cisplatin sensitivity. Through molecular manipulations of PC4, we were able to recapitulate the events following cisplatin treatment of chemosensitive cells. We have provided evidence that over-expression of PC4 protects chemosensitive cells from cisplatin-induced apoptosis while inhibition or down-regulation of the protease sensitizes the resistant counterpart cells to the chemotherapeutic agent. Moreover, we have demonstrated that these effects may be mediated through the processing, activation and subsequent signaling of mature IGF-II (Figure 25). These findings provide novel insight into the intracellular mechanisms of cisplatin resistance and provide new molecular targets for therapeutic intervention of drug-resistant ovarian cancer.

In addition to the future experiments described in the discussion, it is necessary to establish that the observations of the current study are not the result of a cell line-specific phenomenon. A single pair of cisplatin-sensitive (OV2008) and –resistant (C13*) ovarian cancer cells were employed and it will be imperative to examine additional pairs of sensitive and resistant strains. In addition, a major criticism of using cell lines is that

Figure 25: Hypothetical model of PC4-mediated chemoresistance

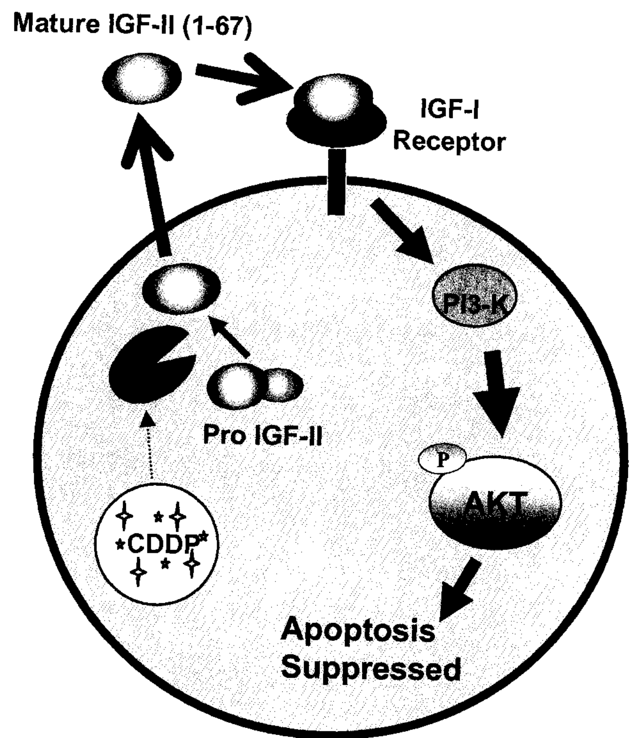
Hypothetical model for the involvement of PC4 in the chemoresistance of human ovarian cancer cells. (A) In chemosensitive cells, cisplatin down-regulates PC4 thus decreasing the processing of pro IGF-II to mature IGF-II and ultimately suppressing cell survival signals in the cell. (B) In the chemoresistant cells, cisplatin has no effect on PC4 and IGF-II is processed efficiently, contributing to a potent induction of cell survival cascades within the cell.

A



Chemosensitive Cell

B



Chemoresistant Cell

Figure 25

they may have acquired certain adaptive changes for growth in culture that no longer makes them representative of the physiological tumour cells. While the experiments presented in this thesis made use of established ovarian cancer cell lines, it will be important to examine the effects of PC4 manipulation in primary culture of ovarian cancer cells to confirm if the same phenomena are observed. Specifically, down-regulation of PC4 or inhibition of its activity in primary culture cells would lend further support for a role of PC4 in cell survival while the same experiments in primary culture of recurrent chemoresistant tumour cells would establish its role in determination of chemosensitivity. Although this model has a number of limitations including a finite proliferative potential of cultures, the inability to generate large amounts of cells, and phenotypical instability and variance, it will provide clear evidence for the involvement of PC4 in this disease.

Furthermore, it will be important to demonstrate the proof of principle that PC4 is indeed a determinant of chemoresistance *in vivo*. This hypothesis could be tested using a xenograft mouse model in which ovarian cancer cells are injected into nude mice to induce tumour formation, and then examine whether treatment with the PC4 inhibitor leads to chemosensitization and tumour regression. Particular attention should be made to which cell lines are used, as evidence for variations in histopathological features of the derived tumours from various ovarian cancer cell lines have been reported (Shaw *et al*, 2004). Several cell lines (A2780s, A2780cp, ES-2, HEY, and OCC1) produced undifferentiated adenocarcinomas, while others produced histologically distinct tumours including clear cell (SKOV3, OVCA 429) and endometrioid tumours (OV2008). In

addition, it will be of interest to examine tumour specimens or cells from ascites fluid of patients with recurrent disease who have previously undergone chemotherapy and compare them with the PC4 levels seen in chemoresponsive specimens. These studies will provide important insight into the pathophysiological relevance of PC4 in human ovarian cancer that cannot be achieved through cell line experiments.

The results of the present study suggest that PC4 represents a novel therapeutic target for molecular intervention and ideally, treatment of drug-resistant ovarian cancer. However, in order for this to occur, the above mentioned studies are necessary and further characterization of the PC4-inhibitor in humans is required. Development of methods to target the PC4-inhibitor selectively to the ovarian tumour may be one mechanism by which processing of key substrates by PC4 could be interrupted. Additionally, we have demonstrated that IGF-II can alter the chemotherapeutic response of ovarian cancer cells to cisplatin, and it may represent another upstream mediator of known cell survival signaling cascades that could be targeted to disrupt anti-apoptotic signals in ovarian malignancies.

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CURRICULUM VITAE

Hillary M. Boulay

EDUCATION

Sept. 2003-Feb. 2006	M.Sc. Cellular and Molecular Medicine (Physiology), University of Ottawa, Canada
Sept. 1998-Dec. 2002	B.Sc. (Hon.) Biomedical Toxicology, University of Guelph, Canada

RESEARCH AND PROFESSIONAL EXPERIENCE

Sept. 2003 - present

Graduate Student

Ottawa Health Research Institute

Investigated the role and regulation of proprotein convertases in chemoresistance of human ovarian cancer cells.

Jan. 2003 - Aug. 2003

Research Assistant

Agriculture and Agri-Food Canada, ECORC

Investigated and described a new fungal species (*Hirsutella uncinata*) using molecular techniques (PCR based assays) and microscopy.

May 2002 - Aug. 2002

Research Assistant

Agriculture and Agri-Food Canada, ECORC

Enhanced molecular skills and microbiology techniques through isolation, extraction, purification and DNA sequencing of diverse fungal isolates for a multi-partner research project.

May 2001 – Aug. 2001	**Environmental Management Systems Coordinator** *Communications Research Centre (Industry Canada)* Reviewed and assessed existing environmental management practices of the organization to build a foundation for ISO 14001 compliant EMS.
Sept. 2000 – Dec 2000	**Fungal Pathogens Research Assistant** *Agriculture and Agri-Food Canada, ECORC* Investigated and organized data collection for preliminary investigations for the development of a microchip-based DNA microarray for crop fungal pathogens.
Jan. 2000 – April 2000	**Fungal Pathogens Research Assistant** *Agriculture and Agri-Food Canada, ECORC* Development of molecular skills (DNA extraction, PCR, sequencing) while researching fungal pathogens in agriculture ecosystems.
May 1999 – Aug. 1999	**Analyst** *Transport Canada, Environmental Affairs* Preparation of Annual Glycol De-icing Reports and Aircraft De-icing Service License and Glycol Mitigation Plan for national airports. Promotion and implementation of Green Commute Program for employees.

AWARDS AND ACCOMPLISHMENTS

Sept. 1998- 2002	University of Guelph, Dean's Honor List, in recognition of academic excellence

LABORATORY SKILLS

Technical skills include: RNA and DNA extraction, RT-PCR, Real-Time PCR, quantification and separation by gel electrophoresis, plasmid cDNA purification and amplification using *E.coli,* Western analysis, protein extraction, immunohistochemistry, mammalian cell culture, cellular manipulations (transfection of cDNA and siRNA), fungal culture preparation for growth, storage and preservation, phylogenetic systematics for molecular data, basic microscopy.

2003- present Completion of WHMIS and MSDS training
(Ottawa Health Research Institute)

Post secondary courses in molecular biology, pharmacology, toxicology, human physiology and biochemistry.

COMPUTER SKILLS

Regular use of Microsoft windows, Microsoft Office, GraphPad Prism 3 statistical analysis software, Scion Imaging, Adobe Acrobat and Photoshop, MegAlign and Sequencher sequence editing software, Internet (including NCBI Pubmed search engine)

PUBLICATIONS

Boulay HM, Qiu Q, Fraser M, Senterman M, Basak A, Tsang BK. Proprotein convertase 4 is a cell survival mediator and determinant of chemoresistance in human ovarian cancer cells. (Submitted to Cancer Research)

Seifert KA, and **Boulay H.** (2004) *Hirsutella uncinata*, a new hyphomycete from Australia. *Mycologia* 96: 929-934.

Sholberg PL, Haag PD, Hambleton S and **Boulay H**. (2003) First report of brown rot in wine grapes caused by *Monolinia fructicola* in Canada. *Plant Disease* 87: 1268.

RESEARCH PRESENTATIONS

Nov. 2005	**Boulay HM** Proprotein convertase 4: a cell survival mediator and determinant of chemoresistance in human ovarian cancer cells. (Oral presentation) Ottawa Health Research Institute Research Day, Ottawa, Ontario, Canada
May 2005	**Boulay HM**, Qiu Q, Fraser M, Basak A, Tsang BK. Proprotein convertase 4: a cell survival mediator and determinant of chemoresistance in human ovarian cancer cells. Canadian Workshop on Human Reproduction and Reproductive Biology, Annual Meeting, Ottawa, Ontario, Canada
May 2004	**Boulay HM,** Khatib AM, Tsang BK. Proprotein convertases and cell survival in chemoresistant human ovarian cancer. Canadian Workshop on Human Reproduction and Reproductive Biology: From Physiology to Genes to Therapy, Ottawa, Ontario, Canada