The Role and Regulation of Mitochondrial Dynamics in Cisplatin Resistance in Human Gynecologic Cancer Cells

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Contribution of Co-Authors

All studies were carried out under the supervision of Dr. Benjamin Tsang. All experimental work was conducted by Mr. Bao Kong, except those noted below.

Figure 4.1E. Influence of CDDP on contents of mitochondrial fission proteins (Drp1 and Fis1) and mitochondrial fusion proteins (Mfn1, Mfn2 and Opa1) in CECA cells. This experiment was initiated by Mr. Bao Kong. Ms. Ella Fung was an undergraduate summer student in Dr. Tsang's laboratory, under the direct supervision of Mr. Bao Kong. She performed 80% of the experiments documented in this figure.

Figure 4.3. Comparison of Opa1 splice forms in CECA cells. Ms. Qi Wang was a Ph.D student in Dr. Tsang's laboratory. She performed the cell culture and qPCR for this experiment as documented in this figure.

Figure 4.4C. Forced expression of Oma1 in CECA cells. Mr. Kai Xue was an exchange Ph.D student in Dr. Tsang's laboratory. He amplified the myc tag-Oma1 cDNA and control cDNA (Origene), which were used in this experiment.

Abstract

Cervical cancer (CECA) and ovarian cancer (OVCA) rank first and third in the number of new cases diagnosed among gynecologic cancers，and chemoresistance severely limits their treatment success. The underlying mechanism of chemoresistance is multi-factorial and partly due to defects in drug-induced apoptosis. Cisplatinum (CDDP) -induced, p53-mediated mitochondrial cell death is controlled by Akt and is a determinant of chemosensitivity in gynecologic cancer cells. Mitochondria dynamics (fusion and fission) are involved in the regulation of mitochondria-mediated apoptosis. The tumor suppressor prohibitin 1 (Phb1) is involved in long from Opa1 (L-Opa1) processing and p53-regulated apoptosis. Whether mitochondrial fusion protein Opa1 and its protease Oma1 as well as Phb1 are involved in the regulation of chemoresistance in CECA and OVCA cells are not known.

The overall objective of my research is to increase the current understanding on the regulation of mitochondrial dynamics and on its role in chemoresistance in gynecologic cancer cells. We hypothesize that CDDP induces Phb1 binding to phosphorylated p53 (p-p53) and Bak, resulting in Bak activation and mitochondrial outer membrane permeabilization (MOMP). These responses also induce Oma1-mediated Opa1 processing, mitochondrial fragmentation and apoptosis but are inhibited by high Akt level in chemoresistant cells.

Here we present evidence that CDDP induces Oma1 activation, L-Opa1 processing and mitochondrial fragmentation in chemosensitive but not in chemoresistant cells. Silencing p53 expression attenuated CDDP-induced L-Opa1 loss, mitochondrial fragmentation and apoptosis in chemosensitive cells, while reconstitution of p53 in p53-

deficient (mutant or null) chemoresistant cells induced Oma1 activation, L-Opa1 processing and changes in mitochondrial dynamics irrespective of the presence of CDDP. In response to CDDP, p-p53 (ser15) dissociates Phb1 from Opa1-Phb1 complex and binds to Bak in chemosensitive but not chemoresistant cells. Inhibition of Akt is required for CDDP to induce L-Opa1 processing, mitochondrial fragmentation and apoptosis in chemoresistant cells.

Our study suggests a mechanism that p53 regulates L-Opa1 processing and mitochondrial fragmentation in chemosensitive cells induced by CDDP, while this pathway is suppressed in chemoresistant cells. Dysregulated mitochondrial dynamics may in part be involved in the pathophysiology of CDDP resistance. Inhibiting Akt activity and inducing Opa1 processing may serve as novel therapeutic strategies for these gynecologic cancers.

THESIS FORMAT

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

Chapter 1 (Introduction) provides a review of the current knowledge so as to provide the background and rationale of our study, which covers the following aspects: 1) overview of ovarian cancer and cervical cancer, 2) Cisplatin and its anticancer effects, 3) the apoptosis pathways, 4) the tumor suppressor p53, 5) the role of Akt in cell survival, and 6) the regulation of mitochondrial dynamics.

Chapter 2 (Objectives and Hypotheses) contains the overall and specific objectives, as well as the overall and specific hypotheses.

Chapter 3 (Materials and Methods) describes the reagents and experimental procedures used in the experiments designed to test our hypothesis.

Chapter 4 (Results) contains the findings that we have obtained during our study on how mitochondrial dynamics are regulated and contribute to chemoresistance in gynecologic cancer cells.

Chapter 5 (Discussion) presents an in depth analysis of our results, including how these results contribute to the related field, experimental limitations and shortcomings of current results, as well as future plans that may be taken to further our understanding of this field.

Chapter 6 (References) provides the detailed information of citations in this thesis.

Chapter 7 (Appendices) contains the tables summarizing the characteristics of the cell lines, the antibodies and primers used in this thesis.

Chapter 8 (Curriculum Vitae) is an overview of the author's education, work experience, publications and skills.

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LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette Family
AIF	Apoptosis inducing factor
Akt	v-akt murine thymoma viral oncogene homolog
Apaf-1	Apoptotic protease activating factor-1
ARF	Adenosine Diphosphate Ribosylation Factors
ATF6	Activating transcription factor 6
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia mutated and Rad3-Related protein
BAD	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
Bcl-XL	B-cell lymphoma-extra large
BER	base excision repair
BH	Bcl-2 homology
Bid	BH3-interacting domain death agonist
Bim	Bcl-2 interacting mediator of cell death
BRAF	B-raf murine sarcoma viral oncogene homolog
BRCA 1	Breast cancer susceptibility gene 1
BRCA 2	Breast cancer susceptibility gene 2
BRCP	breast cancer resistance protein

CA-125	Cancer antigen 125
CDDP	Cis-diamminedichloroplatinum (II)
Cdk1	Cyclin-dependent kinase 1
cDNA	complementary DNA
CECA	cervical cancer
CHK1	checkpoint kinase 1
CIN	cervical intraepithelial neoplasia
CSC	cancer stem cell
CTL	control
Ctr1	copper transporter protein 1
Cu	copper
DDR	DNA damage response
DIABLO	Direct inhibitor of apoptosis protein binding protein with low pI
DISC	death-inducing signaling complex
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DN-Akt	dominant negative Akt
dNTP	Deoxyribonucleotide triphosphate
DR	direct repair
Drp1	dynamin-related protein 1
EDTA	Ethylenediaminetetraacetic acid
ER	endoplasmic reticulum

FADD	Fas-Associated protein with Death Domain
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FIGO	International Federation of Gynecology and Obstetrics
Fis1	Fission 1
FLICE	FADD-like interleukin-1 beta-converting enzyme
FLIP	FLICE-like inhibitory protein
FOXO	Forkhead transcription factor
5-Fu	5-Fluorouracil
G2 phase	Gap 2 phase of the cell cycle
GAPDH	Glyceraldehyde phosphate dehydrogenase
GPCR	G-protein-coupled receptors
HA	Hemagglutinin
HDM2	Human Double Minute 2
HPV	Human papilloma virus
HR	homologous recombination
HtrA2	High Temperature Requirement Protein A 2
IF	Immunofluoresence
IP	immunoprecipitation
IP3R	inositol 1,4,5-triphosphate receptor
IV	intravenous
kDa	kilodalton
KRAS	Kirsten rat sarcoma viral oncogene homolog

L-Opa1	long form of Opa1
MDM2	mouse Double Minute 2
MDR	Multi-drug resistance
MEF	mouse embryonic fibroblast
Mfn1	Mitofusion 1
Mfn2	Mitofusion 2
MMR	mismatch repair
MOI	multiplicity of infection
MOMP	mitochondrial outer membrane permeabilization
MPP	mitochondrial processing peptidase
mRNA	messenger RNA
MRP	non-P glycoprotein MDR
mtDNA	mitochondrial DNA
mTOR	mechanistic target of rapamycin
Na_3VO_4	sodium orthovanadate
NER	nucleotide excision repair
NOXA	NADPH oxidase activator
NuMA	Nuclear Mitotic Apparatus
OHRI	Ottawa Hospital Research Institute
Oma1	overlapping activity with M-AAA protease 1
Opa1	optic atrophy 1
OVCA	Ovarian cancer
p53AIP1	p53-regulated apoptosis-inducing protein 1

PAGE	Polyacrylamide gel electrophoresis
Pap	Papanicolaou
PARC	p53-associated Parkin-like cytoplasmic protein
PARP	Poly (adenosine diphosphate) polymerase
PERK	PRKR-like endoplasmic reticulum kinase
Pgp	P-glycoprotein
Phb1	Prohibitin 1
Phb2	Prohibitin 2
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKA	protein kinase A
PKB	protein kinase B
PMSF	phenylmethylsulfonyl fluoride
p-p53	phosphorylated p53
PPM1D	Protein phosphatase magnesium-dependent 1D
PTEN	Phosphatase and tensin homolog
PUMA	p53-upregulated mediator of apoptosis
qPCR	quantitative PCR
Ras	Rat sarcoma
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecylsulfate

SEER	Surveillance, Epidemiology, and End Results program
Ser	Serine
siRNA	small interfering RNA
Slp-2	synaptotagmin-like protein 2
Smac	Second mitochondria-derived activator of caspases
S phase	Synthesis phase of the cell cycle
rtTA	reverse tetracycline-controlled transactivator
SV40	Simian vacuolating virus 40
Thr	Threonine
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Tom20	translocase of outer mitochondrial membrane 20 homolog
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPR	unfolded protein response
USA	the United States of America
WB	Western blot
wt-p53	wild type p53
XIAP	X-linked inhibitor of apoptosis protein

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CHAPTER I: INTRODUCTION

1. Ovarian cancer and cervical cancer

1.1 Ovarian cancer

Ovarian cancer (OVCA) is the most lethal gynecological malignancy, ranking fifth in cancer deaths among women, and resulting in the death of approximately 1,750 women per year in Canada (among 2,800 new cases per year, www.cancer.ca 2015). The most common symptoms of OVCA include: bloating, pelvic or abdominal pain, trouble eating or feeling full easily, and urinary symptoms. However, these symptoms are not organ specific, and can either be caused by non-cancerous disease or by cancers of other organs. The diagnosis of OVCA often needs a physical exam, imaging test (e.g., ultrasound), and blood test (usually cancer antigen 125), and the confirmation of the diagnosis needs a biopsy, which is usually taken during laproscopic surgery.

1.1.1 Histological subtypes

Classically, there are three main types of OVCA, which are defined by where the cancer cell originated in the ovary. 1) About 85% to 90% of OVCA are epithelial ovarian carcinomas, which include serous, mucinous, endometrioid, clear cell and undifferentiated epithelial ovarian carcinomas(Gore 1994). 2) Less than 10% of OVCA are germ cell tumors, which include teratomas, dysgerminomas, endodermal sinus tumors, and choriocarcinomas(Gore 1994). 3) The third type of OVCA is stromal cell tumor, which contains granulosa cell tumors (the most common type), granulosa-theca tumors and Sertoli-Leydig cell tumors.

The two type system of OVCA proposed by Shih and Kurman in 2004 is now becoming accepted (Shih Ie and Kurman 2004). Type 1 OVCA, encompasses the low grade serous, clear cell, low grade endometrioid, and mucinous carcinomas. They are relatively slowly growing with a low mitotic index, confined to the ovary or peritoneum upon diagnosis, and believed to be poorly responsive to platinum-based chemotherapy (first-line chemotherapy for OVCA). These cancers are genetically stable and have wild type *TP53* and Breast cancer susceptibility gene (*BRCA) 1* and *2* genes, but are characterized by mutations in a number of genes, most commonly Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and *BRAF* murine sarcoma viral oncogene homolog (*BRAF*) (Kohn and Hurteau 2013).

Type 2 OVCA encompass the high grade serous cancers, malignant mixed mullerian tumors, and undifferentiated carcinoma, which have frequent late stage diagnosis and worse outcome (Vang, Shih Ie et al. 2009, Matulonis, Hirsch et al. 2011). Type 2 tumors contain mutant p53 and frequently have abnormalities in homologous recombination DNA repair pathways, including BRCA1/2 mutations, resulting in genomic instability and varied genomic signatures (Cancer Genome Atlas Research 2011, Kang, D'Andrea et al. 2012). They grow rapidly with a high mitotic index and are responsive to platinum-based chemotherapy. Provocative data suggest that these cancers arise from the distal fallopian tube (Medeiros, Muto et al. 2006, Lee, Miron et al. 2007).

1.1.2 Risk factors

Several risk factors have been identified for epithelial OVCA, although those for ovarian germ cell cancer and ovarian stromal cancer are not well known.

1) Age. The risk of developing OVCA increases with age. OVCA is more common in women aged 50-79 (Byers, Marshall et al. 1983).

2) Family history. OVCA risk increases when a close relative has (or has had) OVCA. Interestingly, a family history of some other types of cancer such as colorectal and breast cancer is linked to an increased risk of OVCA, which is because these cancers can be caused by an inherited mutation in certain genes (e.g. *BRCA1*, *BRCA2*, *TP53*) that causes a family cancer syndrome (Lynch, Silva et al. 2008).

3) Reproductive history. Women who have never carried a pregnancy to term or who have a history of having difficulty in getting pregnant, have a higher risk of OVCA. Breast feeding may lower the risk even further (Feng, Chen et al. 2014).

4) Oral contraceptives/hormone replacement. OVCA risk is lower in women who have used oral contraceptives. On the contrary, risk may be higher if one has taken hormone replacement, especially for serous and endometrioid ovarian cancers (Feng, Chen et al. 2014).

1.1.3 Staging and Treatment

OVCA is most often staged using the International Federation of Gynecology and Obstetrics (FIGO) system, which relies on the results of surgery to determine the spread of the primary tumor, the absence or presence of metastasis to nearby lymph nodes, and the absence or presence of distant metastasis. This information is combined to determine the final stage. Ovarian cancers have different prognoses at different stages and are treated differently.

Briefly, in stage I, the tumor is confined to the ovaries. In stage II, the tumor involves one or both ovaries with pelvic extension (below the pelvic brim). In stage III, the tumor involves one or both ovaries with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes. In stage IV, distant metastasis is found excluding peritoneal metastasis (www.sgo.org, 2015).

In the treatment of OVCA, one needs to consider the following: the stage of ovarian cancer, the type and size of the tumor, patient's age, general health condition, and whether it is a newly diagnosed or a recurrent case.

Surgery is the main treatment for most OVCA patients. The purpose of the surgery is staging and debulking. Staging can be done once the tumor spread is confirmed during surgery. Debulking is done to remove as much of the tumor as possible. For this reason, removing part or all other organs, such as colon, bladder or omentum may be required, along with uterus, both ovaries and fallopian tubes.

Chemotherapy is needed for most cases of OVCA. It is used sometimes before the surgery to reduce the tumor size and make it easier to remove. More often, it is used after the surgery to kill any remaining cancer cells. A combination of drugs rather than just one drug alone seems to be more effective in the initial treatment of OVCA. The standard approach is the combination of a platinum compound, such as cisplatin or carboplatin, and a taxane (diterpenes produced by the plants of the genus *Taxus*), such as paclitaxel (Taxol) or docetaxel (Taxotere) (Rooth 2013). The typical course of chemotherapy for epithelial OVCA involves 3 to 6 cycles of intravenous injection. In some cases, chemotherapy may also be injected directly into the abdominal cavity

(intraperitoneal) to acquire the most concentrated dose of the drugs to the cancer cells in the abdominal cavity.

1.1.4 Prognosis

OVCA is the most lethal gynecological malignancy. For all types of OVCA, the 5-year survival is 45% (National Cancer Institute, SEER Data Base; Canadian Cancer Society). The treatment outcome depends on a number of factors, including the patient's age, type and stage of tumor, and how much of the tumor has been removed during the initial surgery. Women diagnosed when they are younger than 65 have a better prognosis than older women.

If OVCA is found (and treated) before the cancer has spread outside the ovary (stages I), the 5-year survival rate is 92%. However, only 15% of all OVCA cases are found at this early stage. For patients at stages III and IV (61% of all ovarian cancers), the 5-year survival rate is only 34% and 18% respectively (www.cancer.ca, 2015). Unfortunately, due to the lack of screening method and non-specific symptoms, the majority of ovarian cancer patients are diagnosed at advanced stage with metastasis.

Chemoresistance also contributes to the high mortality of ovarian cancer. Clinical recurrences that take place within 6 months of completion of a platinum-containing treatment regimen are considered platinum-refractory or platinum-resistant recurrences. Although more than 80% of these women benefit from first-line therapy, tumor recurrence occurs in almost all these patients at a median of 15 months from diagnosis (Hennessy, Coleman et al. 2009). Second-line treatments (such as Topotecan,

Liposomal Doxorubicin) can improve survival and quality of life but are not curative (Hennessy, Coleman et al. 2009). Chemoresistance severely limits treatment success. However, the mechanisms of chemoresistance still need to be further elucidated.

1.2 Cervical cancer

Cervical cancer (CECA) forms in tissues of the cervix. It is a common reproductive cancer, with 1,450 new cases per year, resulting in approximately 380 deaths per year in Canada (2014 Canadian Cancer Statistics). Early cervical cancer may not exhibit symptoms. When the tumor grows bigger, the patient may present with abnormal vaginal bleeding and pelvic pain.

In order to diagnose CECA, the following steps are usually taken: 1) Lab tests. The Papanicolaou (Pap) test is a widely used method for early detection of pre-cancer and cervical cancer. Cells are collected from the exterior of the cervix and the endocervix, and examined microscopically for abnormalities. The test aims to detect potentially pre-cancerous changes (called cervical intraepithelial neoplasia (CIN) or cervical dysplasia). The human papilloma virus (HPV) test is used to find any of the high-risk types of HPV that are most commonly found in CECA in the cells collected from the cervix. 2) Cervical exam. The cervix is examined in an illuminated, magnified view through colposcopy directly. Premalignant and malignant lesions can be distinguished from normal appearing tissue. 3) Pathological examination of tissue sample. Tissue sample can be collected for further pathological examination when necessary through biopsy.

Malignant cells/tissues and the spread range are assessed microscopically, as well as the histopathologic type of the tumor.

1.2.1 Histologic subtypes

Squamous carcinoma and adenocarcinoma are the most frequent histologic types of CECA. Other histologic types include: endometrioid adenocarcinoma, clear cell adenocarcinoma, adenosquamous carcinoma, adenoid cystic carcinoma, small cell carcinoma and undifferentiated carcinoma.

1.2.2 Risk factors

The most important risk factor for cervical cancer is infection by the human papilloma virus (HPV). More than 100 types of HPVs have been identified, including 13 high-risk types, which are responsible for cervical cancer and other anogenital and oropharyngeal cancers. HPV types 16 and 18 account for roughly 70% of all CECA (Crosbie, Einstein et al. 2013). HPVs infect epithelial cells. Once they enter the cell, HPVs disrupt normal cell-cycle control, promoting uncontrolled cell division and the accumulation of genetic damage, resulting in the formation of the tumor (Ghittoni, Accardi et al. 2010).

Other factors are also believed to be associated with developing CECA after infection of high risk HPVs, including smoking, having a weakened immune system, increased number of sexual partners, young age at time of first sexual intercourse, or long term oral contraceptive use (Coffey, Beral et al. 2015).

1.2.3 Staging and Treatment

CECA can also be staged using the FIGO system, based on the extent of the tumor, and the absence or presence of metastasis to lymph nodes and distant organs. Briefly, in stage 0, carcinoma is in situ (pre-invasive carcinoma). In stage I, cervical carcinoma is confined to the uterus. In stage II, the tumor invades beyond the uterus but not to the pelvic wall or to the lower third of the vagina. In stage III, the tumor extends to pelvic wall and/or involves the lower third of vagina and/or causes hydronephrosis or non-functioning kidney. In stage IV, it invades the mucosa of the bladder or rectum and/or extends beyond the true pelvis, or has a distant metastasis （www.sgo.org, 2015）.

Treatment plans are made based on several factors, including size of the tumor, the stage, and personal factors, such as the desire for future fertility. Different surgeries are performed depending on the situation. A cone biopsy is used to treat very early cervical tumors in women who want to preserve their fertility. A radical hysterectomy is done when the cervical tumor is large, during which the cervix, uterus, part of the vagina, some of the structures and tissues near the cervix and the pelvic lymph nodes are removed. Pelvic exenteration is sometimes done when CECA recurs locally (within the pelvis) after being treated with radiation therapy. The cervix, uterus, vagina, ovaries, fallopian tubes, lymph nodes, rectum and/or bladder will be removed in this case.

Radiation therapy is a primary treatment option to kill cancer cells, or is used after the surgery to prevent the recurrence of the tumor, with or without the combination of chemotherapy (Inoue 2004). External beam radiation therapy or internal radiation

therapy (brachytherapy, a radioactive substance is placed right next to the tumor) can be used depending on the tumor and the overall health condition of the patient.

Chemotherapy is commonly used in treating CECA, sometimes combined with radiation therapy. Cisplatin, 5-fluorouracil and ifosfamide are the most common chemotherapy drugs for CECA.

1.2.4 Prognosis

The overall mortality from CECA has dramatically decreased during the past few decades, since the early screening tests (Pap and HPV test) are used for the detection of CECA (Reade and Elit 2012). However, CECA remains an important health problem, especially in developing countries. The most important prognosis factor of CECA is clinical stage. The 5 year survival rate is much lower in advanced stage, partly because the relapse of tumor and the acquired resistance to radiotherapy and chemotherapy (Scatchard, Forrest et al. 2012).

2. Cisplatin and its anticancer actions

Cisplatin (CDDP), also called cisplatinum, or cis-diamminedichloroplatinum (II), is a metallic (platinum) coordination compound with a square planar geometry (www.chemicalbook.com, 2015). It is composed of a doubly charged platinum ion surrounded by four ligands, two amine ligands and two chloride ligands.

2.1 Mechanism of cytotoxicity

DNA has been implicated as the main target of CDDP (Fraval, Rawlings et al. 1978). CDDP is transported into the cell by the copper transporter protein 1 (Ctr1) in mammals (Ishida, Lee et al. 2002). Once inside the cell, the chloride atoms of CDDP are displaced by water molecules and CDDP becomes a potent electrophile, allowing the platinum atom to bind to DNA and form CDDP-DNA adducts, thereby inducing intra-strand and inter-strand cross-linking. The cross-linking distorts and abnormally unwinds the DNA duplex, which is recognized by multiple repair pathways (Perego, Giarola et al. 1996). When the extent of damage is limited, an arrest in the S and G2 cell cycle phases will be induced, allowing reestablishment of DNA integrity through DNA repair. However, if DNA damage is beyond repair, several cellular pathways culminating in apoptosis will be activated (Perego, Giarola et al. 1996, Jamieson and Lippard 1999). p53 phosphorylation at serine 15 and serine 20 by ataxia telangiectasia mutated- and Rad3-related protein (ATR) or checkpoint kinase 1 (Chk1) kinase is a key event during this process (Shieh, Ahn et al. 2000, Appella and Anderson 2001). p53 is a short lived protein, and it is stabilized by phosphorylation (Damia, Filiberti et al. 2001). p53 then activates apoptosis by transcription-dependent and transcription-independent pathways (Kruse and

Gu 2009).

2.2 Treatment of cancer

Cisplatin has been used as a chemotherapeutic agent in pre-clinic trials since the 1960s. It was approved by the FDA for cancer treatment as the first platinum compound in 1978 (Kelland 2007). It is one of the most effective anticancer drugs and widely used to treat different types of cancers, including sarcomas, lung cancer, ovarian cancer, breast cancer, cervical cancer, and head and neck squamous cell carcinoma (Kelland 2007).

It has been reported that CDDP is associated with several toxic side effects, including cardiotoxicity, hepatoxicity, nephrotoxicity and some reproductive toxic effects, when it is administered intravenously, hence also affect non-cancer cells (Hartmann, Fels et al. 2000, Hartmann and Lipp 2003, Al-Majed 2007). Lowering the dose of CDDP and combination with other compounds, such as doxorubicin and 5-Fluorouracil (5-Fu), are often used to reduce side effects (Al-Majed 2007).

2.3. CDDP resistance

Even though the initial responsiveness to CDDP treatment is high, after an unpredictable period, some patients will relapse, with the tumor often resistant to further CDDP treatment (Kelland 2007). The effect of CDDP relies on its uptake and retention in the cells, causing DNA damage, and inducing apoptosis. The mechanism of CDDP resistance is multi-factorial, and associated with the following aspects (Figure 1.1).

Figure 1.1

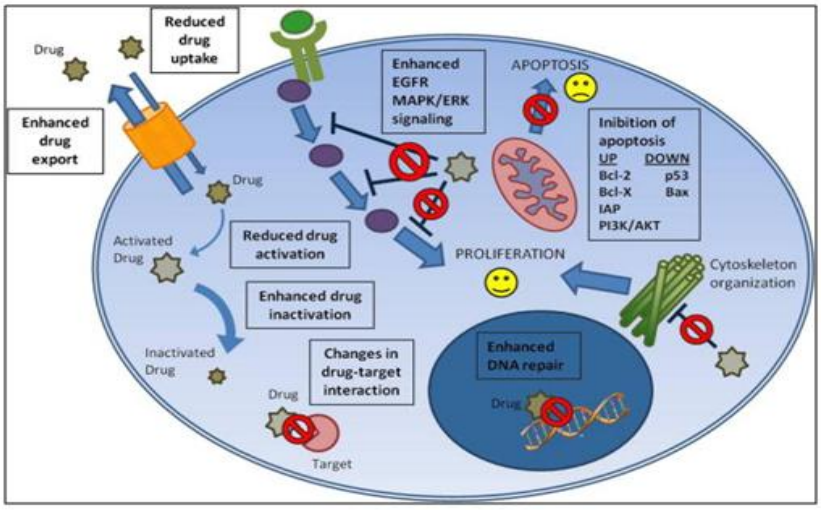

Drug
Reduced drug uptake
Enhanced drug export
Drug
Enhanced EGFR MAPK/ERK signaling
APOPTOSIS
Inibition of apoptosis
UP DOWN
Bcl-2 p53
Bcl-X Bax
IAP
PI3K/AKT
Activated Drug
Reduced drug activation
PROLIFERATION
Cytoskeleton organization
Enhanced drug inactivation
Inactivated Drug
Changes in drug-target interaction
Enhanced DNA repair
Drug
Drug
Target

Figure 1.1 Multiple mechanisms are involved in chemoresistance. These include alterations of drug uptake, export and metabolism, derangement of intracellular pathways' signaling, cross-talk between different membrane receptors, modification of apoptotic signaling, and interference with cell replication (Fodale, Pierobon et al. 2011).

2.3.1 Decreased uptake

Chemoresistant cells may have reduced CDDP uptake and accumulation in the cell. Evidence shows that chemoresistant OVCA and CECA cells exhibit much lower intracellular platinum concentrations than their chemosensitive parental cells (2.5-2.9 fold lower), due to decreased drug uptake or increased drug efflux (Zisowsky, Koegel et al. 2007). As mentioned previously, Ctr1 transports platinum-based drugs (e.g., CDDP) into the cells. CDDP uptake decreased almost 80% with Ctr1 knock-down (Ishida, Lee et al. 2002). Ctr1 expression was also found associated with CDDP sensitivity and prognosis in women with OVCA. Patients with high Ctr1 levels had a better response to CDDP and progression-free survival (Lee, Choi et al. 2011). Moreover, chemoresistance was overcome by enhancing the expression of Ctr1 (Chen and Kuo 2013).

Notably, even though over-expression of Ctr1 significantly enhanced accumulation of CDDP in OVCA cell lines, it only induced a marginal increase in sensitivity to CDDP, and failed to increase the extent of CDDP-DNA adduct formation (Holzer, Samimi et al. 2004), suggesting that chemoresistance may also depend on other mechanisms.

2.3.2 Increased drug efflux

The ATP Binding Cassette Family (ABC protein) is the largest transporter protein family. Several ABC proteins have been found to be involved in the development of multi-drug resistance (MDR) as efflux pumps, which include P-glycoprotein (Pgp, also called MDR1 or ABCB1), non-P glycoprotein MDR (MRP) and breast cancer resistance

protein (BRCP) (Liang, Li et al. 2015). They are able to translocate drugs across cellular membranes through the hydrolysis of ATP, inducing less drug effect and chemoresistance. Patients with tumors expressing MDR1 were three times more likely to fail to respond to chemotherapy than patients with MDR1-negative tumors, suggesting MDR1 expression is associated with chemoresistance (Trock, Leonessa et al. 1997). Sensitivity to CDDP has also been demonstrated to be associated with MDR1 levels in ovarian cancer cells (Januchowski, Wojtowicz et al. 2013). Another ABC family member, MRP was also found to be over-expressed in a CDDP-resistant human osteosarcoma cell line (Han, Zhu et al. 2014).

2.3.3 Increased drug inactivation

Xenobiotic detoxification mediated by metabolic enzymes influence drug response and resistance. This effect is controlled in 3 steps: 1) Cytochrome P450 catalyzes the formation of the oxidation of organic substances; 2) These substances form conjugates with glutathione, glucuronic acid or sulfate; and 3) Metabolized substances are exported out of the cells and into extracellular fluid through transmembrane pumps, including MDR-1 and MRP (Fodale, Pierobon et al. 2011).

Enhanced CDDP inactivation may contribute to chemoresistance. In fact, the level of glutathione was found to be higher in chemoresistant cancer cells, and depletion of glutathione sensitized the resistant cancer cells to CDDP treatment (Meijer, Mulder et al. 1992, Jansen, Brouwer et al. 2002). Interestingly, glutathione was also involved in other factors of chemoresistance, such as increasing DNA repair and inhibition of apoptosis (Stordal and Davey 2007), further suggesting that chemoresistance is an

outcome of multiple pathways, and these pathways crosstalk, making chemoresistance a difficult puzzle to solve.

2.3.4 Increased DNA damage repair

Normal cells are protected by intrinsic DNA damage response (DDR) from endogenous and exogenous DNA damage inducers. The DDR system initiates different processes depending on the damage, such as DNA repair, cell cycle arrest, cell senescence or apoptosis. At least five major DNA repair mechanisms are involved in these processes, including direct repair (DR), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and base excision repair (BER).

NER is the main system that cells use to remove CDDP lesions from DNA (Dijk, Typas et al. 2014). A correlation between NER proficiency and CDDP resistance has been reported in ovarian cancer cells (Li, Gardner et al. 1998). Excision Repair Cross-Complementation Group 1 (ERCC1, a single-strand DNA endonuclease involved in NER) expression has been shown to be negatively correlated with survival and responsiveness to CDDP in several cancer types, including bladder (Bellmunt, Paz-Ares et al. 2007), colorectal (Shirota, Stoehlmacher et al. 2001), gastric (Metzger et al., 1998), esophageal (Kim et al., 2008), and head and neck (Handra-Luca, Hernandez et al. 2007).

CDDP-induced inter-strand adducts also leads to double-strand breaks, which are normally repaired by the machinery of HR. BRCA1 and BRCA2, two critical components of the HR system, are frequently mutated in inherited breast and ovarian cancers (Venkitaraman 2002, Narod and Foulkes 2004). These BRCA1/2 deficient tumors are normally hypersensitive to DNA-crosslinking agents (e.g. CDDP), but could develop CDDP resistance by wild type BRCA2 restoration through secondary mutations

(Edwards, Brough et al. 2008, Sakai, Swisher et al. 2008).

2.3.5 Inhibition of apoptosis

One of the main objectives of chemotherapy is to induce apoptosis in tumor cells. Chemoresistant tumors exhibit lower rate of apoptosis, as a result of the down-regulation of pro-apoptotic proteins/pathways and/or up-regulation of anti-apoptotic proteins/pathways (Scatchard, Forrest et al. 2012). Four of these proteins/pathways are described here after. The Phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway is a key regulator of cell survival, apoptosis, DNA repair, proliferation, and angiogenesis. This pathway is frequently dysregulated in cancer and implicated in oncogenesis and tumor progression (Saal, Johansson et al. 2007, Liu, Cheng et al. 2009). Increased PI3K/Akt pathway activity also contributes to drug resistance through different mechanisms, including the regulation of apoptosis. Our laboratory has shown that Akt inhibits CDDP-induced apoptosis and promotes chemoresistance by inhibiting the CDDP-induced p53 phosphorylation in OVCA and CECA cells (Yang, Fraser et al. 2006, Fraser, Bai et al. 2008).

p53 is an important tumor suppressor, involved in the regulation of cell cycle, proliferation and apoptosis. p53 mutations (hence p53 loses the normal function) are found in almost half of the malignancies from a wide range of human tumors (Kirsch and Kastan 1998). p53 mutation is associated with CDDP resistance, demonstrated by comparing the sensitivity of CDDP in a wide panel of p53 wild type and p53 mutant tumor cell lines (O'Connor, Jackman et al. 1997). Frequency of p53 mutation is much higher in CDDP resistant epithelial OVCA patients compared to CDDP sensitive patients

(83% vs. 16%) (Kigawa, Sato et al. 2001). Our previous studies have suggested that p53 phosphorylation and its action in mitochondria and nucleus are important determinants for CDDP-induced apoptosis, and this mechanism is inhibited in chemoresistant cells (Yang, Fraser et al. 2006, Fraser, Bai et al. 2008, Woo, Xue et al. 2012).

B-cell lymphoma 2 (Bcl-2) family is a group of proteins that regulate apoptosis by governing the mitochondrial outer membrane permeabilization (MOMP) complex, which contain pro-apoptotic proteins (Bak, Bax, and Bad among others) and anti-apoptotic proteins (Bcl-2 and Bcl-xL among others). Alterations of these proteins may also contribute to chemoresistance (Karnak and Xu 2010). Decreased levels of pro-apoptotic proteins Bak and Bax, and elevated levels of their anti-apoptotic counterparts have been reported to be associated with CDDP resistance and tumor recurrence in different cancer types, including lung cancer (Han, Hong et al. 2003), and OVCA (Williams, Lucas et al. 2005).

X-linked inhibitor of apoptosis protein (XIAP) is an important apoptosis suppressor via inhibition of caspase-3, -7 and -9 activity, as well as apoptosis inducing factor (AIF) via polyubiquitination (Zhang, Iqbal et al. 2013). Previous work from our lab has showed that XIAP is decreased in chemosensitive cells, but stabilized in chemoresistant cells when challenged with CDDP (Reddy, Reddy et al. 2011). High levels of XIAP in chemoresistant cells promote chemoresistance partly by protecting other anti-apoptotic proteins, including Akt, FLICE-like inhibitory protein (FLIP) and Gelsolin (DiSaia, Sinkovics et al. 1972, Andrews, Murphy et al. 1985, Escobar-Henriques and Anton 2013).

2.3.6 Cancer stem cell model

Recently the cancer stem cell (CSC) model has been proposed to explain the origin of chemotherapy resistant cells. CSCs are a small portion of cancer cells, which have similar properties to stem cells, such as self-renewal capacity, the ability to develop into different lineages (Qiu, Fang et al. 2015). CSCs are also more resistant to chemotherapy compared with normal cancer cells, due to their slow cycling, efflux of drugs by ATP binding cassette transporters, and up-regulation of anti-apoptotic genes(Abdullah and Chow 2013). CSCs may serve as a major source of chemoresistance in tumors.

Cancer cells become chemoresistant due to these described mechanisms. Notably, accumulating evidence suggest that CDDP resistance is often multifactorial, since inhibition of a single pathway usually fails to restore chemosensitivity. Further elucidation of the mechanism of CDDP resistance is still required, which may lead to the discovery of new prognostic and predictive markers, and development of more efficient and less toxic anticancer drugs.

3. Apoptosis pathway

Apoptosis or “programmed cell death” is a genetically regulated mechanism of cell turn-over that occurs during development and aging. It is considered a homeostatic mechanism to maintain cell populations in tissues, or as a defense mechanism occurring in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson 2001). Apoptosis can be caused by wide range of physiological and non-physiological stimuli. It is a strictly controlled and energy-dependent event that is

characterized by morphologic changes including cell shrinkage, plasma membrane blebbing, chromatin condensation and DNA fragmentation (Samali, Gorman et al. 1996, Hacker 2000). Apoptotic cells also exhibit biochemical features, such as protein cleavage, cross-linking and degradation, DNA breakdown, and phagocytic recognition (Hacker 2000). Several apoptosis pathways have been suggested to explain the mechanisms of apoptosis, including the extrinsic pathway (also called death receptor pathway), the mitochondria-mediated pathway (often referred to as the intrinsic pathway) and the endoplasmic reticulum (ER)-mediated pathway.

3.1 The extrinsic pathway

The extrinsic pathway of apoptosis is initiated by the interaction between ligand and corresponding transmembrane receptor, such as Fas ligand and Fas ligand receptor, or tumor necrosis factor (TNF) and TNF receptor (TNFR). Upon ligand binding, transmembrane receptors which carry a death domain, will recruit cytoplasmic adaptor proteins, such as Fas-Associated protein with Death Domain (FADD), which carry corresponding death domains. FADD will further recruit procaspase-8, and form a death-inducing signaling complex (DISC), resulting in the auto-catalytic activation of procaspase-8 and subsequent activation of caspase-3 and induction of apoptosis (Kischkel, Hellbardt et al. 1995, Kischkel, Lawrence et al. 2001) (Figure 1.2).

Figure 1.2

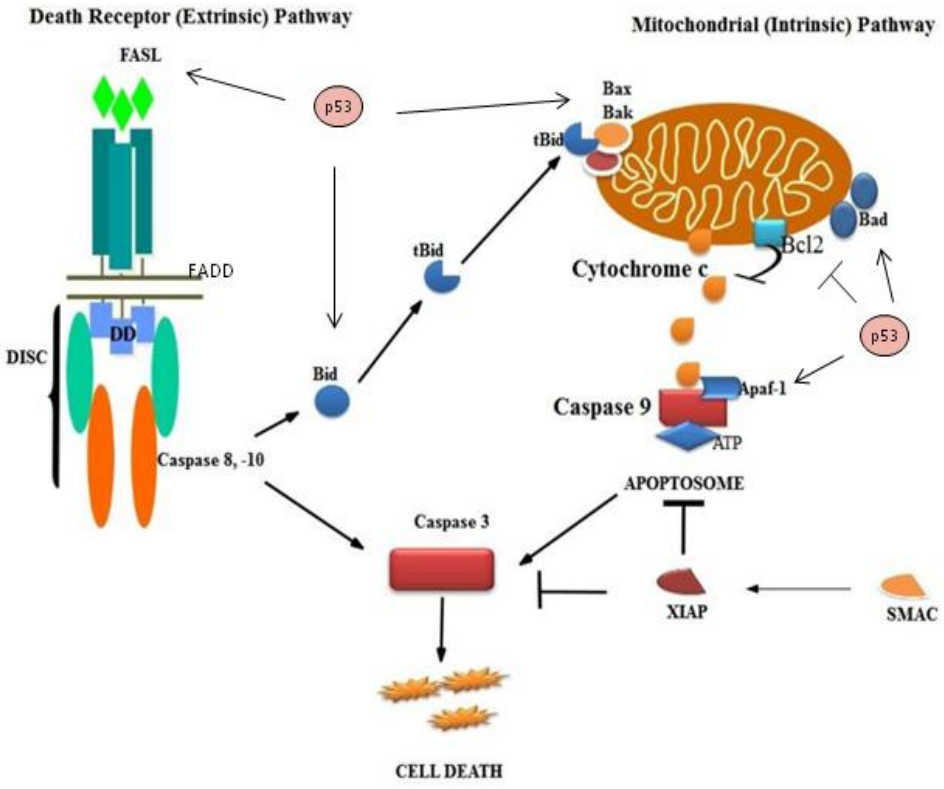

Death Receptor (Extrinsic) Pathway
Mitochondrial (Intrinsic) Pathway
FASL
p53
Bax
Bak
tBid
tBid
Bad
Bcl2
Cytochrome c
FADD
DD
DISC
Bid
p53
Apaf-1
Caspase 9
ATP
Caspase 8, -10
APOPTOSOME
Caspase 3
XIAP
SMAC
CELL DEATH

Figure 1.2 The intrinsic (mitochondrial) and extrinsic (death receptor) -mediated pathways of apoptosis. ***Extrinsic pathway:*** The Fas receptor (FasR) is stimulated by Fas ligand (FASL), recruiting death adapter proteins, like FADD to the FasR via an interaction between the death domains (DD). Caspase-8/10 is also recruited to the complex, forming DISC (death inducing signaling complex) and resulting in the activation of caspase-8/10. Caspase-8/10 cleaves and activates the executioner caspase-3, induces apoptosis. Caspase-8/10 also cleaves a proapoptotic protein Bid, and the truncated Bid (tBid) activates intrinsic pathway by activating Bak or Bax. ***Intrinsic pathway:*** The pro-apoptotic Bcl-2 family members Bax and Bak are activated by apoptotic stimuli or tBid, and form an oligomeric pore in the outer mitochondrial membrane, allowing the release of cytochrome c and other pro-apoptotic factors from the mitochondria to the cytosol. Cytochrome c triggers the assembly of the apoptosome (consisting of Apaf-1, caspase-9 and also ATP). Subsequently, the apoptosome activates caspase-3 and cell death (Kalimuthu and Se-Kwon 2013). p53 can transcriptionally activate Fas ligand (FASL), Bid, Bad, Bax and Apaf1 (Nakamura 2004, Riley, Sontag et al. 2008). Anti-apoptotic protein Bcl-2 is transcriptionally repressed by p53 (Miyashita, Krajewski et al. 1994, Sugars, Budhram-Mahadeo et al. 2001).

3.2 The mitochondria-mediated pathway

The mitochondria-mediated pathway of apoptosis is induced by stimuli that produce intracellular signals acting directly on the targets within the cell, and does not work through transmembrane receptors. These stimuli include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals (Xiong, Mu et al. 2014)(Figure 1.2).

All of these stimuli cause changes in the membrane of mitochondria, inducing MOMP, which allows for the release of pro-apoptotic proteins from the mitochondria into the cytosol, such as cytochrome c, Smac/Direct IAP-Binding Protein With Low PI (DIABLO), Apoptosis Inducing Factor (AIF), endonuclease G and the serine protease HtrA2/Omi (Du, Fang et al. 2000, Garrido, Galluzzi et al. 2006). Released cytochrome c binds Apaf-1 and procaspase-9, forming an "apoptosome", which leads to activation of caspase-9 (Hill, Adrain et al. 2004) and subsequently of caspase-3, the executioner caspase. AIF and endonuclease G are released from mitochondria and translocate to the nucleus, causing DNA fragmentation and condensation of nuclear chromatin (caspase independent pathway) (Joza, Susin et al. 2001, Li, Luo et al. 2001).

MOMP is the irreversible point in the apoptotic pathway, which is tightly controlled by the Bcl-2 family. The Bcl-2 family is a group of proteins which contain at least one Bcl-2 homology (BH) region, highly conserved stretches of homology found in Bcl-2 family members. They can be sub-grouped into anti-apoptotic multi-domain proteins, which contain four BH domains (BH 1-4), such as Bcl-2 and Bcl-xL, and pro-apoptotic multi-domain proteins (BH 1-3), including Bax, Bak, and pro-apoptotic BH-3 only proteins Bid, Bad, Bim, Puma, and Noxa (Czabotar, Lessene et al. 2014).

Accumulating evidence suggests that when the cell receives a death signal, the pro-apoptotic proteins Bak and Bax, which are normally inhibited by anti-apoptotic Bcl-2 family members, such as Bcl-xL, are activated and homo-oligomerize at the outer membrane of mitochondria, forming a pore and inducing MOMP (Chen, Willis et al. 2005, Willis, Chen et al. 2005). Cells lacking Bak and Bax are resistant to MOMP with different apoptotic stimuli (Wei, Zong et al. 2001), suggesting Bak and Bax are key effectors of MOMP. Even though our knowledge has expanded substantially over the past few years, the detailed mechanism of MOMP by Bax or Bak oligomers remains unclear (Bender and Martinou 2013).

Caspase-6 and caspase-7 also function as executioner caspases like caspase-3, which cleave cytokeratins, Poly ADP-Ribose Polymerase (PARP), the plasma membrane, cytoskeletal protein alpha fodrin, the nuclear protein NuMA and Gelsolin (Slee, Adrain et al. 2001), leading to the morphological and biological changes in apoptotic cells.

3.3 The ER-mediated apoptosis pathway

The ER is a highly dynamic membranous network composed of sac-like structures and tubules, which spans from the nuclear envelope to the plasma membrane. The ER is implicated in multiple cellular functions, including folding and maturation of newly synthesized proteins, bioenergetic processes such as lipid synthesis, and calcium storage.

ER stress is induced by perturbations in ER function, such as hypoxia, disturbed Ca^{2+} homeostasis, accumulation of misfolded proteins, oxidative stress, nutrient deprivation, metabolic changes and acidosis (Dugue, Rebolj et al. 2013). Unfolded proteins accumulate within the ER lumen due to impaired ER function. Unfolded protein

response (UPR) therefore is initiated to counteract ER stress and restore cellular homeostasis by clearing the unfolded proteins (Sano and Reed 2013). Three ER transmembrane proteins Inositol-requiring transmembrane kinase/endonuclease-1 (IRE1α), RNA-dependent protein kinase (PERK), and Activating transcription factor 6 (ATF6) have been proposed to act as sensors for ER stress and activate UPR (Sano and Reed 2013). However, when ER stress is chronically prolonged or too severe, UPR signaling shifts from pro-survival to apoptosis(Sano and Reed 2013).

ER-mediated apoptosis involves multiple mechanisms. Several BH3-only proteins (pro-apoptotic Bcl-2 family members) have been reported to be activated by ER stress, including Bid and Bim (Scatchard, Forrest et al. 2012, Liang, Li et al. 2015). UPR also directly activates caspase-2 and caspase-12, thereby promoting apoptosis (Dijk, Typas et al. 2014, Qiu, Fang et al. 2015).

Calcium is mainly localized in the ER of the cell. High concentrations of calcium are essential for proper folding of proteins in the ER. Recent evidence suggests that UPR stimulates inositol 1,4,5-triphosphate receptor (IP3R), the major release channel for Ca^{2+} from the ER, inducing Ca^{2+} release into the cytoplasm (Abdullah and Chow 2013). Massive amounts of Ca^{2+} further translocates into the mitochondria and induces cell death by caspase-dependent and -independent pathways (Flusberg and Sorger 2015). For example, Ca^{2+}-dependent activation of the protease Calpain has been implicated in the activation of several pro-apoptotic proteins, including AIF, caspases and Bax (Xiong, Mu et al. 2014).

The last stage of apoptosis is the phagocytic uptake of apoptotic cells. Phosphotidylserine appears on the outer leaflet of apoptotic cells, which help them to be

recognized by phagocytes (Fadok, de Cathelineau et al. 2001). The apoptotic cells are eliminated without release of cellular contents, thereby eliciting no inflammatory responses (Fadok, de Cathelineau et al. 2001).

4. Tumor suppressor p53

4.1 Overview

p53 is considered the "guardian of the genome" due to its ability to suppress tumors through monitoring and responding to a diverse array of cellular stress signals, including DNA damage, abnormal oncogenic events, hypoxia, oxidative and nutrional stress, and disruption of nucleolar function (Horn and Vousden 2007, Vlatkovic, Boyd et al. 2014). p53 is involved in the regulation of cell cycle progression, DNA repair, metabolism, senescence and apoptosis, via transcriptionally activating or suppressing a wide range of genes in the nucleus (transcription-dependent pathway), or directly binding to certain proteins in the cytosol or mitochondria (transcription-independent pathway) (Vousden and Lane 2007, Lanni, Racchi et al. 2012, Rufini, Tucci et al. 2013).

4.2 Control and activation of p53

p53 is a short-lived protein (Kubbutat, Jones et al. 1997). Its protein level is kept low in normal cells, with functions repressed mainly by Human Double Minute 2 (HDM2, mouse ortholog is MDM2) (Kubbutat, Jones et al. 1997, Kruse and Gu 2009). HDM2/MDM2 is a member of the RING finger family of E3 ligases, which contain a p53

binding domain. Upon binding to HDM2/MDM2, p53 loses its transcription ability, and is translocated to cytoplasm and degraded by proteasomes (Lee and Gu 2010, Hu, Feng et al. 2012).

p53 contains serine phosphorylation sites, and the sites 15, 20, 33, 37, and 46 are rapidly phosphorylated by ataxia telangiectasia mutated (ATM) or ATR following cellular stress, which reduces the interaction between p53 and MDM2, leading to p53 stabilization (Banin, Moyal et al. 1998, Tibbetts, Brumbaugh et al. 1999, Ashcroft, Taya et al. 2000). Our lab has shown that p53 is phosphorylated at serine 15 and serine 20 by CDDP in chemosensitive CECA cells, but not in chemoresistant cells, and mutation of these two sites significantly inhibits CDDP-induced apoptosis in ovarian cancer cells, suggesting that p53 phosphorylation is needed for p53 function in inducing apoptosis (Fraser, Bai et al. 2008). p53 can also be stabilized through the inhibition of MDM2 activity by the ARF protein, which is activated in response to abnormal proliferative signals (Palmero, Pantoja et al. 1998, Zindy, Eischen et al. 1998). Our previous work has also suggested that chemoresistant gynecologic cancer cells lose their response to CDDP partly due to deactivated p53 by protein phosphatase magnesium/manganese dependent 1 D (PPM1D) through site-specific dephosphorylation (Ali, Abedini et al. 2012, Ali, Kim et al. 2014).

4.3 p53 and tumor suppression

p53 is one of the most prominent tumor suppressors. Activation of p53 in response to aberrant oncogenic expression leads to cell cycle arrest, senescence or apoptosis, and these cellular outcomes are important for tumor suppression (Fodale,

Pierobon et al. 2011). Tumor cells escape from the monitoring of p53 through p53 mutation or inhibition (Levine and Oren 2009). p53 mutation is found in more than half of the human cancer cases, thus acknowledged as the most common genetic event in cancer (Levine and Oren 2009). Mutation of p53 can not only abolish normal p53 function in suppressing tumor formation by monitoring oncogenic events, but also function as dominant negative p53 through tetramer formation with wild type p53 (hence inhibit p53 normal function) (Kalimuthu and Se-Kwon 2013). Mutant p53 even behaves as an oncogene and promotes cell survival by transcriptionally activating new target genes or via inappropriate interaction with other proteins (Matsumoto, Furihata et al. 2006).

In the tumor cells that retain wild type p53, its function can also be attenuated by different mechanisms. For instance, HPV type 16 and 18 (account for roughly 70% of all CECA) produce the E6 oncoprotein, which can bind and inactivate wild type p53 (Levine and Oren 2009). MDM2, the negative regulator of p53, is also found over-expressed in a variety of tumors (Marine, Francoz et al. 2006).

4.4 p53 and apoptosis

p53 plays a central role in cell fate decisions (cell survival versus cell death) in response to cell stresses by transcription-dependent and -independent pathways. A wide range of pro-apoptotic proteins involved in the apoptosis pathways can be transcriptionally activated by p53, including Fas/Fas ligand, p53-upregulated mediator of apoptosis (PUMA), Noxa, Bid, Bad, p53AIP1, Bax and Apaf1 (Nakamura 2004, Riley, Sontag et al. 2008). Anti-apoptotic proteins, Bcl-2 and Bcl-xL, are transcriptionally

repressed by p53 (Miyashita, Krajewski et al. 1994, Sugars, Budhram-Mahadeo et al. 2001). Evidence also suggests that p53 induces apoptosis directly through its actions on the mitochondria, independent of its transcription ability. In this model, p53 rapidly translocates to the mitochondria and binds to anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL, resulting in the release of pro-apoptotic Bak and Bax (Westermann 2010).

Our laboratory has also found that p53 phosphorylation of serine 15 and serine 20 are needed for CDDP-induced apoptosis in gynecologic cancer cells (Fraser, Bai et al. 2008). Recently, p-p53 (ser15) was reported to interact with Bak, a pro-apoptotic member of Bcl-2 family, leading to Bak activation, MOMP and apoptosis (Nieminen, Eskelinen et al. 2013). Whether the interaction of p-p53 (ser15) and Bak is associated with CDDP-induced apoptosis, and how this pathway is involved in the regulation of chemoresistance in gynecologic cancer need to be investigated.

5. The role of PI3K/Akt in cell survival

5.1 Overview

The phosphoinositol-3-kinase (PI3K)/Akt pathway plays a central role in regulating cell metabolism, growth, proliferation and survival, and is frequently dysregulated in cancer (Martelli, Tabellini et al. 2012). Understanding the mechanism of how this pathway promotes cell survival and tumor progression is important for identifying more powerful tools for cancer treatment.

5.2 Activation of PI3K/Akt pathway

PI3K is a member of lipid kinases, which are activated by multiple signals, including activated tyrosine kinase growth factor receptors, cell adhesion molecules, such as integrins and G-protein-coupled receptors (GPCR), and oncogenes, such as Ras (Martelli, Tabellini et al. 2012). PI3K will further generate phosphatidylinositol-3,4,5-trisphosphate (PIP3) from phosphatidylinositol-4,5-bisphosphate (PIP2), inducing the activation of Akt with phosphorylation at Thr 308 and Ser 473 (Scheid and Woodgett 2001).

Akt, also known as protein kinase B (PKB), is a serine/threonine kinase. To date, over 100 Akt substrates have been identified (Martelli, Tabellini et al. 2012). Akt regulates a wide range of cellular processes, such as protein synthesis, cell survival, proliferation, and metabolism by phosphorylating these substrates.

Akt suppresses apoptosis and promotes cell survival. Akt phosphorylates pro-apoptotic BH3-only protein BAD, therefore inhibits its binding to its target protein (Datta, Katsov et al. 2000). Akt can also inhibit the pro-apoptotic function of BH3-only protein by inhibiting their transcriptional factor, such as FOXO (Tran, Brunet et al. 2003). Akt also inhibits p53 by phosphorylating MDM2, which facilitates the translocation of MDM2 to the nucleus for p53 degradation (Mayo and Donner 2001). Our lab has also found that Akt stabilizes phosphatase PPM1D, which dephosphorylates p53 and Chk1, therefore suppressing the phosphorylation and stabilization of p53 (Ali, Kim et al. 2014).

5.2 PI3K/Akt pathway and cancer

The phosphatase and tensin homolog (PTEN), which dephosphorylates membrane phosphatidylinositols and therefore negatively regulates the PI3K/Akt pathway, is the second most frequently mutated tumor suppressor gene (Stokoe 2001). The activity of PTEN is inhibited by different mechanisms, such as mutations, loss of heterozygosity, methylation, and protein phosphorylation in many cancer types, resulting in the activation of PI3K/Akt pathway (Meric-Bernstam and Gonzalez-Angulo 2009). Other alterations of the PI3K/Akt pathway, which lead to the activation of the pathway, have also been reported in human cancer, such as mutations or amplification of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), or over-expression of Akt (Liu, Cheng et al. 2009). High level of Akt, which inhibits p53 phosphorylation and action in the nucleus and mitochondria, is a determinant of chemoresistance in gynecologic cancer cells (Yang, Fraser et al. 2006, Fraser, Bai et al. 2008).

6. Regulation of mitochondrial dynamics

6.1 Mitochondrial biology

The most prominent roles of mitochondria are to produce the energy currency (ATP) through respiration and regulate cellular metabolism. The central set of reactions involved in ATP production is the citric acid cycle. However, the mitochondria have many other functions in addition to the production of ATP, including storage of calcium ions, and regulation of programmed cell death and cell proliferation (Skulachev 2001, Aon, Cortassa et al. 2004, Frieden, James et al. 2004, Lee, Jeong et al. 2004).

6.2 Mitochondrial dynamics and its function

Mitochondria are highly dynamic organelles that are constantly dividing and elongating to form a network. The dynamic nature of mitochondrial networks is due to two opposing processes, mitochondrial fission and fusion. The dynamic change of mitochondria allows the adjustment of mitochondrial morphologies to specific cellular processes (Rambold, Kostelecky et al. 2011). Mitochondrial fusion has been suggested as a route for the rapid exchange of metabolites. Before cells enter the energy-costly DNA replication phase (S phase), mitochondria become hyperfused and increase their ATP production. Fission is thought to facilitate the segregation of mitochondrial DNA (mtDNA) and isolation of mitochondria from the network to allow their degradation. Thus, mitochondrial fission and fusion appear to influence nearly all aspects of mitochondrial function, including respiration, calcium buffering and apoptosis (Chen and Chan 2009) (Figure 1.3). Disruption of mitochondrial fission and fusion has been linked to the development and progression of some diseases, including Alzheimer's disease, Parkinson disease, hereditary optic atrophy, and Charcot-Marie-Tooth neuropathy (Westermann 2010).

6.3 Mitochondrial dynamics and apoptosis

Numerous studies have shown that mitochondria fragments form filamentous tubules into numerous small punctate particles during early apoptosis, at approximately the same time as MOMP and before activation of effector caspase (Frank, Gaume et al. 2001, Fannjiang, Cheng et al. 2004, Jagasia, Grote et al. 2005, Goyal, Fell et al. 2007). Inhibition of mitochondrial fragmentation reduces cytochrome c release and delays cell

Figure 1.3

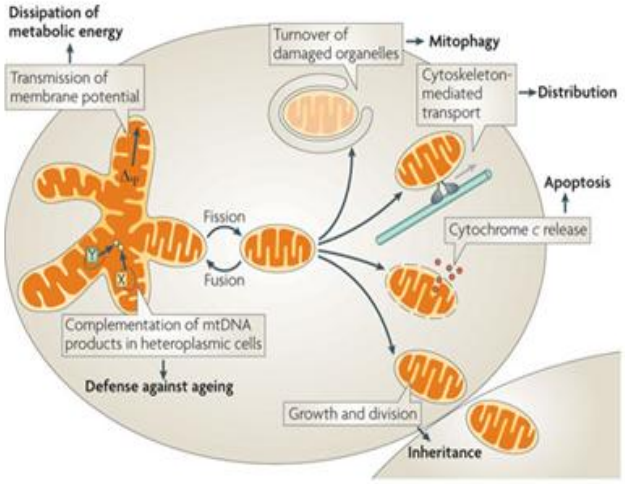

Dissipation of metabolic energy
Transmission of membrane potential
Turnover of damaged organelles
Mitophagy
Cytoskeleton-mediated transport
Distribution
Apoptosis
Cytochrome c release
Fission
Fusion
Complementation of mtDNA products in heteroplasmic cells
Defense against ageing
Growth and division
Inheritance
Nature Reviews | Molecular Cell Biology

Figure 1.3 Fusion and fission of mitochondria are important for cellular biological functions. Fission is needed for mitosis, for the release of cytochrome c from mitochondria during apoptosis, for distribution of mitochondria by cytoskeleton-mediated transport and for mitophagy. Fusion is required for the dissipation of metabolic energy along mitochondrial filaments and for the complementation of mitochondrial DNA (mtDNA) gene products. (Westermann 2010)

death (Frank, Gaume et al. 2001). Mitochondrial fragmentation may promote apoptosis by facilitating cytochrome c release through the remodeling of mitochondrial cristae (Dhingra and Kirshenbaum 2014). These observations suggest that mitochondrial dynamics are involved in the regulation of apoptosis.

Moreover, extensive cross-talks of the signaling pathways regulating mitochondrial dynamics and apoptosis proteins have been reported. Pro-apoptotic Bak and Bax were found to regulate mitochondrial dynamics, since these responses were altered in Bak or Bax deficient-cells, while the fission protein Drp1 and the fusion protein Opa1 were involved in the regulation of apoptosis through processing of its long forms (Frank, Gaume et al. 2001, Karbowski, Lee et al. 2002, Jagasia, Grote et al. 2005, Brooks, Wei et al. 2007). However, the temporal sequence and causative links between mitochondrial fragmentation and apoptosis are still unclear, and further work is needed to elucidate the detailed mechanism by which mitochondrial dynamics and related proteins contribute to the induction of apoptosis. Whether mitochondrial dynamics are dysregulated in chemoresistant cells and play a role in the mechanism of CDDP sensitivity is not known.

6.4. Key players in mitochondrial dynamics

The balance between fission and fusion is controlled by the actions of several large GTP-binding proteins, which are members of the dynamin family. Drp1 mediates mitochondrial fission by wrapping around constricted parts of mitochondria, where they control the fission process. Mitochondrial fusion is mediated by some other dynamin-related proteins in the outer (Mfn 1 & Mfn 2) and inner (Opa1) membrane (Figure 1.4).

These four proteins form the core of the mitochondrial dynamics machinery, and are the targets of different regulatory mechanisms, including modulation of expression, proteolytic processing and post-translational modifications (Westermann 2010).

6.4.1 Fission protein Drp1

Drp1 is the master regulator of mitochondrial fission. Cells transfected with dominant negative Drp1 show highly interconnected mitochondria instead of tubular as a result of the absence of fission activity (Smirnova, Shurland et al. 1998). Drp1 is mainly present in cytoplasm. However, during mitochondrial fission, it translocates to the mitochondria outer membrane and binds to its partner protein Fis1, assemble into rings and spirals that encircle and constrict the mitochondrial tubule (Yoon, Krueger et al. 2003).

Several post-translational modifications of Drp1 have been reported to be involved in the regulation of its translocation. Phosphorylation of serine 616 by

Figure 1.4

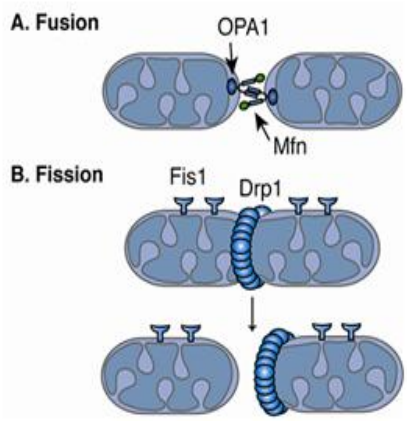


Figure 1.4 Mitochondrial dynamics are controlled by key players, including the fusion proteins Opa1 (mitochondrial inner membrane), Mfn 1&2 (mitochondrial outer membrane), and the fission proteins Fis1 and Drp1.(Chen and Chan 2006)

Cdk1/CyclinB induces Drp1 translocation to the mitochondria, whereas phosphorylation of serine 637 by protein kinase A (PKA) stimulates Drp1 translocation to the cytosol (Chang and Blackstone 2010). On the contrary, dephosphorylation of serine 637 by Calcineurin promotes Drp1 mobilization to mitochondria (Pennanen, Parra et al. 2014). Both sumoylation and nitrosylation have also been suggested to trigger Drp1-mediated mitochondrial fission (Zorzano, Liesa et al. 2010). Several Sumoylation proteins, including Ubc9 and Sumo1, were reported to interact with Drp1. Overexpression of Sumo1 specifically protected Drp1 from degradation, resulting in a more stable and active pool of Drp1 (Reddy, Reddy et al. 2011).

6.4.2 Fusion protein Opa1

In humans, there are 8 splice variants of Opa1. Each of these splice variants carries two to three cleavage sites for serine proteases (Ishihara, Fujita et al. 2006). These sites are designated MPP (Mitochondrial processing peptidase site), S1 and S2. Once the gene product of a splice variant is imported into mitochondria, its mitochondrial localization signal peptide is cleaved at the MPP site, generating mature membrane-bound Opa1 protein (long form). Subsequently, second and possibly third cleavages take place at S1, S2 or both, at a fraction of long forms, producing equimolar amounts of shorter forms (Ishihara, Fujita et al. 2006, Griparic, Kanazawa et al. 2007). Although the function of each form is not clear, long and short forms of Opa1 functionally complement each other, and the combination of at least one short form and one long form are necessary for the generation of fusion competent mitochondria (Song, Chen et al. 2007). Different isoforms of Opa1 form homo and hetero-oligomers, which promote tethering of

the two opposing mitochondria (Collins, Berridge et al. 2002).In addition to its fundamental role in mitochondrial fusion, Opa1 also promotes mitochondrial cristae formation via oligomeric self-interactions (Amutha, Gordon et al. 2004, Griparic, van der Wel et al. 2004). Cytochrome c is mainly located in cristae. Cristae remodeling and increased cytochrome c release have been observed with loss of Opa1 (Cipolat, Rudka et al. 2006, Frezza, Cipolat et al. 2006) (Figure 1.5).

The S1 site of Opa1, which is common to all variants, seems to be the primary target of regulated cleavage upon the presence of apoptotic signals (Song, Chen et al. 2007). Long forms become cleaved at site S1, thereby disrupting the balance of long and short forms and abolishing mitochondrial fusion. In mammalian cells, the metallopeptidase Oma1 is responsible for the processing of long form of Opa1 (L-Opa1) at the S1 site. Significant L-Opa1 stabilization has been reported upon down-regulation of Oma1 (Ehses, Raschke et al. 2009, Head, Griparic et al. 2009). Oma1 small interfering RNA inhibits inducible cleavage of L-Opa1, which helps retain mitochondrial fusion competence, and slows the onset of apoptosis. If and how Oma1 regulates L-Opa1 processing and controls chemosensitivity in gynecologic cancer cells are not known.

6.4.3 Prohibitin and its role in mitochondrial dynamics

The tumor suppressor Prohibitin 1 (Phb1) is a multifunctional protein and is implicated in different cellular processes, including the regulation of cell cycle progression, apoptosis and gene transcription (Merkwirth, Dargazanli et al. 2008, Merkwirth and Langer 2009, Osman, Merkwirth et al. 2009). Phb1 and Phb2 form a complex in the mitochondrial inner membrane, which has also been proposed to function

as a scaffold that recruits membrane proteins to a specific lipid environment (Osman, Haag et al. 2009).

L-Opa1 processing and mitochondrial dynamics have recently been reported to be regulated by Phb1, although the mechanism involved seems not clear (Merkwirth, Dargazanli et al. 2008, Merkwirth and Langer 2009). Interestingly, Phb1 is also involved in p53-induced apoptosis. Recent publications showed that p53 interacts with Phb1 during apoptotic signaling, and that the function of p53 is attenuated in the absence of Phb1, suggesting that Phb1 may play an important role in p53-regulated apoptosis (Chander, Halpern et al. 2010, Liu, Peck et al. 2012). Whether Phb1 is involved in p53 regulated mitochondrial dynamics and apoptosis in gynecologic cancer cells is not known.

Figure 1.5

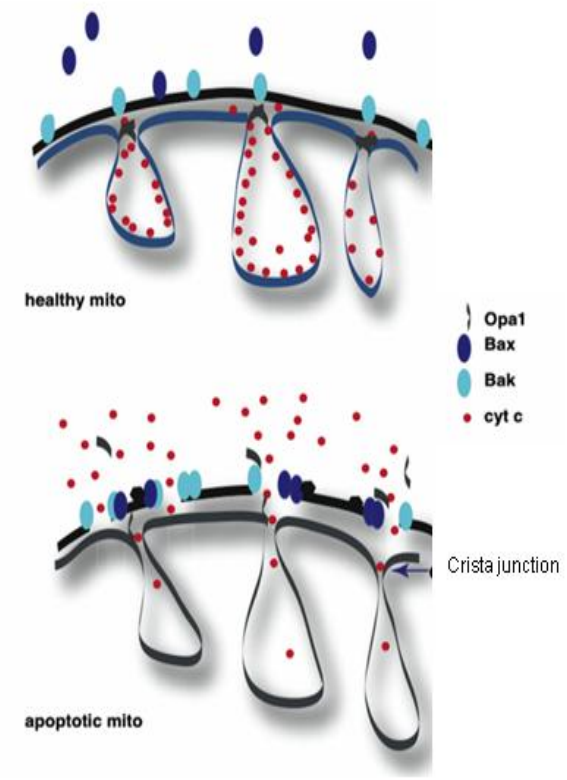

healthy mito
Opa1
Bax
Bak
cyt c
Crista junction
apoptotic mito

Figure 1.5 A model showing the mechanism by which Bak/Bax and Opa1 regulate cytochrome c (cyt c) release. In non-apoptotic cells, Bak (in mitochondrial outer membrane) and Bax (in cytosol) are not activated. Oligomerized Opa1 blocks the cristae junction, preventing the release of cyt c. During apoptosis, L-Opa1 is processed, inducing the destabilization of Opa1 complex and opening of cristae junction. Activated Bak/Bax oligomerize and form a pore in mitochondrial outer membrane. Cyt c is released through cristae junction and mitochondrial outer membrane pore into cytosol and induces apoptosis. (Yamaguchi and Perkins 2009)

Chapter 2: Objectives and Hypotheses

Platinum derivatives are the first line chemotherapeutic drugs in the treatment of OVCA and CECA patients. Chemoresistance severely limits treatment success in these two cancers. The underlying mechanism of chemoresistance is multi-factorial and partly due to defects in drug-induced apoptosis (Li, Feng et al. 2001).

Mitochondria are highly dynamic organelles. Mitochondria fusion and fission are involved in the regulation of mitochondria-mediated apoptosis. The long form of Opa1, a key player for mitochondrial fusion, is processed by Oma1 during mitochondrial fragmentation and apoptosis. Whether Oma1-mediated L-Opa1 processing and mitochondrial dynamics are involved in the regulation of chemoresistance in gynecologic cancer cells is not known.

In addition to its genomic action, p53 induces apoptosis in a transcription-independent manner by targeting mitochondria. p53 phosphorylation and targeting mitochondria are determinants of sensitivity of OVCA and CECA cells to CDDP (Yang, Fraser et al. 2006), although whether p53 regulates apoptosis by modulating Oma1-mediated L-Opa1 processing and mitochondrial dynamics is not known. The tumor suppressor Phb1 regulates Oma1-mediated Opa1 processing, but the mechanism involved is not known. Recent publications have shown that p53 interacts with Phb1 upon apoptotic signaling, and the function of p53 is attenuated in the absence of Phb1 (Chander, Halpern et al. 2010, Liu, Peck et al. 2012), suggesting that Phb1 may play an important role in p53-regulated apoptosis. Whether Phb1 plays a role in p53-regulated apoptosis by regulating mitochondrial dynamics in gynecologic cancer cells needs to be further explored. Interestingly, p-p53 (ser15) has been reported to interact with Bak, a

pro-apoptotic member of the Bcl-2 family, leading to Bak activation and apoptosis (Nieminen, Eskelinen et al. 2013). Whether the interaction of p-p53 (ser15) and Bak is associated with Oma1 mediated L-Opa1 processing, mitochondrial fragmentation, and how Phb1 is involved in the interaction needs to be investigated.

Akt is an important cell survival factor and is found to be activated or over-expressed in different cancer types (Bauer, Patel et al. 2014). Previous publications from our laboratory showed that Akt promotes chemoresistance through inhibition of p53 phosphorylation and its action on the caspase-dependent mitochondrial death pathway (Yang, Fraser et al. 2006, Fraser, Bai et al. 2008). Whether Akt also modulates the action of p-p53 in regulating mitochondrial dynamics is not known.

1. Hypotheses

The overall hypothesis is that chemoresistance in gynecologic cancer cells is determined by dysregulated mitochondrial dynamics induced by the action of p53 and a series of downstream molecules. The specific hypotheses are:

In chemosensitive cells, CDDP induces activation of p53 (by phosphorylation at ser15), which targets mitochondria and competitively binds Phb1, therefore destroying the complex of Phb1-Opa1. The complex of p-p53 and Phb1 binds to Bak, which induces Bak activation and MOMP, leading to activation of Oma1 and L-Opa1 processing. In chemoresistant cells, p53 phosphorylation and targeting mitochondria are suppressed by high Akt activity. Consequently, the complex formation of p-p53, Phb1 and Bak is inhibited, and L-Opa1 and mitochondrial dynamics are stabilized (Figure 2.1).

2. Objectives

The overall objective of my research was to increase the current understanding of the regulation of mitochondrial dynamics and its role in chemoresistance in gynecologic cancer cells. My specific objectives were to examine:

1) whether Oma1 processes L-Opa1 during CDDP-induced apoptosis, and whether this is associated with mitochondrial dynamics and chemosensitivity in gynecologic cancer cells
2) whether p53 regulates Oma1-Opa1 pathway by interacting with Phb1 and Bak
3) whether Akt promotes chemoresistance by suppressing the action of p53 in Oma1-mediated L-Opa1 processing and mitochondrial dynamics.

Figure 2.1

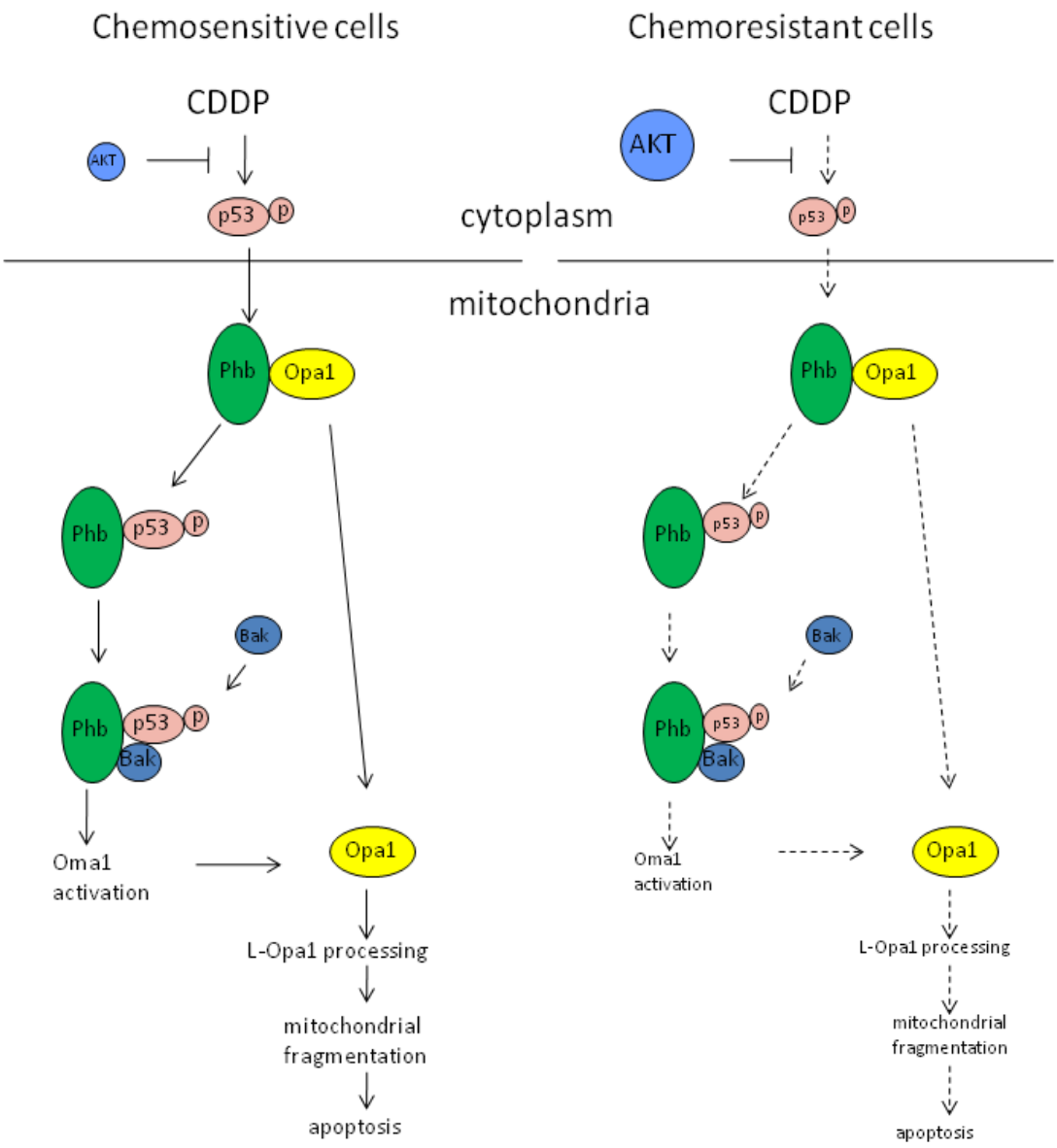

Chemosensitive cells
Chemoresistant cells
CDDP
CDDP
AKT
AKT
p53
P
cytoplasm
mitochondria
Phb
Opa1
Bak
Oma1
activation
L-Opa1 processing
mitochondrial
fragmentation
apoptosis

Figure 2.1 A hypothetical model illustrating the involvement of Akt, p-p53, Phb1, Bak, Oma1 and Opa1 in the regulation of mitochondrial fragmentation, apoptosis, and chemosensitivity in gynecologic cells. In chemosensitive cells, CDDP induces p53 phosphorylation (ser15), which accumulates in the mitochondria and targets the Phb1- Opa1complex. p-p53 (ser15) binds to Phb1 and releases Opa1. The p-p53 (ser15)-Phb1 complex binds to Bak, inducing Bak activation and mitochondrial outer membrane permeabilization (MOMP). Oma1 is activated, inducing L-Opa1 processing and mitochondrial fragmentation, and subsequent apoptosis. In chemoresistant cells, CDDP-induced p53 phosphorylation is inhibited by Akt, stabilizing the Phb1-Opa1complex and inhibiting Oma1 activation. L-Opa1 is protected from being processed, leading to the failure of CDDP to induce mitochondrial fragmentation and apoptosis.

CHAPTER 3: MATERIALS AND METHODS

1. Reagents

CDDP, DMSO, Hoechst 33258, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), and aprotinin were purchased from Sigma-Aldrich (St Louis, MO, USA). Mouse monoclonal GAPDH, mouse monoclonal p-p53 (ser20), mouse monoclonal HA tag antibody, rabbit monoclonal anti-Bak, mouse monoclonal Mfn1, mouse monoclonal Mfn2 and rabbit polyclonal Oma1 antibody were from Abcam (Cambridge, MA, USA). Mouse monoclonal translocase of outer mitochondrial membrane 20 (Tom20) antibody, mouse monoclonal p53 antibody, rabbit polyclonal p-p53 (ser15) antibody, rabbit polyclonal Histone H3 and rabbit polyclonal Phb1 antibody were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Mouse monoclonal Opa1 and mouse monoclonal Drp1 were from BD (Franklin Lakes, NJ, USA). Rabbit polyclonal Fis1 antibody was purchased from Life Span Biosciences (Seattle, WA, USA). A list of antibodies used in the present studies is provided in Table 7.1.

Oma1 plasmid, p53 plasmid, Oma1 siRNA, Phb1 siRNA, scramble siRNA and mouse monoclonal Myc-tag antibody were from Origene (Rockville, MD, USA). p53 siRNA was from Qiagen (Valencia, CA, USA). RNeasy Mini kits were purchased from QIAGEN (Mississauga, Canada). Random decamer primers were from Ambion (Austin, USA). M-MLV Reverse Transcriptase was from Promega (Madison, USA). Ribonuclease inhibitor and dNTP were from Fermentas (Burlington, Canada). qPCR primers were from Invitrogen (Burlington, Canada). HA-tagged, triple-A mutated (K179A, T308A, S473A) kinase-dead DN-Akt and LacZ adenoviral constructs were synthesized at the University of Ottawa Adenoviral Core Facility (Ottawa, ON, Canada).

2. Cell Lines and Cell Culture

The CDDP-sensitive cancer cell line OV2008 (wt-p53) is of cervical origin. CDDP-resistant C13* (wt-p53) cell line is the isogenic resistant counterpart to OV2008, selected by chronic exposure of increasing concentrations of CDDP *in vitro*. CDDP-sensitive A2780s (wt-p53), its resistant variant A2780cp (p53 mutant), and CDDP-resistant HEY (wt-p53) human ovarian cancer cell lines were derived from ovarian moderately differentiated papillary cystadenocarcinoma. SKOV3 cells (p53 null) are of clear cell carcinoma origin (Shaw, Senterman et al. 2004). These cell lines were gifts from Drs. Rakesh Goel and Barbara Vanderhyden (Ottawa Regional Cancer Centre, Ottawa, Ontario, Canada), and were cultured as previously reported (Abedini, Qiu et al. 2004, Abedini, Muller et al. 2010, Woo, Xue et al. 2012). Please see Table 7.2 for a summary of the characteristics of these cell lines.

Cells were maintained in RPMI 1640 (OV2008, C13* and HEY) and DMEM/F12 (A2780s, A2780cp and SKOV3). RPMI 1640 and DMEM/F12 media contained heat-inactivated FBS (10%), streptomycin (50,000 µg/L), penicillin (50,000 U/L) and Fungizone (625 µg/L). Cells were plated (60 mm culture dishes) at 40% confluence unless stated otherwise and cultured at 37ºC with 5% CO_2/95% O_2.

3. Protein Extraction and Western Blot Analysis

Protein extraction and western blot analysis were performed as previously described (Fraser, Leung et al. 2003). Cells were sonicated in lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM sodium fluoride, 10

mM NaPPi, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM PMSF, 10 µg/µl aprotinin, and 1mM Na_3VO_4. Proteins were isolated and quantified. Equivalent amounts of total protein were loaded onto acrylamide gels (8–10%), separated by PAGE, and transferred to nitrocellulose membranes. Ponceau-S staining was used to confirm even loading between groups. The membranes were blocked for 1 h in Blotto (5% skim milk in Tris-buffered saline-Tween). Unless indicated otherwise, membranes were incubated overnight at 4°C, with antibodies against Opa1 (1:1000), Mfn1 (1:1000), Mfn2 (1:1000), Drp1 (1:1000), Fis1 (1:1000), Oma1(1:1000), Phb1 (1:2000), p-p53 (ser15) (1:2000), p-p53 (ser20) (1:2000), p53 (1:5000), Bak (1:1000), Histone H3 (1:5000), Tom20 (1:5000), and HA tag (1:5000), and 1 h at room temperature for anti-GAPDH (1:10,000), Vinculin (1:10,000) and horseradish peroxidase-conjugated rabbit or mouse secondary antibodies (1:5000-1:10,000), and signal intensity (using enhanced chemiluminescene kit from Amersham Pharmacia Biotech, Arlington Heights) was analyzed densitometrically (Scion Image software; Scion Corporation, Frederick, MD).

4. Fluorescence Microscopy and Determination of Mitochondrial Phenotype

The procedures were performed as previously described (Woo, Xue et al. 2012). Cells were plated on poly-D-lysine-coated (0.05% w/v; Sigma) 8-well glass culture slides (BD Biosciences) and cultured in growth media (48 h) prior to CDDP treatment. For immunocytochemistry, cells were fixed in paraformaldehyde (4%, 1 h, RT), washed in PBS, and blocked with 1% BSA. Mitochondria were visualized by immunofluorescence microscopy, using a mouse monoclonal antibody anti-human Tom20 (1:100) and Alexa Fluor 488 goat anti-mouse secondary antibody (1:500; Invitrogen). Confocal images were

obtained (×100 objective) on an Olympus IX81 inverted microscope with appropriate argon lasers (488 nm). Mitochondrial phenotype of each cell was categorized as tubular, intermediate or completely fragmented as previously described (Taguchi, Ishihara et al. 2007, Mai, Klinkenberg et al. 2010, Rambold, Kostelecky et al. 2011). Cells displaying an intact network of tubular mitochondria were classified as tubular. When this network was disrupted and mitochondria appeared predominantly rod-like or sphere, they were classified as intermediate. Cells with only spherical mitochondria (no tubular or rod-like mitochondria) were classified as completely fragmented. The mitochondrial morphology of at least 100 cells per treatment group was determined in a blinded manner, *i.e.* the researcher was blind to treatment status. Quantifications were based on at least three independent experiments.

5. RNA Interference

For gene knock-down studies, cells were transfected with p53 siRNA (0 - 100 nM; 48h), Oma1 siRNA (0 - 40 nM; 48 h), Phb1 siRNA (0 - 100 nM; 24h), or control siRNA (scrambled sequence) using Lipofectamine 2000 (Invitrogen), and were treated with CDDP (0 - 10 μM; 24 h), and harvested for further analysis.

6. Transient cDNA Transfection

OV2008 were transfected with C-terminus Oma1-myc cDNA (0-1 μg, 24 h; Origene) as previously described (Woo, Xue et al. 2012). Empty vector served as a control. Following transfection, cells were treated with CDDP (10 μM) or vehicle (DMSO, 1:1000) and harvested for analyses or fixed for immunofluorescence studies.

A2780cp and SKOV3 cells were transfected with p53 cDNA (0 - 2 μg, 24 h). Empty vector served as a control. Following transfection, cells were treated with CDDP (10 μM, 0 - 24 h) or vehicle (DMSO at 1:1000, 0 - 24 h) and fixed for immunofluorescence studies or protein extracted for western blot.

HA-tagged wild type p53 in pcDNA3 was used as a template for site-directed mutagenesis, using the QuickChange Site-Directed Kit from Stratagene (La Jolla, CA) as previously described (Fraser, Bai et al. 2008). The presence of mutations was confirmed by direct sequencing at the Ottawa Hospital Research Institute (OHRI) sequencing facility.

7. Reverse Transcriptase Polymerase Chain Reaction

Total RNAs were extracted according to the manufacturer's instructions, using the Qiagen RNeasy Mini kit. One μg total RNAs were reverse transcribed into cDNA and the mRNA abundances of target genes were analyzed by real-time quantitative PCR using LightCycler® 480 SYBR Green I Master (Roche Diagnostics, Quebec). The variants of Opa1 were amplified using specific primer pairs based on literature (Table 7.3) (Olichon, Elachouri et al. 2007). Data were analyzed by the 2-ΔΔCT method (Livak and Schmittgen 2001). The data are presented as the fold change in mRNA abundance normalized against the β-actin gene (commercial primers from Invitrogen, sequence showed in Table 7.3) and expressed relative to the respective control.

8. Immunoprecipitation

One mg of protein sample was incubated (1h, RT) with 50 µL Protein G Dynabeads (Invitrogen) coated with rabbit polyclonal Phb1 antibody (2µg, 1 h, RT) or rabbit monoclonal Bak antibody (1µg, 1 h, RT) and immunoprecipitated (4ºC, overnight). The beads were pelleted, and resuspended in Laemmli sample buffer (2X; 35 µL; Bio-Rad Laboratories Ltd., Mississauga, ON), boiled (10 min), and loaded onto 9% SDS-PAGE gels. After protein was transferred to nitrocellulose, Phb1, phosphorylated p53, Opa1 were examined by western blotting, using Clean Blot IP Detection Reagent (Thermo Scientific).

9. Determination of apoptosis

Depending on experimental design and preference of individual laboratory, apoptosis could be assessed by different methods, such as TUNEL assay, flow cytometry, or PARP cleavage. Our laboratory uses Hoechst staining routinely to identify apoptotic cells, which is a well-established and widely accepted method and is based on the morphology of the cell undergoing apoptosis, including nuclear shrinkage, fragmentation and chromatin condensation. We have also assessed apoptosis by flow cytometry and determining PARP cleavage and compared them with Hoechst staining, which showed consistent results and confirmed its reliability (Ali, Abedini et al. 2012, Woo, Xue et al. 2012). Apoptosis rate was assessed morphologically with Hoechst 33258 nuclear dye (6.25 ng/ml). Cells with fragmented DNA or condensed nucleus were considered as

apoptotic. At least 400 cells/treatment groups were assessed in randomly selected fields with the counter blinded to the sample group to avoid experimental bias.

10. Adenoviral infection

C13* cells were infected with adenoviral HA-tagged "triple-A" (K179A, T308A, S473A) dominant-negative Akt (DN-Akt, MOI = 0 - 80, 24 h) as previously described (Fraser, Bai et al. 2008). Adenoviral LacZ served as a control. DN-Akt expression was confirmed by western blot with anti-HA antibody.

11. Cellular fractionation

Mitochondrial, nuclear and cytosolic fractions were prepared as previously described (Dimauro, Pearson et al. 2012). Cells were washed with cold PBS, followed by centrifugation (200 xg; 7 min). All samples were resuspended in 300-500 µl of STM buffer (comprising 250 mM sucrose, 50 mM Tris-HCl pH 7.4, 5 mM MgCl2, protease and phosphatase inhibitor cocktails). After 60 strokes in a Dounce homogenizer, the homogenate was decanted into a centrifuge tube and maintained on ice (30 min), vortexed (15 s) and then centrifuged (800 xg, 15 min). The pellet was labeled as P_0, the supernatant was labeled as S_0 and used for subsequent isolation of mitochondrial and cytosolic fractions.

The pellet P_0 was resuspended in 300-500 µl STM buffer, vortexed (15 s) and centrifuged (500 xg, 15 min). The pellet was washed in STM buffer (300-500 µl), vortexed (15 s) and then centrifuged (1,000 xg, 15 min). The washed pellet was resuspended in 200-500 µl NET buffer (comprising: 20 mM HEPES pH 7.9, 1.5 mM

MgCl2, 0.5 M NaCl, 0.2 mM EDTA, 20% glycerol, 1%Triton-X-100, protease and phosphatase inhibitors). Pellet was vortexed (15 s), incubated on ice for 30 minutes, and sonicated at high setting for 10 - 15 seconds. The lysate was centrifuged (9,000 xg, 30 min, 4°C) and the resultant supernatant was the final "nuclear fraction".

Cytosolic and mitochondrial fractions were extracted from S_0 by centrifugation (800 xg, 10 min). The supernatant S_1 was then centrifuged (11,000 xg,10 min) and the supernatant S_2 was precipitated in cold 100% acetone at -20°C for at least 1 hour followed by centrifugation (12,000 xg,5 min), the new pellet was then resuspended in 100-300 µl STM buffer and labeled as "cytosolic fraction". The pellet P_2 (separated from S_2) was again resuspended in 100-200 µl STM buffer and centrifuged (11,000 xg, 10 min). Once centrifuged, the mitochondrial pellet (P_3) was resuspended in 100 µl SOL buffer (comprising: 50 mM Tris HCl pH 6.8, 1 mM EDTA, 0.5% Triton-X-100, protease and phosphatase inhibitors) by sonication on ice (5–10 s) and labeled as "mitochondrial fraction".

12. Statistical Analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was carried out by two-way analysis of variance, using PRISM software (Version 5.0; GraphPad, San Diego, CA, USA). Differences between multiple experimental groups were determined by the Bonferroni post-hoc test. Statistical significance was inferred at $p<0.05$.

CHAPTER 4: RESULTS

Objective 1: Examine the mechanism of action of Oma1 in processing L-Opa1 in the regulation of mitochondrial dynamics and chemosensitivity

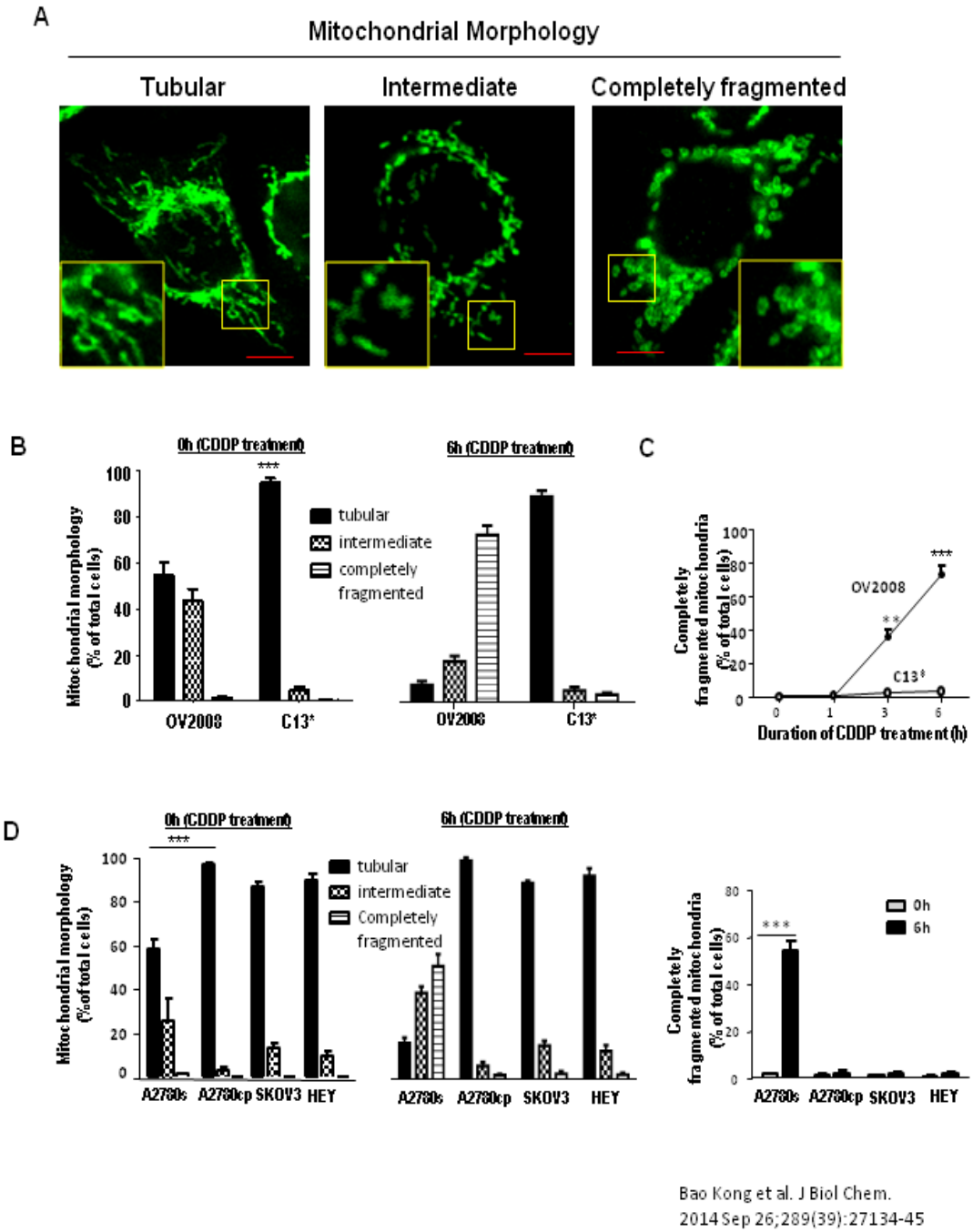
4.1 CDDP decreases mitochondrial fusion protein Opa1 (long form) and increases mitochondria fragmentation in chemosensitive but not resistant gynecologic cancer cells.

CDDP induces apoptosis in chemosensitive but not chemoresistant gynecologic cancer cells (Fraser, Leung et al. 2003, Yang, Fraser et al. 2006, Woo, Xue et al. 2012). To determine if changes in mitochondrial dynamics play a role in the regulation of chemosensitivity in CECA cells, OV2008 and C13* cells were cultured in the absence and presence of CDDP (10 μM, 0 - 6 h) and mitochondrial phenotype was examined by immunofluorescence (IF) confocal microscopy. Three major mitochondrial phenotypes were found irrespective of the presence of CDDP: tubular, intermediate, completely fragmented (Fig 4.1A). Although only a few completely fragmented mitochondria were observed in these chemosensitive and chemoresistant cancer cells in the absence of CDDP, the percentage of cells bearing tubular mitochondria was much higher in chemoresistant C13* cells than in chemosensitive OV2008 cells (0 h, $p<0.001$, Fig 4.1B). CDDP induced mitochondrial fragmentation in the chemosensitive cells in time-dependent manner, as evident by an increase in the percentage of cells with completely fragmented mitochondria as early as 3 h of CDDP exposure ($p<0.01$ at 3h, Fig 4.1C). In contrast, in chemoresistant cells, CDDP failed to significantly influence these

mitochondrial structures, implying the presence of stabilized and fused mitochondria in chemoresistant cells (Fig 4.1 B&C).

To determine whether mitochondrial dynamics are dysregulated in chemoresistant OVCA cancer cells, and whether p53 status affects mitochondrial dynamics, we examined mitochondrial dynamics in chemosensitive A2780s cells (wt-p53) and its chemoresistant counterpart A2780cp cells (p53 mutant), SKOV3 cells (p53 null) and HEY cells (wt-p53) in the absence and presence of CDDP (10 μM, 6 h). The percentage of cells bearing tubular mitochondria was much higher in chemoresistant A2780cp cells than in chemosensitive A2780s cells in the absence of CDDP (0 h, $p<0.001$, Fig 4.1D). Most SKOV3 cells and HEY cells also exhibit tubular mitochondria (Fig 4.1D). CDDP increased the number of cells with complete mitochondrial fragmentation in A2780s cells ($p<0.001$, Fig 4.1D), but not in A2780cp, SKOV3 and Hey cells, suggesting that mitochondrial fragmentation is associated with chemosensitive but not chemoresistant OVCA cells in response to CDDP.

Figure 4.1



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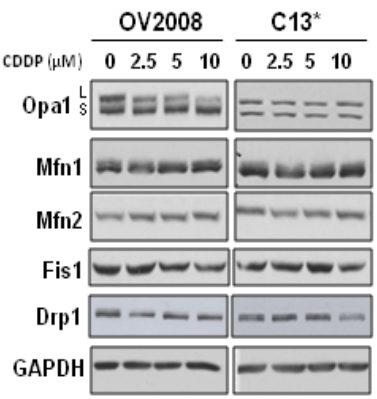
2014 Sep 26;289(39):27134-45

Figure 4.1 Mitochondrial dynamics in chemosensitive and chemoresistant gynecologic cancer cells. (**A**) Mitochondrial phenotypes (tubular, intermediate, and completely fragmented) in CECA cells (OV2008&C13*). Mitochondria were immuno-stained with Tom20 antibody. Mitochondrial phenotypes were examined by immunofluorescence confocal microscopy. Inset is enlarged image of boxed area. (**B**) Comparison of mitochondrial phenotypes in chemosensitive and chemoresistant CECA cells treated with CDDP. OV2008 and C13* cells were cultured with and without CDDP (10 μM, 0-6 h). Percentage of cells with tubular mitochondria in chemoresistant cells (C13*) is higher than in chemosensitive cells (OV2008) at 0 h. More than 100 cells were assessed in each experimental group (n=3, ***$p<0.001$). (**C**) Comparison of completely fragmented mitochondrial phenotype in chemosensitive and chemoresistant CECA cells treated with CDDP. OV2008 and C13* cells were cultured with CDDP (10 μM, 0-6 h). Mitochondrial phenotypes were examined by immunofluorescence confocal microscopy. OV2008 cells exhibited much higher level of completely fragmented mitochondria than C13* cells with CDDP treatment (n=3, **$p<0.01$ at 3 h and ***$p<0.001$ at 6 h). At least 100 cells were assessed for each time point in each replicate. (**D**) Comparison of mitochondrial phenotypes in different OVCA cell lines treated with CDDP. A2780s, A2780cp, SKOV3 and HEY cells were cultured with and without CDDP (10 μM, 0-6 h). Chemoresistant A2780cp cells had more tubular mitochondria than chemosensitive A2780s cells (0 h, n=3, ***$p<0.001$). Most SKOV3 and HEY cells exhibited tubular mitochondria. CDDP induced a significant increase in mitochondria complete fragmentation in A2780s (***$p<0.001$, n=3) but not in A2780cp, SKOV3 and HEY cells. Over 100 cells were assessed in each experimental group. Scale bars are all 5μm.

To further examine the regulation of mitochondrial dynamics in CECA cells and its possible involvement in the control of chemosensitivity, we examined several key regulators of mitochondrial fission and fusion by western blotting, including the abundance of mitochondrial fusion proteins Opa1, Mfn1, and Mfn2, and mitochondrial fission proteins Fis1 and Drp1 in OV2008 and C13* cells following exposure to CDDP (0-10 μM, 0-48 h). Mitochondrial fusion protein Opa1 contents are higher in OV2008 cell than C13* cells. CDDP decreased L-Opa1 in OV2008 cells in a time- and concentration-dependent manner ($p<0.001$, Fig 4.2 & 4.3). This response was however, not apparent in C13* cells. The contents of other intracellular regulators of mitochondrial fission and fusion were not affected by CDDP under same experimental conditions.

Figure 4.2


OV2008
C13*
CDDP (μM)
0 2.5 5 10 0 2.5 5 10
Opa1 L S
Mfn1
Mfn2
Fis1
Drp1
GAPDH

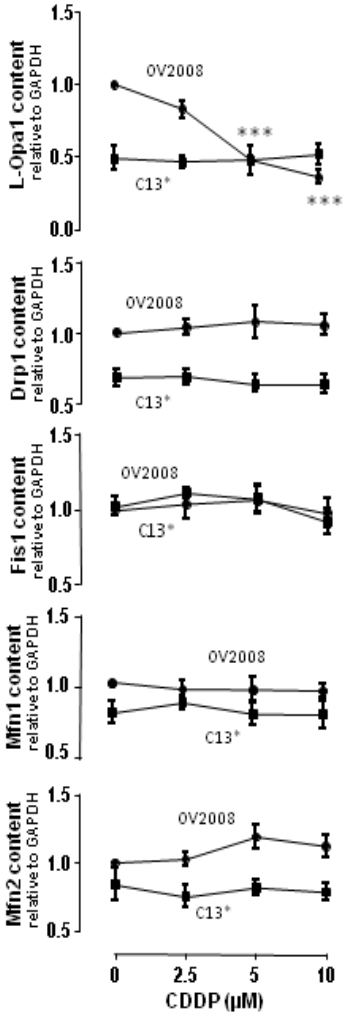

L-Opa1 content relative to GAPDH
Drp1 content relative to GAPDH
Fis1 content relative to GAPDH
Mfn1 content relative to GAPDH
Mfn2 content relative to GAPDH
OV2008
C13*
***
***
1.5
1.0
0.5
0.0
0
2.5
5
10
CDDP (μM)

Figure 4.2 Influence of CDDP on contents of mitochondrial fission proteins (Drp1 and Fis1) and mitochondrial fusion proteins (Mfn1, Mfn2 and Opa1) in CECA cells. OV2008 and C13* cells were cultured with CDDP (0 - 10 μM, 24h), Opa1, Mfn1, Mfn2, Drp1 and Fis1 contents were examined by Western blot. Opa1 content (long form) significantly decreased in a concentration-dependent manner in chemosensitive (OV2008) but not in chemoresistant (C13*) cells when treated with CDDP**.** Contents of Mfn1, Mfn2, Drp1 and Fis1 were not altered by CDDP treatment. Representative Western blots of four independent experiments are shown.

Fig4.3

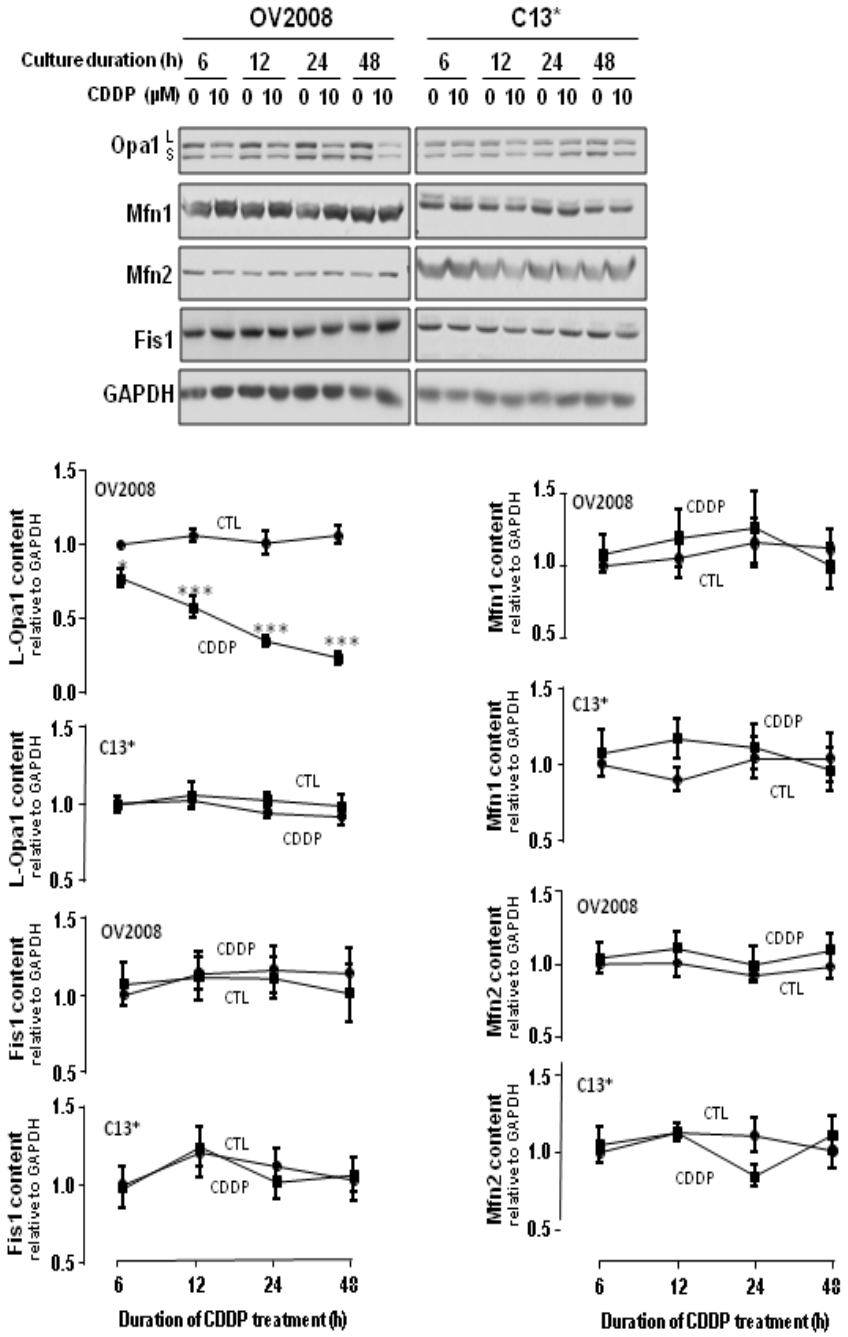

OV2008
C13*
Culture duration (h) 6 12 24 48 6 12 24 48
CDDP (μM) 0 10 0 10 0 10 0 10 0 10 0 10 0 10 0 10
Opa1 L S
Mfn1
Mfn2
Fis1
GAPDH
L-Opa1 content relative to GAPDH
OV2008
CTL
CDDP
*
***
***
***
C13*
Fis1 content relative to GAPDH
Mfn1 content relative to GAPDH
Mfn2 content relative to GAPDH
1.5
1.0
0.5
0.0
6 12 24 48
Duration of CDDP treatment (h)

Fig 4.3 Influence of CDDP on contents of mitochondrial fission proteins (Fis1) and mitochondrial fusion proteins (Mfn1, Mfn2 and Opa1) in CECA cells. OV2008 and C13* cells were cultured with CDDP (0 or 10 μM, 6 - 48 h), Opa1, Mfn1, Mfn2 and Fis1 contents were examined by Western blot. Opa1 content (long form) significantly decreased in a time- dependent manner in chemosensitive (OV2008) but not in chemoresistant (C13*) cells when treated with CDDP. Contents of Mfn1, Mfn2 and Fis1 were not altered by CDDP treatment. Representative Western blots of four independent experiments are shown.

4.2 Chemosensitive CECA cells exhibit five Opa1 forms whereas only three are present in the chemoresistant counterpart.

At least five forms of Opa1 have been reported in mammalian cells (Guillery, Malka et al. 2008). We thus examined the differences in the contents of different Opa1 forms when chemosensitive and chemoresistant CECA cells were challenged with CDDP (0-10 μM, 0-24 h). Interestingly, with over running of the western blot, five forms of Opa1 (95, 92, 88, 84, 81kDa) could be recognized in OV2008 cells, while only three in C13* cells (95, 92, 84 kDa) (Fig 4.4). CDDP decreased the contents of Opa1 (95, 92 kDa) forms ($p<0.001$ and $p<0.01$, respectively), but increased the Opa1 (81 kDa) form ($p<0.01$) in OV2008 cells in a time- and concentration-dependent manner (Fig 4.4A and 4.4B, respectively). However, the content of the Opa1 forms in C13* cells were not affected by the CDDP treatment.

Figure 4.4

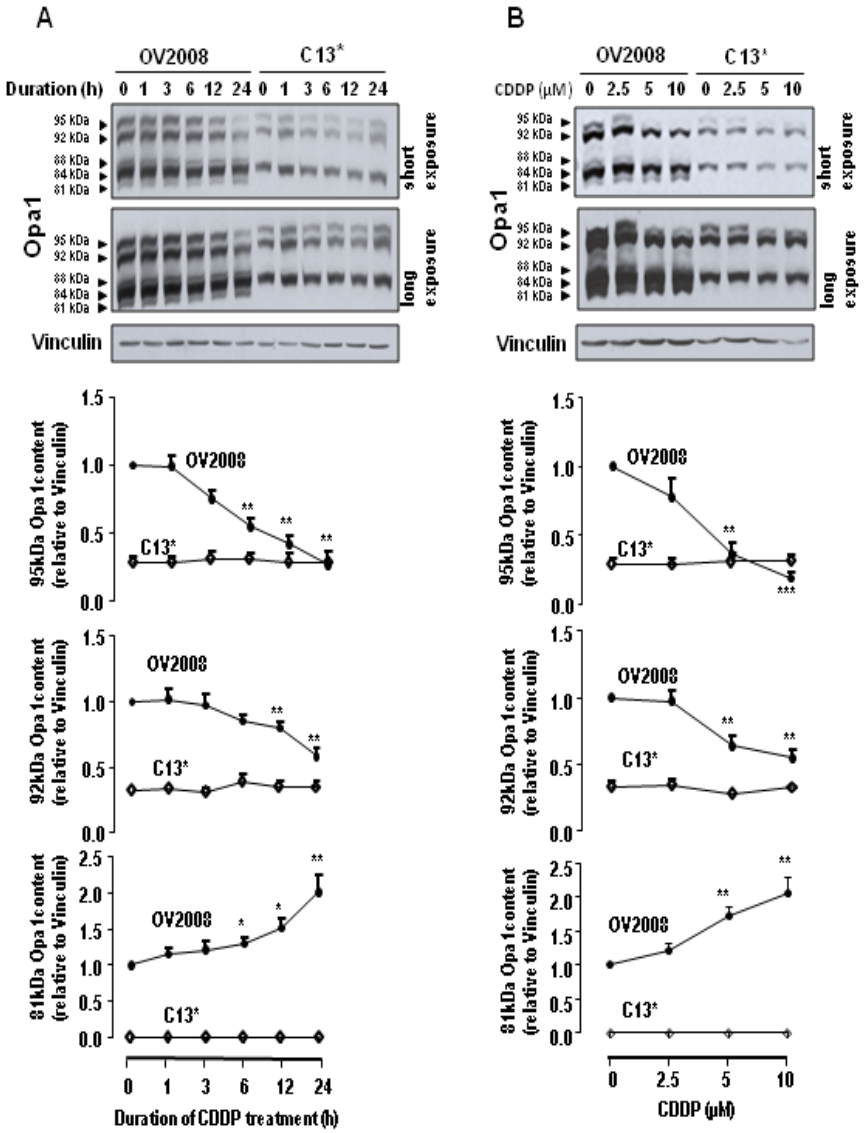
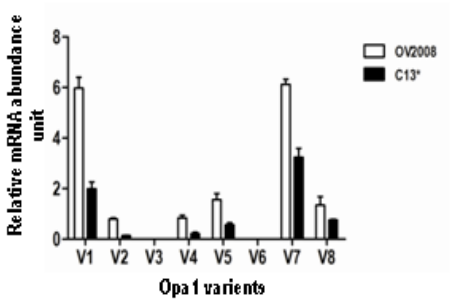

A
B
OV2008
C13*
Duration (h)
0 1 3 6 12 24
CDDP (μM)
0 2.5 5 10
95 kDa
92 kDa
88 kDa
84 kDa
81 kDa
Opa1
short exposure
long exposure
Vinculin
95kDa Opa1 content (relative to Vinculin)
92kDa Opa1 content (relative to Vinculin)
81kDa Opa1 content (relative to Vinculin)
Duration of CDDP treatment (h)
CDDP (μM)

Figure 4.4 Different forms of Opa1 in CECA cells. Chemosensitive (OV2008) and Chemoresistant (C13) CECA cells were cultured with CDDP for different durations (**A**; 10 μM, 0 - 24 h) or at different concentrations (**B**; 0 - 10 μM, 24 h). Contents of different forms of Opa1 and Vinculin (loading control) were examined by Western blot. Five forms of Opa1 (95, 92, 88, 84 and 81 kDa) were recognized in OV2008 cells, whereas only 3 forms (95, 92 and 84 kDa) in C13* cells. CDDP significantly decreased contents of L-Opa1 forms (95 and 92 kDa) while significantly increased S-Opa1 content (81 kDa) in a time- (**A**; *$p<0.05$, **$p<0.01$, $n = 4$) and concentration- (**B**; **$p<0.01$ ***$p<0.001$, $n = 3$) dependent manner in OV2008 cells, but not in C13* cells.

Opa1 has eight mRNA splice forms. The abundance of each mRNA splice form and their response to CDDP treatment in chemosensitive and chemoresistant CECA cells were examined by qPCR. Variants 1, 2, 4, 5, 7 and 8 were present in OV2008 and C13* cells and variants 1 and 7 are the most predominant forms. The abundance of the above variants are higher in the sensitive cells (OV2008) than in its resistant counterpart (C13*) (Fig 4.5A). CDDP significantly decreased the abundance of variants 1, 2, 4, 5, 7 and 8 in OV2008 cells, but not in C13* cells (Fig 4.5B).

Figure 4.5

A


Relative mRNA abundance unit
8
6
4
2
0
V1
V2
V3
V4
V5
V6
V7
V8
Opa1 varients
OV2008
C13*

B

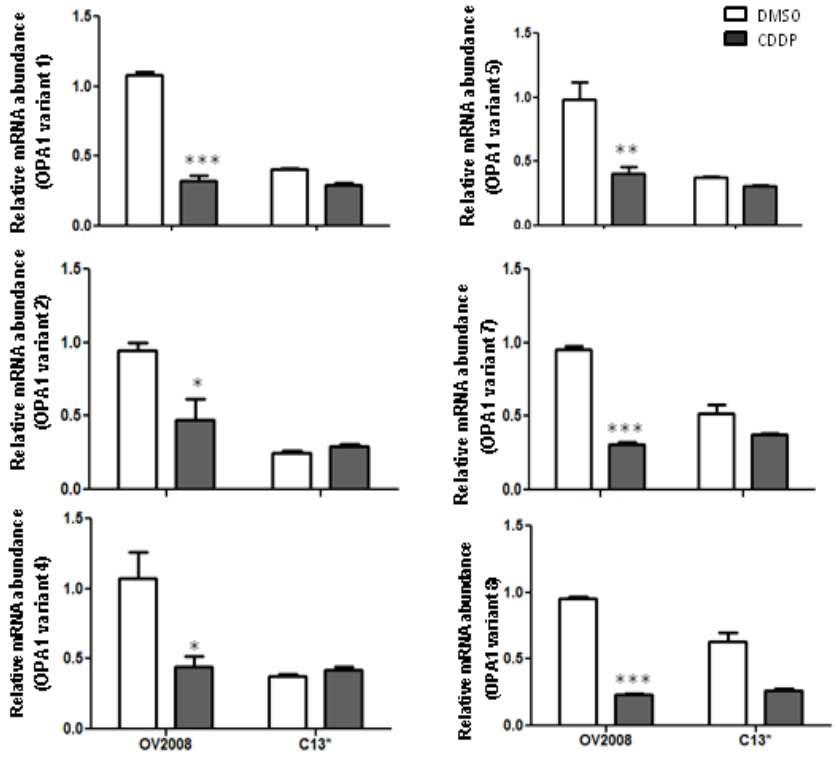

Relative mRNA abundance (OPA1 variant 1)
Relative mRNA abundance (OPA1 variant 5)
Relative mRNA abundance (OPA1 variant 2)
Relative mRNA abundance (OPA1 variant 7)
Relative mRNA abundance (OPA1 variant 4)
Relative mRNA abundance (OPA1 variant 3)
1.5
1.0
0.5
0.0
***
**
*
***
*
***
DMSO
CDDP
OV2008
C13*

Figure 4.5 Comparison of Opa1 splice forms in CECA cells. OV2008 and C13* cells were cultured with CDDP (10 μM, 6h; DMSO as control) and the abundance of each Opa1 splice form was examined by qPCR. (A) Form 1, 2, 4, 5, 7, 8 were expressed in OV2008 and C13* cells, and the abundance of the above forms are higher in OV2008 cells than C13* cells (n=3). V with number represents specific form of Opa1. (B) CDDP decreased all Opa1 splice forms detected in OV2008 cells, not in C13* cells (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n=3).

4.3 CDDP increased Oma1 content (40 kDa) in time- and concentration-dependent manner in chemosensitive but not chemoresistant CECA cells.

Oma1 is a protease believed to be involved in L-Opa1 processing in mammalian cells (Ehses, Raschke et al. 2009, Head, Griparic et al. 2009, Quiros, Ramsay et al. 2012). To examine expression of Oma1 and its possible role in regulating chemosensitivity, OV2008 cells and C13* cells were cultured with CDDP (0 - 10 μM, 0 - 24 h), and Oma1 content was examined by western blot. Two Oma1 forms (55 kDa and 40 kDa) were found in OV2008 cells, while only one Oma1 (55 kDa) in C13* cells. Oma1 content (40 kDa) significantly increased in a concentration- (Fig 4.6A, $p<0.001$) and time- (Fig 4.6B, $p<0.001$) dependent manner in OV2008 cells in respond to CDDP, but not in C13 * cells. In addition, CDDP failed to influence Oma1 content (55 kDa) in both OV2008 and C13* cells. To examine the relationship between the two Oma1 forms in CECA cells, Oma1-myc construct was transfected in OV2008 cells which were subsequently challenged with CDDP. The expression of Myc-tagged protein was investigated by western blot. While two bands (55 kDa and 40 kDa) were recognized using an anti-Myc antibody, CDDP increased the ratio of 40 kDa band to 55 kDa band ($p<0.001$, Fig 4.6C).

Figure 4.6

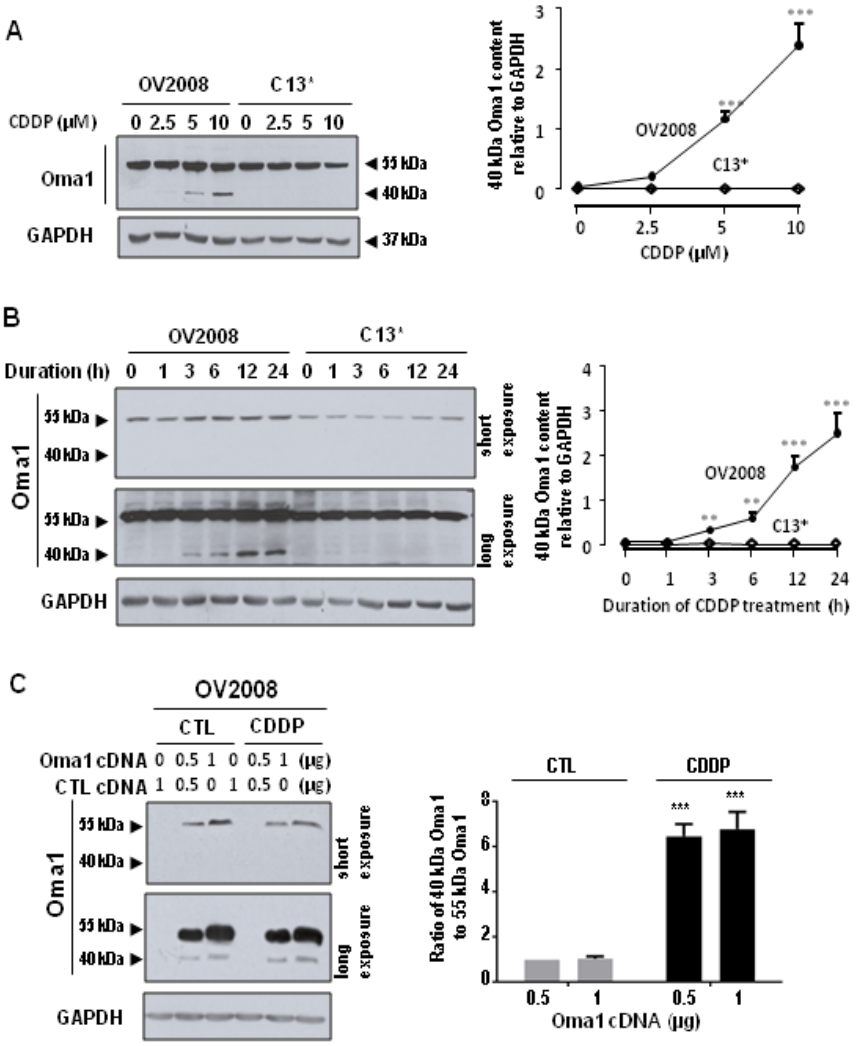

A
OV2008
C13*
CDDP (μM)
0 2.5 5 10 0 2.5 5 10
Oma1
GAPDH
55 kDa
40 kDa
37 kDa
40 kDa Oma1 content relative to GAPDH
OV2008
C13*
CDDP (μM)
B
OV2008
C13*
Duration (h)
0 1 3 6 12 24 0 1 3 6 12 24
Oma1
55 kDa
40 kDa
short exposure
long exposure
GAPDH
40 kDa Oma1 content relative to GAPDH
OV2008
C13*
Duration of CDDP treatment (h)
C
OV2008
CTL
CDDP
Oma1 cDNA 0 0.5 1 0 0.5 1 (μg)
CTL cDNA 1 0.5 0 1 0.5 0 (μg)
Oma1
55 kDa
40 kDa
short exposure
long exposure
GAPDH
CTL
CDDP
Ratio of 40 kDa Oma1 to 55 kDa Oma1
0.5 1 0.5 1
Oma1 cDNA (μg)

Figure 4.6 Different forms of Oma1 in chemosensitive and chemoresistant CECA cells. (A and B) OV2008 and C13* cells were cultured with CDDP at different concentrations (A: 0-10 μM, 24 h) or for different duration (B: 0 - 24 h, 10 μM). Contents of Oma1 and GAPDH (loading control) were examined by western blotting (n=3). Two forms of Oma1 (55 kDa and 40 kDa) were present in OV2008 cells but only one (55 kDa) in C13* cells when cultured in the presence of CDDP. Oma1 contents (40 kDa) in OV2008 cells but not in C13* cells significantly increased in the presence of CDDP in a concentration- and time-dependent manner. (**C**) OV2008 cells were transfected with myc tag-Oma1 cDNA or CTL cDNA (vector) and subsequently cultured with CDDP (10 μM, 24 h) or CTL (DMSO). Two myc bands (55 kDa and 40 kDa) were detected by Western blot with anti-myc antibody, with the ratio of 40kDa band to 55kDa band significantly increased in the presence of CDDP (***$p<0.001$, $n = 3$).

4.4 Oma1 is responsible for CDDP-induced L-Opa1 processing, mitochondrial fragmentation and apoptosis in chemosensitive CECA cells.

To determine whether Oma1 is required for CDDP-induced L-Opa1 processing in chemosensitive CECA cells, Oma1 expression in OV2008 cells was silenced by RNA interference (scramble siRNA as control) and then treated with CDDP *in vitro* (0-10 μM, 24 h). Oma1 and L-Opa1 contents were examined by western blot. Treatment of OV2008 cells with Oma1 siRNA significantly decreased CDDP-induced L-Opa1 loss (Fig 4.7A) and inhibited CDDP-induced mitochondrial fragmentation by 45% (a reduction of apoptotic rate from 42% to 25%) at 6 h ($p<0.001$, Fig 4.7B) and apoptosis by 40% at 24 h (Fig 4.7B).

Figure 4.7

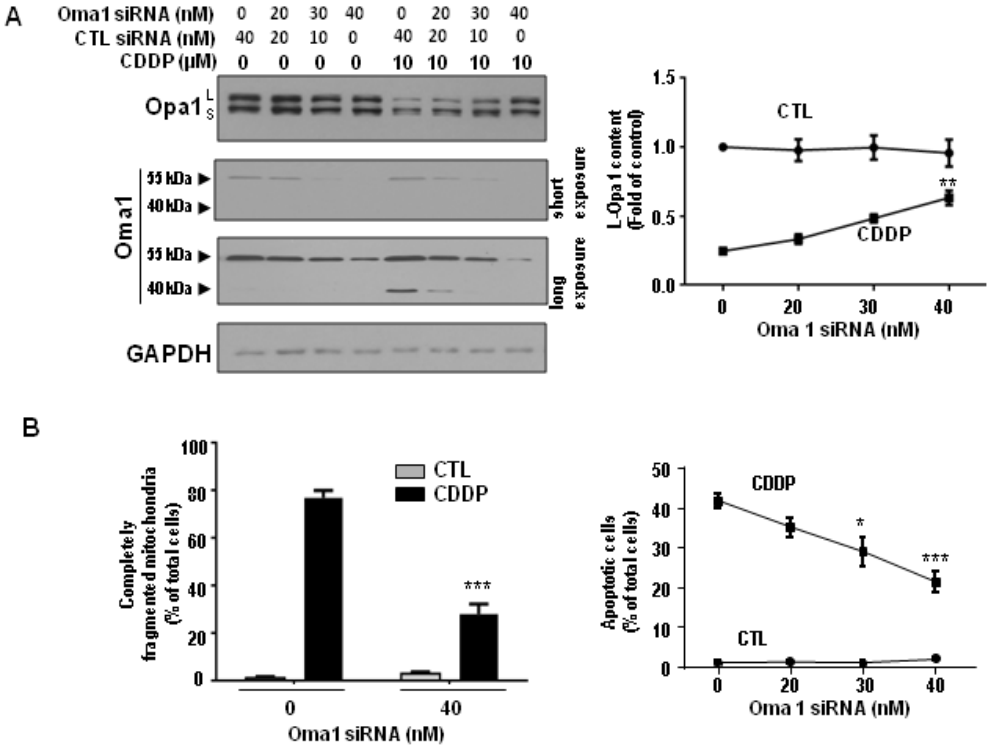

A
Oma1 siRNA (nM) 0 20 30 40 0 20 30 40
CTL siRNA (nM) 40 20 10 0 40 20 10 0
CDDP (μM) 0 0 0 0 10 10 10 10
Opa1 L S
Oma1
55 kDa
40 kDa
short exposure
long exposure
GAPDH
L-Opa1 content (Fold of control)
CTL
CDDP
Oma 1 siRNA (nM)
B
Completely fragmented mitochondria (% of total cells)
CTL
CDDP
Oma1 siRNA (nM)
Apoptotic cells (% of total cells)
CDDP
CTL
Oma 1 siRNA (nM)

Figure 4.7 Oma1 knock-down attenuated CDDP-induced loss in L-Opa1 content, mitochondrial fragmentation and apoptosis in OV2008 cells. (A) OV2008 cells were treated with Oma1 siRNA or CTL siRNA (scramble siRNA) (0-40 nM, 48 h) and cultured with CDDP (10 μM, 24 h) or CTL (DMSO). L-Opa1 and Oma1 contents were examined by Western blot. Oma1 siRNA (40 nM) significantly attenuated the loss of L-Opa1 content induced by CDDP (**$p<0.01$, versus 0 nM, n=3). (B) Left: OV2008 cells were treated with Oma1 siRNA or CTL siRNA (scramble siRNA) (0-40 nM, 24h) and subsequently with CDDP (10 μM, 6 h) or CTL (DMSO). Mitochondria were immunostained with anti-Tom20 antibody and examined by immunofluorescence confocal microscopy. Oma1 siRNA significantly decreased mitochondrial completely fragmentation induced by CDDP (***$p<0.001$, versus 0 nM, n=3). More than 100 cells were assessed in each experimental group. Right: OV2008 cells were treated with Oma1 siRNA or CTL siRNA (scramble siRNA) (40 nM, 24h) and subsequently with CDDP (10 μM, 24 h) or CTL (DMSO). Apoptosis was examined by Hoechst assay. Oma1 siRNA significantly decreased apoptosis induced by CDDP (*$p<0.05$, ***$p<0.001$, versus 0 nM n=3). More than 300 cells were assessed in each experimental group.

Objective 2: Examine the role of p53 in regulating this pathway by interacting with Phb1 and Bak

4.5 p53 is required for CDDP-induced, Oma1-mediated L-Opa1 processing, mitochondrial fragmentation and apoptosis.

Since p53 targets mitochondria rapidly during CDDP-induced apoptosis in gynecologic cancer cells (Yang, Fraser et al. 2006, Woo, Xue et al. 2012), we hypothesized that p53 is required for CDDP-induced Oma1 (40 kDa) content increase, L-Opa1 processing and mitochondria fragmentation in OV2008 cells. To test this hypothesis, OV2008 cells were transfected with p53 siRNA (scramble siRNA as control), followed by CDDP treatment (0-10 μM, 24 h), and changes in p53, Oma1 and L-Opa1 contents were examined. Knockdown of p53 by siRNA inhibited the CDDP-induced increase in Oma1 content (40 kDa) and L-Opa1 loss (Fig 4.8A&B). Treatment of p53 siRNA also inhibited CDDP-induced mitochondrial complete fragmentation at 6 h (Fig 4.8C) and apoptosis at 24 h (Fig 4.8B).

Figure 4.8

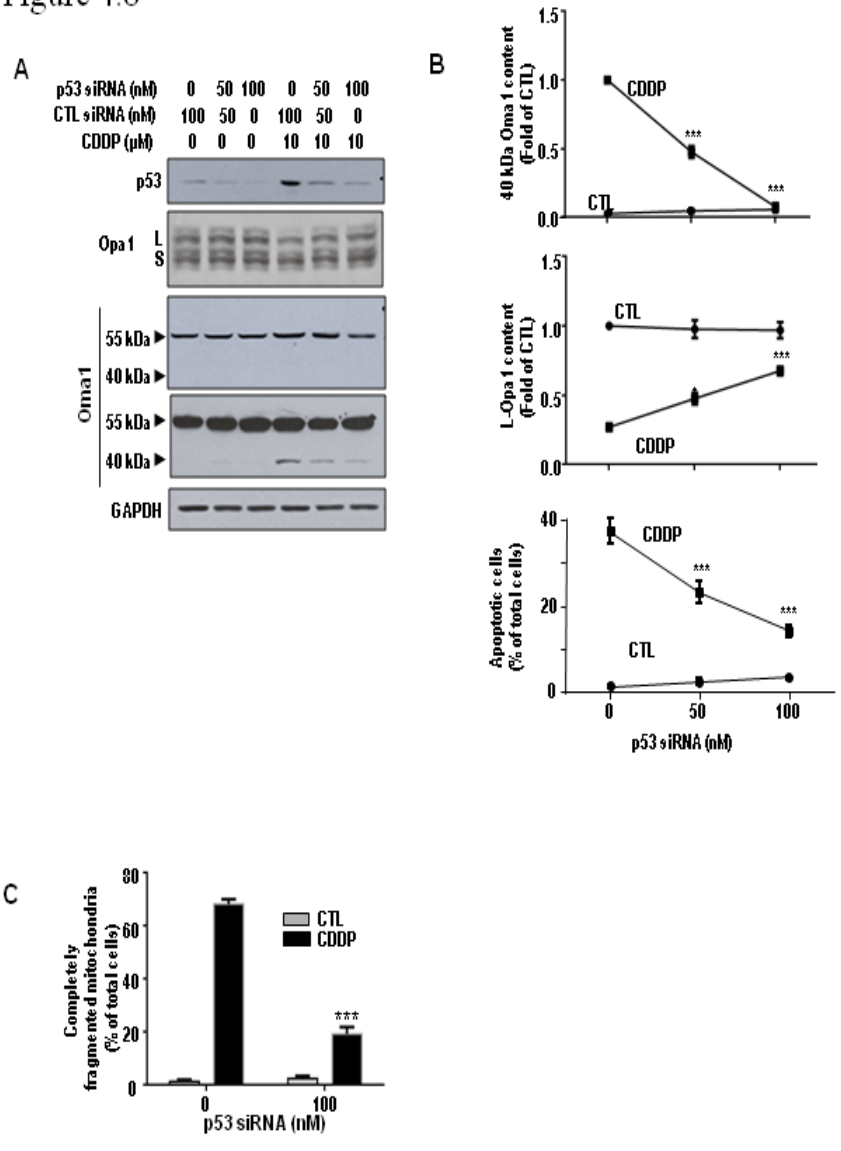

A
p53 siRNA (nM) 0 50 100 0 50 100
CTL siRNA (nM) 100 50 0 100 50 0
CDDP (μM) 0 0 0 10 10 10
p53
Opa1
L
S
Oma1
55 kDa
40 kDa
55 kDa
40 kDa
GAPDH
B
40 kDa Oma1 content (Fold of CTL)
CDDP
CTL
***
***
L-Opa1 content (Fold of CTL)
CTL
CDDP
***
Apoptotic cells (% of total cells)
CDDP
CTL
***
***
p53 siRNA (nM)
C
Completely fragmented mitochondria (% of total cells)
CTL
CDDP
***
p53 siRNA (nM)

Figure 4.8 p53-dependent action of CDDP on Oma1 (40 kDa) and L-Opa1 contents, mitochondrial fragmentation and apoptosis in OV2008 cells. (A&B) Chemosensitive CECA cells (OV2008) were transfected with p53 siRNA or CTL siRNA (scramble siRNA) (0 - 100 nM, 48 h), and subsequently cultured with CDDP (10 μM, 24 h) or CTL (DMSO). The contents of p53, Oma1, Opa1 and GAPDH (loading control) were examined by Western blotting. Apoptosis was examined by Hoechst assay. Knock-down of p53 inhibited CDDP-induced increase in Oma1 content (40 kDa; *** $P<0.001$, versus 0 nM, n=3) and loss in L-Opa1 content (*** $P<0.001$, versus 0 nM, n=3). p53 siRNA significantly decreased apoptosis induced by CDDP (***$p<0.001$, versus 0 nM, n=3). More than 300 cells were assessed in each experimental group. (C) OV2008 cells were treated with p53 siRNA or CTL siRNA as in (A) and subsequently cultured with CDDP (10 μM, 6 h) or CTL (DMSO). Mitochondria were immunostained with Tom20 antibody. Mitochondrial phenotypes were examined by immunofluorescence confocal microscopy. Knock-down of p53 significantly decreased CDDP-induced mitochondrial completely fragmentation (***$p<0.001$, versus 0 nM, n=3). More than 100 cells were assessed in each experimental group.

To further explore the requirement of p53 in CDDP-induced mitochondria fragmentation in OVCA cells, wt-p53 was over-expressed in chemoresistant A2780cp (p53 mutant) and SKOV3 (p53 null) cells with transfection of p53 cDNA. p53 over-expression induced Oma1 increase (40 kDa), L-Opa1 processing, complete mitochondrial fragmentation and apoptosis in these two cell lines (Fig 4.9A&B). Whereas CDDP alone had no effects on mitochondrial morphology in these p53 deficient CDDP resistant cells, reconstitution of wild type-p53 sensitized them to CDDP treatment (apoptosis, $p<0.001$, Fig 4.9A&B).

Figure 4.9

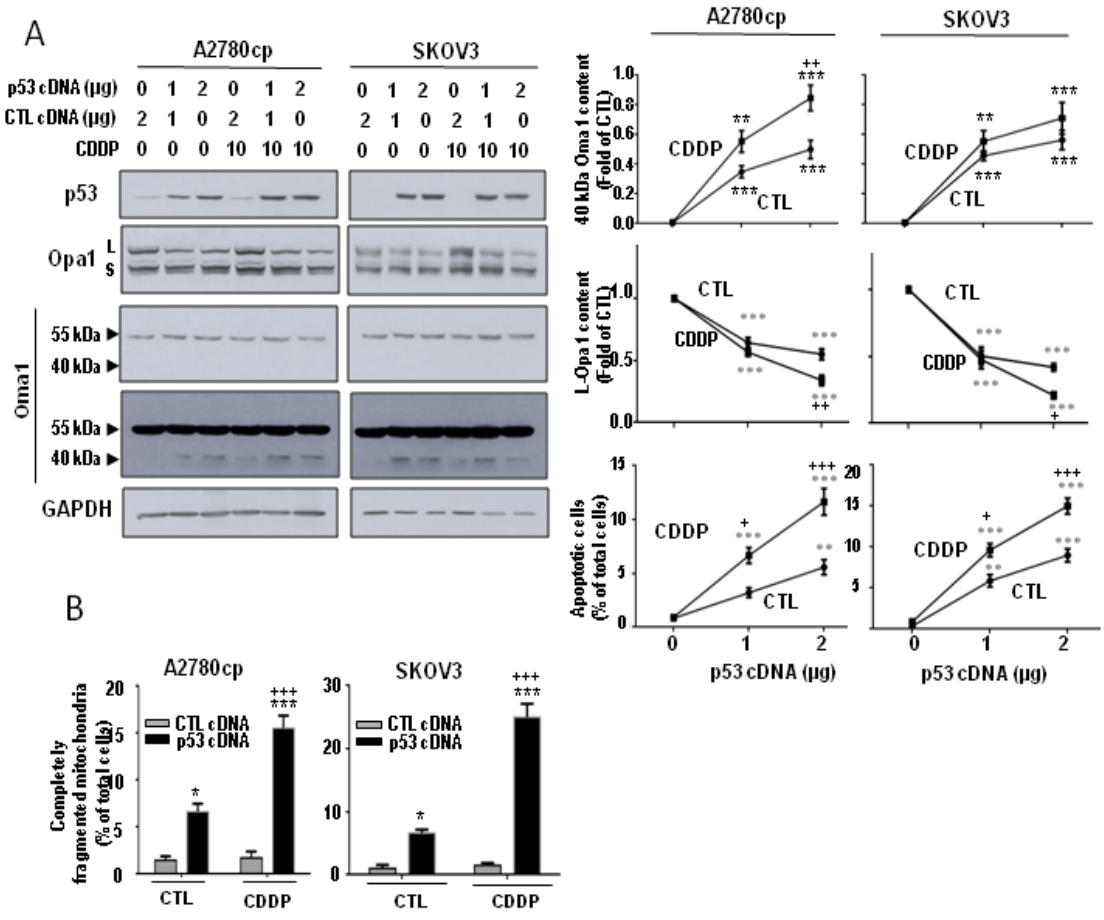

A
A2780cp
SKOV3
p53 cDNA (μg) 0 1 2 0 1 2 0 1 2 0 1 2
CTL cDNA (μg) 2 1 0 2 1 0 2 1 0 2 1 0
CDDP 0 0 0 10 10 10 0 0 0 10 10 10
p53
Opa1 L S
Oma1
55 kDa
40 kDa
55 kDa
40 kDa
GAPDH
B
A2780cp
SKOV3
CTL cDNA
p53 cDNA
Completely fragmented mitochondria (% of total cells)
CTL
CDDP
A2780cp
SKOV3
40 kDa Oma1 content (Fold of CTL)
L-Opa1 content (Fold of CTL)
Apoptotic cells (% of total cells)
CDDP
CTL
p53 cDNA (μg)

Figure 4.9 p53 reconstitution induced Oma1-mediated L-Opa1 processing, mitochondrial fragmentation and apoptosis in p53-mutant or –null chemoresistant OVCA cells. (A) Chemoresistant OVCA cells [A2780cp (p53 mutant) and SKOV3 (p53 null)] were transfected with wt-p53 cDNA or CTL cDNA (empty vector) (0-2 μg, 24 h) and subsequently treated with CDDP (10 μM, 24 h) or CTL (DMSO). Contents of p53, L-Opa1, Oma1 were examined by Western blot. Apoptosis was examined by Hoechst assay. Reconstitution of wt-p53 not only induced Oma1 cleavage, L-Opa1 processing and apoptosis in both CDDP resistant OVCA cell lines (**$p<0.01$, ***$p<0.001$, versus 0 μg, n=3), but markedly sensitized them to CDDP-induced Oma1 cleavage, L-Opa1 processing and apoptosis ($^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$, versus CTL). More than 300 cells were assessed in each experimental group). (B) A2780cp (p53 mutant) and SKOV3 (p53 null) cells were transfected with wt-p53 cDNA or CTL cDNA (empty vector) (0.44 μg/well on 8-well chamber slide, 24 h) and subsequently treated with CDDP (10 μM, 6 h) or CTL (DMSO). Mitochondria were immunostained with Tom20 antibody and examined by immunofluorescence confocal microscopy. Reconstitution of wt-p53 not only increased percentage of cells with completely fragmented mitochondria in both CDDP resistant OVCA cell lines (***$p<0.001$, *$p<0.05$, versus CTL cDNA, n=3), but markedly sensitize them to CDDP-induced mitochondrial completely fragmentation ($^{+++}p<0.001$, versus CTL). More than 100 cells were assessed in each experimental group.

4.6 In response to CDDP, phosphorylated p53 binds to Phb1 and dissociates Phb1 from Opa1-Phb1 complex in response to CDDP in chemosensitive but not chemoresistant CECA cells

Our previous publications have shown that CDDP-induced p53 phosphorylation at Ser15 and Ser20 is essential for apoptosis in chemosensitive CECA cells (Fraser, Leung et al. 2003, Fraser, Bai et al. 2008). A publication suggested a link between Phb1 and the p53 apoptotic pathway (Chander, Halpern et al. 2010). We therefore investigated the interaction between Phb1 and p-p53. OV2008 and C13* cells were treated with CDDP (10 μM, 6 h). Phb1, p-p53 (ser15), p-p53 (ser20), Opa1 and GAPDH contents were examined in the whole cell lysates. CDDP increased the content of both p-p53 (ser15 and ser20) in the chemosensitive cells but not in their resistant counterpart. Phb1 immuno-precipitates from OV2008 and C13* cells were immune-blotted with anti-p-p53 (ser15 and ser20). CDDP increased the interaction of p-p53 (ser15) and Phb1 in OV2008 cells but not in C13* cells ($P<0.001$, Fig 4.10). In contrast, p-p53 (ser20) did not interact with Phb1 irrespective of the presence of CDDP (Fig 4.10). Interestingly, Opa1 was also detected in both OV2008 and C13* cells without CDDP treatment, and CDDP decreased the interaction of Opa1 and Phb1 in OV2008 cells but not in C13* cells ($P<0.001$, Fig 4.10).

Figure 4.10

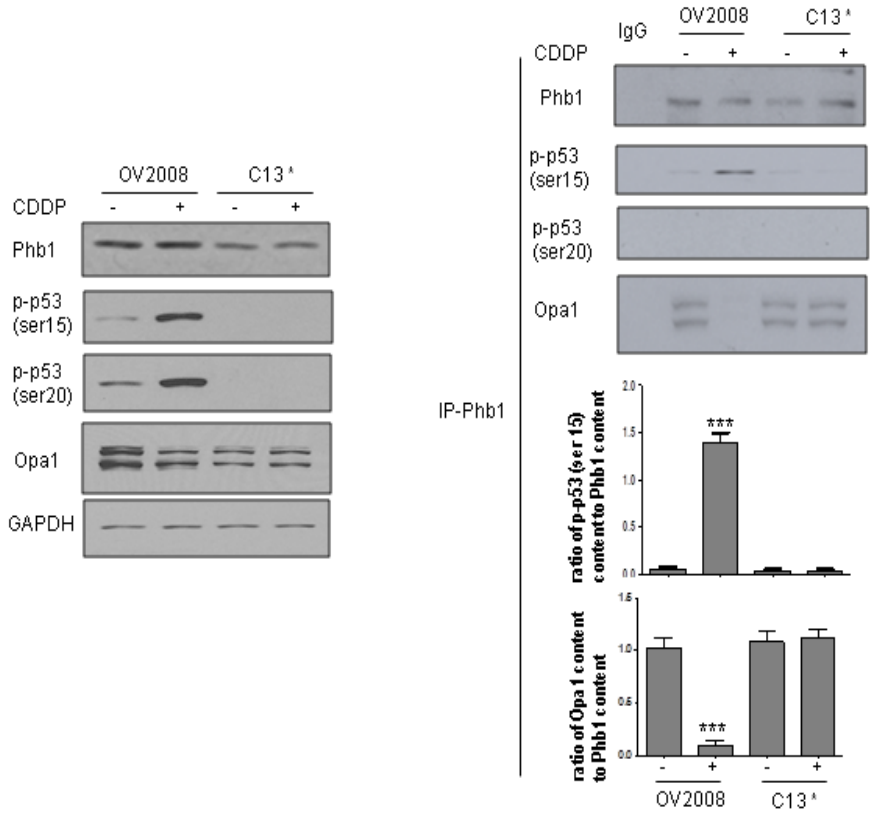
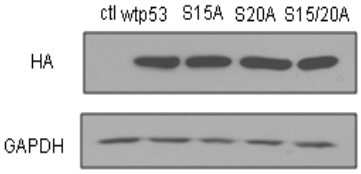

OV2008
C13*
CDDP
- + - +
Phb1
p-p53 (ser15)
p-p53 (ser20)
Opa1
GAPDH
IgG
IP-Phb1
ratio of p-p53 (ser15) content to Phb1 content
ratio of Opa1 content to Phb1 content
***

Figure 4.10 Phosphorylated p53 (serine 15) dissociates Phb 1 from Opa1-Phb1 complex. OV2008 and C13* cells were treated with CDDP (10 µM, 6 h) or DMSO. Contents of Phb1, p-p53 (ser15), p-p53 (ser20), Opa1 and GAPDH were examined by western blot. CDDP increased the content of both phospho-p53 (serine 15 and 20) in the chemosensitive cells but not in their resistant counterpart (n=3). Cell lysates were immunoprecipitated with IgG (control; lanes 1) or Phb1 antibody. Protein-protein interaction was determined by IP-western. Phb1 immunoprecipitates were immunoblotted [IP: Phb1, WB: Opa1, phosphorylated p53 (serine 15 and serine 20)]. CDDP increased the interaction of phospho-p53 (serine 15, not serine 20) and Phb1 in OV2008 cells, not in C13* cells (***$p<0.001$, versus DMSO, n=3). Results show representative images from 3 independent experiments.

To further test whether p-p53 (ser15 and ser20) is required for the CDDP-induced dissociation of Opa1 from the Opa1-Phb1 complex, we transfected OV2008 cells with wild type p53, ser15 mutant p53, ser20 mutant p53, ser15 plus ser20 mutant p53 plasmid, and treated the cells with CDDP (10 μM, 6h). Overexpression was confirmed by probing HA tag in the whole cell lysates. Phb1 immuno-precipitates were immunoblotted and interactions between Phb1 and Opa1, Phb1 and p-p53 (ser15 and ser20) were examined. The interaction of Phb1 with p-p53 (ser15) was significantly reduced ($p<0.001$, Fig 4.11) while that between L-Opa1 and Phb1 was significantly enhanced ($p<0.001$, Fig 4.9) by ser15 mutation and ser15 plus ser20 mutation. In contrast, mutation of p53 at ser20 alone had no significant effect on p-p53 (ser15)-Phb1 and Phb1-Opa1 interactions ($p> 0.05$, Fig 4.11).

Figure 4.11


ctl wtp53 S15A S20A S15/20A
HA
GAPDH

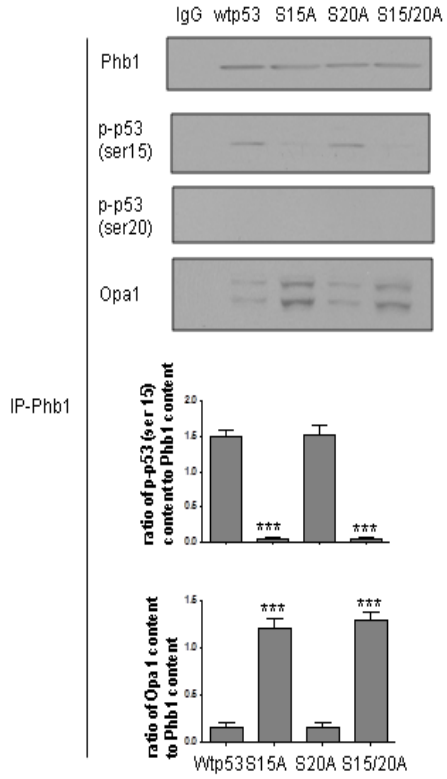
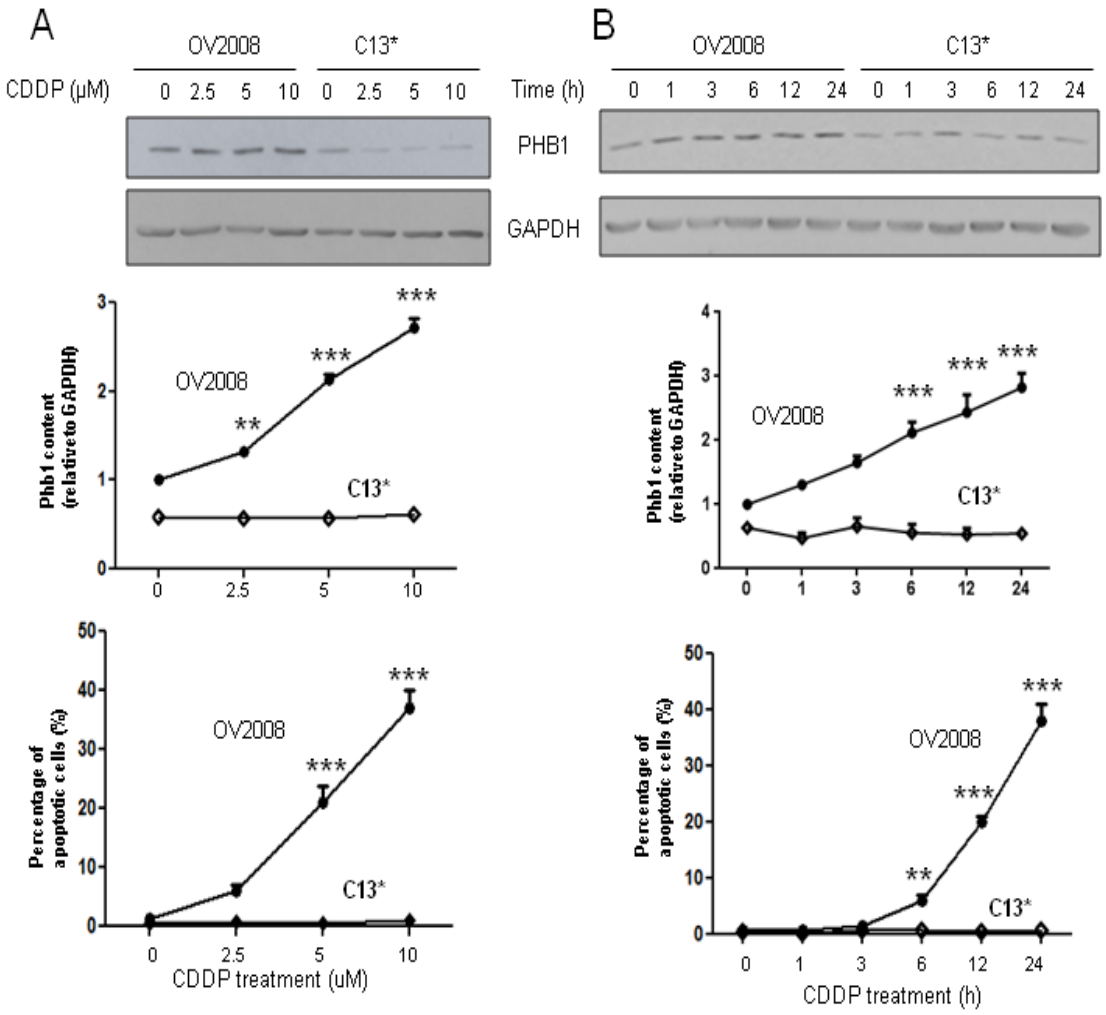

IgG wtp53 S15A S20A S15/20A
Phb1
p-p53 (ser15)
p-p53 (ser20)
Opa1
IP-Phb1
ratio of p-p53 (ser 15) content to Phb1 content
***
***
ratio of Opa1 content to Phb1 content
***
***
Wtp53 S15A S20A S15/20A

Figure 4.11 OV2008 cells were transfected with wild type p53, serine 15 mutant p53, serine 20 mutant p53, serine 15 plus serine 20 mutant p53 plasmid, and treated with CDDP (10 μM, 6h). The overexpression was confirmed by probing HA tag in the whole cell lysates. Protein-protein interaction was determined by IP-Western. Phb1 immunoprecipitates were immunoblotted [IP: Phb1, WB: Opa1, p-p53 (serine 15 and serine 20)]. Serine 15 mutant p53 and serine 15 plus serine 20 mutant p53 significantly enhanced the interaction of Opa1 and Phb1, and decreased that of Phb1 and p-p53 (serine 15) (***$p<0.001$, versus wild type p53, n=3). Results show representative images from 3 independent experiments.

4.7 CDDP increased Phb1 content and apoptosis in a time- and concentration-dependent manner in OV2008 cells, but not in C13 cells.*

Although Phb1 is believed to be involved in the regulation of L-Opa1 processing and mitochondrial dynamics (Merkwirth, Dargazanli et al. 2008, Merkwirth and Langer 2009, Osman, Merkwirth et al. 2009), whether it also plays a role in mitochondrial fragmentation and apoptosis in CECA cells by CDDP is not known. OV2008 and C13* cells were cultured with CDDP (0 - 10 μM, 0 - 24 h) and the changes in Phb1 contents were examined by western blot. Phb1 increased in a time- and concentration-dependent manner in response to CDDP in OV2008 but not in C13* cells (Figure 4.12 A&B). CDDP induced apoptosis, as determined morphologically by Hoechst staining, in chemosensitive OV2008 cells, but not in chemoresistant C13* cells (Figure 4.12 A&B).

Figure 4.12



Fi

gure 4.12. CDDP increased Phb1 content and apoptosis in OV2008 cells, but not in C13* cells. (A&B) Comparison of Phb1 contents and apoptosis in OV2008 and C13* cultured with CDDP at different concentrations (A: 0-10 μM, 24 h) or for different duration (B: 0 - 24 h, 10 μM). Contents of Phb1 and GAPDH (loading control) were examined by Western blotting. Phb1 contents in OV2008 cells but not in C13* cells significantly increased in the presence of CDDP in a concentration- (A; **$p<0.01$ ***$p<0.001$, $n = 3$) and time- (B; ***$p<0.001$, $n = 3$) dependent manner. Apoptosis was examined by Hoechst assay. OV2008 cells exhibited higher apoptosis than C13* cells when treated with CDDP (A&B; **$p<0.01$ ***$p<0.001$, $n = 3$).

4.8 Chemoresponsiveness in CECA cells is associated with increased p-p53 (ser15)-Phb1-Bak interaction following CDDP treatment.

We have previously found that p-p53 (ser15) interacts with Phb1 in response to CDDP treatment in OV2008 cells (Kong, Wang et al. 2014). A recent publication shows that p-p53 (ser15) accumulates in mitochondria, and binds to Bak, leading to spontaneous Bak oligomerization and changes in MOMP (Nieminen, Eskelinen et al. 2013). Whether p-p53 (ser15) binds to Bak during CDDP-induced apoptosis in CECA cells is not known. To investigate this possibility, OV2008 and C13* cells were treated with CDDP (10 μM, 6 h). Bak immunoprecipitates from OV2008 and C13* cells were immune-blotted with anti-p-p53 (ser15) or anti-p-p53 (ser20). CDDP increased the interaction of p-p53 (ser15) and Bak in OV2008 cells ($p<0.001$), but not in C13* cells. Interestingly, CDDP also increased Phb1 and Bak interaction in OV2008 cells ($p<0.001$), but not in C13* cells. Although CDDP also increased p-p53 (ser20) in OV2008 cells, p-p53 (ser20) was not detected in Bak immunoprecipitates from OV2008 and C13* cells, regardless of CDDP treatment. (Figure 4.13)

Figure 4.13

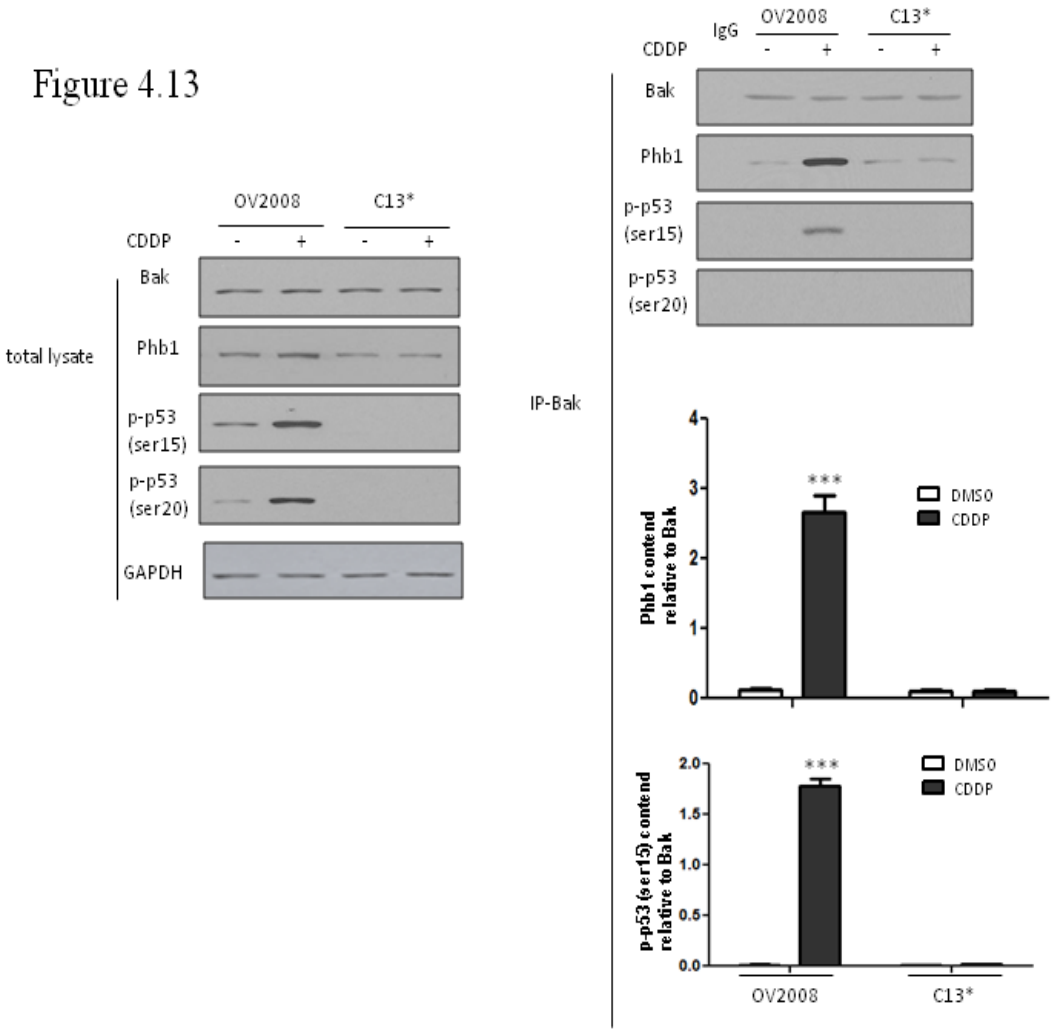

OV2008
C13*
CDDP
- + - +
Bak
Phb1
p-p53 (ser15)
p-p53 (ser20)
GAPDH
total lysate
IP-Bak
IgG
Phb1 contend relative to Bak
***
DMSO
CDDP
p-p53 (ser15) contend relative to Bak
OV2008
C13*

Figure 4.13 p-p53 (ser15) interacts with Phb1 and Bak in response to CDDP in chemosensitive CECA cells, but not in chemoresistant cells. OV2008 and C13* cells were treated with CDDP (0-10 μM, 6 h). Contents of Phb1, p-p53 (ser15), p-p53 (ser20), Bak and GAPDH were examined by Western blot. CDDP increased the content of both p-p53 (serine 15 and serine 20) in the chemosensitive cells but not in their resistant counterpart (n=3). Protein-protein interaction was determined by IP-Western. Cell lysates were immunoprecipitated with IgG (control; lanes 1) or Bak antibody. Bak immunoprecipitates were immunoblotted [IP: anti-Bak, WB: anti-Bak, Phb1, p-p53 (serine 15 and serine 20)]. CDDP increased the interaction of p-p53 (serine 15, not serine 20) -Bak (***$p<0.001$, versus DMSO, n=3) and Phb1-Bak (***$p<0.001$, versus DMSO, n=3) in OV2008 cells but not in C13* cells. Results show representative images from 3 independent experiments.

4.9 Phb1 is required for CDDP-induced p-p53 (ser15)-Bak interaction, mitochondrial fragmentation and apoptosis.

Since Phb1 is involved in CDDP-induced p53 action in inducing apoptosis (Chander, Halpern et al. 2010), and Phb1 increases in OV2008 cells with CDDP treatment, we hypothesize that Phb1 is required for Oma1-mediated L-Opa1 processing and apoptosis, which are regulated by p53. To test this hypothesis, OV2008 cells were treated with Phb1 siRNA (0 - 100 nM, 24 h; scramble siRNA as control), and followed with CDDP (0 - 10 μM, 24 h). The contents of Oma1, Phb1, Opa1, p-p53 (ser15) and GAPDH (loading control) were determined by Western blotting. Phb1 knock-down inhibited CDDP-induced Oma1 (40 kD) content, L-Opa1 loss and apoptosis. The increase of p-p53 (ser15) induced by CDDP was not affected by Phb1 knock-down (Figure 4.14A). The involvement of Phb1 in CDDP-induced mitochondrial fragmentation was also examined by confocal-microscopy with staining of Tom20. Knock-down of Phb1 inhibited the CDDP-induced complete mitochondrial fragmentation (Figure 4.14B).

The involvement of Phb1 in CDDP-induced Bak-p-p53 (ser15) interaction was examined by immunoprecipitation (IP-Bak)/western blot. Knock-down of Phb1 inhibited the CDDP-induced interactions between Phb1, Bak and p-p53 (ser15) ($p<0.001$, Figure 4.15).

Figure 4.14

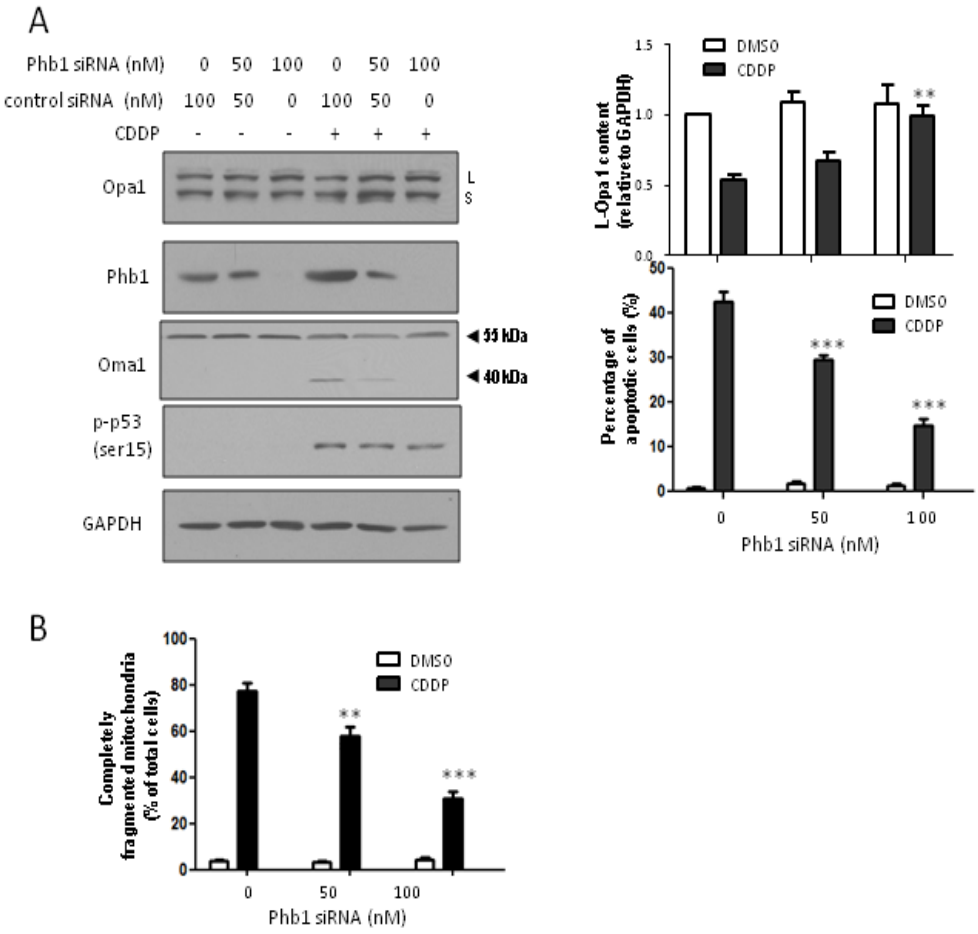

A
Phb1 siRNA (nM) 0 50 100 0 50 100
control siRNA (nM) 100 50 0 100 50 0
CDDP - - - + + +
Opa1
L
S
Phb1
Oma1
◀ 55 kDa
◀ 40 kDa
p-p53 (ser15)
GAPDH
L-Opa1 content (relative to GAPDH)
DMSO
CDDP
Percentage of apoptotic cells (%)
Phb1 siRNA (nM)
B
Completely fragmented mitochondria (% of total cells)
Phb1 siRNA (nM)

Figure 4.14 Phb1-dependent effect of CDDP on Oma1 cleavage, L-Opa1 processing, mitochondrial fragmentation in OV2008 cells. (A) OV2008 cells were transfected with Phb1 siRNA (0 - 100 nM, 24 h; scrambled siRNA as control), and subsequently with CDDP (0 - 10 μM, 24 h). The contents of Oma1, Phb1, Opa1, p-p53 (ser15) and GAPDH (loading control) were determined by Western blotting. Phb1 knock-down attenuated CDDP-induced Oma1 (40 kD) content and L-Opa1 loss (**$p<0.01$, ***$p<0.001$. n=3), but not of p-p53 (ser15). Apoptosis was examined by Hoechst assay. Phb1 significantly inhibited CDDP-induced apoptosis (***$p<0.001$, n = 3). (B) OV2008 cells were treated with control siRNA or Phb1 siRNA (0 - 100 nM, 24 h), followed by CDDP (0 - 10 μM, 6 h). Mitochondrial phenotypes were examined by immunofluorescence confocal microscopy. Knock-down of Phb1 inhibited CDDP-induced mitochondrial completely fragmentation ((**$p<0.01$, ***$p<0.001$, n=3). At least 100 cells were assessed for each time point in each replicate.

The involvement of Phb1 in CDDP-induced Bak-p-p53 (ser15) interaction was examined by immunoprecipitation (IP-Bak)/Western blot. Knock-down of Phb1 inhibited the CDDP-induced interactions between Phb1, Bak and p-p53 (ser15) ($p<0.001$, Figure 4.15).

Figure 4.15

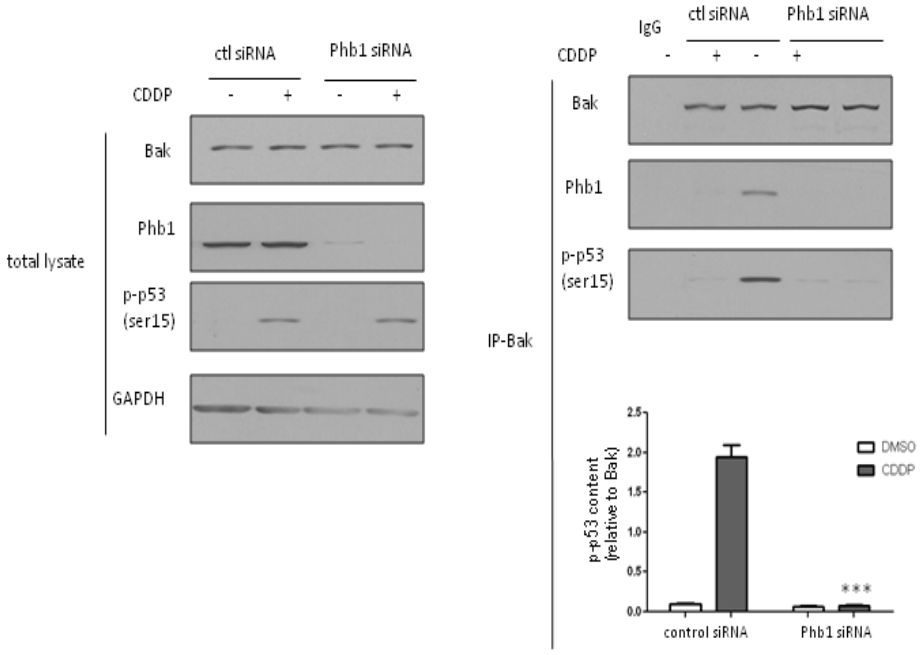

ctl siRNA
Phb1 siRNA
CDDP
- + - +
Bak
Phb1
p-p53 (ser15)
GAPDH
total lysate
IgG
ctl siRNA
Phb1 siRNA
CDDP
- + - +
Bak
Phb1
p-p53 (ser15)
IP-Bak
p-p53 content (relative to Bak)
DMSO
CDDP
***
control siRNA
Phb1 siRNA

Figure 4.15 Phb1-dependent effect of CDDP on p-p53 (ser15)-Phb1-Bak interaction. OV2008 cells were forced expressed with Phb1 siRNA (0 - 100 nM, 24 h; scrambled siRNA as control) and then treated with CDDP (0 - 10 μM, 6 h). Contents of Phb1, p-p53 (ser15), Bak and GAPDH were examined by Western blot. Cell lysates were immunoprecipitated with IgG (control; lanes 1) or Bak antibody. Protein-protein interaction was determined by IP-Western. Bak immunoprecipitates were immunoblotted [IP: anti-Bak, WB: Anti-Bak, Phb1, phosphorylated p53 (serine 15)]. Phb1 knock-down inhibited CDDP-induced interaction of Bak with p-p53 (ser15) ($p<0.001$, $n=3$).

To determine if the observed effects of CDDP on molecular interactions, mitochondrial dynamics and chemosensitivity in CECA cells are shared with OVCA cells, we extended the above studies to include the chemosensitive and chemoresistant OVCA cells, A2780s and A2780cp. CDDP induced apoptosis in A2780s cells, but not in A2780cp cells (Figure 4.16). This response was associated with increased contents of p-p53 (ser15), Oma1 (40 kD) and Phb1, as well as L-Opa1 loss (Figure 4.16). CDDP also increased Phb1-Bak-p-p53 (ser15) interaction in A2780s ($p<0.001$) but not in A2780cp cells (Figure 4.17).

Figure 4.16

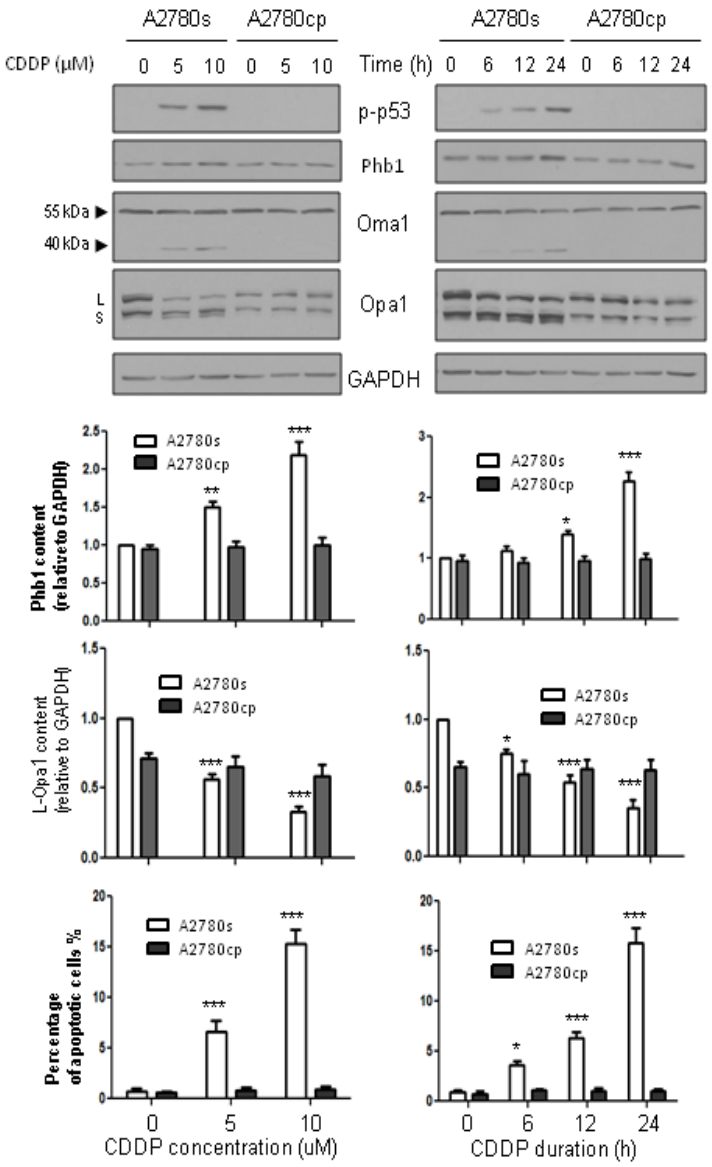

A2780s
A2780cp
CDDP (μM)
0 5 10 0 5 10
Time (h)
0 6 12 24 0 6 12 24
p-p53
Phb1
55 kDa
40 kDa
Oma1
L
S
Opa1
GAPDH
Phb1 content (relative to GAPDH)
L-Opa1 content (relative to GAPDH)
Percentage of apoptotic cells %
CDDP concentration (uM)
CDDP duration (h)

Figure 4.16 CDDP also induced Oma1 cleavage, L-Opa1 processing, mitochondrial fragmentation and apoptosis in A2780s cells, but not in A2780cp cells. A2780s and A2780cp cells were cultured with CDDP at different concentrations (0-10 μM, 24 h) or for different duration (0 - 24 h, 10 μM). Contents of Opa1, Oma1, p-p53 (ser15), Phb1 and GAPDH (loading control) were examined by Western blotting. Phb1 contents in A2780s cells but not in A2780cp cells significantly increased in the presence of CDDP in a concentration- (**$p<0.01$ ***$p<0.001$, $n = 3$) and time- (*$p<0.05$ ***$p<0.001$, $n = 3$) dependent manner. CDDP induced L-Opa1 loss in a concentration- (***$p<0.001$, $n = 3$) and time- (*$p<0.05$ ***$p<0.001$, $n = 3$) dependent manner in A2780s cells but not in A2780cp cells. Apoptosis was examined by Hoechst assay. A2780s cells exhibited higher apoptosis than A2780cp cells when treated with CDDP (*$p<0.05$, ***$p<0.001$, $n = 3$).

Figure 4.17

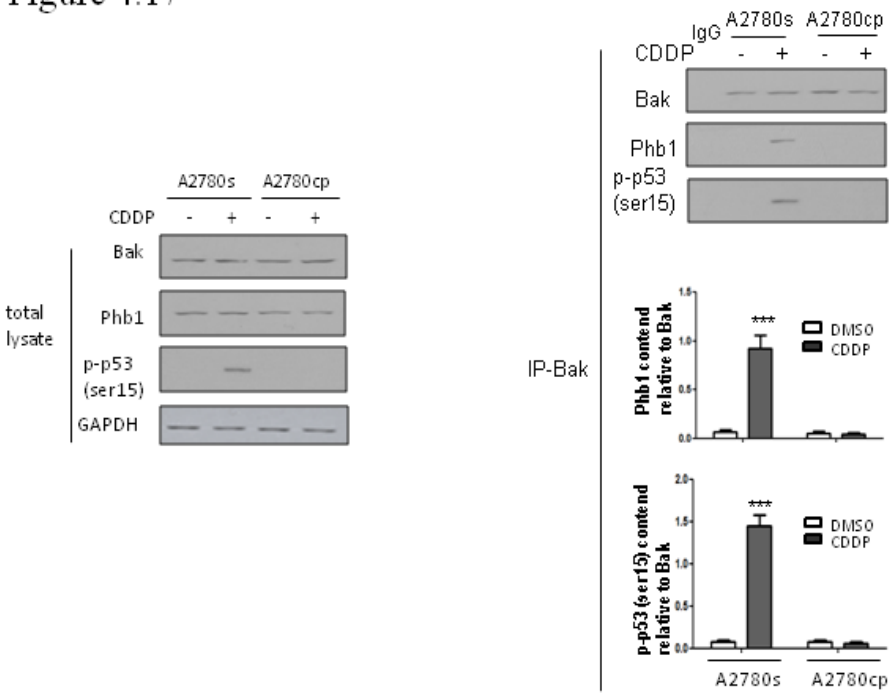

A2780s
A2780cp
CDDP - + - +
Bak
Phb1
p-p53 (ser15)
GAPDH
total lysate
IgG
A2780s
A2780cp
CDDP - + - +
Bak
Phb1
p-p53 (ser15)
IP-Bak
Phb1 contend relative to Bak
***
DMSO
CDDP
p-p53 (ser15) contend relative to Bak
***
DMSO
CDDP
A2780s
A2780cp

Figure 4.17 CDDP also induced p-p53 (ser15)-Phb1-Bak interaction in A2780s cells, but not in A2780cp cells. A2780s and A2780cp cells were treated with CDDP (0-10 μM, 6 h). Contents of Phb1, p-p53 (ser15), Bak and GAPDH were examined by Western blot. CDDP increased the content of phospho-p53 (serine 15) in the chemosensitive cells but not in their resistant counterpart (n=3). Protein-protein interaction was determined by IP-Western. Cell lysates were immunoprecipitated with anti-IgG (control; lanes 1) or anti-Bak antibody. Bak immunoprecipitates were immunoblotted [IP: anti-Bak, WB: anti-Bak, Phb1, p-p53 (serine 15)]. CDDP increased Bak-phospho-p53 (serine 15) and Bak-Phb1 interactions in A2780s cells, not in A2780cp cells (***$p<0.001$, versus DMSO, n=3). Results show representative images from 3 independent experiments.

4.10 CDDP induced mitochondrial p-p53 (ser15) accumulation and binding to Phb1 and Bak.

We have previously reported that p53 phosphorylation is a determinating factor of CDDP-induced apoptosis in gynecologic cancer cells (Fraser, Bai et al. 2008). We therefore investigated if CDDP induces mitochondrial p-p53 (ser15) accumulation and Phb1-Bak interaction, and activates Oma1-mediated L-Opa1 processing. OV2008 and C13* cells were cultured with CDDP (0 - 10 μM, 6 h). Fractions of cytoplasm, nucleus and mitochondria were prepared and the contents of Opa1, Oma1, Bak, Phb1 and p-p53 (ser15) were determined by western blot. Histone-H3, GAPDH and Tom20 were used as the marker and loading controls of nuclear, cytoplasmic and mitochondrial fractions, respectively. Opa1, Oma1, Bak and Phb1 were primarily located in mitochondria in OV2008 and C13* cells, irrespective of the presence of CDDP. CDDP increased p-p53 (ser15) in cytoplasm and mitochondria in OV2008 cells, but not in C13* cells. Oma1 (55 kD) is present in mitochondria fraction in both OV2008 and C13* cells, with and without CDDP treatment. CDDP increased mitochondrial Oma1 (40 kD) content in OV2008 cells, but not in C13* cells (Figure 4.18A). The interaction of Phb1, Bak and p-p53 (ser15) was further confirmed by IP-Bak in mitochondria fraction. CDDP increased Phb1-Bak and p-p53 (ser15)-Bak interactions in OV2008 cells ($p<0.001$), but not in C13* cells (Figure 4.18B).

Figure 4.18

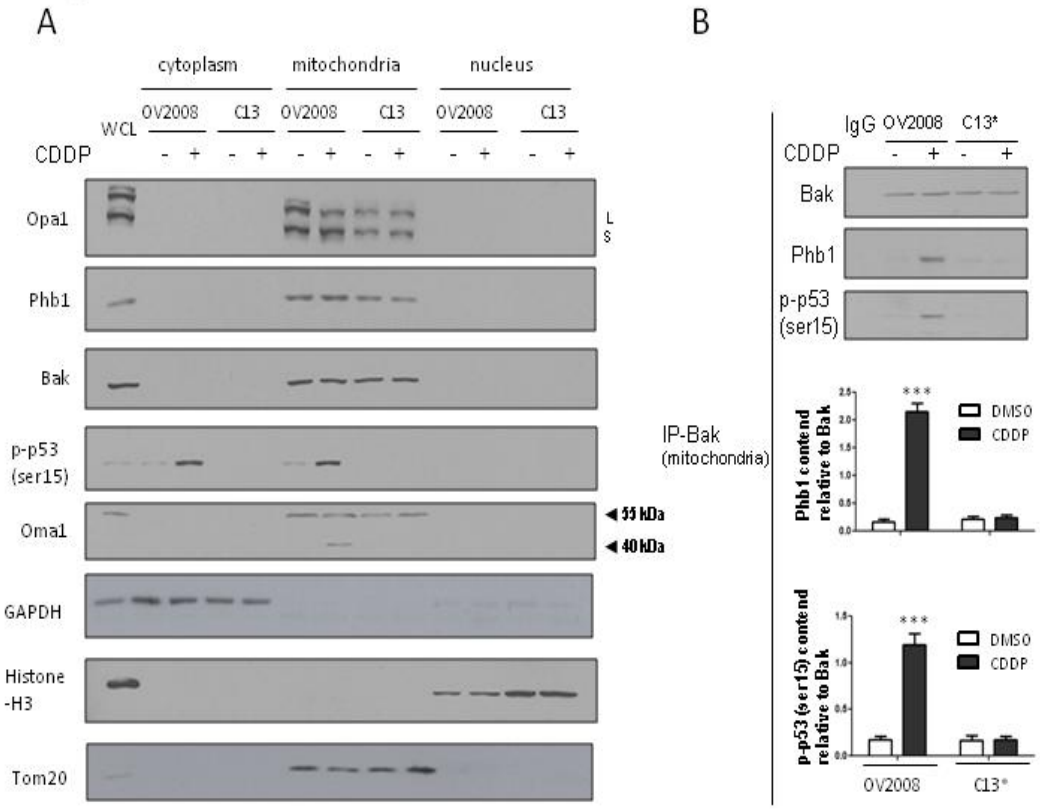

A
B
cytoplasm
mitochondria
nucleus
WCL
OV2008
C13
CDDP
Opa1
L
S
Phb1
Bak
p-p53
(ser15)
Oma1
55 kDa
40 kDa
GAPDH
Histone
-H3
Tom20
IP-Bak
(mitochondria)
IgG
C13*
Phb1 contend
relative to Bak
p-p53 (ser15) contend
relative to Bak
DMSO
CDDP
***

Figure 4.18 CDDP induced mitochondrial p-p53 (ser15) accumulation and binding to Phb1 and Bak. A. OV2008 and C13* cells were treated with CDDP (0-10 μM, 6 h). Mitochondrial, cytoplasmic and nuclear fractions were prepared. Contents of Opa1, Oma1, Phb1, p-p53 (ser15), Bak, Histone H3 (nuclear loading control), Tom20 (mitochondria loading control) and GAPDH (cytoplasm loading control) were examined by Western blot. CDDP increased the content of p-p53 (serine 15) in cytoplasm and mitochondria in the chemosensitive cells but not in their resistant counterpart (n=3). B. OV2008 and C13* cells were treated with CDDP (0-10 μM, 6 h). Mitochondria fraction was separated and immunoprecipitated with anti-IgG (control; lanes 1) or anti-Bak antibody. Protein-protein interaction was determined by IP-Western. Bak immunoprecipitates were immunoblotted [IP: anti-Bak, WB: anti-Bak, Phb1, p-p53 (serine 15)]. CDDP increased Bak-p-p53 (serine 15), Bak-Phb1 interactions in A2780s cells, not in A2780cp cells (***$p<0.001$, versus DMSO, n=3). Results show representative images from 3 independent experiments.

Objective 3: Determine whether Akt promotes chemoresistance by suppressing the action of p53 in L-Opa1 processing and mitochondrial dynamics

4.11 Akt confers resistance by suppressing CDDP-induced interaction of p-p53 (ser15), Phb1 and Bak, Oma1 cleavage, L-Opa1 processing, mitochondrial fragmentation and apoptosis.

Our previous study has showed that Akt confers chemoresistance by suppressing p53 phosphorylation induced by CDDP in chemoresistant cells (Fraser, Bai et al. 2008). We therefore hypothesize in the present study that increased p-p53 (ser15) content leads to Oma1 activation, L-Opa1 processing, and mitochondrial fragmentation in chemosensitive cells. Further Akt attenuates CDDP-induced p-p53 (ser15) contents, subsequent L-Opa1 processing and mitochondrial fragmentation, resulting in chemoresistance. To test these possibilities, chemoresistant C13* cells were infected with HA-tagged dominant negative Akt (HA-DN-Akt, MOI= 0 - 80, 24 h), and then treated with CDDP (0 - 10 μM, 24 h). The success of the infection was confirmed by Western blot (anti - HA), as were the contents of Oma1, Phb1, Opa1, p-p53 (ser15) and GAPDH (loading control). DN-Akt expression enhanced CDDP-induced p-p53 (ser15) and Oma1 (40 kD) contents and L-Opa1 processing. HA-DN-Akt also significantly sensitized C13* to CDDP-induced apoptosis (Figure 4.19A). The role of Akt in regulating the mitochondrial fragmentation induced by CDDP was also examined. C13* cells were infected with HA-tagged DN-Akt (0 - 80 MOI, 24 h) and subsequently treated with CDDP (10 μM, 6 h). DN-Akt expression markedly enhanced CDDP-induced mitochondrial fragmentation (Figure 4.19B).

Figure 4.19

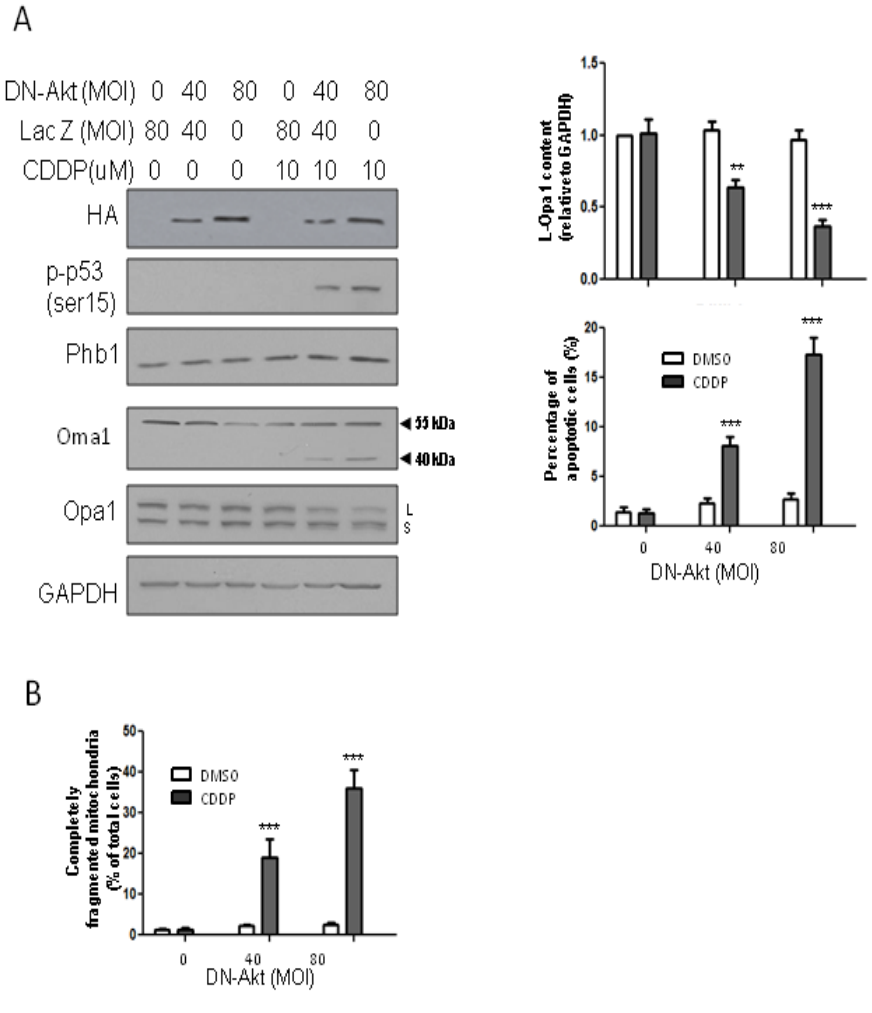
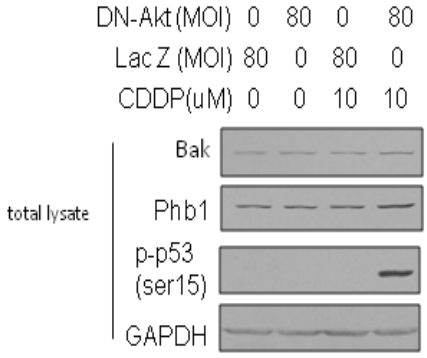

A
DN-Akt (MOI) 0 40 80 0 40 80
Lac Z (MOI) 80 40 0 80 40 0
CDDP(uM) 0 0 0 10 10 10
HA
p-p53 (ser15)
Phb1
Oma1
55 kDa
40 kDa
Opa1
L
S
GAPDH
L-Opa1 content (relative to GAPDH)
1.5
1.0
0.5
0.0
**
***
Percentage of apoptotic cells (%)
20
15
10
5
0
DMSO
CDDP
***
***
0
40
80
DN-Akt (MOI)
B
Completely fragmented mitochondria (% of total cells)
50
40
30
20
10
0
DMSO
CDDP
***
***
0
40
80
DN-Akt (MOI)

Figure 4.19 Akt confers resistance by suppressing Oma1 cleavage, L-Opa1 processing, mitochondrial fragmentation and apoptosis. **(**A) C13* cells were infected an adenoviral HA-DN-Akt or Lac Z (MOI = 0-80, 24 h) and treated with CDDP (0 - 10 μM, 24 h). The contents of HA tag, Oma1, Phb1, Opa1, p-p53 (ser15) and GAPDH (loading control) were determined by western blot. HA-DN-Akt treatment enhanced CDDP-induced p-p53 (ser15), Oma1 (40 kD) and L-Opa1 processing (**p<0.01, ***p<0.001, n = 3). Apoptosis was examined by Hoechst assay. HA-DN-Akt significantly sensitized C13* to CDDP-induced apoptosis (***p<0.001, n = 3). (B) C13* cells were treated with HA-DN-Akt or Lac Z (MOI = 0-80, 24 h), and then with CDDP (0 - 10 μM, 6 h). Mitochondrial phenotypes were examined by immunofluorescence confocal microscopy. HA-DN-Akt treatment significantly enhanced CDDP-induced mitochondrial completely fragmentation (***p<0.001, n=3). At least 100 cells were assessed for each time point in each replicate.

The role of Akt in regulating the interaction between Phb1, Bak and p-p53 (ser15) induced by CDDP was also examined. C13* cells were infected with a HA-tagged DN-Akt (0 - 80 MOI, 24 h), and subsequently treated with CDDP (10 uM, 6 h). DN-Akt expression enhanced CDDP-induced interaction of Phb1 with Bak and p-p53 (ser15) (Figure 4.20).

Figure 4.20


DN-Akt (MOI) 0 80 0 80
Lac Z (MOI) 80 0 80 0
CDDP(uM) 0 0 10 10
total lysate
Bak
Phb1
p-p53 (ser15)
GAPDH

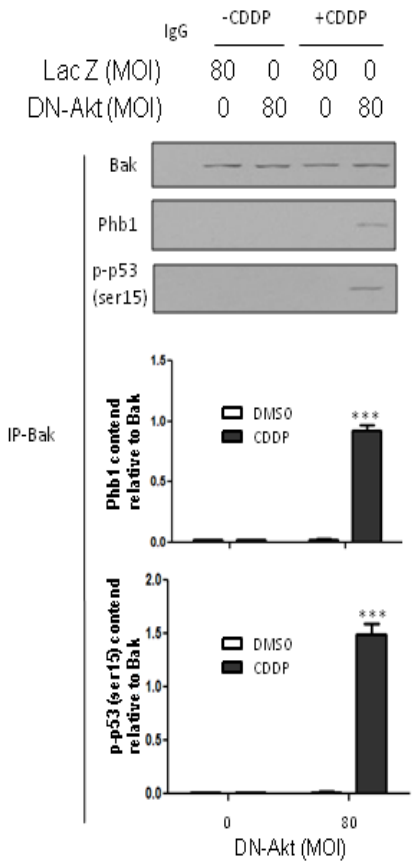

IgG
-CDDP
+CDDP
Lac Z (MOI) 80 0 80 0
DN-Akt (MOI) 0 80 0 80
Bak
Phb1
p-p53 (ser15)
IP-Bak
Phb1 contend relative to Bak
DMSO
CDDP
***
p-p53 (ser15) contend relative to Bak
0
80
DN-Akt (MOI)

Figure 4.20 Akt confers resistance by suppressing CDDP-induced interaction of p-p53 (ser15), Phb1 and Bak. C13* cells were infected with an adenoviral HA-DN-Akt or Lac Z (MOI 0-80, 24 h), and then treated with CDDP (0 - 10 μM, 6 h). Contents of Phb1, p-p53 (ser15), Bak and GAPDH were examined by Western blot. Cell lysates were immunoprecipitated with IgG (control; lanes 1) or Bak antibody. Protein-protein interaction was determined by IP-Western. Bak immunoprecipitates were immunoblotted [IP: anti-Bak, WB: anti-Bak, Phb1, p-p53 (serine 15)]. HA-DN-Akt treatment significantly enhanced CDDP-induced interaction of Bak, p-p53 (ser15) and Phb1 (n=3).

Chapter 5: DISCUSSION

5.1 Overview and significance

Chemoresistance is a major hurdle for treatment success of OVCA and CECA. The mechanisms of chemoresistance are multifactorial and partly due to decreased apoptosis (Abdullah and Chow 2013). Mitochondrial dynamics and its regulatory machineries are involved in the regulation of apoptosis (Chen and Chan 2009). Whether chemoresistant cells have dysregulated mitochondrial dynamics and altered expression of mitochondrial machineries are not known. In the present study, we have shown for the first time dysregulated mitochondrial dynamics in chemoresistant OVCA and CECA cells. We also compared the main machineries of mitochondrial dynamics between chemosensitive and chemoresistant cells.

L-Opa1 processing is essential for mitochondrial fragmentation and subsequent cell death (Yamaguchi and Perkins 2009). Therefore, it is important to understand the molecular mechanisms by which L-Opa1 processing is regulated, and how is it involved in apoptosis. In the present study, we demonstrated that Oma1 mediated L-Opa1 processing is an essential step for CDDP-induced mitochondrial fragmentation and apoptosis in chemosensitive cancer cells, however, this pathway is inhibited in chemoresistant cells.

p53 phosphorylation at different sites (ser15 and ser20) induced by CDDP is crucial for CDDP-induced apoptosis, however, the function of each p53 phosphorylation form is not clear. Our current work has provided the novel observation supporting the notion that p53 plays an important role in regulating CDDP-induced Oma1-mediated L-Opa1

processing and mitochondrial fragmentation in these chemosensitive gynecologic cancer cells through activation of p-p53 at ser15 but not ser20. Furthermore, the action of p-p53 (ser15) in these processes involves its binding to and displacement of Opa1 from the Phb1-Opa1 complex.

The present studies also attempt to provide a better understanding on how p53 induces Oma1 activation, leading to subsequent L-Opa1 processing and cell death. We have shown that p-p53 (ser15) binds to Bak in sensitive cells in response to CDDP, a step important for Bak activation. Activation of Bak is needed for induction of MOMP, and subsequent Oma1 activation (Jiang, Jiang et al. 2014, Zhang, Li et al. 2014).

Phb1 is involved in the regulation of L-Opa1 processing and apoptosis, however, the mechanism is not clear. We also showed for the first time that Phb1 is involved in the action of p-p53 (ser15) by facilitating its binding to Bak and promoting mitochondrial fragmentation.

Moreover, our results suggest that Akt confers CDDP resistance by regulating mitochondrial dynamics in gynecologic cancer cells, in part through inhibiting the binding of p-p53 (ser15) to Bak, thereby attenuating Oma1-mediated L-Opa1 processing.

5.2 Experimental advantages of matched pair gynecologic cancer cell lines

In this study, two matched pairs of chemosensitive parental cell lines (OV2008 and A2780s) and their isogeneic resistant counterparts (C13* and A2780cp) were used. The chemosensitive OV2008 cell lines were obtained from cervical carcinoma origin with squamous differentiation (Andrews, Murphy et al. 1985, Ali, Kim et al. 2014). The chemosensitive A2780s cells were obtained from ovarian serous cystodenocarcinoma cells (DiSaia, Sinkovics et al. 1972, Fraser, Bai et al. 2008). The chemoresistant variants were

developed by culture in the presence of CDDP with increased concentration (Brown, Clugston et al. 1993), which provide the chemosensitive and chemoresistant cells the same genetic background, except those alterations that contribute to chemoresistance. Therefore, the differences we observed between chemosensitive and chemoresistant cells in response to CDDP are more relevant to chemoresistance, and less likely due to the random differences between different cell lines which have various genetic profiles. It is more efficient for us to find the important phenomenon or molecules which are related to chemoresistance, and subsequently can be used to examine their regulation and function in chemoresistance by manipulations (up- or down-regulation).

5.3 Dysregulation of apoptosis and CDDP sensitivity in chemoresistant cancer cells

CDDP and its analog (such as Carboplatin), which cause apoptosis in cancer cells, are important chemotherapy reagents in treating OVCA and CECA. As previously mentioned, dysregulation of the apoptosis pathways is an important mechanism of chemoresistance. Understanding the dysregulated apoptosis pathway may provide important clues for targeting chemoresistant OVCA and CECA cells.

CDDP induce minimal apoptosis in chemoresistant OVCA and CECA cells, as has been shown in the present studies and our previous reports (Ali, Abedini et al. 2012). Different molecules and pathways have been shown to contribute to chemoresistance in these cancer cells, such as high Akt level, altered expression of Bcl-2 family members, stabilized PARC and PPM1D (Fraser, Bai et al. 2008, Woo, Xue et al. 2012, Yang, Wang et al. 2012, Ali, Kim et al. 2014). Our study here focuses on the involvement of

mitochondrial dynamics and related proteins in the regulation of apoptosis and CDDP sensitivity.

5.4 Regulation of mitochondrial dynamics and chemoresistance

Mitochondrial fission and fusion influence nearly all aspects of mitochondrial function, including respiration, calcium buffering and apoptosis (Skulachev 2001, Aon, Cortassa et al. 2004, Frieden, James et al. 2004, Lee, Jeong et al. 2004). However, to our knowledge, whether mitochondrial dynamics are dysregulated in chemoresistant cells has not been reported.

Here, for the first time, our results indicate an imbalanced mitochondrial fusion and fission in chemoresistant gynecologic cancer cells compared to their chemosensitive counterparts, as evident by a higher percentage of cells with tubular mitochondria (Figure 4.1B&D). The large mitochondrial networks generated by fusion are also found in metabolically active cells (Skulachev 2001). In contrast, most mitochondria present as small spheres or short rods in quiescent cells (Collins, Berridge et al. 2002). Evidence also showed that mitochondrial elongation can be induced by starvation in MEFs, which protected the cells from apoptosis, with increased mitochondria cristae surface (the privileged compartments for ATP synthesis), oligomerization of the ATPase and maintenance of mitochondrial ATP production. Conversely, when mitochondria elongation is blocked, cells are more sensitive to starvation-induced cell death (Gomes, Di Benedetto et al. 2011).

Interestingly, Tondera et al. also reported mitochondria hyperfusion could be induced by selective stresses and conferred cellular resistance to stress with higher cellular ATP level (Tondera, Grandemange et al. 2009). Together with our observations,

which showed that chemoresistant cells with higher level of mitochondrial fusion status are resistant to CDDP treatment, these findings indicate that fused mitochondria promote cell survival, which could be due to better mitochondria function, especially more efficient ATP production and transport.

Our results also showed that fused mitochondria in chemoresistant cells appeared incapable of undergoing fragmentation in response to CDDP treatment (Figure 4.1C&D). Mitochondria dynamics are controlled by the action of key regulating proteins, which include fission proteins Drp1 and Fis1, and fusion proteins Mfn1, Mfn2 and Opa1. However, with the exception of Opa1, these regulatory molecules in both sensitive and resistant CECA cells appeared not to be affected by CDDP treatment (Figure 4.2).

Our findings do not exclude the possible involvement of these key mitochondrial fission/fusion proteins in the regulation of mitochondrial dynamics and CDDP responsiveness through post-translational modification, such as phophorylation. In fact, our laboratory has found that piceatannol, a functional food compound, induced mitochondrial fragmentation and apoptosis in chemosensitive CECA cells, associated with decreased phosphorylated Drp1 (ser637) content, which promotes Drp1 mobilization to mitochondria and mitochondrial fission (Farrand, Byun et al. 2013). Whether these molecules regulate mitochondrial dynamics and CDDP sensitivity by post-translational modifications in gynecologic cancer cells still needs to be investigated.

Interestingly, contrary to our initial hypothesis, the chemoresistant cells (C13*) have lower expression of fusion protein Opa1 (Figure 4.2), whereas they exhibit higher activity of fusion and resistance to CDDP-induced mitochondrial fragmentation. These findings suggest that Opa1 level is not a “rate-limiting” step or determinant of

mitochondrial fusion, and that other molecules or mechanisms, such as phosphorylated Drp1 status (at different sites) or Slp-2 (Tondera, Grandemange et al. 2009), could be more important.

5.5 Opa1 and its regulation in apoptosis and chemoresistance

Five forms of Opa1, two long ones and three short ones, have been found in most reported mammal cells, which are generated from proteolytic processing of eight splicing variants (Merkwirth, Dargazanli et al. 2008). In our current study, we found five Opa1 protein forms and six splice forms (variant 3&6 undetectable) in chemosensitive CECA cells (OV2008) (Figure 4.4&5).

Although the requirement of Opa1 in generating mitochondrial fusion is well established, the function of each Opa1 form in promoting mitochondria fusion remains unclear. Studies that introduced individual Opa1 variants and their mutants into MEFs from an Opa1 null mouse model demonstrated that co-expression of at least one short Opa1 form and one long form is necessary and essential for the generation of mitochondria fusion (Song, Chen et al. 2007). However, evidence also suggests that long forms of Opa1 may be more important for mitochondrial fusion, since the cells losing all the long isoforms of Opa1, such as found in patients suffering from mitochondrial myopathies or MEFs harbouring an error-prone mitochondrial mtDNA polymerase α, exhibited fragmented mitochondria, as well as reduced mitochondrial respiration rates (Duvezin-Caubet, Jagasia et al. 2006).

In this thesis, we have shown for the first time chemoresistant cells (C13*) with hyperfused mitochondria do not have two Opa1 forms (88 and 81kDa) (Figure 4.4),

suggesting that these two short forms are less important for generation of mitochondria fusion. Interestingly, these two Opa1 forms were also found to be either undetectable or significantly decreased in MEFs from Oma1$^{-/-}$ mouse (Quiros, Ramsay et al. 2012), strongly suggesting the lack of these two forms in C13* cells may be due to the absence of certain proteases (likely Oma1) involved in the processing of long forms to short forms. This notion requires further investigation.

We also found L-Opa1 isoforms are processed in chemosensitive CECA cells (OV2008) during CDDP-induced apoptosis (Figure 4.4), which is consistent with other reported observations (Guillery, Malka et al. 2008). CDDP induces L-Opa1 processing, mitochondrial fragmentation and apoptosis in chemosensitive CECA cells (OV2008) but not in its resistant variant (C13*), confirming that stabilized L-Opa1 is needed for mitochondrial fusion and cell survival. Moreover, our results also suggest that the abundance of different Opa1 splice forms is associated with chemosensitivity of the CECA cells, and CDDP decreases L-Opa1 contents partly by down-regulating the abundance of Opa1 mRNA (Figure 4.5).

Opa1 is needed for mitochondrial fusion, as indicated by the observation that cells with Opa1 silenced by siRNA exhibiting fragmented mitochondria. Notably, these cells are also largely devoid of cristae structure (Griparic, Kanazawa et al. 2007). It is reported that the majority of cytochrome c resides within mitochondrial cristae, and the remodeling of cristae is a critical regulatory step in the release of cytochrome c (Scorrano, Ashiya et al. 2002, Scorrano and Korsmeyer 2003). Accumulating evidence suggests that Opa1 complex through self-assembly is involved in the control of the cristae junction, which are narrow tubular tunnels connecting cristae to the space between the outer and

inner membranes (inter-membrane space), and become wider to allow the passage of cytochrome c from cristae to the inter-membrane space and cytosol sequentially. However, the loss of L-Opa1 seems to destabilize Opa1 complexes that eventually lead to the loss of cristae structures, and cytochrome c release (Merkwirth, Dargazanli et al. 2008). Whether Opa1-regulated cristae structure is deregulated in chemoresistant cells and how it is involved in chemoresistance is not known and needs to be further studied.

The function of Opa1 in regulating mitochondrial fusion and cristae structure may work through distinct mechanisms (Frezza, Cipolat et al. 2006), although these mechanisms can be highly correlated and both contribute to regulation of apoptosis. Understanding the regulation of L-Opa1 processing in chemosensitive and chemoresistant cancer cells is of great interest in elucidating the mechanisms of chemoresistance.

5.6 Oma1 and its role in processing L-Opa1

Consistent with earlier reports (Ehses, Raschke et al. 2009, Head, Griparic et al. 2009, Zhang, Li et al. 2014), our results also suggest that Oma1 is responsible for L-Opa1 processing, as evidenced by the fact that L-Opa1 is stabilized with CDDP treatment with the absence of Oma1 (Figure 4.7). Moreover, our results are also consistent with the notion that Oma1-mediated L-Opa1 processing is the key step for mitochondrial fragmentation and subsequent cell death, since Oma1 knock-down significantly inhibited CDDP-induced mitochondrial fragmentation and apoptosis in chemosensitive cells. More

importantly, Oma1 seems to play an important role in determining chemosensitivity of gynecologic cancer cells by controlling L-Opa1 processing, evidenced by the observation that Oma1 (40 kDa) and L-Opa1 processing are absent in chemoresistant cells, irrespective of CDDP treatment (Figure 4.6). The reported characteristics of Oma1$^{-/-}$ MEFs, such as increased mitochondrial tubular connections, and resistance to stress stimuli induced L-Opa1 processing, mitochondrial fragmentation and apoptosis (Quiros, Ramsay et al. 2012), are consistent with our findings in chemoresistant cells, further demonstrating the key role of Oma1-Opa1 in determining chemosensitivity.

However, certain issues remain unclear. By using a commercial antibody to Oma1 and a commercial Oma1 construct, we found Oma1 exists as 55 kDa and 40 kDa forms in chemosensitive cancer cells, the latter evident only with CDDP treatment, although at a much lower level compared to the 55 kDa form (Figure 4.6). Interestingly, the endogenous 40 kDa form of Oma1 increased with CDDP treatment, a phenomenon absent in chemoresistant cells irrespective of the presence of CDDP. We thus propose that the 40 kDa form is a functional and activated form of Oma1 and is derived from the 55 kDa form.

This notion is contrary to the opinion of the Bliek group who, by using Oma1-HA construct in Hela cells, showed that Oma1 exists as a 60 kDa and a 40 kDa form (Head, Griparic et al. 2009) and that the 60 kDa form increased during mitochondrial fragmentation. Interestingly, by using a commercial Oma1 antibody and Oma1-Flag construct, a more recent report showed that two forms of Oma1 (40 kDa and 34 kDa) were found in Hela cells (Zhang, Li et al. 2014). Oma1 34 kDa form increased corresponding with L-Opa1 processing during apoptosis. Their results suggest that the

Oma1 40 kDa form is autocatalyzed into 34 kDa form in response to stress stimuli, and the 34 kDa form can induce L-Opa1 processing. These results suggest that Oma1 short forms (34 kDa), which is processed from the long form (40 kDa) through self-cleavage, is the functional form for L-Opa1 processing in their system (Zhang, Li et al. 2014). Whether the observed differences in Oma1 size and form pattern reported by different groups is due to differences in the experimental methods or in cell types, is not clear and requires further investigations.

Notably, in our study, silencing of Oma1 failed to completely abolish L-Opa1 loss induced by CDDP (Figure 4.7). One possibility is that CDDP also decreases Opa1 mRNA abundance, which also contributes to L-Opa1 loss, however, it cannot be rescued by Oma1 knock-down. Another possible mechanism is that other proteases are also activated or still functioning in processing L-Opa1 when Oma1 is knocked down, such as the matrix AAA (m-AAA) protease AFG3L1&2, and this needs to be further investigated (Ehses, Raschke et al. 2009).

Interestingly, in addition to its role in the processing of L-Opa1, Oma1 has also been found to be involved in the regulation of metabolism (Quiros, Ramsay et al. 2012). Oma1 deficiency in mice is associated with increased body weight due to increased adipose mass, hepatic steatosis, decreased energy expenditure and impaired thermogenesis, and these changes are more significant under metabolic stress conditions such as high-fat diet or cold-shock (Quiros, Ramsay et al. 2012). These findings indicate that Oma1 is important for the development of appropriate adaptive responses to different metabolic stressors (Quiros, Ramsay et al. 2012). As we mentioned earlier, hyperfused mitochondria in chemoresistant cells are associated with altered metabolism, whether and

how Oma1 regulates chemosensitivity through regulating metabolism may be an interesting area for future investigation.

5.7 The role of p53 in regulating mitochondrial fragmentation and apoptosis

Although it is known that p53 mediates apoptosis in a transcription-independent manner by targeting mitochondria and interacting with Bcl-2 family members (Moll, Marchenko et al. 2006, Shibue, Suzuki et al. 2006, Wolff, Erster et al. 2008), limited evidence indicates that p53 is involved in the regulation of mitochondrial dynamics (Chen and Chan 2006, Yamaguchi and Perkins 2009). Our present study strongly suggests that p53 is involved in the regulation of CDDP-induced mitochondrial fragmentation by controlling L-Opa1 processing, since L-Opa1 processing and mitochondrial fragmentation induced by CDDP treatment were significantly inhibited when a functional p53 was absent.

More importantly, our results also suggest that p53 regulates Opa1 processing by modulating Oma1 (40 kDa) product (Figure 4.8). As we mentioned earlier, evidence shows that Oma1 produces short forms through self-cleavage (Zhang, Li et al. 2014). Taken together, these data suggest that p53 is important in regulating the activity of Oma1 self-cleavage, although the mechanism involved is unknown. A requirement for p53 in the regulation of mitochondrial dynamics is further supported by the observation that p53 reconstitution not only induced mitochondrial fragmentation in p53 mutant or null chemoresistant OVCA cells, but also sensitized the cells to CDDP-induced mitochondrial fragmentation (Figure 4.9).

Phosphorylation of p53 is crucial for its stability and ability to induce apoptosis (Banin, Moyal et al. 1998, Tibbetts, Brumbaugh et al. 1999, Ashcroft, Taya et al. 2000). Our previous publication showed that p53 phosphorylation at serine 15 and serine 20 is needed for CDDP-induced apoptosis (Fraser, Bai et al. 2008), however, the function and mechanism of each phosphorylation form in inducing apoptosis is not clear. Our current study strongly suggests that p-p53 (ser15, not ser20) is involved in CDDP-induced mitochondrial fragmentation by interacting with Phb1 and Bak (Figure 4.10, 4.11, 4.13, 4.15, 4.17, 4.18).

These data also provide direct evidence demonstrating p-p53 (ser15) targets mitochondria during apoptosis by showing that p-p53 (ser15) increases in the mitochondrial fraction in chemosensitive cells, but not in chemoresistant cells after CDDP treatment (Figure 4.18). Interestingly, a very recent report shows that fission protein Drp1, which resides in cytosol and translocates to mitochondria during apoptosis, facilitates p53 translocation to the mitochondria under conditions of oxidative stress (Guo, Sesaki et al. 2014). Whether this is also the mechanism for CDDP-induced p-p53 (ser15) translocation to mitochondria needs to be validated.

5.8 The role of Phb1 in regulating apoptosis and chemoresistance

Phb1 is mainly localized in mitochondria (Osman, Merkwirth et al. 2009). Accumulated evidence suggests that it is involved in the regulation of L-Opa1 processing (Merkwirth, Dargazanli et al. 2008, Osman, Merkwirth et al. 2009), although the underlying mechanism is not clear. Our results strongly suggest Phb1 plays a critical role in the regulation of L-Opa1 processing by L-Opa1 sequestration and protection from

proteolysis (Figure 4.10, 4.11, 4.12). We have shown that the targeting of p-p53 (ser15) to the mitochondria is required for the induction of apoptosis by CDDP in chemosensitive cells (Fraser, Bai et al. 2008). Our current study suggests that phosphorylated p53 (ser15, but not ser20) induces apoptosis by regulating mitochondrial dynamics through competitive binding to Phb1, therefore exposing L-Opa1 to its protease. To our knowledge, this is the first study showing the mechanism by which Phb1 regulates L-Opa1 processing and mitochondrial dynamics.

Phb1 is an evolutionarily conserved protein and is involved in different cellular processes, such as cellular signaling and transcriptional control, apoptosis, cellular senescence, and mitochondrial biogenesis (Merkwirth, Dargazanli et al. 2008). The role of Phb1 in apoptosis remains controversial. Our results shows that Phb1 content increases in response to CDDP in chemosensitive cells, but not in chemoresistant cells, and that Phb1 knock-down attenuates CDDP-induced apoptosis in chemosensitive cells (Figure 4.12 & 4.14), suggesting that Phb1 is a pro-apoptotic protein. Our findings are consistent with the report of Yu-Huei et al, which shows that Abrin upregulates Phb1 in the induction of apoptosis in Jurkat T cells, whereas down-regulation of Phb1 delays Abrin-induced apoptotic response (Liu, Peck et al. 2012). However, Chowdhury and colleagues report that Phb1 acts as a survival factor during apoptosis in rat granulosa cells (Chowdhury, Branch et al. 2011). Whether these differences in the role of Phb1 (cell survival or death) are due to differences in cell type is not clear, and awaits further investigation.

Phb1 is also found aberrantly expressed in different cancer types. It is over-expressed in gastric cancer and esophageal squamous cell carcinoma, and correlated with

tumor differentiation, metastasis and worse prognosis (Kang, Zhang et al. 2008, Ren, Wang et al. 2010). On the contrary, another study found Phb1 plays a tumor suppressing role in ovarian cancer, by showing that Phb1 was highly expressed in luteinized ovarian stromal cells, follicular cells, and fallopian tube, with a trend of gradual loss from benign ovarian tumors, to borderline tumors and to carcinomas (Jia, Ren et al. 2014). Similarly, a significant decrease in Phb1 was observed in chemoresistant OVCA patients than chemosensitive patients (Dai, Yin et al. 2010), which is consistent with our observation that Phb1 contents are lower in chemoresistant C13* cells than chemosensitive OV2008 cells (Figure 4.10). Whether the function of Phb1 in cancer formation and chemoresistance depends on the organ specificity is not known and needs to be further explored.

p53 activation (phosphorylation at serine 15 and serine 20) and mitochondrial targeting is a determining factor of CDDP-induced apoptosis in CECA cells (Yang, Fraser et al. 2006, Fraser, Bai et al. 2008). In the current study, we have shown that p-p53 (ser15, but not ser20) binds to Phb1 in chemosensitive but not chemoresistant CECA and OVCA cells (Figure 4.10, 4.13, 4.15, 4.17, 4.18), suggesting Phb1-p-p53 (ser15) interaction may be important for apoptosis and chemosensitivity. This notion is supported by several studies. For instance, it has been demonstrated that the ability of Phb1 to suppress tumor formation is associated with increased p53-mediated apoptotic response, and Phb1 directly interacts with p53 in HCT116 cells and in colon mucosa (Kathiria, Neumann et al. 2012). p53-Phb1 interaction has also been reported in MCF-7 cells, and p53 transcription activity is lower in the absence of Phb1 (Chander, Halpern et al. 2010). Direct interaction of p53 and Phb1 is also increased in response to stress stimuli, and

Phb1 is involved in p53-mediated transcriptional regulation of Bax in Jurkat T cells (Liu, Peck et al. 2012). Together with our results, these data suggest that Phb1 interacts with p53 and is involved in the p53 transcription-dependent and -independent pathway. Moreover, this current thesis presents a novel mechanism that Phb1 facilitates p-p53 interaction with Bak to promote apoptosis.

5.9 The role of Bak in regulating MOMP

The integrity of the outer mitochondrial membrane (OMM) is controlled by BCL-2 family members (Bender and Martinou 2013). The pro-apoptotic BCL-2 family member, Bak or Bax, which are normally inhibited by anti-apoptotic Bcl-2 family members through interaction, such as Bcl-XL, are activated and oligomerize into proteolipid pores, leading to increased MOMP, cytochrome c release and apoptosis. p-p53 (ser15) has been shown to target mitochondria and bind to Bak, inducing Bak activation (Nieminen, Eskelinen et al. 2013). Consistent with this, we also found that increased mitochondrial p-p53 (ser15) binds to Bak in chemosensitive cells during CDDP-induced mitochondrial fragmentation and apoptosis, but not in chemoresistant cells (Figure 4.13, 4.15, 4.17, 4.18). These data suggest that the binding of p-p53 (ser15) to Bak may be the mechanism for CDDP-induced Bak activation and subsequent MOMP in chemosensitive cells. However, in chemoresistant cells, p-p53 phosphorylation and binding to Bak are suppressed.

Furthermore, our results also provide new mechanistic insight into the role of Phb1 in the regulation of the p-p53 (ser15)-Bak complex caused by CDDP in gynecologic cancer cells, by showing that knock-down of Phb1 dramatically inhibited CDDP-induced

interaction between p-p53 (ser15) and Bak in chemosensitive cells (Figure 4.15). However, the mechanism for the requirement of Phb1 in the interaction of p-p53 (ser15) and Bak is not known. One possible mechanism is the binding of Phb1 to p-p53 (ser15) changed the 3D structure of p-p53 (ser15), therefore promoting the affinity of the p-p53 (ser15) and Bak, but this possibility needs to be tested.

Both MOMP and Oma1-mediated L-Opa1 processing are important cellular events during apoptosis. However, the correlation between these two important apoptotic steps seems not clear. Interestingly, very recent reports demonstrated that Bak activation and increased MOMP cause Oma1 activation through self-cleavage, inducing subsequent L-Opa1 processing, mitochondrial fragmentation and cell death (Jiang, Jiang et al. 2014, Zhang, Li et al. 2014). Although these novel findings significantly advanced our understanding of how these important apoptotic steps happen sequentially, certain issues remain to be further explored, such as how Oma1 senses MOMP changes, and what is the mechanism of regulation of the activity of Oma1 self-cleavage.

Notably, our study only explored if p-p53 regulates apoptosis through interaction with Bak, and does not exclude the possibility that p53 may regulate mitochondrial dynamics and apoptosis through the pro-apoptotic factor Bax, another pro-apoptotic Bcl-2 family member involved in the regulation of MOMP. Whether and how Bax is involved in p53 regulated Oma1-mediated L-Opa1 processing remains unclear.

5.10 Akt promotes CDDP resistance by controlling p53 actions in mitochondria

Akt activation or over-expression has been reported in different cancer types and is believed to be a major determinant of chemoresistance. Akt protects cells from stress

stimuli-induced apoptosis and contributes to chemoresistance through different mechanisms, including cytoskeletal rearrangement, inhibiting apoptosis, cell cycle arrest and increased DNA repair (Martelli, Tabellini et al. 2012, Bauer, Patel et al. 2014). Our previous publication showed that Akt confers chemoresistance in gynecologic cancer cells by inhibiting p53 phosphorylation and mitochondrial translocation (Yang, Fraser et al. 2006, Fraser, Bai et al. 2008).

Our current results show that inhibiting Akt in chemoresistant cells increased p-p53 (ser15) content and sensitized the cells to CDDP (Figure 4.18). These responses are associated with interaction of p-p53 (ser15), Phb1 and Bak, increased Oma1 cleavage (activation), L-Opa1 processing and mitochondrial fragmentation (Figure 4.19 & 4.20). We have therefore demonstrated for the first time that Akt confers chemoresistance at least partly via inhibiting p-p53 (ser15)-regulated Oma1-mediated L-Opa1 processing and mitochondrial fragmentation.

As mentioned earlier, mitochondria hyperfusion protects cells from different stress stimuli. Akt may directly promote mitochondria fusion to favor cell survival, in addition to its role of inhibiting p53 action. Interestingly, a recent publication shows that Akt increases Opa1 protein levels, mitochondria fusion and mitochondrial ATP production in cardiomyocytes through Akt downstream effectors mTOR and NF-κB (Parra, Verdejo et al. 2014), suggesting Akt may have other unknown mechanisms in regulating mitochondrial dynamics. How Akt regulates mitochondrial proteins to promote mitochondria fusion and chemoresistance, including Drp1, Fis1, Mfn1&2 and Opa1, will be an interesting future research topic.

Activated PI3K/Akt pathway disturbs the control of cell growth and survival, leading to a competitive growth advantage, tumor formation, metastasis and therapy resistance. Targeting this pathway has drawn great interest in the field of cancer therapy and chemoresistance. Our data suggest that inhibiting Akt activity is effective in overcoming CDDP resistance in gynecologic cancer cells *in vitro* (Figure 4.19). Future studies need to focus on targeting Akt or related molecules in xenograft models and pre-clinical trials by using safer and more tolerable agents.

In fact, several novel Akt inhibitors have been developed and tested in different xenograft models or different stages of pre-clinical or early clinical trials, including Miltefosine, Perifosine, MK2206 and RX-0201 (Porta, Paglino et al. 2014). Although these studies provided some encouraging data, resistance to novel Akt inhibitors, as well as PI3K or mTOR inhibitors, has also been observed and limits clinical efficacy, as with all anti-neoplastic agents (Tan and Yu 2013). The possible mechanisms include secondary target mutations, activation of alternative, parallel signaling pathways, and amplification of downstream alterations within the same pathway (Tan and Yu 2013). Further understanding of the mechanisms of each Akt inhibitor in killing cancer cells is still required, so that they can be applied to individual patients more specifically.

5.11 Future directions

The current studies provide significant evidence supporting the concept that Oma1-mediated L-Opa1 processing and mitochondrial dynamics are dysregulated in chemoresistant cancer cells, that p-p53 (ser15) targets mitochondria and binds to Bak. The latter is facilitated by Phb1 and may be responsible for CDDP-induced L-Opa1 processing and mitochondrial fragmentation. Additionally, the current studies also

provide evidence that Akt controls chemoresistance partly by inhibiting the regulation of mitochondrial dynamics by p53.

As such, the following approaches should be considered as future directions in order to further address the mechanism of dysregulated mitochondrial dynamics and provide stronger evidence for the hypothesis that dysregulated mitochondrial dynamics contribute to chemoresistance in gynecologic cancers.

5.11.1 Xenograft model

Although results obtained from cultured cell lines are informative, they may not represent the behavior of cancer cells *in vivo*. Therefore, the above studies should be extended to include the xenograft models of CECA and OVCA. Xenografts of CDDP-sensitive and -resistant OVCA cell lines should be grown in female Swiss nude mice to study the involvement of mitochondrial dynamics in the regulation of apoptosis *in vivo*.

To assess whether mitochondrial dynamics are dysregulated in chemoresistant cancer cells and CDDP induces mitochondrial fragmentation in chemosensitive cells *in vivo*, chemosensitive A2780s cells and chemoresistant A2780cp cells would be inoculated in nude mice as described (Farrand, Byun et al. 2013). Subcutaneous cell inoculation would be selected since tumor growth can be easily measured. Sufficient cells ($5\text{-}10\times10^6$) would be injected to ensure tumor growth in >95% of the mice. Animals would be monitored on alternate days to check tumor formation. When the tumor has developed to 0.5cm in diameter, CDDP (1.5 mg/kg, IP, clinical dose equivalent) would be administered. Animals would be sacrificed when tumor diameter reaches 1 cm. After sacrifice, tumor volume would be measured. A portion would be fixed in formalin and

embedded in paraffin for immunohistochemical analysis of apoptosis (TUNEL assay) and mitochondrial morphology (staining of Tom20). A portion would be used for WB to measure the contents of p-p53 (ser15), Oma1, Opa1 and Phb1. Interaction of p-p53 (ser15), Phb1 and Bak would also be determined through IP-WB. We expect CDDP will decrease the size of the tumor from chemosensitive cells, but not chemoresistant cells. We also expect mitochondrial morphology is more fused in chemoresistant tumor than chemosensitive tumor, and that CDDP induces mitochondrial fragmentation, apoptosis, p-p53 (ser15) and Phb1 increase, Oma1-mediated L-Opa1 processing, and interaction between p-p53 (ser15), Phb1 and Bak in chemosensitive tumor, not in chemoresistant tumor, as we observed *in vitro*.

To further confirm the role of Akt in controlling p53 regulated mitochondrial fragmentation induced by CDDP, the Tet-on regulated gene expression system (BD Biosciences) would be used, which is more efficient and provides longer-lasting gene manipulation effects than transient transfection method (Shaikh and Nicholson 2006). The cell lines stably expressing the reverse tetracycline-controlled transactivator (rtTA) under control of the CMV promoter would be generated, and then subjected to a second stable transfection with a vector containing the recombinant gene of interest, under the control of the Tet-responsive element. The gene of interest can be induced by the addition of doxycycline in drinking water. Under this system, gene expression can be induced only in the appropriate cells, and can be maintained over time through the doxycycline administration. Nude mice harbouring A2780cp-[Flag-CTL]-[HA-CTL-Tet], A2780cp-[Flag-DN-Akt]-[HA-CTL-Tet], A2780cp-[Flag-CTL]-[HA-p53-Tet] or A2780cp-[Flag-DN-Akt]-[HA-p53-Tet] would be treated with CDDP and doxycycline. We expect reconstitution of wild type p53 will sensitize the tumor to CDDP only when Akt activity is

inhibited by DN-Akt. These experiments can be extended to include time-course and dose-response effects of doxycycline, to further examine the effect of p53 on mitochondrial dynamics, apoptosis and tumor suppression.

5.11.2 Clinical samples

The key cellular events that we found contribute to chemoresistance in cell lines could also be extended to include primary cultures of OVCA cells from ascites fluids and solid tumors from CECA and OVCA patients of stage III/IV. The cells would be cultured, followed by CDDP treatment. The sensitivity of these tumor cells to CDDP treatment would be determined by apoptosis rate. Mitochondrial morphology changes with CDDP treatment will be examined using confocal microscopy. The contents of p-p53 (serine 15), Phb1, Oma1 and Opa1 would be determined by WB. The interaction of p-p53 (serine 15), Phb1 and Bak would be examined through IP-WB.

Furthermore, p53 mutational status (exons 5-9; DNA Binding Domain) would be checked by direct PCR-based sequencing among the cells which don't respond to CDDP treatment (chemoresistant). p53 wild type chemoresistant cells would be treated with DN-Akt, followed by CDDP treatment. We expect DN-Akt treatment will sensitize these cells to CDDP treatment, therefore enhance CDDP-induced L-Opa1 processing and mitochondrial fragmentation. p53 mutant chemoresistant cells will be treated with DN-Akt and transfected with p53 cDNA, followed by CDDP treatment. We expect DN-Akt will have no effect on chemosensitivity in these cells unless wt-p53 is reconstituted.

5.12 Conclusions

In conclusion, we have demonstrated that mitochondrial dynamics, CDDP-induced L-Opa1 processing and mitochondrial fragmentation are differentially regulated in chemosensitive and chemoresistant gynecologic cancer cells. Processing of L-Opa1 is mediated by Oma1, under the control of p53 and Akt, and involving the interaction of different molecules, including p-p53 (ser15), Phb1 and Bak. Determining the molecular mechanisms by which p53 controls Oma1-mediated L-Opa1 processing may contribute to the current understanding of mitochondrial dynamics and apoptosis, and ultimately of the mechanisms of chemoresistance in human gynecologic cancer. Targeting defective mitochondria pathways in chemoresistant cells may shed light on new cancer treatment strategies.

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Chapter 7: APPENDICES

Table 7.1 Antibodies used in this study

Antibodies that were used in the experiments for current thesis are listed in this table, as well as the resources and species.

Table 7.1

Abcam							Santa Cruz					BD		Life Span
Mouse						Rabbit	Mouse		Rabbit			Mouse		Rabbit
GAPDH	p-p53 (ser20)	HA tag	Bak	Mfn1	Mfn2	Oma1	Tom20	p53	p-p53 (ser15)	Histone H3	Phb1	Opa1	Drp1	Fis1

7.2 Characterization of experimental cell lines

In the present thesis, two human cervical cancer cell lines and four ovarian cancer cell lines were used to investigate the role and regulation of mitochondrial dynamics in the development of CDDP resistance in human gynecologic cancer. Supplemental information in reference to the tumors of origin, p53 status and characterization of tumor formation from intraperitoneal xenograft model are listed in Table 7.2.

Table 7.2 Characterization of experimental cell lines

Cell line	Tumor origin	p53 status	IP tumors from Xenograft (nude mice)
OV2008	Cervical squamous carcinoma (1,2)	WT (3)	Endometrioid adenocarcinomas-squamous differentiation (4)
C13*	Cervical squamous carcinoma (5)	WT (3)	Failed to form IP tumor (4)
A2780s	Ovarian serous cystadenocarcinoma (6)	WT (3)	Undifferentiated tumor (4)
A2780cp	Ovarian serous cystadenocarcinoma (6)	Mutant (3)	Undifferentiated tumor (4)
SKOV3	Ovarian clear cell carcinoma (7)	Null (7)	Clear cell adenocarcinoma (4)
Hey	Ovarian moderately differentiated papillary cystadenocarcinoma (8)	WT (9)	Undifferentiated tumor (4)

WT: wild-type; IP: intraperitoneal

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2. Andrews PA, Murphy MP, Howell SB.(1985) Differential potentiation of alkylating and platinating agent cytotoxicity in human ovarian carcinoma cells by glutathione depletion. Cancer Res. 45(12 Pt 1):6250-3.
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7.3 Primer sequences for Opa1 variants and β-Actin

To determine the physiologic role of Opa1 in the regulation of mitochondrial dynamics and chemoresistance, we compared the mRNA abundances of Opa1 variants in chemosensitive cells and chemoresistant cells by real-time quantitative PCR. The variants of Opa1 were amplified using specific primer pairs based on literature (1).

Table 7.3

Gene	Accession No.	Forward primer Sequence (5´–3´)	Reverse primer Sequence (5´–3´)
Opa1 variant 1	NM_015560	CTTTTTTACCTCAGGTTCTCC	CTCTTTGTCTGACACCTTTCT
Opa1 variant 2	NM_130831	GAGTATATCGATTTTGGTTCTC	CTCTTTGTCTGACACCTTTCT
Opa1 variant 3	NM_130832	GAGTATATCGATTTTGGTCACA	CTCTTTGTCTGACACCTTTCT
Opa1 variant 4	NM_130833	GAGTATATCGATTTTGGTTCTC	TCACCAAGCAGACCCTTTCT
Opa1 variant 5	NM_130834	GACTTTTTTACCTCAGGTCAC	CTCTTTGTCTGACACCTTTCT
Opa1 variant 6	NM_130835	GAGTATATCGATTTTGGTCACA	TCACCAAGCAGACCCTTTCT
Opa1 variant 7	NM_130836	CTTTTTTACCTCAGGTTCTCC	TCACCAAGCAGACCCTTTCT
Opa1 variant 8	NM_130837	GACTTTTTTACCTCAGGTCAC	TCACCAAGCAGACCCTTTCT
β-Actin	NM_001101	GATCAGCAAGCAGGAGTATG	AAGGGTGTAACGCAACTAAG

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CHAPTER 8 CURRICULUM VITAE

Bao Kong
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University of Ottawa
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EDUCATION INFORMATION

Ph.D. candidate, Cellular and Molecular Medicine, University of Ottawa, (Sep/2009 - Feb/2015 expected)

MSc, School of Medicine, Shandong University, (Aug/2006 – Jul/2009), major in Gynecology.

MBBS, School of Medicine, Shandong University, (Aug/1998 - Jul/2003).

WORK EXPERIENCE

Lab reagents and supplies ordering duty: Dr Benjamin Tsang's lab in Ottawa Hospital Research Institute (Sep/2012 - present)

Obstetrician and Gynecologist: Traffic Hospital of Shandong Province in China (Aug/2003 - Jul/2006).

AWARDS AND SCHOLARSHIPS:

2012 Recipient of the Quota Club of Ottawa Scholarship Award

2012 Travel Award, 6th Canadian Conference on Ovarian Cancer Research

2010 International Student Admission Scholarship, University of Ottawa

2009 Doctoral Scholarship, China Scholarship Council

2008 President Scholarship, Shandong University

RESEARCH EXPERIENCE

Participated in two studies：

1. Experimental study of the involvement and mechanism of mitochondrial dynamics in the regulation of chemoresistance in ovarian and cervical cancer cells (2009-2015, in University of Ottawa, supported by CIHR).

2. Experimental study of the expression of SF-1 and StAR in endometriosis (2006-2009, in Shandong University, supported by a grant from natural science foundation of Shandong Province, P.R. China).

The skills mastered on experiment： cell culture, immunohistochemistry, knock-down of the target protein with siRNA or over-expression with cDNA, western-blot, cellular fractionation, immunofluoresence, confocal-microscopy and immunoprecipitation.

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PUBLICATION

1. **Kong B,** Wang Q, Fung E, Xue K, Tsang BK. p53 is required for cisplatin-induced processing of the mitochondrial fusion protein L-Opa1 that is mediated by the mitochondrial metallopeptidase Oma1 in Gynecologic Cancers. J Biol Chem. 2014 Sep 26;289(39):27134-45

2. **Kong B**, Tsang BK. Prohibitin 1 is involved in CDDP-induced mitochondrial fragmentation and apoptosis in Gynecologic cancers. (To be submitted)

3. Tian Y, **Kong B**, Zhu WW, etc. Expression of steroidogenic factor 1 (SF-1) and steroidogenic acute regulatory protein (StAR) in endometriosis is associated with endometriosis severity. J Int Med Res. 2009 Sep-Oct;37(5):1389-95.

4. **Kong B**, Tian YJ, Zhu WW, etc. A pure nongestational ovarian choriocarcinoma in a 10-year-old girl: case report and literature review. J Obstet Gynaecol Res. 2009 Jun;35(3):574-8.

5. **Kong B**, Tian Y, Zhu W, Su S, Kan Y. Effects of celecoxib and nimesulide on the proliferation of ectopic endometrial stromal cells in vitro. J Int Med Res. 2008 Sep-Oct;36(5):1032-8

POSTER AND ORAL PRESENTATION

Participated in 2014 Ottawa Hospital Research Institute Research Day held in Ottawa, Ontario, Canada with a oral presentation titled:
The role and regulation of mitochondrial dynamics in chemoresistance in ovarian cancer cells. **Bao Kong**, Tsang BK.

Participated in the 2013 (46^{th}) Annual Society for the Study of Reproduction conference in Montreal, Quebec, Canada with a poster titled:
p53 is required for CDDP-induced L-Opa1 Processing and Mitochondrial Fission in Ovarian Cancer Cells. **Bao Kong**, Tsang BK.

Participated in the 6th Canadian Conference on Ovarian Cancer Research (2012) in Quebec City, Quebec, Canada with a poster titled:
Suppressed Opa1 Processing and Mitochondrial Fission in Chemoresistance in Ovarian Cancer Cells. **Bao Kong**, Benjamin K. Tsang.

Participated in 2010, 2011, and 2012 Ottawa Hospital Research Institute Research Day held in Ottawa, Ontario, Canada with a poster titled:
The role and regulation of mitochondrial dynamics in chemoresistance in ovarian cancer cells. **Bao Kong**, Tsang BK.

Participated in the 27th and the 28th Annual Reproductive Biology Workshop of the University of Ottawa held in Ottawa, Ontario, Canada with a poster titled:
The role and regulation of mitochondrial dynamics in chemoresistance in ovarian cancer cells. **Bao Kong**, Tsang BK.

ASSIGNED MENTORSHIP

2012 Ella Fang, Summer Student, Department of Cellular and Molecular Medicine, University of Ottawa