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APM2 Is a Novel Mediator of Cisplatin Resistance in a Variety of Cancer Cell Types *in vitro* and in Xenograft Models of Cancer

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APM2 IS A NOVEL MEDIATOR OF CISPLATIN RESISTANCE IN A VARIETY OF CANCER CELL TYPES *IN VITRO* AND IN XENOGRAFT MODELS OF CANCER

Bradley James Scott

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
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LIST OF ABBREVIATIONS

5-Fu	5-Fluorouracil
AF594	Alexa Fluor 594
Akt	Protein kinase B
ANOVA	Analysis of variance
APM2	Adipose most abundant transcript 2, Adipose specific 2
AR	Adaptive response
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
ATP7A	ATPase, Cu++ transporting, alpha polypeptide
ATP7B	Wilson's disease protein
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR interacting protein
Bcl-2	B cell lymphoma 2
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosourea
bp	Basepair
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer type 2 susceptibility protein
BSA	Bovine serum albumin
C10orf116	Chromosome 10 open reading frame 116
cDNA	Complementary DNA
cGy	Centigray
cm	Centimeter
cMOAT	Canalicular multispecific organic anion transporter
CSC	Cancer stem cells
CTR1	Copper transporter 1
d	Day
DAPI	4',6-diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dCTP-32P	Radioactive dCTP
dGTP	Deoxyguanosine triphosphate
DMEM/F12	Dulbecco's modified eagle medium/nutrient mix F12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTP	Deoxynuceloside triphosphate
DSB	Double strand break(s)
DTT	Dithiothreitol
dTTP	Deoxythreonine triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ERCC1	Excision repair cross complementing 1
EST	Expressed sequence tag
FLIP	Flice-like inhibitory protein
FOLFOX	Folinic acid/5-FU/Oxaliplatin colorectal cancer treatment regimen
GAPDH	Glyceraldehyde-3-phophate dehydrogenase
GBM	Glioblastoma multiforme

G-NER	Global genomic nucleotide excision repair
GSH	Glutathione
GST	Glutathione S transferase
Gy	Gray
h	Hour(s)
H460	Human lung carcinoma cell line
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HCT116	Human colorectal tumour cells
hMLH1	Human MutL homolog 1
hMSH2	Human MutS homolog2
HR	Homologous recombination
HRP	Horse radish peroxidase
IAP	Inhibitor of apoptosis
ICP-MS	Inductively coupled plasma mass spectrophotometry
ID50	Inhibitory dose 50
IR	Ionizing radiation
JNK/SAPK	c-Jun NH2-terminal kinase/Stress activated protein kinase
JR8	Human melanoma cell line
Kg	Kilogram
KOH	Potassium hydroxide
kVp	Kilovolt potential
LDH	Lactate dehydrogenase
LDS	Lithium dodecyl sulfate
LRP	Lung-resistance related protein
lwd	Length, width, depth
M	Molar
M14	Human melanoma cell line
MCF-7	Human breast carcinoma cell line
MCL1	Myeloid cell leukemia sequence 1
MES	2-(N-morpholino)ethanesulfonic acid
mg	Milligram
MgCl2	Magnesium chloride
min	Minute(s)
MKN-28	Human gastric cancer cell line
MKN-45	Human gastric cancer cell line
ml	Millilitre
mM	Millimolar
MMR	Mismatch repair
mRNA	Messenger ribonucleic acid
MRP1/2	Multi-drug resistance proteins 1 and 2
MT	Metallothionein
MWF	Monday, Wednesday, Friday
N.S.	Not significant
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NBS1	Nijmegan breakage syndrome 1
NCBI	National Center for Biotechnology Information
NER	Nucleotide excision repair
ng	Nanogram

NHEJ	Non-homologoug end joining
nm	Nanometer
nM	Nanomolar
NSCLC	Non-small cell lung cancer
°C	Degrees Celsius
OV2008	Human ovarian carcinoma cell line
PAGE	polyacrylamide gel electrophoresis
PBS	Phosohate buffered saline
PC-3	Human prostate carcinoma cell line
pM	Picomolar
pmoles	Picomoles
Pt	Platinum
PTEN	Phosphatase and tensin homolog on chromosome 10
PVDF	Polyvinylidine fluoride
RNA	Ribonucleic acid
RS	Correlation co-efficient (Spearman's rank test)
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
S phase	Synthesis phase
SDS	Sodium Dodecyl Sulfate
SEM	Standard error of the mean
SF	Surviving fraction
shRNA	Short hairpin RNA
siRNA	Short interfering ribonucleic acid
SSC	Saline-sodium citrate buffer
SW620	Human colorectal carcinoma cell line
T	Threonine
T24	Human bladder cancer cell line
T25	25 cm tissue culture flask
TBS	Tris-buffered saline
TBS-T	TBS + 1% Tween-20
TC-NER	Transcription coupled nucleotide excision repair
TFIIH	Transcription factor II H
Tp53	Tumour protein 53
tRNA	Transfer RNA
UV	Ultraviolet light
XIAP	X-linked *inhibitor of apoptosis*
XPA	Xeroderma pigmentosum complementation group A
XPC	Xeroderma pigmentosum complementation group C
XPF	Xeroderma pigmentosum complementation group F
XPG	Xeroderma pigmentosum complementation group G
XR	X-radiation
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
β	Beta
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar

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'No great discovery was ever made without a bold guess' – Sir Isaac Newton

STATEMENT OF CO-AUTHORSHIP

All studies appearing in this manuscript were performed by Bradley J. Scott under the supervision of Dr. Cheng E. Ng with the exception of the following: the isolation of clones having different responses to various chemotherapeutics and X-radiation was performed in our laboratory by Dr. Sami S. Qutob [Qutob et al., 2004]. Additionally, the performance of the microarray experiments was completed in the laboratory of Dr. Qing Liu by Dr. Sami S. Qutob. Analyses and RT-PCR were performed by Bradley J. Scott and Dr. Sami S. Qutob, supervised by Dr. Cheng E. Ng.

ABSTRACT

Resistance to traditional modes of cancer therapy is a major obstacle facing cancer patients. The untreated HCT116 cell line was subcloned and each clone was assessed for its response to several drugs and X-radiation. One clone HCT116-K was resistant to cisplatin/etoposide/topotecan but sensitive to XR while another HCT116-2 was sensitive to cisplatin but resistant to XR. HCT116-10, a clone that responded to drugs and radiation in a manner similar to parental HCT116 cells was used as a control clone. Comparing gene expression between HCT116-K, or HCT116-2, vs. HCT116-10, we were able to identify several genes whose expressions were altered in clones that were resistant/sensitive to various insults. Each identified gene's expression was then examined in a panel of HCT116 clones to determine whether the expression observed in the resistant/sensitive clone was outside the normal expression distribution within the population. One gene APM2 was significantly upregulated in HCT116-K. Upon follow-up, APM2, was found to promote cisplatin resistance when overexpressed in sensitive HCT116 clones. Furthermore, silencing APM2, using sequence specific siRNAs, in a panel of cell lines encompassing all combinations of p53 status and MMR proficiency (HCT116-K, HCT116, SW620, MCF7, PC-3, and OV2008) resulted in sensitization regardless of these two factors. In addition, silencing APM2 stably, using shRNAs, also resulted in the sensitization of cells to cisplatin. More importantly, cisplatin inhibited the growth of APM2 silenced tumour xenografts (HCT116-K or OV2008 cells) significantly better than it inhibited the growth of xenografts carrying non-targeting control shRNAs. Additionally, APM2 was found to be localized to the cytoplasm and membrane. Further investigation revealed that HCT116 cells secrete APM2 to the extracellular space representing a possible mechanism by which

APM2 promotes cellular resistance to cisplatin, by binding to and carrying cisplatin out of the cell. These findings represent a novel strategy that could be exploited to overcome cisplatin resistance in patients regardless of p53 status or ability to perform MMR.

CHAPTER 1

INTRODUCTION

1.1. – CANCER AND THERAPEUTIC RESISTANCE

The American Cancer Society describes cancer as "a group of diseases characterized by uncontrolled growth and spread of abnormal cells". The spread of abnormal cells throughout the body eventually results in death. According to the Canadian Cancer Society 73,800 cancer related deaths will occur in Canada in 2008 [Canadian Cancer Society, 2008]. In addition, 166,400 Canadians will be diagnosed with cancer during 2008 [Canadian Cancer Society, 2008]. While cancer incidence appears to be on the rise, the rate of mortality due to many types of cancer has slowed or declined. One such example of this is prostate cancer where mortality rates began to decline in the 1990's and was actually down by 2.9 % from 1995 to 2004 [Canadian Cancer Society, 2008]. These improvements are likely due to a combination of improved detection methods, which allow for earlier detection and improved treatment options.

Despite the many forms in which cancer manifests itself, modern medical research has provided us with a number of useful treatment modalities that, in many cases, are

applied successfully. Generally, treatment of cancer involves surgical removal of cancerous tissues, treatment with ionizing radiation (X-radiation), or treatment with chemotherapeutic agents, or a combination of these methods [Zhivotovsky et al., 1999]. Regrettably, with the exception of surgical resection, all of these options are susceptible to failure due to resistance of cancer cells to the particular type of treatment. Resistance to drugs and/or radiotherapy is seen in many different types of cancer. For example, treating ovarian cancers with platinum (cisplatin, carboplatin) in combination with paclitaxel is a very effective first-line treatment with approximately 75% of patients responding [Richardson et al., 2008]. However, the majority of patients will experience disease recurrence. The recurring disease is almost always resistant to re-treatment with the same regimen [Richardson et al., 2008]. To complicate matters further, recurrent ovarian cancers, treated initially with platinating agents, may also become cross-resistant to a number of other agents including X-radiation [Godwin et al., 1992]. Because of these factors, patients with recurrent, treatment resistant, ovarian cancer are treated with a variety of different agents in the hopes that they will be active against the disease. The goal of these treatments is usually intended to improve and extend quality of life rather than cure the disease [Bhoola and Hoskins, 2006; Richardson et al., 2008].

Ovarian cancer is a good example of how cancers can become resistant to chemotherapy and/or radiation regimens. However, there are many other cancers that are either inherently or subsequently develop resistance to chemotherapeutics and radiation. For instance, lung cancers are known to be refractory to several therapies including treatment with various chemotherapeutics and radiotherapy [Brognard et al., 2001]. Because lung cancers are prevalent and response to traditional therapies is poor, lung cancer has become the most lethal form of cancer in both the United States and Canada

[Brognard et al., 2001; Canadian Cancer Society, 2008; Landis et al., 1999]. The reasons for this resistance are not completely understood, but a great deal of research has been conducted to uncover the genes and pathways that allow lung cancers to be insensitive to chemotherapeutics and radiation (see, for example:[Brognard et al., 2001; Cao et al., 2004; Deng et al., 2004; Hansen et al., 2003; Rosell et al., 2003; Whiteside et al., 2004]). As a consequence of this research several targeted therapies have been designed to help overcome resistance. One notable targeted therapy currently being used in the treatment of non-small cell lung cancers (NSCLC) is gefitinib [Paez et al., 2004]. Gefitinib is a selective inhibitor of the epidermal growth factor receptor (EGFR) [Paez et al., 2004]. The development of gefitinib was based on the observation that the EGFR is more highly expressed in the NSCLCs than in adjacent normal tissues [Rusch et al., 1993]. Activation of the EGFR begins signalling cascades that ultimately result in cell proliferation and survival, therefore, inhibition of the receptor is capable of preventing these responses and slowing tumour growth [Sordella et al., 2004]. Unfortunately, overexpression of the EGFR does not correlate with drug responsiveness and the drug is only active in a subset of patients that harbour EGFR activating mutations [Rusch et al., 1993; Sordella et al., 2004]. Thus, it is necessary to uncover new strategies to combat therapy resistance in lung cancer.

Another important example of drug resistance occurs in patients with glioblastoma multiforme (GBM). This particular type of cancer is highly resistant to a wide range of chemotherapeutics and X-radiation. Research to understand the mechanisms that account for this resistance has been intense (see examples:[Maxwell et al., 2008; Virrey et al., 2008; Ziegler et al., 2008]). One of the main factors confounding GBM treatment is the existence of intrinsically resistant subpopulations of cells within the primary tumour. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) is the chemotherapeutic most commonly employed to

treat and control GBM. However, these intrinsically resistant cells, termed cancer stem cells (CSC), are able to avoid BCNU induced death and initiate or repopulate a tumour [Kang and Kang, 2008]. Therefore, much of the research in this area has been focused on identifying targets that can be exploited to sensitize CSC to the various treatment regimens in use. One such study identified the chloride channel CLIC1 as overexpressed in BCNU resistant cells when compared BCNU sensitive cells. When a the chloride channel was blocked using a known chloride channel blocker, in combination with BCNU, resistant cells became significantly more sensitive than when they were treated with BCNU alone [Kang and Kang, 2008]. Other chemotherapeutics are also heavily prescribed for the treatment of GBM, especially where patients have proven to be unresponsive to BCNU. In fact, temozolomide, a drug that causes DNA mismatch, was recently shown to preferentially kill CSC in GBM [Beier et al., 2008]. Hence, a combination therapy utilizing BCNU and temzolomide may be an effective strategy to extend survival in patients with GBM.

While significant advancements have been made in treating a number of cancers, drug resistance, whether intrinsic or acquired, remains a serious obstacle. As was illustrated above, several cancers remain very refractory to current treatment regimens. Some of these cancers initially respond quite well while others tend to be resistant from the beginning. Thus, identifying methods of sensitizing cancer to current treatment protocols are potentially very important in cancer therapeutics. This thesis investigates primarily X-radiation and cisplatin resistance and the search for factors that contribute to these phenotypes. Furthermore, it will describe the discovery of a little known gene, APM2, which appears to play a novel role in cellular resistance to cisplatin. The following sections will be an attempt to summarize the vast amount of literature pertaining to X-radiation and

cisplatin resistance. The goal of this literature review is to highlight some of the key factors that contribute to treatment failure and progress that has been made to overcome these failures. As well, a review of the literature pertaining to the contribution of DNA microarray to the field of drug/radiation resistance will be described. Finally, literature regarding APM2 characterization and function will be presented.

1.2. – IONIZING RADIATION

1.2.1. - MODE OF ACTION AND TREATMENT OBSTACLES

Ionizing radiation (IR) is one of the key components of many modern, multi-modal strategies currently in use for treating a wide variety of malignancies. In the medical community, it is commonly accepted as one of the most effective treatments available for a wide variety of malignancies originating from different tissues and presenting at different stages. High-energy photons (gamma rays, x-rays) and charged particles (electrons) are used to deliver IR to target tissues causing the ionization of atoms within the biological tissue [Gudkov and Komarova, 2003]. The traditional model of IR induced cell killing is that IR induces cell death through the induction of DNA damage of different types leading to mitotic cell death (Figure 1). Further evidence for this model is the correlation between nuclear size and morphology (DNA content) and radiation sensitivity, demonstrating that DNA is likely to be the most important target of IR [Hawkins, 2005]. The more severe types of damage include single and double strand breaks (DSB) and sites with multiple types of damage such as DNA-DNA and DNA-protein cross-links. Lesser damages such as small-base and nucleotide damage may also occur [McMillan et al., 2001]. Of the specific

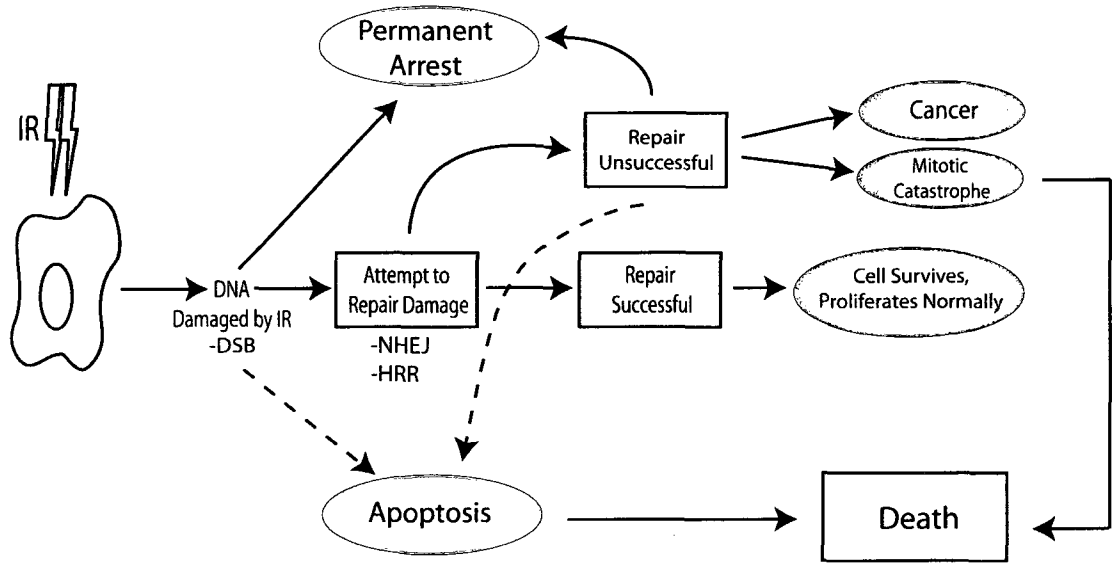

Figure 1: The effect of ionizing radiation on mammalian cell fate. DNA damage occurs after receiving an acute dose of ionizing radiation (IR), usually in the form of DSBs. The mammalian cell can follow several pathways that result in either permanent arrest, or death. If the cell can successfully repair the IR induced DNA damage the cell may survive and proliferate normally. Normal cells may perform repair incorrectly, which could lead to either mitotic catastrophe or cancer. Solid lines represent the more prominent pathways. Dashed line represent less prominent pathways Adapted from: Nat. Rev. Canc. 3, 117-129 (February, 2003).

types of DNA damage occurring through treatment with IR, DSB appear to be the most important due to the observation that DSB levels correlate with cell killing. In addition, DSB produced by digestion with restriction enzymes cause phenotypes similar to those presented by IR treated cells. [Bryant and Liu, 1994].

Malignancies that are routinely treated with radiation therapy include cancers of the head and neck, breast, bladder, brain and others. However, several problems can arise during or after the treatment of patients with radiotherapy. One such problem is the occurrence of radiation related second cancers. Secondary cancers are especially prevalent in paediatric patients receiving radiotherapy [Levin et al., 2005]. Children that have been cured of their primary cancer stand a greater chance of developing a secondary cancer sometime during their lives. The fact that most secondary cancers caused by radiotherapy occur within 10-15 years of primary cancer treatment, may make radiation treatment applied to young children more of a concern than treatment given to adults [Miralbell et al., 2002]. Another common drawback to radiation therapy is repopulation of the IR treated tumour with rapidly multiplying cancer cells that have survived the treatment (some of these are thought to be tumour stem cells) [Bao et al., 2006].

Radiotherapy generally involves the administration of small doses of IR over a period of several weeks. This type of schedule allows for the recovery of normal tissues from sub-lethal damage incurred from the radiation treatment. This also prevents an accumulation of normal tissue damage, which can lead to severe toxicity [Kim and Tannock, 2005]. The major disadvantage of allowing recovery periods throughout the IR treatment is that it also allows for repopulation of the tumour with rapidly multiplying cells that, before treatment, may have been destined to die due to a lack of oxygen and nutrients [Kim and Tannock, 2005]. Repopulation of tumours with rapidly growing and often

radioresistant cells is a major problem that limits the effectiveness of IR. In addition, normal tissue toxicity limits the dose that clinicians are able to administer to their patients. Several classical genetic defects exist that result in severe hypersensitivity to radiation including *Ataxia telangiectasia* (AT) and Nijmegen breakage syndrome. These diseases are the result of mutations in genes (ATM and NBS1) involved in the recognition, and mediation of cellular response to DSBs [Crompton et al., 2001]. However, even patients without recognizable genetic defects can have unpredictable normal tissue toxicity. Normal tissue toxicity is variable from patient to patient and, thus far, no clinical test has been developed that can reliably predict a patient's normal tissue or tumour radiosensitivity. However, strong efforts are being made to uncover an indicator of normal tissue radiosensitivity [Borgmann et al., 2002; Hill et al., 2001; Russell and Begg, 2002]. The last, but certainly not least, important obstacle to radiotherapy is the development of, or inherent radioresistance of tumour cells. Many tumour types display inherent radioresistance. For example, radiotherapy is the post-operative standard of care for patients afflicted with a glioblastoma multiforme (GBM). However, GBMs tend to be resistant to radiation and almost always recur (>90 %) [Hovinga et al., 2005]. Induced radioresistance also seems to play an important role in the resistance of tumours to IR [Lambin et al., 1996; Wolff, 1998]. Inducible radiation resistance occurs as part of the cellular stress response seen during periods of genotoxic stress and is known as the adaptive response (AR). Adaptive response generally refers to the ability of a single priming IR dose to induce subsequent cellular radioresistance to later doses of IR that are often larger in magnitude [Olivieri et al., 1984; Sasaki et al., 2002; Wolff, 1998]. This is important clinically as radiation treatment is usually given in small doses over a period of days. Therefore, it is possible that

the initial doses, given during a regimen of fractionated radiotherapy, will act as primers of AR causing an increase in the radioresistance of the tumour.

The factors mentioned above represent a significant roadblock to effective radiotherapy. Radiosensitization seems to be the key to overcoming these roadblocks. For decades, the search for radiosensitizers has been one of the primary goals of radiation research. It has already been shown that combined treatment of cancers with chemotherapy (oxaliplatin, cisplatin, camptothecin derivatives) and radiotherapy can increase the efficacy of radiation treatment by sensitizing cancer cells to radiation [Chen et al., 1997; Mattern et al., 1991]. More recently, the focus has turned to a search for an understanding of the molecular pathways that lead to radioresistant phenotypes within cancer. It is hoped that the elucidation of these pathways will lead to the identification of molecular targets suitable for modulation leading to radiosensitization.

1.2.2. - ACQUIRED VS. INTRINSIC RADIORESISTANCE

At least half of all patients diagnosed with cancer will receive radiation therapy as at least part of their treatment strategy [American Cancer Society, 2008]. Many patients respond well to this method of treatment. However, some patients present with cancers that do not respond to radiotherapy at all. Others will respond initially and become resistant to radiotherapy later on. Cancers that do not respond well to radiotherapy are said to be intrinsically resistant to radiation. Intrinsic radioresistance is often observed in cancers for which ionizing radiation is a first-line treatment. Studies aimed at uncovering the mechanisms responsible for intrinsic radioresistance have shown that the presence of oncogenic ras mutations within a tumour can account for increased resistance to radiation when compared to cells that do not harbour ras mutations [Sklar, 1988]. One study, showed

that transfection of NIH 3T3 cells with activated ras mutants (H-Ras, K-Ras, N-Ras) significantly altered the shape of radiation survival curves increasing the cell line's radioresistance [Sklar, 1988]. Further studies investigating the effects of activating ras mutations on the response to ionizing radiation revealed that transfection of cells with H-ras ultimately resulted in the upregulation of the DSB repair protein Ku-80. In addition, the number of DSB present decreased in cells expressing H-ras and this effect was reversed when Ku-80 expression was disrupted by siRNAs [Chang et al., 2005a]. This example represents one mechanism by which cancers may be intrinsically resistant to cell killing by radiation. TP53 status has also been shown to be a determinant of intrinsic radioresistance. Pardo et al illustrated the effects of p53 mutation on radio resistance in rat embryos. This set of experiments demonstrated that rat embryo clones expressing a mutant form of p53 were significantly more resistant to ionizing radiation than non-transfected rat embryo cells [Pardo et al., 1994]. When taken together it can be appreciated that cancer cells harbouring dominant mutations in genes involved in pathways related to survival (DNA repair, apoptosis etc.), will be intrinsically more, or less, resistant to radiation therapy than cancer cells that do not harbour these mutations. The p53 protein is a transcription factor that is involved in numerous facets of cellular regulation including DNA repair, cell cycle, and apoptosis [Kuerbitz et al., 1992; Shaw et al., 1992; Smith et al., 1995], so it is no surprise that cells carrying a dominant mutated version of p53 react differently to stressors, such as irradiation, than their wild-type counterparts.

Intrinsic resistance occurs due to changes in cellular physiology that occur before the actual event of radiation treatment. Acquired resistance, on the other hand, occurs when initially sensitive cancers become resistant to radiation after one, or several radiation treatments. The concept of acquired resistance has been debated [Bristow and Hill, 1990;

Leith and Michelson, 1990; Yaes, 1989]. One explanation for acquired radioresistance is the cell selection model, which states that sensitive cells are killed off by the initial treatments with radiation. Consequently, only cells that are inherently more resistant to radiation remain. These cells can then repopulate the tumour, giving rise to an overall more resistant malignancy [Pearce et al., 2001]. However, there is increasing evidence to support the argument that radiation can induce molecular changes in cancer cells that result in more resistant phenotypes. One study that seems to support this model examined the role of survivin in radioresistance. Survivin is a member of the inhibitor of apoptosis (IAP) family [Fortugno et al., 2002]. In this study it was shown that radiation can promote increased expression and increased phosphorylation of survivin leading to stabilization [Chakravarti et al., 2004]. Cells that had increased expression of survivin became more resistant to ionizing radiation. In addition blocking the function of survivin by introduction of a dominant mutant reversed this sensitivity. It should also be noted that the observed radioresistance was not related to survivin's ability to inhibit apoptosis but rather, was attributed to involvement in DSB repair and increased metabolic activities [Chakravarti et al., 2004]. While this is an example of acquired resistance at the molecular level, it is likely that both of the presented scenarios are important in driving resistance to ionizing radiation. That is, selection of radioresistant cancer cells, and radiation induced activation of molecular mechanisms that control responses to radiation are both responsible for acquired radioresistance.

1.2.3. - MECHANISMS OF RADIORESISTANCE

Several key molecular pathways are important during the response of cells to IR. These pathways can generally be broken up into three categories: DNA repair, DNA damage signalling, and Survival regulation [Elshaikh et al., 2005]. The regulation of theses pathways involve a vast number of proteins and protein networks and that are still not well understood. However, many proteins involved in each of these categories of cell signalling have been implicated in the response of cells to IR and more and more are being added to the list as the search for radiosensitizing molecular targets continues.

1.2.3a - DNA Repair

Already, several key proteins involved in DNA repair pathways have been implicated as important molecules with respect to the sensitization of cells to IR. This is not surprising, as the importance of DNA damage, mainly DSB, is an important factor in IR induced cell death [Alsbeih et al., 2004; Bryant and Liu, 1994; Chang et al., 2005a; Kienker et al., 2000; Omori et al., 2002; Seno and Dynlacht, 2004; Wang et al., 2004a]. Two pathways are responsible for the repair of DSB. The first, known as homologous recombination (HR), only repairs DNA damage once the cell has moved through the S phase of the cell cycle. This pathway ensures the proper joining of broken DNA strands so that only DNA that was attached before the damage is rejoined [Sancar et al., 2004]. Proteins involved in this method of DNA repair include Mre11, Rad50, and Nbs1 all of which form a protein complex necessary for the initiation of DNA end joining. Also involved is the Rad51 protein, whose role involves mediation of strand invasion and branch migration reactions, as well as the BRCA1 and BRCA2 proteins, whose roles are still

unclear [Elshaikh et al., 2005]. Interestingly, inhibition or inactivation of any of these proteins leads to an increase in cellular radiosensitivity. This is likely due to decreased overall DNA repair. Normally, the Mre11/Rad50/Nbs1 protein complex is evenly distributed throughout the nucleus. However, heat shocking cells at 42.5 ° C leads to rapid relocalization of the complex to the cytoplasm, effectively disabling the complex's ability to participate in HR. When the complex is disabled, cells become more sensitive to IR [Dynlacht et al., 2004; Seno and Dynlacht, 2004]. The inactivation of BRCA1, BRCA2 or Rad51 also is capable of increasing radiation sensitivity [Ismail et al., 2004]

An alternative pathway involved in the repair of DSB is known as the non-homologous end-joining pathway (NHEJ) and is active during the G1 phase of the cell cycle [Hefferin and Tomkinson, 2005]. This mechanism of DNA repair is not as reliable as HR and occasionally joins two pieces of DNA that were not previously joined. The result is chromosomal rearrangement, which causes mutation and other problems for the cell [Hefferin and Tomkinson, 2005]. The process of NHEJ involves the recruitment of a complex of proteins to the site of the DNA DSB and, like HR, inactivation of these proteins often results in increased sensitivity to IR. The first step in the process of NHEJ involves the binding of the Ku complex, made up of Ku70 and Ku86 to the broken ends of the DNA. This is followed by the recruitment of DNA-PKcs and Artmeis. The ends of the DNA strands are then bridged by the interaction of DNA-PKcs and the phosphorylation of Artemis on each side of the gap. The DNAligaseIV/XRCC4 complex along with PNK is then recruited to each of the strands to be joined and processing of the gap occurs with the help of a DNA polymerase [Hefferin and Tomkinson, 2005]. Recently, one study tested the inhibition of the NHEJ complex as a method of radiosensitizing breast cancers to IR [Jones et al., 2005]. It was found that adenoviral infection of MDA-MB-231 cells with an inactive

fragment of XRCC4, that is able to bind to ligase IV in the NHEJ complex, was able to radiosensitize the breast cancer cell line [Jones et al., 2005]. Inactivating XRCC4 could be a useful strategy for radiosensitizing tumour cells for several reasons. First, the authors suggest that XRCC4/ligaseIV interference should only affect DSB repair efficiency and the process of V(D)J recombination which may be tolerable to patients. Interference with other components of NHEJ (Ku, DNA-PK) can result in telomere dysfunction as well as reduced repair, which may lead to more serious side effects. Finally, inhibiting repair proteins is likely to be selective to cells undergoing DNA repair due to targeting with IR, leaving most normal tissues unaffected [Jones et al., 2005].

When the damage to DNA is too great or the NHEJ and/or HR pathways fail in their tasks to repair the DNA, the cell may enter mitosis with damaged DNA. There are several possible outcomes but the preferred outcome when treating cancer cells with IR is cell death, which seems to occur mainly by mitotic catastrophe [Castedo et al., 2004a]. It has been proposed that mitotic catastrophe occurs due to deficient cell cycle checkpoints and cellular damage resulting in the failure to arrest before the cell enters mitosis [Castedo et al., 2004a; Castedo et al., 2004b]. Once mitosis begins, irregular chromosome segregation is attempted, ultimately leading to activation of the apoptotic pathway [Castedo et al., 2004a; Castedo et al., 2004b]. Since entering mitosis with DNA damage likely results in cell death by mitotic catastrophe it stands to reason that inactivation of any molecules participating in DNA repair pathways, particularly DSB repair will result in increased radiosensitization. Conversely, any increase in activity of DNA repair as a whole may lead to cellular radioresistance. Indeed this seems to be the case as numerous studies have compared the radiosensitivity of cells with wild type repair proteins to cells with mutated or absent repair proteins and it seems quite clear that inactivation of proteins involved in HR

or NHEJ confers sensitivity on cells to IR [Friesen et al., 2007; Kienker et al., 2000; Li et al., 2003; Omori et al., 2002].

1.2.3b - DNA damage sensing/signalling

DNA damage signalling/sensing may play as great a role as the process of DNA repair itself in the development of radiosensitive or radioresistant phenotypes among cancer cells. Several key proteins function to sense DNA damage and provoke an appropriate response when damages such as DSB are found. A major factor in this process is the protein kinase ataxia telangiectasia mutated (ATM). It is well known that mutation of this protein leads to the radiohypersensitivity syndrome known as ataxia telangiectasia [McKinnon, 2004]. Patients afflicted with this disease display severe normal tissue sensitivity to radiation. ATM is activated immediately following exposure to IR resulting in the phosphorylation of proteins important in processes ranging from DNA repair to apoptosis, and cell cycle control [Jameel et al., 2004]. For example, the protein p53 is a substrate of activated ATM and, after activation by phosphorylation, induces the expression of a large number of genes involved in cell cycle arrest and apoptosis [Li et al., 2001; Marchetti et al., 2003]. ATM also interacts with Chk2 (cell cycle regulator) and regulators of DSB repair such as Mre11-Rad50-NBS1 which, play major roles in the HR repair pathway [Friesner et al., 2005].

ATM and Rad3-related (ATR) protein kinase is another important sensor of DNA damage [Shiloh, 2001]. ATR along with ATR interacting protein (ATRIP) respond to damage, caused by IR, by blocking DNA replication and activating downstream signalling similar to that activated by ATM (p53, Chk1). This signalling can lead to several possible

outcomes including the resumption of replication, cell cycle arrest, or induction of apoptosis [Abraham, 2001]. Indeed, it has been shown that cells expressing defective ATR are sensitive to IR [Wang et al., 2004a]. Recent studies placed a kinase dead version of ATR under the control of an inducible tetracycline system. It was shown that cells that were induced to express the kinase dead version of ATR were notably more sensitive to IR then those that expressed normal ATR. The authors also suggested that ATR activity does not affect the NHEJ DNA repair pathway but is important in the promotion of HR repair [Wang et al., 2004a]. However, the mechanism by which HR repair is promoted is still not fully understood.

1.2.3c - Survival regulation

Cell survival or the "decision" whether to enter the apoptotic program or continue through the cell cycle is a complex process involving numerous proteins acting on both sides. Members of the Bcl-2 family of proteins can fall into two categories, pro- or anti-apoptotic. Anti-apoptotic members of the Bcl-2 protein family include Bcl-2, Bcl-XL, Bcl-W and MCL1 [van de Donk et al., 2005]. Many of these proteins have been studied for their effect on radiosensitivity and it has been shown that overexpression leads to radioresistance while inactivation leads to radiosensitivity. Recently, it was shown that anti-sense oligonucleotides directed against MCL1 increased the sensitivity of human melanoma cells to IR [Skvara et al., 2005].Also, a recent report indicated that expression of many pro-survival proteins correlate with radioresistance and may be useful in predicting the response of patients to IR [Nix et al., 2005]. Tumours from patients successfully treated with IR were matched and compared to tumours from patients for whom treatment with IR

had failed and expression of several proteins was compared by immunohistochemical staining. It was found that expression of the *anti*-apoptotic proteins Bcl-2 and Bcl-XL were strongly correlated with radioresistance and predicted treatment outcome correctly in 71 % of cases [Nix et al., 2005]. On the other hand, loss of expression of the *pro*-apoptotic protein bax also correlated with increased radioresistance, suggesting a possible mechanism for cells to avoid IR induced death [Nix et al., 2005].

Other inhibitors of apoptosis include the anti-apoptotic proteins XIAP and survivin. Both proteins have demonstrated their ability to inhibit apoptosis through the inhibition of caspases [Cao et al., 2004]. Interestingly, both proteins have also been shown to be upregulated in a variety of tumour types and, clinically, are related to tumour recurrence, decreased patient survival, and radiation sensitivity [Cao et al., 2004; Holcik et al., 2000]. Additionally, it was demonstrated that inactivation or downregulation of XIAP and/or survivin, members of the inhibitors of apoptosis family, using anti-sense oligonucleotides resulted in the sensitization of the human lung carcinoma cell line, H460 [Cao et al., 2004]. When XIAP and survivin were inhibited in combination with radiotherapy the growth of H460 xenografts was significantly delayed in female athymic nude mice [Cao et al., 2004]. Other studies also have shown relationships between the inhibition of XIAP and/or survivin and increased radiosensitivity. For example, Pennati et al. showed that inhibition of survivin, by ribozyme mediation, resulted in the radiosensitization of the human melanoma cell lines; JR8 and M14 [Pennati et al., 2003].

Mediation of cell death, namely apoptosis, is a complex process and describing each participant's role would fill volumes. However, more and more participants in these intricate cellular pathways are being implicated as important molecules with respect to their effects on IR response. For example, the transcription factor E2F-1 which, can act as either

a tumour suppressor or oncogene depending on cellular context, was shown to significantly radiosensitize p53 wild-type and p53 null human prostate cancer cells [Nguyen et al., 2005]. This suggests that delivery of E2F-1 to target tumour cells could potentially be useful in combination with radiotherapy [Nguyen et al., 2005]. This may be partly due to E2F-1's ability to induce tumour regression (which has been shown through gene transfection experiments), but is most likely due to the ability of E2F-1 to enhance apoptosis in response to cellular stress [Nguyen et al., 2005]. The fact that overexpression of E2F-1 effectively radiosensitizes both p53 wild-type and p53 null cells is important because approximately 50% of tumours have p53 mutations and are known to be more aggressive and more resistant to IR [Nguyen et al., 2005]

In recent years, the control of apoptosis and the activity of proteins involved in this complex process have been suggested to play a central role in the development of resistance to radiotherapy [Pearce et al., 2001; Pyo et al., 2001; Skvara et al., 2005]. Strategies that manipulate these proteins to push tumour cells towards apoptosis and ultimately death could be a major step forward in the search for effective radiosensitizers.

1.3. – CISPLATIN

Cis-diamminedichloroplatinum (cisplatin) and other platinum analogs such as carboplatinum and oxaliplatinum are useful for the treatment of a wide range of human malignancies. Platinum based chemotherapy is currently the treatment of choice for treating testicular [di Pietro et al., 2005; Wang and Lippard, 2005], head and neck [Argiris et al., 2005; Reyes Lopez and Navalpotro Yague, 2005], ovarian [Du Bois and Pfisterer, 2005],

and resectable non-small-cell lung cancers [Pisters and Le Chevalier, 2005]. In fact, in the case of testicular cancer, cisplatin treatment is highly effective resulting in an overall cure rate of approximately 90% and nearing 100% when detected early [Wang and Lippard, 2005]. Cisplatin is also one of the primary agents used in the treatment of uterine cervix, bladder, and oesophageal cancers [Ohmichi et al., 2005]. Currently, colorectal cancers are also treated with platinum therapy. Oxaliplatin in combination with 5-FU and folinic acid is now the standard of care this particular disease and is known by the acronym FOLFOX [Goldberg et al., 2004]. Oxaliplatin given in combination with other chemotherapeutics has led to steady increases in the median survival of patients with metastatic disease [de Gramont, 2005]. Platinating agents display significant efficacy against a wide range of cancers, and can actually be curative in some cases. It is for these reasons that platinum therapy is considered one of the most important classes of cancer treatment available today.

Although platinum drugs are effective in controlling and/or eliminating a number of tumour types, their efficacies can be limited by several factors. First, side effects such as emetogenesis, neurotoxicity, and nephrotoxicity are serious problems that patients must be monitored for throughout the course of treatment. Fortunately, management of some platinum associated side effects is possible through proper hydration and the use of diuretics [Wong and Giandomenico, 1999]. However, these types of side effects will obviously limit the maximum dose that can safely be administered to a patient thereby limiting the usefulness of the drug. Therefore, any method of sensitization would be useful in lowering the dose, thus, preventing side effects. Furthermore, the emergence of cellular resistance to platinum therapy, whether it is inherent or acquired, is also a major limiting determinant of platinum efficacy. The following sections will discuss the mechanisms by which platinating agents exert their cytotoxic effects. In addition several routes to cisplatin

resistance will be addressed with special attention paid to the roles of p53 and mismatch repair as these have some relevance to the studies in this thesis. Discoveries that have contributed to the overall understanding of cellular resistance to platinum therapy will also be highlighted.

1.3.1. - MODE OF CYTOTOXIC ACTION

Although cisplatin has been prescribed in the clinic for almost 30 years, the mechanisms by which it induces cell death in cancer cells are still not fully understood. Originally, cisplatin was thought to enter the cell by simple diffusion across the cell membrane. This was supported by the observation that cisplatin uptake was directly proportional to time elapsed and did not seem to saturate even at high concentrations [Binks and Dobrota, 1990; Wang and Lippard, 2005]. However, evidence that is more recent seems to indicate the participation of several transporter proteins in both platinum influx and efflux [Holzer et al., 2004a; Ishida et al., 2002; Samimi et al., 2004a; Samimi et al., 2004b; Song et al., 2004]. These transporters will be discussed later as they represent mechanisms through which cells may become resistant to platinum therapy. Once cisplatin has entered the cell, it must undergo a series of transformations before it becomes active and able to interact with its primary target; DNA [Jamieson and Lippard, 1999]. The transformation of cisplatin into an active DNA damaging agent involves the replacement of either one or both of the *cis*-chloro ligands with water. This reaction is carried out quickly due to the low concentration of chloride ions present within the cell. Once these reactions have taken place, the aquated cisplatin molecules can attack the DNA allowing the Pt atoms to form covalent bonds at the N7 position of purine bases [Jamieson and Lippard, 1999]. Formation of covalent bonds between the platinum atom of cisplatin and purine bases can

lead to several types of DNA damage, which are ultimately responsible for the cytotoxic effects of cisplatin. The most common types of DNA damage seen with cisplatin treatment are 1,2- or 1,3-intrastrand crosslinks in which purines located on the same strand of DNA are covalently linked together through the Pt atom of cisplatin (Figure 2) [Jamieson and Lippard, 1999; Wang and Lippard, 2005]. Cisplatin induced DNA damage can lead to the activation of several cellular responses such as replication arrest, DNA repair, apoptosis, cell-cycle arrest, and transcription inhibition [Wang and Lippard, 2005]. These processes all play important roles in determining the fate of cells that have acquired damage through platinum treatment.

1.3.2. - How Do Cancer Cells Develop Platinum Resistance?

While some forms of cancer respond well to platinum treatment others are inherently resistant, or acquire resistance during treatment. The principles behind the two forms of resistance are generally similar to those discussed in relation to radioresistance in section 2.2. In fact, the mechanisms by which cells become resistant to platinum and ionizing radiation are thought to include some overlap [Frit et al., 1999; Raaphorst et al., 1999]. Much research has focused on elucidating the mechanisms responsible for platinum resistance. The findings are breathtaking in their expanse. To say the least, it is clear that platinum resistance is multifactorial. Several reviews of have attempted to summarize the current understanding platinum resistance [Siddik, 2003; Stewart, 2007; Wang and Lippard, 2005]. Generally, reviewers categorize mechanisms of platinum resistance into three categories; those that affect the intracellular accumulation of platinum, those that affect survival and cell death signalling pathways, and finally, mechanisms that affect DNA repair.

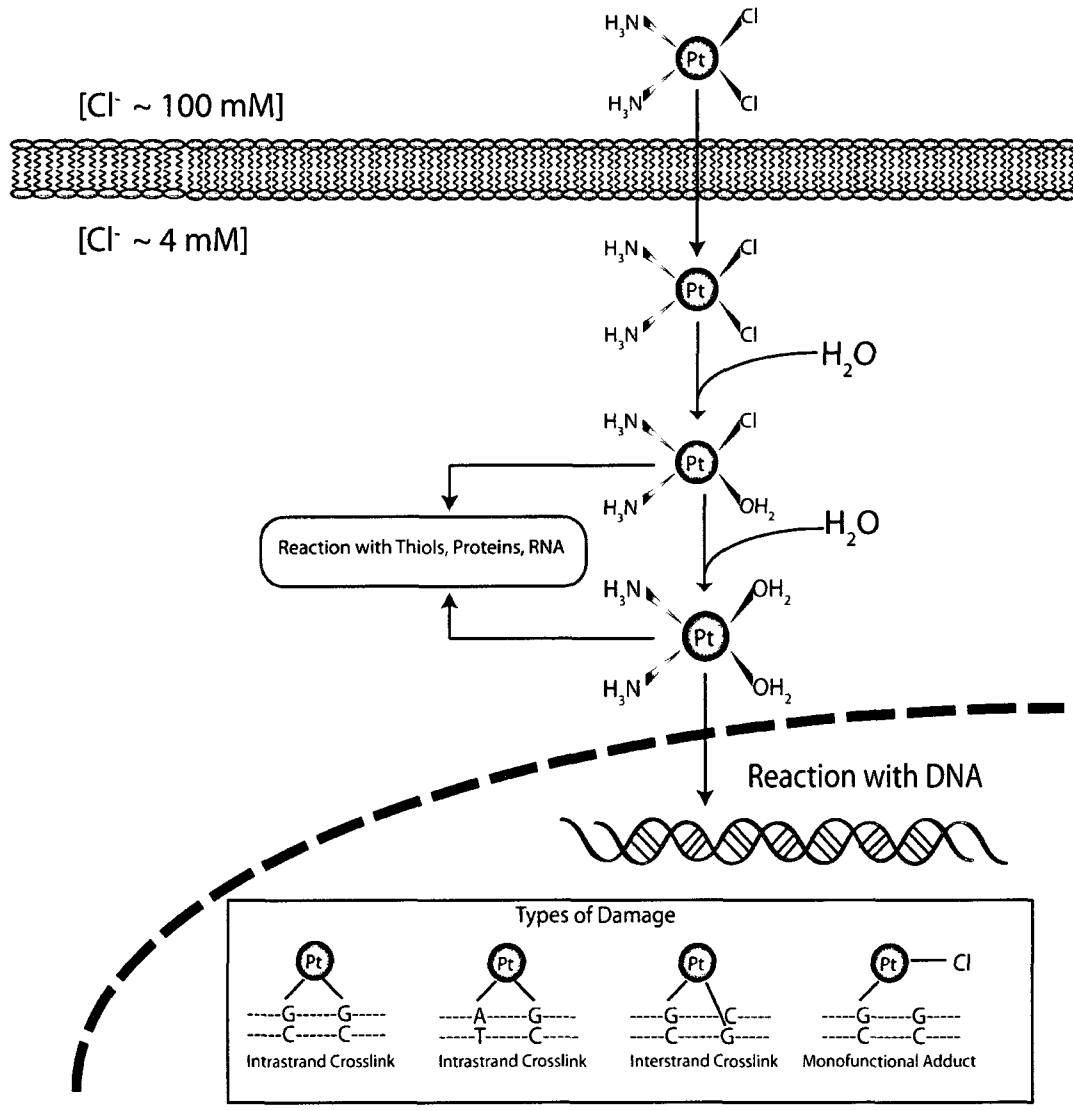

Figure 2: Mechanism of action of cisplatin. Cisplatin enters the cell mainly by passive diffusion. Once inside the cell cisplatin undergoes a series of aquation reactions in which, the Cl- ions are replaced by water molecules. While in the cytoplasm cisplatin may react with proteins and other molecules such as thiols and glutathiones. Once aquated cisplatin enters the nucleus and attacks DNA at the N7 position of purine bases. Several types of damage may occur but the most common type is the intrastrand crossline between neighbouring guanines or guanines and adenines.

1.3.2a - Decreased Intracellular Accumulation

Several mechanisms have been identified that can lead to a decrease in the intracellular accumulation of cisplatin and, therefore, a decrease in the amount of cisplatin available to bind DNA. These involve processes such as drug uptake, drug efflux, drug detoxification, and drug binding [Stewart, 2007]. Also, sequestration of the drug within intracellular vesicles can prevent platinating drugs form entering the nucleus. Until recently, drug uptake was generally accepted to occur by passive diffusion across the cell membrane [Wang and Lippard, 2005]. However, it has become clear that several membrane transporters are actively involved moving cisplatin into and out of the cell. CTR1 is a copper transporter that is able to carry cisplatin into the cell. Overexpression of CTR1 results in increased accumulation of cisplatin in ovarian carcinoma cells [Holzer et al., 2006]. However, this increased accumulation of cisplatin did not affect the sensitivity of this particular cell line to cisplatin. This may be due to other resistance mechanisms that are in place within these cells as ovarian cells are known to become resistant to cisplatin through a multitude of pathways. Another study showed that treatment with cisplatin actually caused a rapid down-regulation of CTR1 in an ovarian carcinoma cell line [Holzer et al., 2004b]. This may represent a mechanism by which cancer cells acquire resistance to cisplatin. Other factors that can affect the uptake of cisplatin include increased cell membrane fluidity [Liang et al., 2004], and defective endocytosis [Liang et al., 2006]. In fact, these two factors were found to affect the response to cisplatin in cisplatin resistant cervical cancer cells with increases in membrane fluidity and defective endocytic recycling both contributing to increased resistance to cisplatin [Liang et al., 2006; Liang et al., 2004].

Intracellular accumulation can also be affected by an increase in cisplatin efflux. Several transporters have been shown to increase platinum resistance when their expression

is elevated in cancer cells. Of note are the copper efflux transporters ATP7A and ATP7B. A great deal of research has been carried out on determining the role of these copper transporters on platinum resistance [Kalayda et al., 2008; Matsumoto et al., 2007; Safaei, 2005; Safaei and Howell, 2005; Samimi et al., 2004a; Samimi et al., 2004b; Samimi et al., 2003; Song et al., 2004; Yoshizawa et al., 2007]. The findings indicate that overexpression of the P-type ATP copper transporters confers cisplatin and carboplatin resistance to a variety of different cancer cell types including ovarian, and head and neck carcinomas [Kalayda et al., 2008; Samimi et al., 2004a; Samimi et al., 2004b; Yoshizawa et al., 2007]. The authors suggest that the observed resistance is due to the sequestration of platinum within vesicles and secretory compartments within the cytoplasm thereby preventing the interaction of cisplatin with nuclear DNA [Safaei et al., 2005; Samimi et al., 2004a; Samimi et al., 2004b]. The prevention of cisplatin from reaching its primary target is likely responsible for the observed increase in resistance. Other transporters may also be involved in cisplatin resistance. These include MRP1/2, p-glycoprotein, cMOAT, and LRP, although, modulation of some of these have given rise to inconsistent results (reviewed in:[Stewart, 2007]). Of particular note is LRP (lung-resistance related protein) which, when down-regulated by transfection with LRP specific anti-sense oligonucleotides, reversed cisplatin resistance in resistant ovarian carcinoma cells [Wang et al., 2004b]. The report also showed that LRP down-regulation resulted in decreased accumulation of cisplatin within the cell [Wang et al., 2004b].

Drug detoxification by biomolecules such as glutathiones (GSH) and metallothioniens (MT) can reduce the effectiveness of platinum therapy [Godwin et al., 1992; Siddik, 2003; Stewart, 2007]. Cisplatin is bound by these molecules through a chemical reaction involving the platinum atom and thiol groups [Kelland, 2007]. The

reaction of molecules, often heavy metals, with thiol containing species, like GSH, is a defence mechanism that aids in the export of toxins. Because cisplatin and other platinum analogues have a strong affinity for thiol containing molecules, increased levels of thiols can result in resistance to platinum due to increased efflux or inactivation [Kelland, 2007; Siddik, 2003; Stewart, 2007]. Studies supporting this have correlated glutathione-S-transferase (GST) gene expression, immunostaining, and plasma levels with clinical resistance to cisplatin [Cabelguenne et al., 2001; Cullen et al., 2003; Kase et al., 1998]. GST is responsible for catalyzing the reaction between glutathione and various xenobiotics [Douglas, 1987]. Other studies also correlated the expression of MT with clinical resistance to platinum therapy and survival [Surowiak et al., 2005; Surowiak et al., 2007; Wulfing et al., 2007]. Thus, it is likely that the expression of GST and/or MT play important roles in promoting resistance to platinum in the clinic.

1.3.2b - Cell Survival, Apoptosis, and Platinum Resistance

Death can occur in response to cisplatin by either necrosis or apoptosis. Necrosis is characterized by cell swelling and the loss of plasma-membrane integrity. Apoptosis, on the other hand, is characterized by cell shrinkage, fragmentation of DNA and the condensation of cellular chromatin [Wang and Lippard, 2005]. However, it is evident that apoptosis is the major mode of cell death occurring after the administration of platinum drugs at normal clinical concentrations. Necrosis appears to be highly active only at very high concentrations of cisplatin (>800μM). Therefore, the process by which cells die after platinum treatment appears to be concentration dependant [Gonzalez et al., 2001]

Within the cancer cell, many proteins, pathways, and or mechanisms can act to activate or inhibit apoptosis. Among these are the pro and anti-apoptotic protein families.

The pro-apoptotic proteins belonging to the Bcl-2 family of apoptosis related proteins include Bax, Bad, Bak, Bik, Bid, Bim, and others [Rogers et al., 2002]. These proteins promote apoptosis by regulating the release of apoptogenic factors from the mitochondria [Cho et al., 2005]. Therefore, it is evident that diminished expression or activity among these proteins could cause a significant decrease in apoptosis in response to platinum therapy. This is indeed the case and has been demonstrated in several reports. One such report used the bladder cancer cell line T24 to demonstrate that increased expression of the anti-apoptotic protein Bcl-2 can actually prevent translocation of Bax in the presence of cisplatin, inhibiting apoptosis, and increasing resistance to cisplatin [Cho et al., 2005]. In addition, treating cisplatin resistant NSCLC cells with Bcl-2 siRNAs reversed their resistance [Huang et al., 2007]. Other pro-apoptotic proteins such as Bax and Bak may also play a role in cisplatin resistance as their expression increases upon treatment with cisplatin [Jones et al., 1998]. In fact, overexpression of the pro-apoptotic Bak was found to sensitize breast cancer cell lines to cisplatin [Sakakura et al., 1997]

Anti-apoptotic proteins also play a role in determining the fate of cancer cells treated with platinum drugs. Anti-apoptotic proteins are a large group and are comprised of Bcl-2 proteins such as Bcl-2, Bcl-X_L, Bcl-w, and Mcl-1 [Rogers et al., 2002]. In addition to Bcl-2 proteins, the inhibitors of apoptosis (IAPs) also play a major role in cisplatin resistance[Asselin et al., 2001; Fraser et al., 2003; Sasaki et al., 2000]. This group of proteins includes survivin and the X-linked inhibitor of apoptosis (XIAP) which is regarded as the most potent inhibitor of apoptosis [Tong et al., 2005]. Several studies have investigated the role of these proteins in promoting platinum resistance. One such study used antisense oligonucleotides to examine the effect of specific downregulation of bcl-2 on cisplatin response. The study found that diminishing bcl-2 protein levels altered the

response of head and neck squamous cell carcinoma to cisplatin by increasing sensitivity [Sharma et al., 2005]. Similarly, antisense oligonucleotides directed at survivin and bcl-XL caused a similar effect [Sharma et al., 2005]. Another study utilized antisense expression vectors aimed at reducing the expression of XIAP in the gastric cancer cell lines, MKN-45 (p53 wild-type) and MKN-28 (p53 mutant). Several studies observed that downregulation of XIAP enhanced the cellular sensitivity of p53 wild-type cells but not p53 mutant-type cells, suggesting that XIAP works in concert with p53 to inhibit apoptosis and therefore cause platinum resistance [Sasaki et al., 2000; Tong et al., 2005].

Mcl-1, another important regulator of apoptosis, also belongs to the bcl-2 family of proteins and is known to be an inhibitor of apoptosis. Naturally, downregulation of this protein was suspected to be an attractive strategy for increasing the response of tumour cells to cisplatin therapy. This was investigated and it was found that antisense oligonucleotides directed at reducing the expression of Mcl-1 mRNA and protein were effective sensitizers of hepatocellular carcinoma (HCC) to cisplatin [Sieghart et al., 2006]. This study also demonstrated that the increased response of HCC to cisplatin was mainly due to the induction of apoptosis, clearly indicating the important role of anti-apoptotic proteins in platinum resistance [Sieghart et al., 2006].

1.3.2c - DNA Repair and Platinum Resistance

Nucleotide Excision Repair

As discussed previously, the primary target of platinum chemotherapeutics is DNA [Jamieson and Lippard, 1999]. It comes as no surprise then, that factors affecting DNA repair can have a major influence on cellular sensitivity to platinating agents. The

mechanisms responsible for the repair of DNA damaged by cisplatin differ from those responsible for DNA damaged by ionizing radiation. This is mainly due to the type of damage induced by each. While ionizing radiation mainly induces DSB, approximately 90 % of the damage caused by cisplatin is in the form of intrastrand crosslinks between neighbouring purine bases (1,2 –ApG, 1,2 – GpG) [Vrána et al., 2007]. Nucleotide excision repair (NER) is the only pathway known to effectively remove inter- and intrastrand crosslinks. The NER complex consists of a large number of proteins (at least 17) that form a complex to remove crosslinks from DNA [Friedberg, 2001; Sancar, 1994]. In order to remove a DNA lesion, the NER pathway relies on the functionality of six key proteins that fall into three groups [Welsh et al., 2004]. The first protein, XPC-HR23B, is responsible for recognition of DNA distortions caused by platinum lesions. Secondly, a pre-incision complex is formed by three NER proteins: XPA, TFIIH and RPA. And finally, ERCC1-XPF and XPG are the nucleases necessary for the removal of the damaged nucleotide [Welsh et al., 2004; Wood et al., 2000]. Increased DNA repair is a mechanism thought to lead to cisplatin resistance (reviewed by [Siddik, 2003; Stewart, 2007; Zamble and Lippard, 1995]. Thus, an increase in the expression of any of the rate limiting proteins is able to increase resistance to cisplatin. For example, increased expression of the excision repair cross complementing 1 (ERCC1) is associated with increased cisplatin resistance as is increased expression of the NER protein XPA [Ferry et al., 2000; Lee et al., 1993]. Conversely, suppression of ERCC1, by RNA interference, was able to sensitize cells to cisplatin, carboplatin, or oxaliplatin [Chang et al., 2005b]. Two types of NER are present within human cells. The first is known as global NER (G-NER) while the other is referred to as transcription coupled NER (TC-NER). TC-NER appears to be important in the maintenance of actively transcribed genes and is activated when RNA polymerase II stalls

at the site of DNA damage while transcribing a gene. TC-NER is likely to be the more important mediator of platinum sensitivity. This was demonstrated by Furuta et al. who observed that cells unable to perform TC-NER are hypersensitive to cisplatin while cells deficient only in G-NER retain their sensitivity to cisplatin [Furuta et al., 2002]. One possible mechanism giving rise to a platinum resistant phenotype may be the upregulation of ERCC1 by oncogenic H-Ras. Ras gene mutations are frequent in cancers and are known to modulate tumour progression as well as chemoresistance. Yuon et al. provided evidence that activated H-Ras signalling promotes cisplatin resistance and increased expression of ERCC1. Suppression of ERCC1 reversed the effect of activated H-Ras implying that increased DNA repair was responsible for the increase in resistance to cisplatin [Youn et al., 2004].

Mismatch Repair

While TC-NER is responsible for removing intrastrand adducts from DNA, there are many other repair factors involved in the response of cancer cells to cisplatin. One such factor is the presence of an intact mismatch repair MMR system. MMR is a mechanism that is responsible for removing and repairing mismatched bases arising from deletions, insertions, or DNA damage [Hsieh and Yamane, 2008]. It has been reported to be an important factor influencing cisplatin response. Although MMR does not actually repair the DNA adducts formed by cisplatin it has been suggested that the MMR complex recognizes cisplatin adducts and attempts to repair them. Unable to do so, ineffective cycles of repair continue eventually leading to activation of apoptotic signalling [Siddik, 2003; Vaisman et al., 1998]. Therefore, loss of MMR, through gene mutation or downregulation, should result in increased resistance to cisplatin, and this has been observed [Fink et al., 1997; Lin and Howell, 2006; Plumb et al., 2000]. This is thought to occur because cells continue to

replicate damaged DNA where they should die by apoptosis. In fact, Vaisman et al. showed that cisplatin treated cells lacking hMLH1 (a critical MMR protein) were able to replicate DNA much more efficiently then those that were MMR proficient [Vaisman et al., 1998]. This data indicates that adduct detection by MMR leads to a block in chain elongation likely at the site of damage. Further investigation revealed that the MMR machinery is able to recruit sensors of DNA damage such as ATM and ATR to the site of DNA damage [Brown et al., 2003]. In addition, MMR, upon treatment with cisplatin, activates c-Abl and JNK/SAPK both of which are important mediators of the apoptotic response [Levy et al., 2008; Nehme et al., 1997; Sanchez-Perez et al., 2000]. These represent mechanisms by which MMR activates DNA damage sensing and subsequent death by apoptosis.

Mismatch repair deficiency due to loss of active hMLH1, hMSH2 or other proteins necessary for complex formation and activation has been shown to increase the resistance of cells to cisplatin two-fold, which is sufficient to result in clinical failure of treatment [Fink et al., 1998]. Often this is due to a loss of hMLH1 expression or hMLH1 mutation [Fedier and Fink, 2004; Strathdee et al., 1999]. Interestingly, downregulation of MMR does not confer resistance to all analogues of cisplatin. Tumours with loss of MMR activity are more resistant to cisplatin and carboplatin, but retain sensitivity to oxaliplatin [Vaisman et al., 1998]. This appears to be due to the fact that the MMR system does not recognize oxaliplatin adducts and replication is allowed to bypass oxaliplatin damage.

1.3.2d - Mismatch Repair and p53 – important regulators of the response to cisplatin

The role of mismatch repair in cisplatin resistance was discussed previously and the role of p53 touched upon. It is well known that p53 is an important regulator of numerous

cellular processes and, therefore, is likely to play a role in various aspects of cisplatin resistance. Already, p53's involvement in apoptotic signalling has been discussed along with the requirement of wild-type p53 expression for sensitization of ovarian cancer cells by XIAP or Akt inhibition [Fraser et al., 2003]. Recently, p53 and mismatch repair have been linked, the following paragraphs will discuss this linkage and the ramifications it has on the response to platinum therapy.

Luo et al. presented work implicating the hMutL MMR complex in p53 regulation in response to DNA damage [Luo et al., 2004]. They demonstrated that upon DNA damage by cisplatin, hMutL proteins accumulate. However this was not the case with hMutL mRNAs, indicating that the increase was due to postranscriptional processing. Further investigation revealed that ATM, a well known participant in the response to DNA damage, is required for increased hMutL protein levels. In addition, the hMutL complex was shown to be necessary for full phosphorylation and activation of p53 in response to DNA damage [Luo et al., 2004]. This represents a mechanism by which MMR is able to initiate cell death signalling in response to DNA damage. It also ties together the role of ATM as a sensor of DNA damage to the actual cellular response. Activation of p53 is necessary for the trans-activation of several modulators of apoptosis including the pro-apoptotic proteins, Bax, PUMA, and $p53^{AIP1}$ [Miyashita and Reed, 1995; Nakano and Vousden, 2001; Oda et al., 2000]. Conversely, p53 is also a potent inhibitor of many anti-apoptotic proteins such as ARC, and Bcl-2 [Li et al., 2008; Marchenko et al., 2000]. Clearly, the linkage between MMR and p53 is an important one that is at least partially responsible for the induction of cell death due to DNA damage. In fact, Lin and Howell showed that in addition to their effects on acute cytotoxicity, p53 and MMR are determinants of the rate at which cells develop resistance to cisplatin [Lin and Howell, 2006]. They demonstrated that the loss of

both components resulted in much earlier development of resistance then the loss of either component alone. The authors concluded that this was due to an increase in HR due to extremely high levels of polymerase-ζ subunits (↑↑ mRNA levels compared to cells missing p53 or MMR alone) which, resulted in high levels of translesion synthesis without induction of cell death signalling [Lin and Howell, 2006].

Clinically, loss of p53 and/or mismatch repair may be important factors with respect to chemotherapy response and disease-free survival. The tumour suppressor p53 is lost or mutated in at least 50% of all cancers [Bond et al., 2004]. An examination of hMLH1 expression (necessary for MMR) revealed that it is significantly reduced in breast cancer patients that had undergone treatment with cisplatin and other drugs. The loss of hMLH1 was strongly associated with shortened disease-free survival [Mackay et al., 2000]. Loss of hMLH1 has also been observed in ovarian cancer cells treated with cisplatin [Strathdee et al., 1999]. Decreased or aberrant MMR activity leads to microsatellite instability and is linked to the development of a number of cancers including colorectal, endometrial, ovarian, and gastric cancers [Imai and Yamamoto, 2008; Miyoshi et al., 2001; Pal et al., 2008; Pal et al., 2004].

Cisplatin and its analogues are important therapeutic agents used in the treatment of many cancers including those mentioned above. Unfortunately, due to factors such as loss of p53, or loss of MMR, cancers can become refractory to treatment with platinating agents. Because factors such as p53 and MMR are lost or mutated in numerous cancers and in a large number of patients, it is difficult to predict whether a particular therapy will be useful for treatment. One method for improving treatment may be to determine the expression of p53 and important MMR proteins immunohistologically before beginning treatment. However, this would be costly and time-consuming. In addition, MMR

functionality may be lost during treatment and resistance acquired making pre-screening irrelevant. Therefore, the identification of molecular targets that can be exploited to sensitize cancers to platinating agents, regardless of these factors, should be an imperative goal in the field of cancer therapy. This thesis describes the identification of such a factor (APM2) and demonstrates that the suppression of this factor results in sensitization of cancer cells to cisplatin both *in vitro* and *in vivo*.

1.4. – MICROARRAY ANALYSIS AND DRUG RESISTANCE

In the past decade the use of microarray analysis has increased substantially. Microarray analysis has become a powerful tool for researchers of drug resistance and allows for rapid determination of differences in gene expression among sensitive and resistant cell types. In order for meaningful data to be generated it is necessary to choose a model that accurately reflects the occurrence drug resistance *in vivo*. Unfortunately, this is difficult, and more likely, impossible to do *in vitro*. However, several approaches have been employed for this purpose. Most often, research has focused on models of acquired drug resistance. The models are generally developed from existing sensitive cancer cell lines. Cancer cell lines are exposed to increasing doses of the drug in question and resistant cells are pooled, giving rise to a resistant variant of the initial cell line. A recent study by Gosepath et Al involving head and neck cancer derived cell lines used microarray technology to identify factors involved in the response of these cells to cisplatin. Microarray analysis revealed that expression of the wnt-pathway inhibitor DKK1 was decreased in the resistant cell line. Further study confirmed that forced overexpression of

DKK1 partially restored sensitivity to the resistant head and neck cancer cells [Gosepath et al., 2008]. Other studies have compared the differential gene expression using numerous models of drug resistance. These models are derived from ovarian [Sakamoto et al., 2001; Varma et al., 2005], gastric [Kang et al., 2004], lung [Whiteside et al., 2004], and head and neck cancer [Gosepath et al., 2008]. While these studies generate large lists of genes, most of the genes are not confirmed as playing an actual role in the resistance to the chemotherapeutic being investigated. In addition microarrays have been used to identify gene signatures. These signatures could be used to identify patients as sensitive or resistant to a particular treatment based on their expression of a given set of genes [Kitahara et al., 2002; Okutsu et al., 2002; Zembutsu et al., 2002]. For example, the gene signature uncovered by Kitahara et Al. consisted of a list of 62 genes identified as differentially expressed in radio-resistant versus radiosensitive cells. They were then able to use this signature to successfully predict the phenotypes of 19 cervical carcinoma samples with respect to their radioresponse. The identification of predictive gene signatures is a popular area of research. This is no surprise, as it could allow for tailored treatments that would save time, prevent unnecessary treatments, reduce undesired side effects, and reduce costs.

Microarray technology has come a long way in the past decade and the applications for which it has been put to use are constantly expanding. One of the most important functions of the microarray is its ability to compare gene expression between normal and cancerous tissues thereby shedding light on the differential expression of genes that may promote or initiate cancers. Similarily, microarrays have allowed for the identification or at least confirmation of a great number of genes that are involved in drug resistance and platinum resistance in particular. One such example would be the microarray research performed by Sakamoto et Al and Varma et Al who showed that DNA repair genes

(XRCC5, XRCC6, ERCC5) and metalloproteinase (MMP3, MMP12) are overexpressed in platinum resistant cell lines, respectively [Sakamoto et al., 2001; Varma et al., 2005]. These groups of genes are well known to be involved in the response to cisplatin and its analogues. Hence, it is clear that microarray technology is capable of detecting genes that are important in mediating the response to cisplatin. The difficulty, however, lies in separating the real resistance factors from the background. This can only be done by rigorous validation followed by functional studies such as overexpression and depletion by methods such as RNA interference.

1.5. – ADIPOSE SPECIFIC 2 AKA (C10ORF116)

The bulk of this thesis will deal with the involvement of Adipose specific 2 (APM2) in resistance to cisplatin. This section is intended to describe what is already known about APM2 based on the current body of literature. The naming of the APM2 gene as adipose specific is somewhat misleading. While APM2 transcripts have been found to be present at high levels in adipose tissue [Sjoholm et al., 2005], many studies have shown that APM2 is also expressed in other tissues [Buchholz et al., 2005; Kawasaki et al., 2003; Lee et al., 2005]. In fact, examining the expression profile of APM2 available at http://symatlas.gnf.org reveals that APM2 is expressed in a wide variety of tissues with the highest expression occurring in adipose, prostate, and lung tissues (Figure 3). This observation may have been partially responsible for the name change to what is now the official gene name, chromosome 10 open reading frame 116 (C10orf116). However, for the purpose of consistency C10orf116 will be referred to as APM2 throughout this text. APM2

is coded for by a gene located on chromosome 10 at cytoband q23.2. The gene is approximately 2.5 kb and is directly downstream of γ-synuclein. The APM2 gene codes for a mRNA of approximately 500 bp for which there are several entries available in the RefSeq database. Each mRNA codes for a small, 76 amino-acid protein of approximately 7.8 kDa. No functional information has been assigned to the protein, thus, to our knowledge; this is the first work to directly study the possible function of APM2. The structure of the protein gives little indication of possible function. A search for conserved domains using the NCBI's conserved domain search tool identifies no known domain. A search for homology shows that homologues of APM2 are expressed by other species. The closest homologue identified thus far is found in *Bos taurus* and shares 85% identity with the human version. Homologues sharing less identity have been identified in *Pan troglodytes*. Other homologues occurring in *Sus scrofus*, and *Monodelphus domestica* have been predicted based on the sequencing of their genomes. These data can be viewed using the BlastP program available at http://blast.ncbi.nlm.nih.gov/Blast.cgi [Altschul et al., 1997; Altschul et al., 2005]. The fact that APM2 has been conserved across species indicates that it may play an important role in cellular physiology. It is likely that once APM2 falls under more intense investigation, more homologues will be identified especially in important research species such as *Mus musculus*.

While there is no available data pertaining directly to the function of APM2, there are numerous gene expression studies in which APM2 has been detected as differentially expressed. Many of these studies are related to cancer. One study examined the global gene expression in patients with uterine leiomyomas and found that APM2 was downregulated

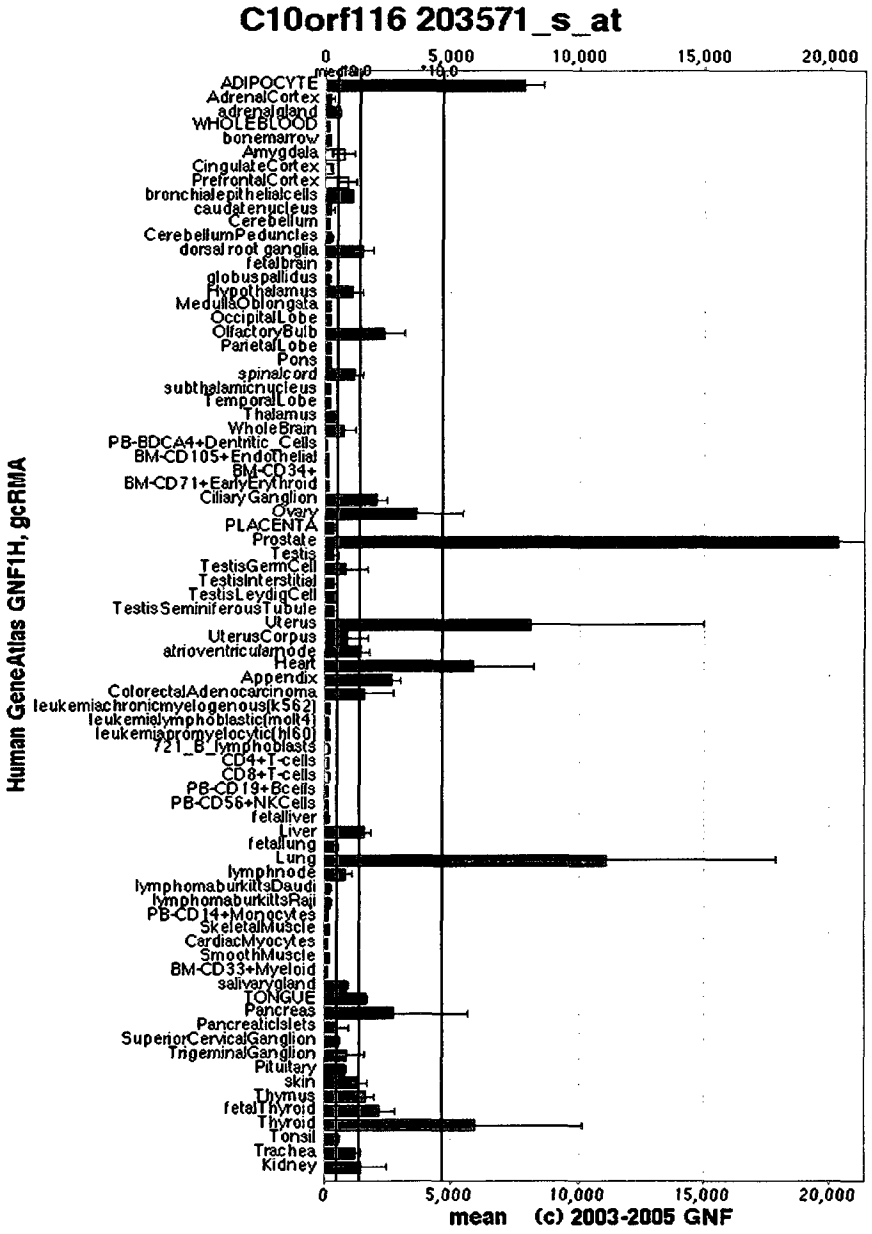

Figure 3: A survey of APM2 gene expression in normal human tissues. Gene expression was surveyed in normal human tissues by Affymetrix microarray. Gene expression is based on the signals obtained using the 203571_s_t probe specific for APM2. Highest expression is observed in prostate followed by adipocyte, lung, and uterus. Figure downloaded from http://symatlas.gnf.org © GNF 2003-2005.

in all of the patients tested [Lee et al., 2005]. Another report showed that APM2 was expressed in normal human epidermal cells. However, APM2 was silenced consistently in melanoma cell lines. Further investigation showed that this was probably due to promoter methylation as the APM2 promoter was methylated in all samples [Yoshimasa Nobeyama, 2007]. The issue of epigenetic silencing of APM2 has been investigated by other groups and the results are conflicting. A report examining the possibility that hypermethylation could actually increase the expression of a subset of genes showed that this indeed was the case with APM2, at least in HCT116 colorectal carcinoma cells [Gius et al., 2004]. Examining the promoter region of APM2 in HCT116 cells revealed complete methylation of its CpG island; however, APM2 expression remained high in these cells. When the same experiments were performed using HCT116 cells that were unable to perform DNA methylation due to knockout of DNA methyltransferases the expression of APM2 decreased. Similar effects were seen when hypomethylation was induced by treatment with chemicals known to induce demethylation [Gius et al., 2004]. This is interesting because it is the opposite of what is generally believed to be an epigenetic mechanism of controlling gene expression. However, the consequence of promoter methylation appears to be dependant on cell type as hypermethylation of the APM2 promoter decreased expression in human melanoma cell lines [Yoshimasa Nobeyama, 2007]. While APM2 was found to be downregulated in uterine leiomyoma and melanoma, its expression appears to be increased in cancer of the prostate [Covell et al., 2003]. An examination of the Unigene expression profiler, available at http://www.ncbi.nlm.nih.gov/UniGene/ , also indicates that APM2 expression is higher in diseased tissue than in normal tissue.

With respect to drug resistance, APM2 has not been directly investigated as a possible drug resistance factor. However, microarray analysis performed by Kang et Al.

revealed that APM2 was one of several genes upregulated in resistant gastric cancer cells [Kang et al., 2004]. Strengthening these findings was the fact that in a set of three cisplatin resistant gastric cancer cell variants, all showed significant overexpression of APM2 [Kang et al., 2004]. Interestingly, the same authors did not observe overexpression of APM2 is gastric cancer cells that were resistant doxorubicin or 5-FU [Kang et al., 2004]. These findings suggest that APM2 may be important in promoting resistance specifically to cisplatin.

1.6. – HYPOTHESIS

Previously, our laboratory characterized the response of several untreated HCT116 clones to X-radiation and several chemotherapeutic drugs [Qutob et al., 2004]. As mentioned in the introduction several studies have been conducted comparing the gene expression profiles of resistant and sensitive cancer cell line variants. These studies, however, only examine resistance that is acquired in a laboratory setting by repeated treatment with various insults. However, we are not aware of any reports comparing the global expression between untreated clones of a cancer cell line with inherent differences in their responses to X-radiation and chemotherapeutics. Since our clones are very closely related and have not previously been treated with any insult (to generate resistant variants) they will be valuable tools for identifying genes involved in the intrinsic response to X-radiation or chemotherapy. *We hypothesize that the differential expression of certain genes are responsible for the occurrence of resistance/sensitivity to cancer therapeutics.*

1.7. – OBJECTIVES

I. To identify genes differentially expressed by HCT116 clones having differing sensitivities to X-radiation and chemotherapeutic drugs [Qutob et al., 2004].

II. To characterize the expression of selected genes found to be differentially expressed between the HCT116 clones, in an expanded panel of HCT116 clones, and determine which genes are expressed in a manner significantly different from the rest of the population (Eliminate genes identified due to clonal variation)

III. In light of the results of objective II - To investigate whether overexpression of APM2 is capable of altering the response of cancer cells to X-radiation or chemotherapeutics.

IV. In light of the results of objective III - To examine whether transient or stable suppression via RNA interference is capable of altering the response of cancer cells to platinating drugs, namely cisplatin, and to investigate whether cell type, p53 status, or MMR status play a role in this response.

V. To evaluate the effect of stable APM2 suppression on the response of human tumour xenografts to cisplatin *in vivo*.

1.8. – IMPORTANCE

As discussed, resistance to X-radiation and cisplatin as well as other chemotherapeutics is multifactorial. Currently, almost all methods of treating cancer are met with intrinsic or acquired resistance in the clinic in at least some patients. Some cancers, such as ovarian cancer, almost always display resistance to chemotherapeutics or

recur as a resistant form after successfully controlling the disease for some time. Thus, the identification of factors that are involved in the response of cancer cells of various types and genetic backgrounds is essential to overcoming resistance to existing cancer treatments. In order to overcome resistance, it will be necessary to base targeted therapeutics on these genes/proteins and combine them with traditional therapies to enhance their effectiveness in a broad range of tumour types.

This work attempts to identify such genes/proteins and investigates the role of one in particular (APM2) in altering the response of cancer cells to X-radiation and chemotherapeutics. Overall this thesis will comprise an important contribution to the field of drug resistance and provides the proof-in-principle that genes involved in therapy resistance can be identified by examining their relative expressions and that modulating the expression of genes can have a significant effect on the sensitivity of cancer cells to traditional cancer therapies.

CHAPTER 2

MATERIALS AND METHODS

2.1. - Cell lines and culture

All cell lines were maintained at 37 °C, 5% CO_2, in DMEM/F12 50:50 mix (Wisent, St. Bruno QC) containing 10% FBS except for the OV2008 cell line, which was maintained in RPMI1640 media (Hyclone) containing 10% FBS. Parental HCT116 cells were a gift from Dr. Bert Vogelstein (Johns Hopkins University). The MCF-7, PC-3, and SW620 lines were provided by the laboratory of Dr. John Bell (Ottawa Health Research Institute). The OV2008 line was provided by the laboratory of Dr. Barbara Vanderhyden (Ottawa Health Research Institute). Cell lines were in the exponential phase of growth at the start of drug/X-radiation treatments or transfections.

2.2. - X-radiation and drug treatments

X-radiation was delivered in various acute doses using a 250 kVp X-ray unit (Pantak, CT) at a dose rate of 150 cGy/min. Fractionated doses were given as daily doses of 2 Gy for up to 10 days followed by immediate incubation under the conditions specified above.

Etoposide was purchased from Sigma (St. Louis, Mo). The drug was dissolved in DMSO according to manufacturer's instruction. Topotecan was received in powder form (SmithKline Beecham, Oakville, ON) and was reconstituted in sterile H_2O as indicated by the manufacturer. Further dilutions were made in PBS to achieve the desired concentrations. Cisplatin was obtained as a clinical formulation of 1 mg/ml cisplatin in 0.9% saline (Bristol-Myers-Squibb, Princeton, NJ). PBS was used for further dilution. Cells were exposed to drugs for 1 h at 37°C at the concentrations indicated.

2.3. - Isolation of HCT116 clones with different drug/X-radiation response phenotypes

Subclones of HCT116 were isolated at the same time, by dilution cloning, from the parental HCT116 cell line [Qutob et al., 2004]. HCT116-K was subsequently characterized as multi-drug resistant/X-radiation sensitive while HCT116-2 was characterized as cisplatin sensitive/X-radiation resistant. HCT116-2 did not respond differently than HCT116-10 or parental cells to topotecan or etoposide. In contrast, HCT116-10 was characterized as having a normal (i.e., similar to parental) response to all treatments and was, therefore, used as the control cell line in subsequent microarray analysis.

2.4. - cDNA microarray – First-strand synthesis, labelling, hybridization

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, CA, U.S.A.). First strand cDNA synthesis, labelling, and array hybridization were performed as described previously [Ng et al., 2007]. Fluorescence-labelled cDNA probes were generated by reverse transcription from 20 μg total RNA. HCT116-K or HCT116-2 cDNA was labelled with

Cy3 fluorescent nucleotides and cDNA from HCT116-10 cells (control RNA) was labelled with Cy5. The Cy5 and Cy3 probes were mixed and hybridized to the human 19K cDNA microarrays from the University Health Network, Ontario Cancer Institute, Toronto, Canada (http://www.microarray.ca). The microarray platform was comprised of two chips, designated A and B, with a total of 19,200 ESTs spotted in duplicate and organized into 24 sub-arrays of 600 spots each. Reciprocal labelling was also performed and experiments were repeated four times.

Fluorescently labelled cDNA from each pool of RNA was generated in a 40 µl reaction mixture containing 1X superscript II first strand buffer (Invitrogen), 150 pmoles AncT mRNA primer (5' T_{20}VN 3'), 20 nmoles each of dATP, dGTP, and dTTP, 200 pmoles dCTP, 100 pmoles Cy3- or Cy5-conjugated dCTP (Amersham Biosciences, Baie d'urfe, QC), 40 nmoles DTT (Invitrogen) and 20 µg total RNA. This mixture was heated to 65°C for 5 min to denature the RNA followed by incubation at 42°C for min to allow annealing of the AncT primer to polyA RNAs. Reverse transcription was performed by adding 2 µl of Superscript II reverse transcriptase (Invitrogen) and 1 µl of RNase inhibitor (Promega) and incubating the mixture at 42°C for 2.5 h. The reaction was terminated by adding EDTA to a final concentration of 5 mM. The template RNA was then hydrolyzed by adding 2 µl of 10 M NaOH followed by incubation at 65°C for 20 min. The reaction was then brought back to neutral pH by adding approximately 4 µl of 5 M acetic acid.

Prior to hybridization, Cy3 and Cy5 labelled cDNA was combined and precipitated with isopropanol. The resulting pellet was washed with 70 % ethanol and air dried in the dark. The pellet was then resuspended in 5 µl H_2O and combined with 80 µl of DIG Easy Hyb buffer (Boehringer Manheim, Germany) containing 0.5 µg/µl yeast tRNA and salmon sperm DNA. The hybridiztion mixture was heated to 65°C for 2 min, allowed to cool to

room temperature and injected between a paired set of the human 19K microarray slides. Hybridization took place in the dark at 37°C for 18 h. After hybridization slides were washed with 1X SSC containing 10% SDS three times for 10 min each. Finally, the slides were washed in 1X SSC alone for 10 min.

2.5. - Microarray data acquisition and processing

Microarray slides were read using a ScanArray 5000 confocal scanner (Packard BioScience, CT) with excitation/emission wavelengths of 543 nm/570 nm for Cy3 and 633 nm/670 nm for Cy 5 at 10 μm resolution. Slides were first scanned in the Cy5 channel to prevent photodegredation of this more sensitive dye. Images were obtained as 16-bit grayscale files for each channel and were quantified together with quantArray v3.0 software (Packard BioScience). An adaptive spot finding method was used to generate spot intensities from mean pixel values. Background intensities were derived using a background mask surrounding each spot. Spots that were of poor quality were manually designated as such and added to the "ignore spot" filter. Further processing of the tab delimited text data output files was performed in Microsoft Excel (Microsoft, WA). Genes were considered up or downregulated if their expression was changed by more than 1.8 fold on 3 out of the four chips after background subtraction and normalization.

2.6. – Real time RT-PCR

For the verification of genes found to be dysregulated by cDNA microarray, cDNAs were synthesized form total RNA using the method described in section 2.4 except that 0.5 nmoles/ul dNTP was used instead of the nucleotide mixture in 2.4. cDNA was purified

using the Wizard PCR purification system (Promega, WI, U.S.A.) from the same total RNA pools used for the microarray analysis. Changes in gene expression were verified in duplicate by quantitative RT-PCR, using the ABI GeneAmp 5700 sequence detection system, using the Quantitect SYBR© Green PCR kit (Qiagen, CA, USA) [Voorhoeve et al., 2006]. Reaction conditions were as follows: 2 ng cDNA was amplified in 25 µl of SYBRgreen PCR master mix (Qiagen, CA, USA) containing 0.15 pM each of gene specific forward and reverse primers (Table 1). All other Real-time PCR (including survey of gene expression in a panel of HCT116 clones) was performed as follows: mRNA was isolated using the RNeasy mRNA isolation kit (Qiagen, CA, USA). cDNA was synthesized using the First-strand cDNA synthesis kit, with oligodT primers, according to the manufacturer's instructions (Fermentas, Burlington, ON). Relative quantification was performed on a Roche LightCycler using primers specific to the transcript of interest at a concentration of 0.1 pmol/µl each. 0.2 µl of Platinum *Taq* was used as the DNA polymerase (Invitrogen, Carlsbad, CA) the reaction also contained 1X PCR reaction buffer with $MgCl_2$ (Qiagen), 1X BSA, a 1:5000 dilution of SYBR green in H_2O to bring the reaction to a final volume of 20 µl. Gene amplification was detected by the incorporation of SYBR green into double stranded DNA (Molecular Probes). All genes were normalized to the expression of β-actin or GAPDH.

Table 1: List of primers used for real time RT-PCR

No.	*Gene Name*	*Forward Primer*	*Reveres Primer*
1	*APM2 (C10orf116)*	CAAGACCACCCAGGAAACCAT	TTTTTTCCCGATCCCAGAGA
2	*ALDH6*	TCCAAGTGGCCTGAGTATTTCA	GAAATTGCACATCATCATTTGGAT
3	*CYP24A1*	TTGGCGTTGGAAAAAGAATGT	ACAATCCAACAAAGAGCCAAATG
4	*GSK-3α*	TGAGGGAACTTGAACTCCGTGTA	TCATCAAGGTGCTGGGAACA
5	*SSAT*	CGGGCCGACTGGTGTTTA	GGAGCGTCCTCTTCTCAGTCA
6	*XRRA1*	CTGACAGAAACATCCCCAACAG	CCTCCTCTCCAACTCATCAGAAG

2.7. – Northern blotting

RNA was extracted from the specified cell lines using the RNeasy RNA extraction kit (Qiagen, CA, USA). 5 -10 μg of total RNA were separated by electrophoresis through a 1.5% formaldehyde agarose gel. When electrophoresis was complete RNA quality and loading were assessed visually by staining with Ethidium Bromide and viewing over a UV gel doc. Images were captured using a GeneGnome gel documentation system (Syngene, Frederick, MD). The gel was then washed four times in deionized water for 20 min each time.

RNA was then transferred from the gel onto a Nylon Hybond N membrane (Amersham). This was achieved by filling a glass dish with 20XSSPE (3M NaCl, 0.25M NaH2PO4 and 0.02M EDTA, adjust to pH 7.4). This dish served as a buffer reservoir. RNA was transferred overnight from the gel onto the membrane by capillary action resulting from the wicking of buffer by the filter paper through the gel and membrane into a 6 cm stack of brown paper towels. The next day the transfer setup was disassembled and the membrane was prepared for hybridization.

The membrane with transferred RNA was rinsed several times with 5X SSPE and then allowed to dry. Once dry, the membrane was placed faced face up in a UV crosslinker and irradiated at a setting of 0.12 joules/cm^2.

Labelled DNA probes specific to APM2 were generated using a full length APM2 PCR product as the template. In order to generate and label the probe the Decaprime Random Priming DNA Labelling Kit was used according to the manufacturer's instructions using dCTP-32P as the labelled nucleotide (Ambion, TX). The resulting probe was purified from unincorporated radioactive dNTPs using a PCR purification column (Qiagen, CA).

Before hybridization membranes were pre-hybridized in 5ml pre-hybridization buffer (2.5ml formamide, 1ml 5x Denhardt's solution, 1.4ml H2O, 0.292g NaCl and 0.1ml salmon sperm DNA) for 6 h at 42°C. Denatured, double-stranded DNA probe was then added directly to the hybridization chamber and allowed to incubate overnight at 42°C while rotating. After hybridization the membranes were washed twice for 10 min each in wash buffer (1% SDS + 0.1% 20X SSPE) at room temperature followed by two 15 min washes in the same buffer at 65°C. A final rinse in 5XSSPE was performed. The membranes were then wrapped in plastic wrap and exposed to a phosphor screen (Amersham) overnight. Images were captured on a Storm phosphorimager (GE lifesciences).

2.8. – Cloning of the APM2 open reading frame

In order to clone the APM2 open reading frame into an expression vector the primers 5'-gaagatcttcatggcaagcaagggcttgc-3' (forward) and 5'-gaagatcttctcatttcaggaggccgaatttttttcc-3' were designed with BglII restriction sites at their 5' ends. PCR was then performed using HCT116-CloneK cDNA as a template. The resulting product was approximately 240 bp in length. The product was then gel purified using the Montage Gel Extraction Kit (Millipore, Billerica, MA). The gel purified DNA fragment was then ligated into a T vector which was subsequently digested with the BglII restriction endonuclease. The resulting 240 bp DNA fragment, containing BglII sticky ends, was then ligated into BglII digested, pCMV-TAG1, vector (Stratagene, La Jolla, CA). Several colonies were screened for the APM2 insert and proper orientation. Positive selections were sent for sequencing and one clone containing the correct sequence was used to perform a

large-scale plasmid preparation. Large-scale plasmid preparation was performed using the QIAfilter Plasmid Maxi Kit according to the manufacturer's instructions (Qiagen, CA). The resulting expression vector produces a FLAG-APM2 fusion protein with the FLAG tag residing at the N-terminal end of the protein. This insert was later excised from pCMV-Flag-APM2 and inserted into pcDNA3.1 resulting in a plasmid coding for the expression of an untagged full-length version of the APM2 protein.

2.9. – Immunoblotting

Cells were grown under the conditions described above. Forty-eight hours after transfections, cells were rinsed with PBS and, lysed by adding ice-cold ProFound lysis buffer (Pierce, IL, USA) with 1X complete protease inhibitor (Roche) directly onto the cells. Cells were incubated on ice for 15 min followed by sonication for 30 sec at 4° C. Proteins in the lysate were separated using 4-12% NuPAGE gels in 1X MES running buffer (Invitrogen, Carlsbad, CA). Proteins were then transferred from the gel to 0.2 μm PVDF (Millipore) using a semi-dry electroblotter. Proteins were fixed to the membrane by incubation for 10 min in 5% glutaraldehyde. Following several rinses in TBS-T, membranes were incubated overnight with a custom APM2 antibody (New England peptide), Flag antibody (1:2000) (Sigma) or β-actin (1:10000) antibody (Sigma) diluted in TBS-T containing 5% milk. No APM2 antibody was available commercially. Therefore, we had a custom polyclonal antibody generated by injecting rabbits with a peptide consisting of the first 12 residues of the APM2 protien (New England Peptide, MA). Membranes were then washed 3 times in TBS-T followed by incubation with an appropriate HRP conjugated secondary antibody diluted in TBS-T containing 5% milk.

After several washes in TBS-T membranes were treated with LumiGlo Reserve chemiluminescent substrate (KPL, MD, USA) and immediately imaged using a GeneGnome HR chemiluminescence capture and analysis system (Syngene).

2.10. – Transient overexpression and knockdown of APM2

APM2 was transiently overexpressed by transfecting cells with pCMV-Tag1-APM2 using GeneJuice (Novagen) as the transfection reagent according to the manufacturer's instructions. pCMV-Tag1 containing no insert was used as a transfection control. pEGFP-C2 was transfected in a separate well to assess plasmid transfection efficiency visually. Transfection efficiencies ranged from 40-60%.

Transient knockdown of APM2 was achieved, in each cell line, by transient transfection of one of two siRNAs (siAPM2 duplex1, siAPM2 duplex4) (Dharmacon) targeting APM2. Each siRNA targeted a discreet region of the APM2 mRNA. The sequences of the siD1 and siD4 are 5'-GCCAAGACCACCCAGGAAAUU-3' and 5'-CGACAAGACUGCUAACCAGUU-3', respectively. siRNAs were delivered using Lipofectamine 2000 according to the manufacturers instructions. A non-targeting siRNA was used as a transfection control (Dharmacon). Transfection efficiency was assessed by transfection, in a separate well with a Cy3 labelled siRNA. siRNA transfection efficiency was >90%.

2.11. – Clonogenic survival assay

Cells were plated at a density of 2 x 10^5 cells/well in six-well plates or T25 flasks. The next day, the cells were transfected with pCMV-Tag1, pCMV-Tag1-APM2 or siRNA

(siNon-Targeting, siAPM2-D1, and siAPM2-D4). After 48 hrs, the cells were treated with drugs at the indicated concentrations for 1hr at 37°C. In the case of X-radiation, cells were treated with 0 - 10 Gy irradiation using a 250 kVp X-ray unit (Pantak, Ct, USA) at a rate of 150 cGy/min. Cells were then washed twice with PBS and trypsinized. Cells were then resuspended, counted and plated on 60 mm dishes at densities yielding approximately 50 colonies per plate for each condition after 10 – 14 d incubation. After 10 – 14 d, plates were removed from the incubator, media was discarded and cells were fixed and stained in a solution containing 50% methanol and 0.5% methylene blue. Colonies containing more than 50 cells were scored and surviving fractions (SF) were calculated for each dose using the following formula: SF = (Colonies counted)/(PE * Colonies on control), where SF = surviving fraction and PE = platin efficiency = (colonies on control)/(cells plated on control). In instances where cells were not transfected prior to clonogenic assay (determining the sensitivities of clones) the same procedures were followed without the transfection steps.

2.12. – shRNAs – Cloning and generation of stably expressing cell lines

shRNA's based on siAPM2-D1 and siAPM2-D4 were developed and inserted into the shRNA vector pSilencer4.1-puro (Ambion). The following pairs of oligos: 5'-GATCCGCCAAGACCACCCAGGAAATTCAAGAGATTTCCTGGGTGGTCTTGGCCAA-3', 5'-AGCTTTGGCCAAGACCACCCAGGAAATCTCTTGAATTTCCTGGGTGGTCTTGGCG-3', and GATCCCGACAAGACTGTCTAACCAGTTCAAGAGACTGGTTAGCAGTCTTGTCGATA, AGCTTATCGACAAGACTGCTAACCAGTCTCTTGAACTGGTTAGCAGTCTTGTCGG-3', were annealed and ligated into pSilencer4.1-puro (Ambion) by incubating at RT with T4 DNA

ligase. A non-targeting sequence was also ligated into pSilencer4.1-puro, and the resulting plasmid was used as the control vector for all experiments. The incorporation of BamHI and HindIII sites in the oligos allowed for the insertion of the annealed oligos into pSilencer4.1. DNA was then transformed into DH5α *E. coli*. Positive clones were verified by DNA sequencing (Stemcore, OHRI). One positive clone for each duplex was used for large-scale DNA preparation as described in section 2.8. The resulting plasmids were designated pSil-shAPM2-D1, pSil-shAPM2-D4, and pSil-shNT. HCT116, HCT116-K and OV2008 were used to generate clones stably expressing short-hairpin RNAs against APM2. HCT116, HCT116-K and OV2008 were transfected with pSil-shAPM2-D1, pSil-shAPM2-D4, or pSil-shNT using GeneJuice according to the manufacturer's instructions. After 24 h cells were passaged at low density and placed in media containing 1 μg/μl puromycin. When individual colonies were visible, single colonies were lifted from the plate and placed in separate wells of a 24 well plate. Colonies were further expanded until there were enough cells to passage regularly. Colonies were then screened for APM2 depletion by western blot and positive clones were frozen down for later use. Several clones carrying the pSil-shNT were also kept to use as controls in later experiments.

2.13. – Tumour xenografts – In vivo studies

5 x 10^6 cells were injected subcutaneously on the backs of CD-1 nude mice and the tumours were allowed to grow for approximately 2 weeks until they reached a diameter of approximately 5 mm. Mice in the untreated group were injected with a volume of 10 ml/Kg PBS. Mice in the treated groups were injected intraperitoneally with cisplatin three times a week for one week at a dose of 4 mg/Kg in a volume of 10 ml/Kg. The total amount of drug

administered to each treated mouse was 12 mg/Kg. Following treatment, the length (*l*), width (*w*), and depth (*d*) of the tumours were measured with callipers three times a week (M, W, F) for the duration of the experiment. Tumour volume at each point was calculated using the following formula: $lwd(\pi/6)$ [Miao et al., 2005].

2.14. – Immunofluorescence

Glass coverslips were cleaned with boiling KOH followed by treatment with D-lysine. Untransfected cells, cells transfected with pCMV-Flag-APM2 or an empty DNA vector were plated on cleaned, coated coverslips in six-well plates and allowed to grow for 24-48 h as described in section 2.1. After this period media was removed from the wells by aspiration and the cells were washed three times with PBS. Cells were then fixed in PBS containing 4% paraformaldehyde for 15 min. Paraformaldehyde was removed and the fixed cells were then washed three times in PBS to remove any residual fixative. Cells were then permeabilized by the addition PBS + 0.25% Triton X-100 to each well for 15 min at room temperature. Triton X-100 solution was removed by aspiration and three washes in PBS. To prevent non-specific binding of antibodies, cells were blocked in 10% BSA overnight at 4°C. After blocking fixed, untransfected cells were incubated with no antibody, anti-APM2 (1:2000) (New England Peptide, MA), or anti-APM2 blocked with an APM2 peptide (New England Peptide, MA). Cells transfected with the Flag-APM2 fusion were incubated with no antibody or anti-Flag (1:2000) (Sigma). Incubation with the primary antibodies was performed at room temperature for 60 min. Following primary antibody incubations, cells were washed 4 times for 5 min each with PBS. Coverslips were then incubated in the dark with the appropriate secondary antibody conjugated to Alexa Fluor 594 (AF594)

(Molecular Probes, CA) at room temperature for 45 min. Once incubation with the secondary antibodies was complete the cells were washed 4 times with PBS for 5 min each time. Finally, the coverslips were washed once with deionized H_2O and mounted on slides with vectashield mounting medium containing DAPI (Vector Labs, Burlington, ON).

Slides were viewed using a Zeiss axiovert fluorescence microscope. Images were captured digitally under the 40X and 63X oil immersion lenses and viewed using Zeiss Axiovision software (Zeiss). Deconvolution was performed on images using Zeiss axiovision software (Zeiss).

2.15. – Immunoprecipitation

Immunoprecipitation of APM2 was performed on HCT116 conditioned media. First, HCT116 cells were plated on 10 cm plates at a density of 5 X 10^5 cells/ml and allowed to grow for 48 h. After this time period conditioned-media was removed from the dishes and placed into 15 ml falcon tubes (Corning). Media was centrifuged at 2000 rpm in a cell culture centrifuge (Becton Dickinson). Media was then passed through a 0.45 μm pore size filter with low protein binding capacity to remove remaining cells and debris (Fisher). Our custom made anti-APM2 antibody (15 μg) (New England Peptide) was then added to 10 ml of media and allowed to incubate overnight at 4°C while rotating. The next day 50 μl of gammabind sepharose (GE lifescieces) was washed three times in wash buffer (50 mM Tris HCl, pH 7.4, with 150 mM NaCl,1 mM EDTA, and 1% Triton X-100) and added to the media. The mixture containing antibody, beads, and media was allowed to incubate at 4°C for 1 h. Following incubation, the media was carefully removed from the tube and discarded. The remaining beads were washed three times in wash buffer. Proteins bound to the beads were eluted by boiling with an equal volume of 2X LDS sample buffer

(Invitrogen) containing 1X DTT. The entire procedure was performed on unconditioned media (fresh media) as a negative control. HCT116 whole cell lysate was used as a positive control for immunoblotting. Immunoblotting was performed as described in section 2.9 with a custom APM2 antibody (1:2000) (New England Peptide).

2.16. – Cellular Fractionation

Nuclear, cytoplasmic, cytoskeleatal, and membranous fractions were obtained from HCT116 cells using the Qproteome kit according to the manufacturer's instruction (Qiagen). Isolated fractions were subject to immunoblotting as described in section 2.9. Resulting blots were probed with a custom APM2 antibody (1:2000) (New England Peptide). Probing with anti-LDH (1:1000) (Chemicon) was performed to confirm the purity of the subcellular fractions.

CHAPTER 3

RESULTS

3.1. – IDENTIFYING FACTORS INVOLVED IN THE RESPONSE OF CANCER CELLS TO X-RADIATION AND CHEMOTHERAPEUTICS

3.1.1. - Isolation and characterization of HCT116 clones having varying sensitivities to drugs and X-radiation

The majority of work pertaining to the isolation and characterization of these clones was performed by a previous graduate student [Qutob et al., 2004]. However, it is necessary to describe these results in some detail here in order to clearly demonstrate how this project was initiated.

Previously, our laboratory isolated clones from an untreated, human colorectal cancer cell line, HCT116 (Figure 4). Subsequently, the isolated clones were subjected to a number of insults including treatment with X-radiation, etoposide, topotecan, and cisplatin (Figures 5a, b, c, d). Figure 4 consists of several phase contrast images. Figure 4a demonstrates the morphology of the parental HCT116 cell line. Figure 4b-c illustrates the morphology of clones HCT116-10, HCT116-K, and HCT116-2, respectively. HCT116-10 and HCT116-K maintain morphologies similar to that of the parental cell line while HCT116-2 has a more fibroblastic character and appears to grow in a less arranged manner.

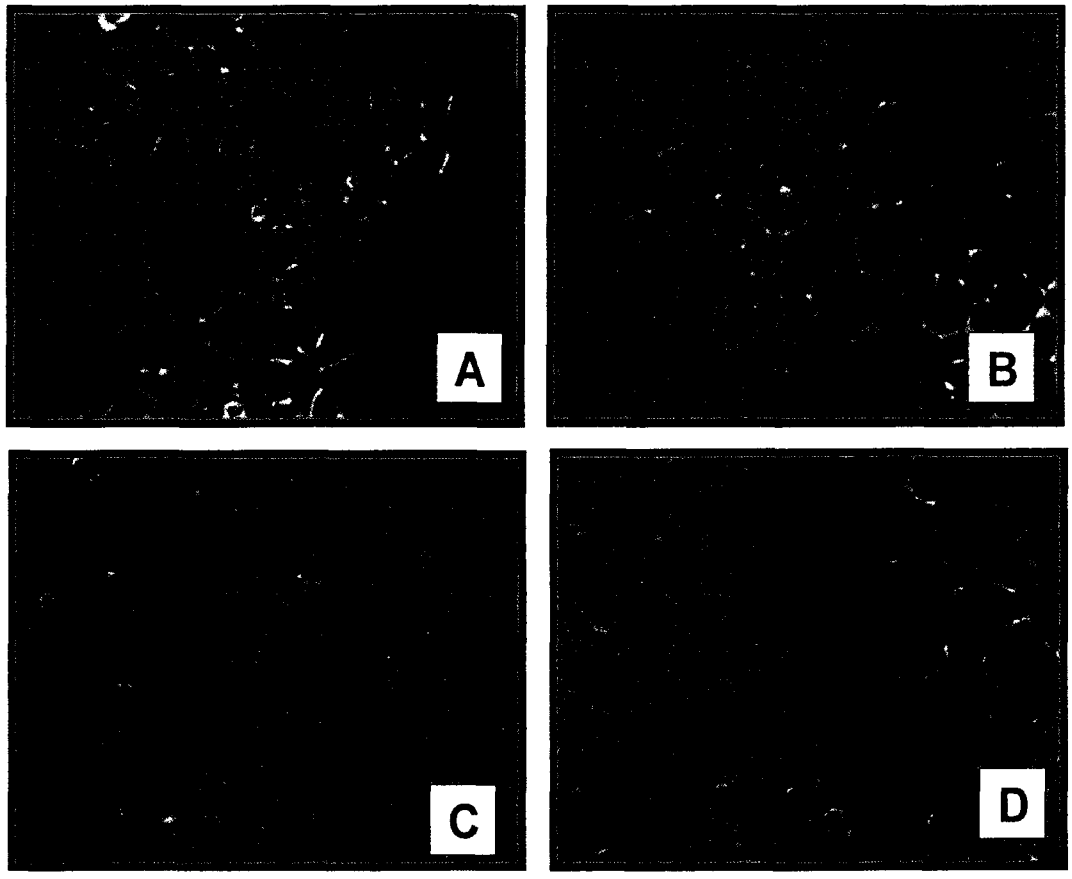

Figure 4: Morphology of HCT116 and HCT116 derived clones. Single cells were isolated from the parental HCT116 and expanded. a) HCT116 b) HCT116-10 c) HCT116-K. d) HCT116-2. Cells were photographed at high density. Note the similar morphologies maintained by clones of the parental HCT116 cell lines.

Figure 5a demonstrates the differences in the response of the clones to treatment with acute doses of X-radiation. HCT116-2 is resistant to dosing with X-radiation when compared to the parental HCT116 cell line. Conversely, the HCT116-K clone displays sensitivity to X-radiation in comparison with parental cells. Finally, HCT116-10 behaves in a manner similar to the original cell line. Interestingly, HCT116-K is relatively resistant to treatment with several drugs including etoposide (Figure 5b), topotecan (Figure 5c), and cisplatin (Figure 5d). HCT116-2 and HCT116-10 do not behave differently than the parental cells when treated with either topotecan (Figure 5c) or etoposide (Figure 5b). However, HCT116-2, which is X-radiation resistant, is relatively sensitive to cisplatin in comparison to HCT116 cells [Qutob et al., 2004].

3.1.2. - Comparison of global gene expression between HCT116 clones with different responses to XR and chemotherapeutics

In an attempt to gain a better understanding of the genetic factors that control the response of cancer cells to X-radiation and/or chemotherapeutics, cDNA microarray analysis was performed. Since HCT116-10 behaves similarly to the parental cells when treated with various insults, it was used as the control cell line to which the expression profiles of HCT116-K and HCT116-2 were compared. Table 2 lists the genes that were found, by microarray, to be upregulated by at least 1.8 fold in at least 3 of 4 experiments in HCT116-K compared to HCT116-10. Table 2 also includes the results of real-time RT-PCR performed on the *same* pools of RNA used for the microarray. The most highly upregulated gene was *APM2* with an average fold change of 2.9 +/- 0.5. Verification of overexpression by real-time RT-PCR revealed that *APM2* was actually highly overexpressed in HCT116-K compared to HCT116-10 with a fold change >2500 (Table 2).

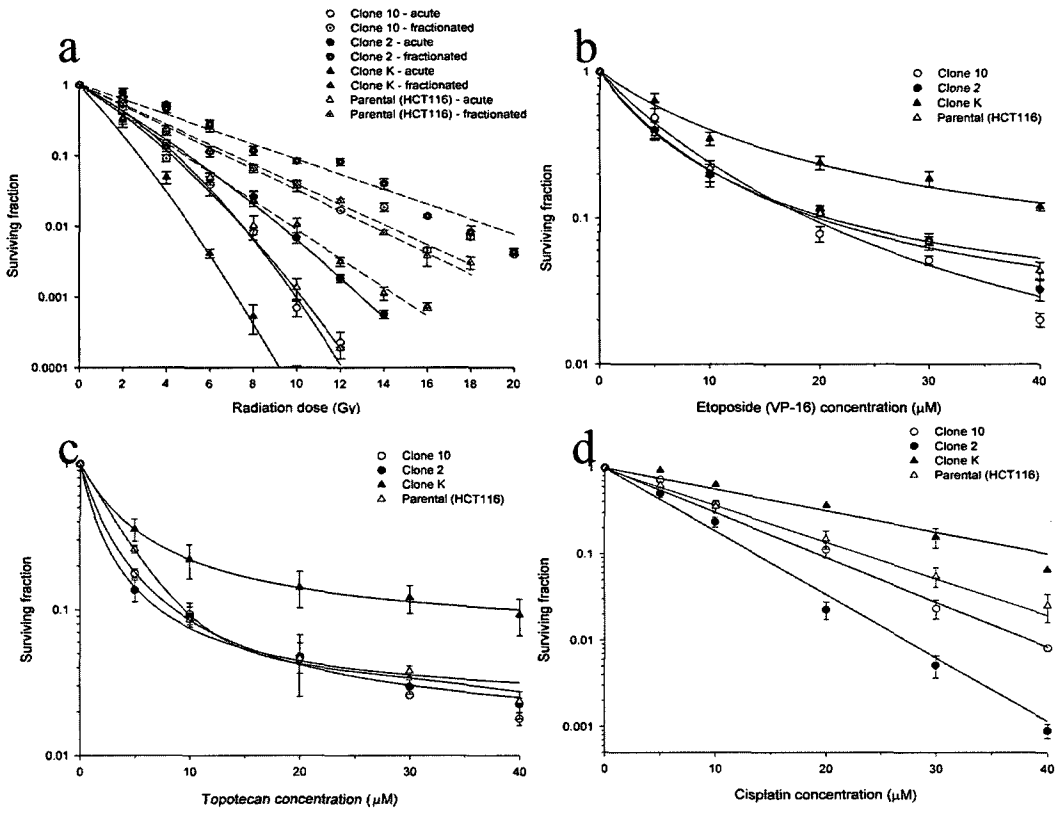

Figure 5: The response of HCT116, HCT116-10, HCT116-2, and HCT116-K to a) fractionated and acute XR, b) etoposide, c) topotecan, and d) cisplatin. Clonogenic assays were used to assess the sensitivities of HCT116 clones to each treatment relative to the parental cell line Note: HCT116-K is sensitive to radiation but the most resistant to all drugs, HCT116-2 is resistant to XR, sensitive to cisplatin and responds "normally" to other drugs tested HCT116-10 responds similarly to the parental and is slightly more sensitive than HCT116 to cisplatin. Points are presented as the mean of 3-5 experiments ± SEM.

Table 2: Genes upregulated by the chemoresistant/radiosensitive clone HCT116-K relative to the cisplatin normal clone, HCT116-10

No.	*Gene Name*	*Symbol*	*Gene ID**	*GO Process†*	*Fold Change by microarray*	*Fold Change by Real time RT-PCR*
1	Adipose Specific 2	*APM2*	10974	Unknown	2.9 ± 0.5	2777 ± 318
2	Spermidine/Spermine N1-Acetyltransferase	*SSAT*	6303	Metabolic process	2.3 ± 0.7	6.3 ± 0.1
3	Glycogen Synthase Kinase 3 alpha	*GSK-3α*	2931	Protein amino-acid phosphorylation	2.5 ± 0.4	1.5 ± 0.1
8	Aldehyde dehydrogenase 6	*ALDH6*	220	Metabolism	1.8 ± 0.1	2.4 ± 0.2

** Gene ID refers to Entrez Gene accession number.*

† GO process is as defined by Entrez Gene.

Several other genes were also identified as overexpressed in HCT116-K versus HCT116-10 (Table 2). These include *SSAT* (FC = 2.3 +/- 0.7), *GSK-3α* (FC = 2.5 +/- 0.4), and *ALDH6* (FC = 1.8 +/- 0.1). Figures 6a, b, c, d show the results of real-time RT-PCR comparing the expression of *APM2, SSAT, GSK-3α*, and *ALDH6* in HCT116-K versus HCT116-10 using several *different* RNA pools. This verified that the expressions of these selected genes were maintained in these clones after storage and/or passaging for a long period of time. It should be noted that this follow up study revealed did not find a significant difference in *GSK-3α* expression between HCT116-K and HCT116-10. Table 3 lists the genes that were found to be downregulated in HCT116-K relative to HCT116-10. Of specific note is the gene *CYP24A1. CYP24A1* was found to be -2.1+/-0.4 fold underexpressed in HCT116-K by microarray. However, real-time PCR performed using RNA from the same pools used for microarray revealed far greater suppression of *CYP24A1* (Table 3). Furthermore, real-time PCR performed on microarray-independent RNA pools confirmed that this gene remained highly suppressed in HCT116-K long after these clones were established (Figure 6e).

Comparisons of global gene expression were also made between the X-radiation resistant HCT116-2 and HCT116-10. The expression of one gene, *XRRA1*, was found to be downregulated by microarray comparison (FC = -2.2 +/- 0.2) and by real-time RT-PCR performed using the same pools of RNA. Real-time RT-PCR comparing the expression of *XRRA1* in HCT116-2 versus HCT116-10 in newly isolated RNA pools showed that *XRRA1* was significantly overesxpressed in HCT116-2 (FC = 2.47 +/- 0.63) (Figure 6f). This was the opposite of what was observed in the initial microarray and RT-PCR results.

Table 3: Genes downregulated by the chemoresistant/radiosensitive clone HCT116-K relative to the cisplatin normal clone, HCT116-10

No.	*Gene Name*	*Symbol*	*Gene ID**	*GO Process†*	*Fold Change by microarray*	*Fold Change by Real time RT-PCR*
1	ELKL motif kinase 1	*MARK2*	2011	Protein phosphorylation, differentiation	2.5 ± 0.3	1.3
2	TAK1 binding protein	*MAP3K7IP1*	10454	Activation of MAPKKK activity	2.4 ± 0.4	1.1
3	Cytochrome P450-CC24	*CYP24A1*	1591	Degradation of 1,25-dihydroxyvitamin D3	2.1 ± 0.4	1726 ± 108

** Gene ID refers to Entrez Gene accession number.*

† GO process is as defined by Entrez Gene

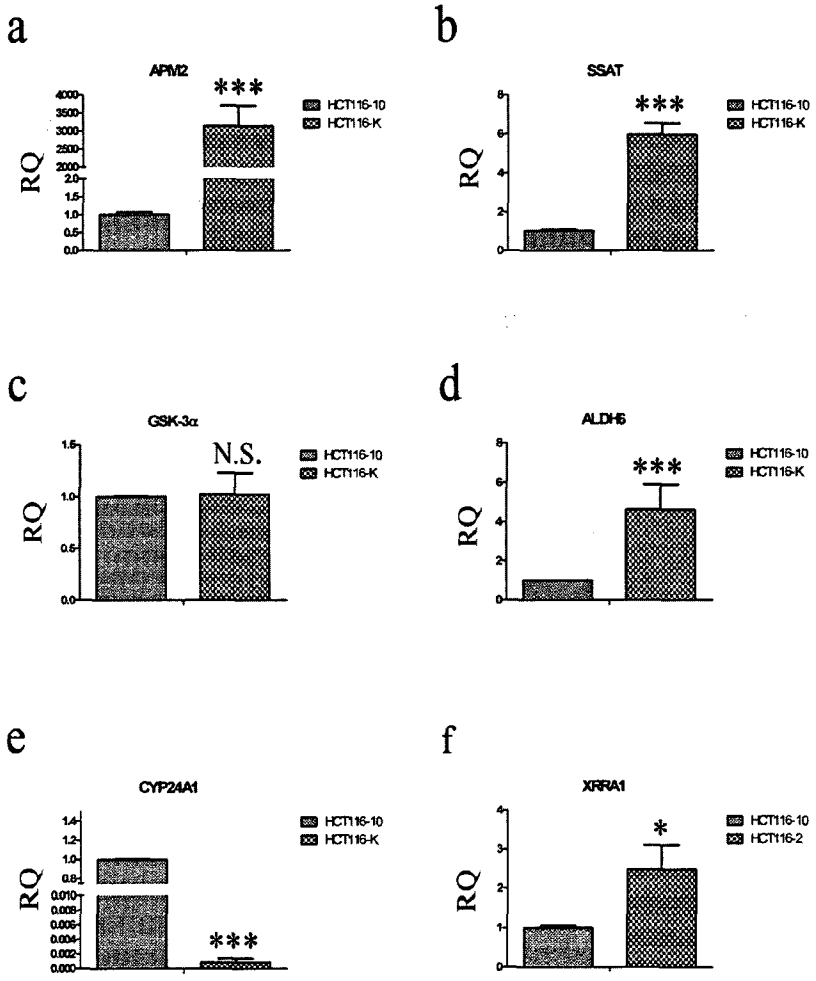

Figure 6: Real-time RT-PCR of genes identified by microarray experiments between HCT116-K and HCT116-10 or HCT116-2 and HCT116-10. Expression of transcripts found to be differentially regulated in both microarray and real-time PCR experiments were further verified in newly isolated RNA pools. HCT116-K vs. HCT116-10: a) APM2, b) SSAT, c) GSK-3α, d) ALDH6, e) CYP24A1. HCT116-2 vs. HCT116-10: and f) XRRA1. N.S. – not significant, * - $p<0.05$, ** - $p<0.01$, ***$p<0.001$, by one-tailed unpaired student's t-test. RQ = Relative quantification normalized to β-actin. Results are presented as the mean of 3 experiments ± SEM.

3.1.3. - Clonal variation may be responsible for some, but not all of the genes identified by cDNA microarray

Clonal variation of gene expression is a well documented phenomenon and can contribute to misleading results when comparing the global gene expression signatures of clones isolated from a single parental cell line [Brem et al., 2001; Stears et al., 2003]. The expressions of several genes identified as dysregulated by our microarray analysis were measured in a panel of HCT116 clones. This was done to minimize the possibility that genes selected for further study with respect to drug/X-radiation resistance were not identified simply due to clonal variation. The expressions of *ALDH6*, *SSAT*, *GSK-3α*, *CYP24A1*, *APM2*, and *XRRA1* were examined in 9 HCT116 clones and in the parental cell line. Significant differences were determined by one-way ANOVA followed by Dunnett's test.

The increased expression of *ALDH6* in HCT116-K was found to be insignificant when compared to its expression in all other clones (Figure 7). Interestingly, the expression of *ALDH6* in all of the selected clones was significantly different from its expression in the parental cell line. However, the fact that no statistically significant difference was observed in HCT116-K versus the panel of HCT116 clones required us to discard this gene as it was likely identified due to clonal variation.

SSAT was identified, by microarray, as overexpressed by HCT116-K versus HCT116-10. Further testing revealed that *SSAT*'s expression was significantly dysregulated in HCT116-K versus all HCT116 clones in the testing panel (Figure 8). Because the expression of SSAT was found to be significantly different in HCT116-K compared to all HCT116 clones, it was selected for further study, by a previous lab member, as a factor in radioresistance [Qutob et al., 2005].

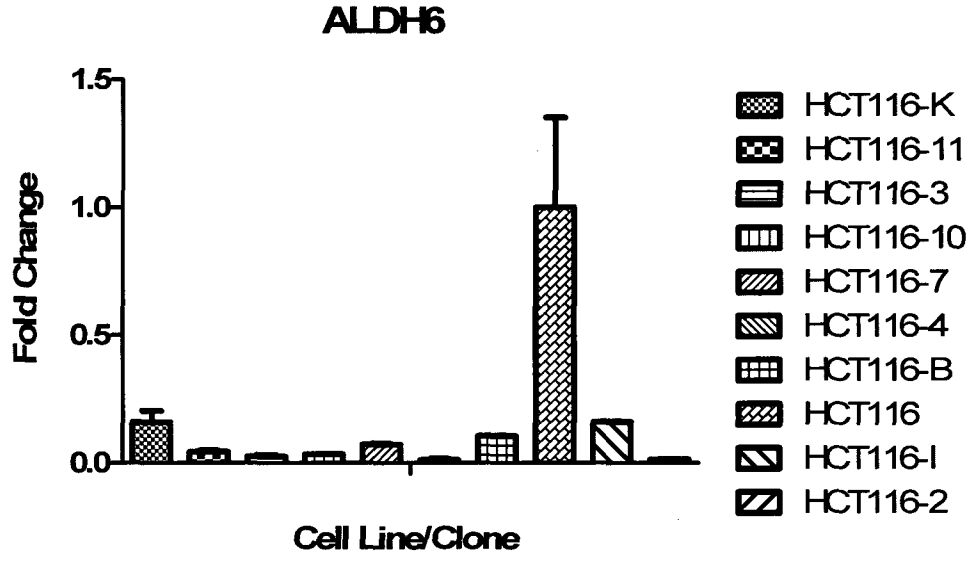

Figure 7: ALDH6 expression in a panel of HCT116 clones, and the parental cell line. Real time RT-PCR was performed to assess the relative expressions of ALDH6 in 9 HCT116 clones and the parental cell line. ALDH6 expression was normalized to the expression of β-actin. *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$, by One-way ANOVA and Dunnet's post-hoc testing vs. expression in HCT116-K. Results are presented as the mean of at least 3 experiments ± SEM.

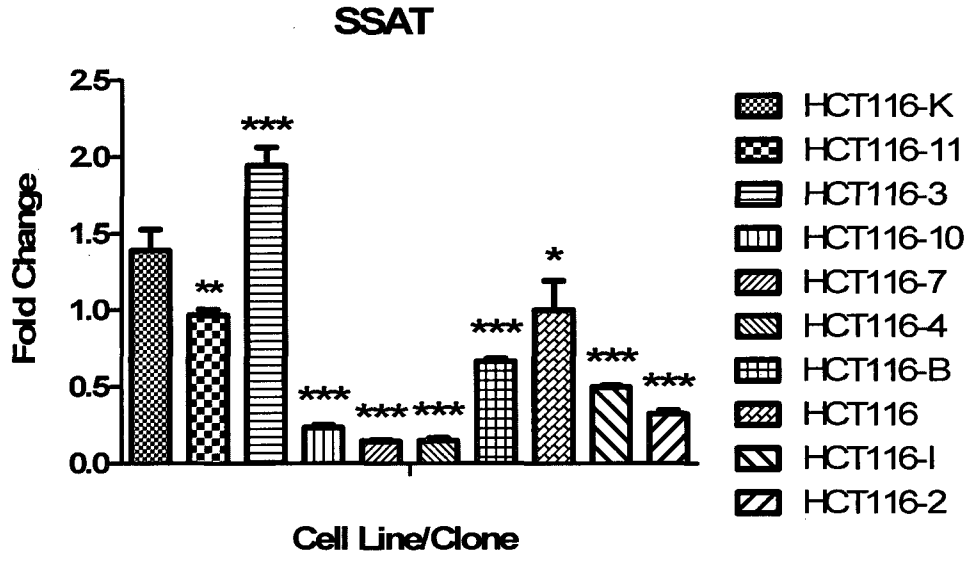

Figure 8: SSAT expression in a panel of HCT116 clones, and the parental cell line. Real time RT-PCR was performed to assess the relative expressions of SSAT in 9 HCT116 clones and the parental cell line. SSAT expression was normalized to the expression of β-actin. *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$, by One-way ANOVA and Dunnet's post-hoc testing vs. expression in HCT116-K. Results are presented as the mean of at least 3 experiments ± SEM.

One-way ANOVA and Dunnett's testing showed that *GSK-3α*, identified as overexpressed in HCT116-K versus HCT116-10, was not significantly dysregulated in HCT116-K compared to the isolated HCT116 clones and, therefore, identification was likely due to clonal variation (Figure 9).

CYP24A1's expression was determined by real time RT-PCR in our panel of HCT116 clones (Figure 10). One-way ANOVA revealed that the group means were significantly different from each other. Dunnett's testing showed that HCT116-K significantly underexpressed *CYP24A1* compared to 6 of the other 8 clones and the parental cell line. However, when comparing *CYP24A1* expression in HCT116-K (chemoresistant/radiosensitive) versus HCT116-2 chemosensitvie/radioresistant) no difference was observed. Since, these clones are on opposite ends of the spectrum with respect to treatment sensitivities, the expression of *CYP24A1* was not pursued as a factor in chemo/radio –resistance.

XRRA1 was downregulated in the chemosensitive/radioresistant HCT116-2. Investigation of the expression of this gene across the panel of HCT116 clones showed that the expression of *XRRA1* in HCT116-2 did not differ signifificantly from the expression observed in any of the other 8 individual HCT116 clones, or in the parental HCT116 cell line (Figure 11). Significance was assessed by one-way ANOVA and Dunnett's post testing. This gene was not selected for further investigation as a factor in chemo/radiation – resistance.

APM2 was identified as the most highly overexpressed gene in the chemoresistant/radiosensitive HCT116-K versus the "normal response" HCT116-10. To strengthen the argument that this was not due to the normal clonal variation of this gene, its expression was assessed in our panel of HCT116 clones. *APM2* was significantly

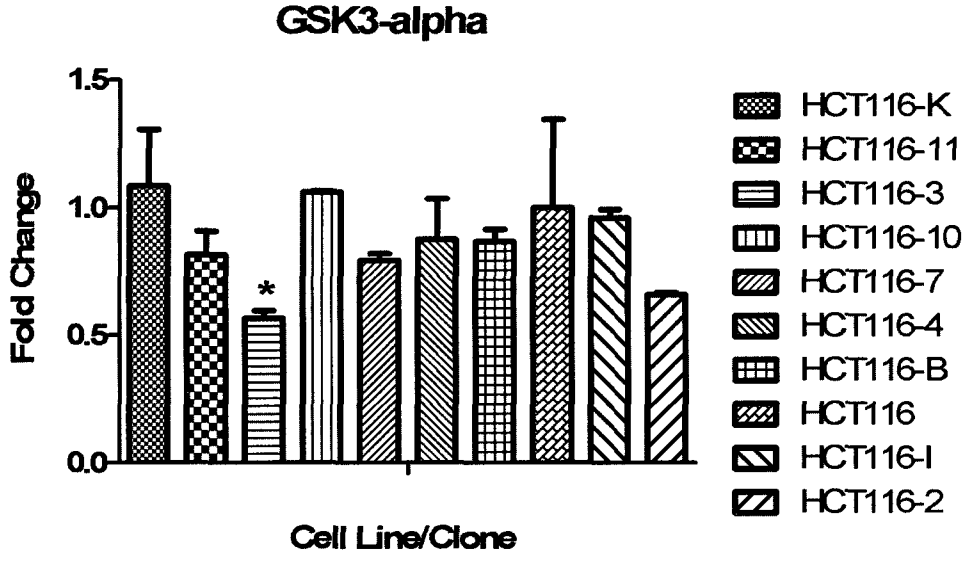

Figure 9: GSK-3α expression in a panel of HCT116 clones, and the parental cell line. Real time RT-PCR was performed to assess the relative expressions of GSK-3α in 9 HCT116 clones and the parental cell line. ALDH6 expression was normalized to the expression of β-actin. *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$, by One-way ANOVA and Dunnet's post-hoc testing vs. expression in HCT116-K. Results are presented as the mean of at least 3 experiments ± SEM.

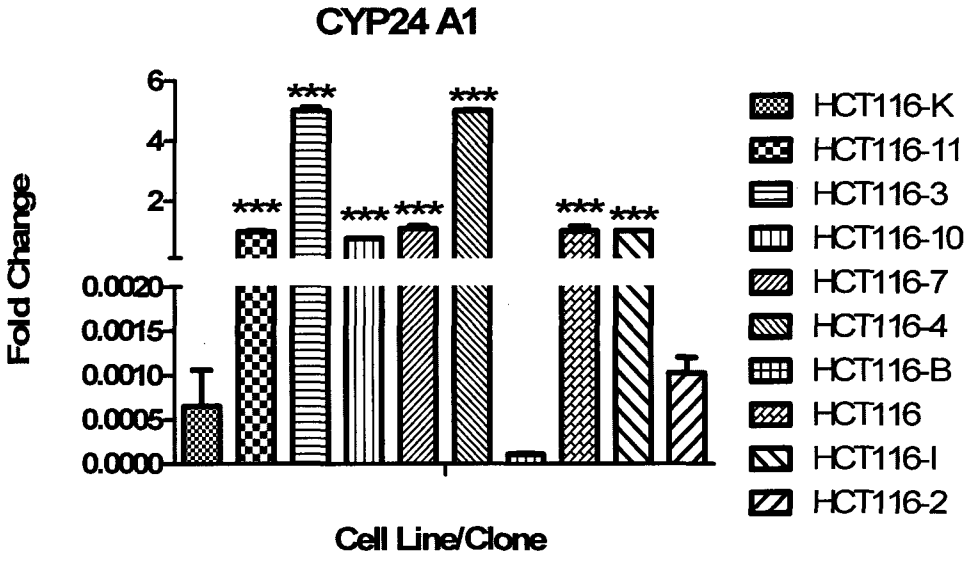

Figure 10: CYP24A1 expression in a panel of HCT116 clones, and the parental cell line. Real time RT-PCR was performed to assess the relative expressions of CYP24A1 in 9 HCT116 clones and the parental cell line. CYP24A1 expression was normalized to the expression of β-actin. *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$, by One-way ANOVA and Dunnet's post-hoc testing vs. expression in HCT116-K. Results are presented as the mean of at least 3 experiments ± SEM.

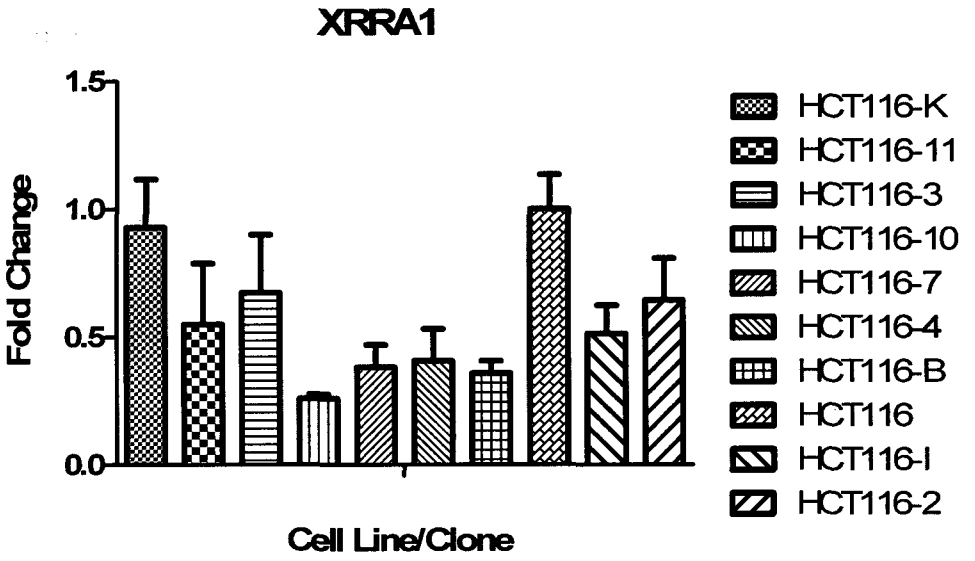

Figure 11: Expression of XRRA1 in a panel of HCT116 clones, and the parental cell line. Real time RT-PCR was performed to assess the relative expressions of XRRA1 in 9 HCT116 clones and the parental cell line. XRRA1 expression was normalized to the expression of β-actin. *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$, by One-way ANOVA and Dunnet's post-hoc testing vs. expression in HCT116-2. Results are presented as the mean of at least 3 experiments ± SEM.

overexpressed when compared to all other clones included in the panel ($p<0.0001$) (Figure 12). Interestingly, the chemosensitive/radioresistant HCT116-2 displayed the lowest expression of this gene. Furthermore, a plot of relative APM2 expression versus cisplatin ID50s for HCT116-2, HCT116-10, HCT116 and HCT116-K revealed a significant correlation between APM2 expression and response to cisplatin ($RS=1.000$, $p<0.05$) (Figure 13). These results indicate that the identification of *APM2,* as being overexpressed in HCT116-K, is not likely due to clonal variation. In light of these results, *APM2* was selected for further study, in this thesis, with respect to its role in chemo/radio–resistance.

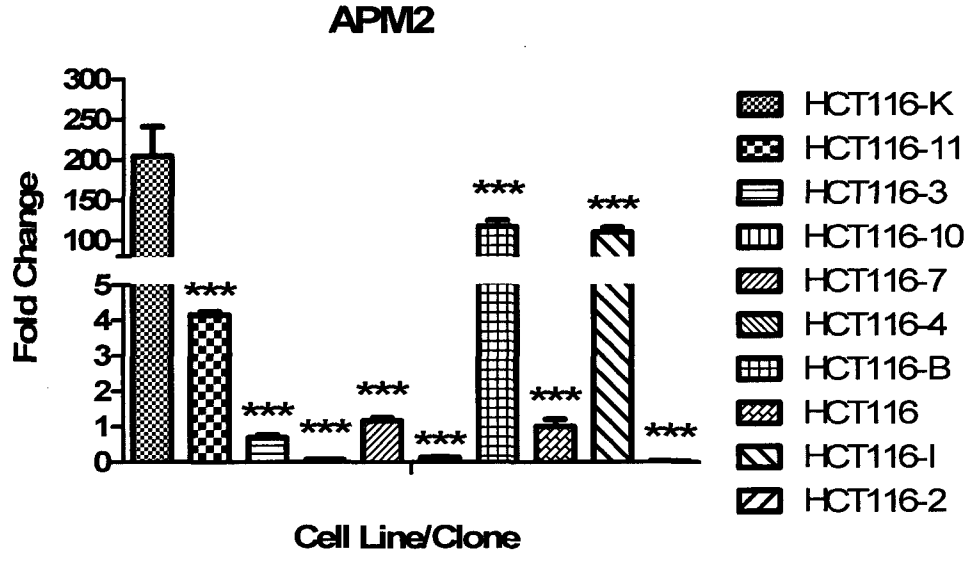

Figure 12: Expression of APM2 in a panel of HCT116 clones, and the parental cell line. Real time RT-PCR was performed to assess the relative expressions of APM2 in 9 HCT116 clones and the parental cell line. APM2 expression was normalized to the expression of β-actin. *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$, by One-way ANOVA and Dunnett's post-hoc testing vs. expression in HCT116-K. Results are presented as the mean of at least 3 experiments ± SEM.

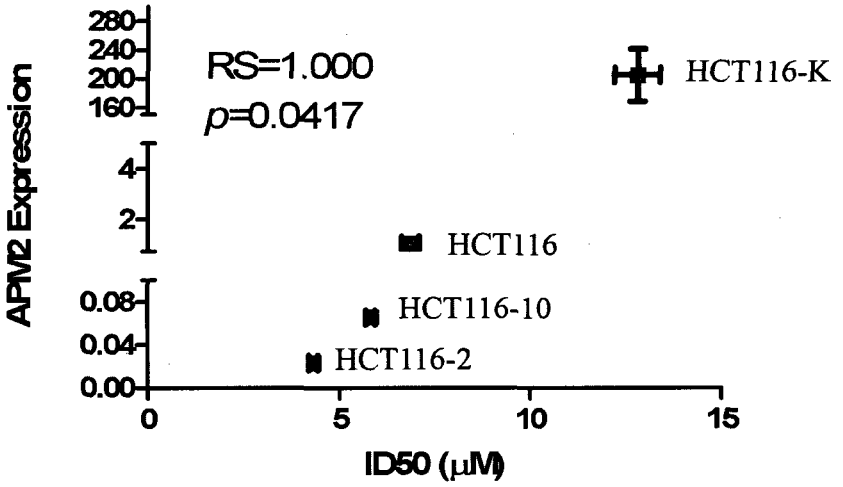

Figure 13: APM2 expression correlates with cisplatin resistance in HCT116-2, HCT116-10, HCT116, and HCT116-K. APM2 expression was measured by real-time RT-PCR and plotted as a function of ID50 to cisplatin. Points are presented as the means of at least 3 experiments ± SEM in both the x and y direction. Correlation was found to be significant by Spearman's rank correlation test. RS = 1.000, $p<0.05$.

3.1.4. - Northern blotting confirms the existence of the APM2 transcript and overexpression by HCT116-K. Western blotting confirms protein expression.

No reports pertaining to APM2 function, transcription, and/or translation exist. Because there have been no studies relating to the function of this gene it was unclear whether the *APM2* gene is actually transcribed and translated. To verify this, northern blotting was performed on total RNA isolated from the parental HCT116 cell line, HCT116-10, HCT116-2, and HCT116-K (Figure 14. a, b). Northern blotting revealed that *APM2* is transcribed and detectable in the parental HCT116 cell line and in HCT116-K. Furthermore, northern blotting reconfirmed the microarray findings that APM2 is highly overexpressed in HCT116-K when compared to HCT116, HCT116-10, or HCT116-2 (Figure 14 a, b). In addition, the mRNA detected by the APM2 DNA probe migrated at approximately 600 bp (Figure 14 b). This corresponds to the expected mRNA size based on entry NM_006829 in the NCBI's nucleotide database.

To determine whether or not this transcript was translatable, immunoblotting was performed. As no antibody specific to APM2 is commercially available, a custom antibody was produced using a 12 amino-acid peptide corresponding to the N-terminal region of APM2. Using this antibody, endogenous expression of the APM2 protein was verified in HCT116 cells (Figure 14 c, lane EV). In order to be sure that this antibody recognized the full length APM2, HCT116 cells were transfected with an expression vector coding for the full length APM2. As can be seen in Figure 14c, transfection with this vector resulted in greatly increased expression of APM2. The fact that the overexpressed protein migrates the same distance as the endogenous protein supports the claim that this antibody is APM2 specific and that the endogenous APM2 is the predicted size.

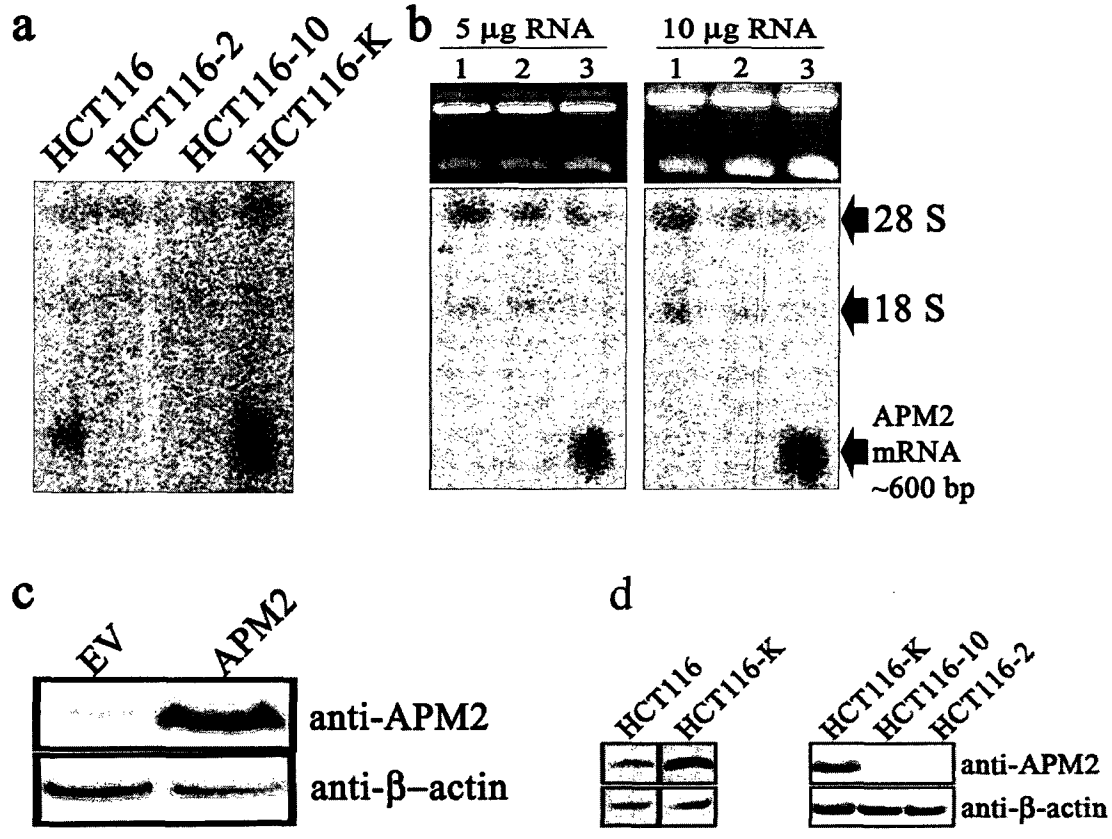

Figure 14: APM2 transcripts are detectable in HCT116 and HCT116-K by northern blotting. APM2 mRNA is translated to protein. a) Northern blot performed using total RNA from HCT116, HCT116-2, HCT116-10 and HCT116-K. APM2 transcript is detectable in HCT116 and HCT116-K b) Formaldehyde gel, showing RNA quality, and northern blots of APM2 in HCT116-10, HCT116-2 and HCT116-K. c) Western blot verifying the expression of APM2 in HCT116 cells, APM2 transfected cells were added as a positive control and to verify the specificity of a custom APM2 antibody. d) Western blots showing the relative expression of APM2 in HCT116, HCT116-K, HCT116-10, and HCT116-2.

3.2. – INVESTIGATING THE EFFECT OF APM2 EXPRESSION ON RADIATION AND CHEMOSENSITIVITY, IN VITRO

3.2.1. - Overexpression of APM2 does not affect the survival of cells treated with X-radiation, etoposide, or topotecan

HCT116-K is resistant to several forms of cancer treatment. These include treatment with topotecan, etoposide, camptothecin, and cisplatin [Qutob et al., 2004]. Conversely, it was characterized as radiosensitive compared to HCT116, HCT116-10 and HCT116-2. APM2 was selected for further investigation with respect chemoresistance and radiosensitivity for several reasons. First, APM2 was identified as the most highly overexpressed gene in HCT116-K, relative to the more chemosensitive/radioresistant HCT116-10. Second, APM2 maintained that high overexpression over a number of passages. Finally, APM2 expression was significantly increased in HCT116-K compared to all other HCT116 clones tested, reducing the chance that this observation was merely due to clonal variation. In addition, HCT116-2, displaying opposite drug and XR responses to HCT116-K, greatly suppressed expression of APM2.

To investigate whether APM2 expression plays a role in cellular resistance to chemotherapeutics or X-radiation, cells were transfected with a Flag-APM2 expression vector. HCT116-2 and HCT116-10 were chosen as transfection hosts as they were both more sensitive to chemotherapeutics, and more resistant to X-radiation than HCT116-K. In addition, APM2 expression was very low in these clones compared to expression in HCT116-K (Figure 12, 14a, b). Figure 15a, b show that transfection of either HCT116-10 or HCT116-2 with the pCMV-Flag-APM2 expression vector results in the expression of a Flag-APM2 fusion protein, at 48 h, in both clones. Furthermore, immunofluorescence, using a monoclonal Flag antibody (Sigma), demonstrated the presence of the Flag-APM2

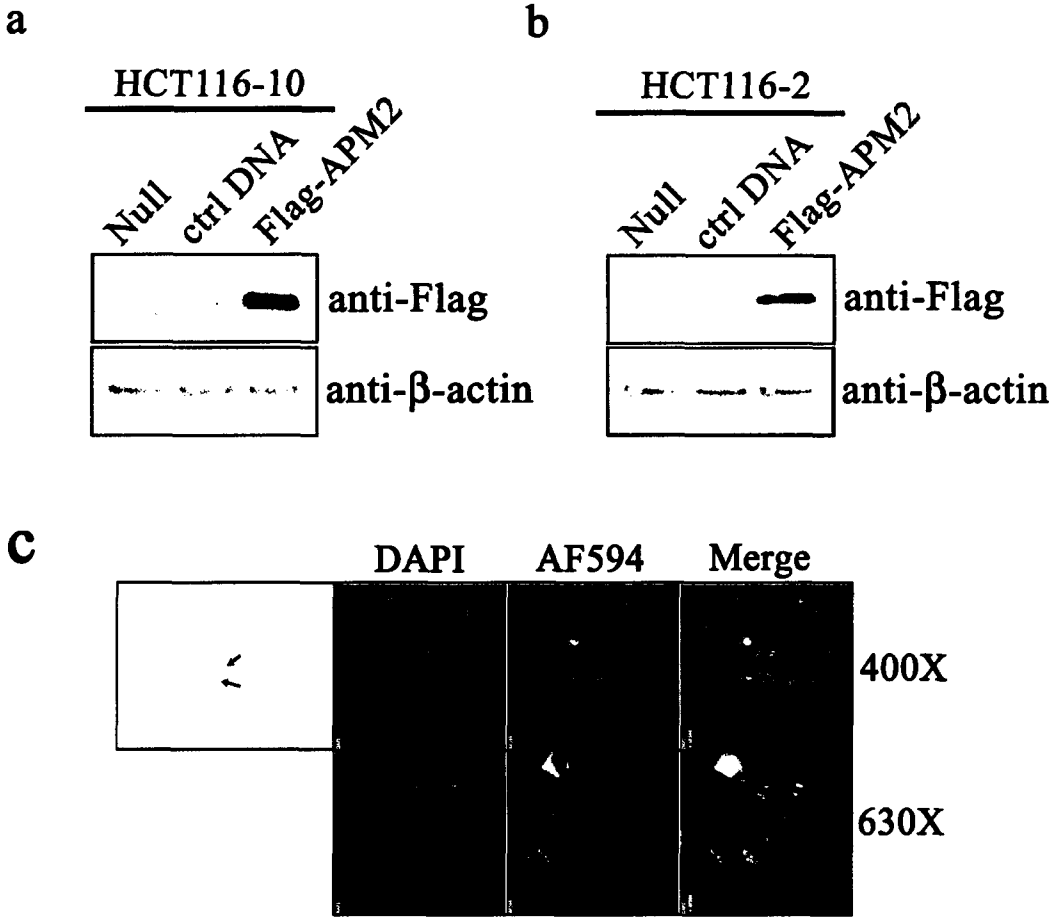

Figure 15: pCMV-Flag-APM2 Transfected HCT116-10 or HCT116-2 express a Flag-APM2 fusion protein. a,b) HCT116-10 and HCT116-2 were transfected with an empty vector or pCMV-Flag-APM2. Expression of the Flag-APM2 fusion was verified by immunoblotting using an anti-Flag antibody. c) Immunofluorescence showing the presence and distribution of the Flag-APM2 fusion in HCT116-10 cells.

fusion protein in the cytoplasm of transfected HCT116-10 cells (Figure 15c). Untransfected cells were used as a negative control and showed no immunofluorescence.

HCT116-10 and HCT116-2 cells, overexpessing APM2, were treated with chemotherapeutics or X-radiation and their survival was measured by clonogenic assay. The response of either clone, treated with X-radiation (0 – 8 Gy), was not affected by increased APM2 expression (Figure 16) when compared to control DNA transfected cells. In HCT116-2 transfection with either control DNA or APM2 appeard to increase the sensitivity of these cells but this was not related to APM2 expression. Survival curves were fit with the linear quadratic model using SigmaPlot 8.0.

These same cell lines were transfected with the APM2 fusion protein, or control DNA, and subjected to treatment with the topoisomerase II inhibitor, etoposide (0-40 μM). Neither HCT116-10's nor HCT116-2's response to etoposide was affected by increased expression of APM2 (Figure 17). Similarly, no differences were observed when the same experiments were performed with the topoisomerase I inhibitor, topotecan (0 – 40 μM) (Figure 18).

3.2.2. - Overexpression of APM2 results in increased resistance to cisplatin in HCT116 clones

Responses to etoposide, topotecan, or X-radiation treatment were not affected by forced expression of APM2 in HCT116-10 or HCT116-2. However, when APM2 was overexpressed in these cells, resistance to cisplatin was significantly increased (Figure 19, Table 4). Table 4 lists the ID50s of control transfected cells and cells transfected with APM2 for all drugs tested. Statistical differences between the control and APM2 transfected cells were assessed by the student's t-test. No significant differences were found between control and APM2 transfected cells for treatments with etoposide, topotecan, or X-

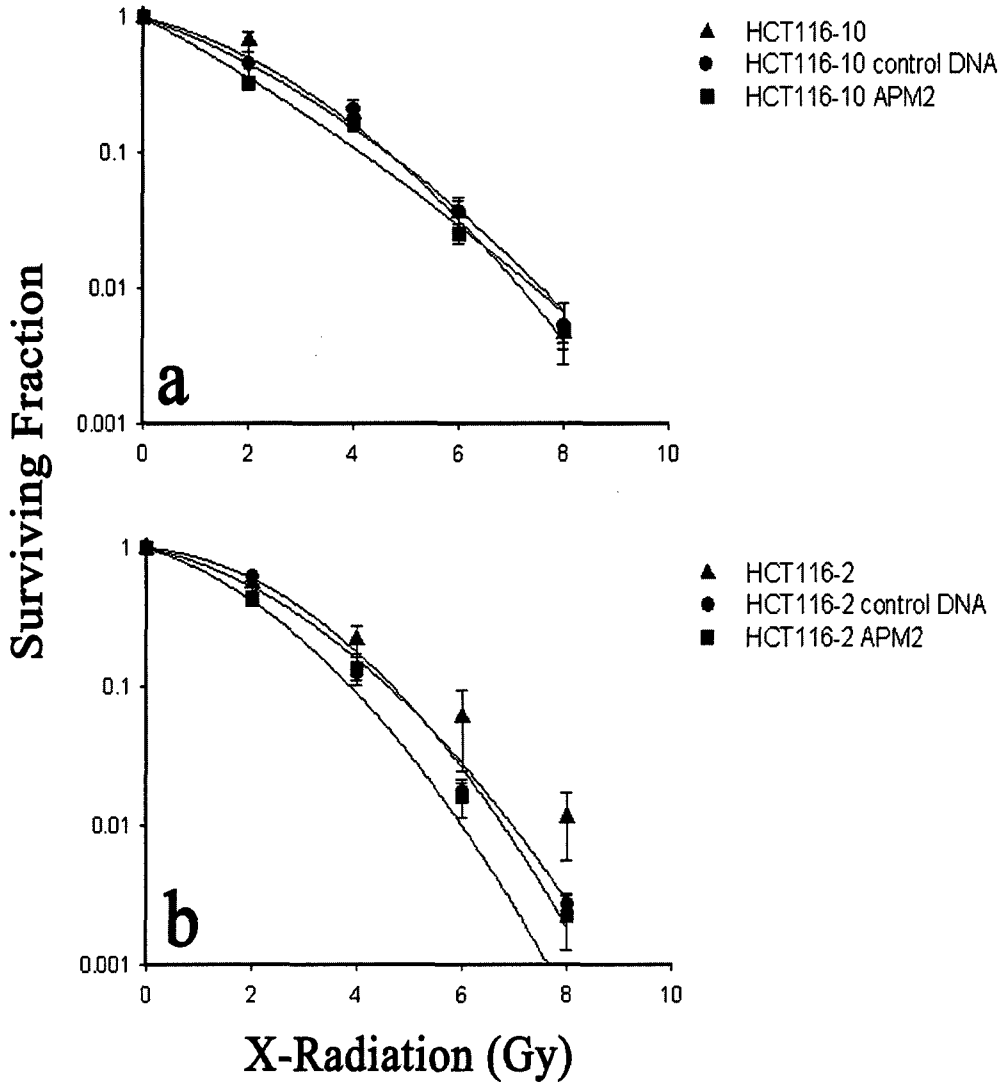

Figure 16: APM2 overexpression does not affect the response of HCT116-10 or HCT116-2 to X-radiation. a) The survival of HCT116-10 cells transfected with an APM2 expression vector and subjected to treatment with X-radation (0-8 Gy). b) same as in a, using HCT116-2 cells. Points represent the mean of 3 – 5 experiments ± SEM.

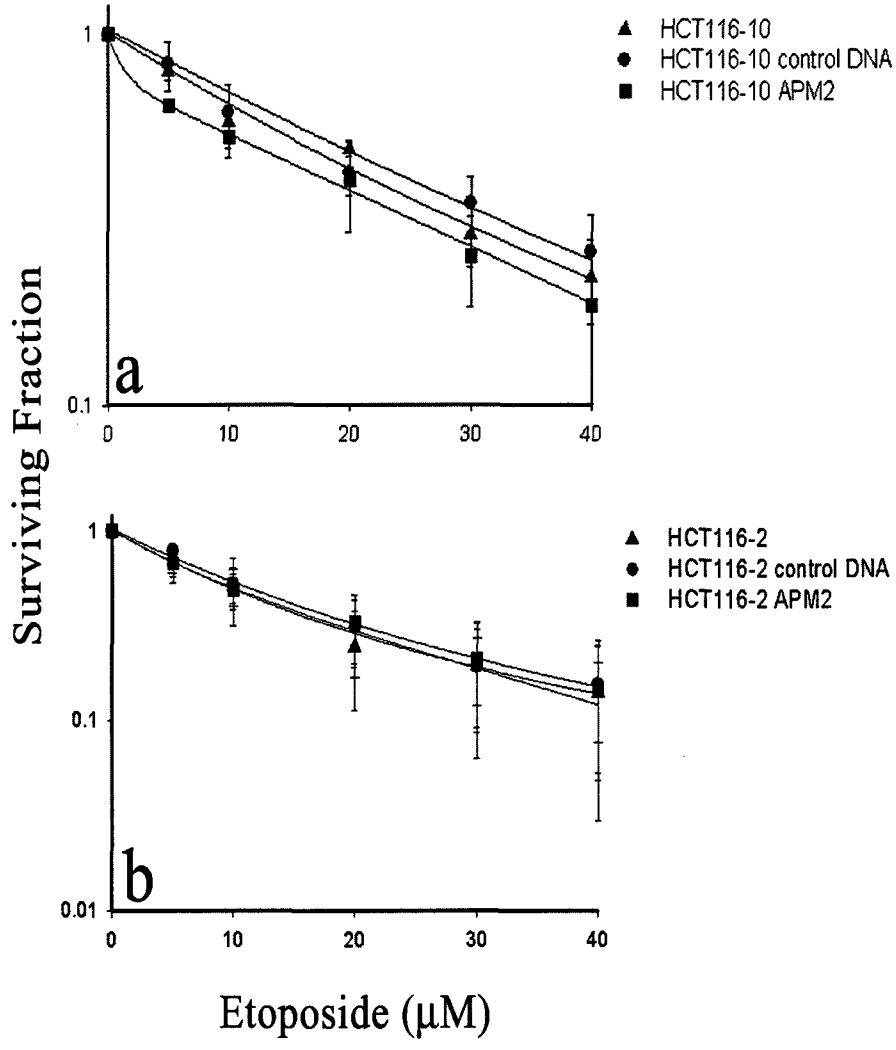

Figure 17: APM2 overexpression does not affect the response of HCT116-10 or HCT116-2 to etoposide. a) The survival of HCT116-10 cells transfected with an APM2 expression vector and subjected to treatment with etoposide (0-40 μM). b) same as in a using HCT116-2 cells. Points represent the mean of 3 – 5 experiments ± SEM.

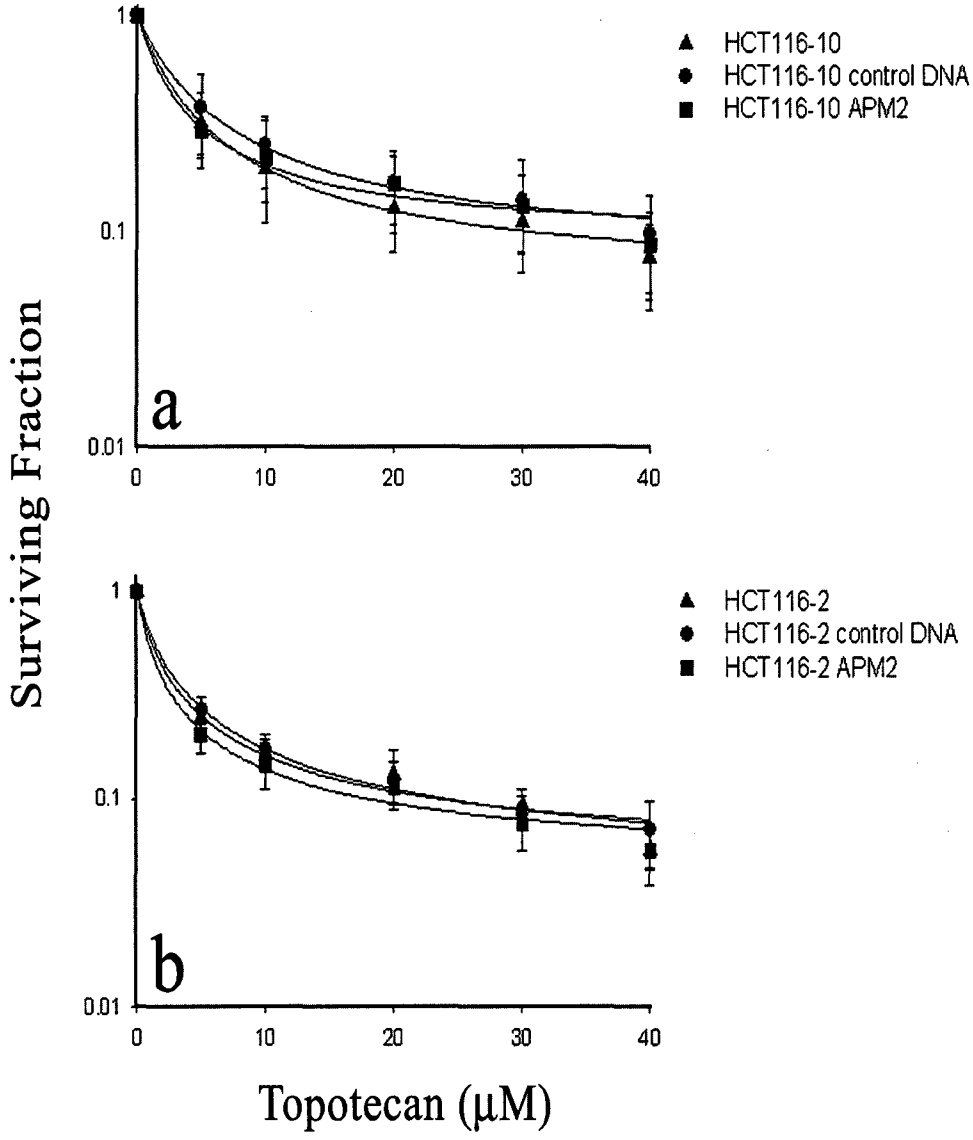

Figure 18: APM2 overexpression does not affect the response of HCT116-10 or HCT116-2 to topotecan. a) The survival of HCT116-10 cells transfected with an APM2 expression vector and subjected to treatment with topotecan (0-40 μM). b) same as in a using HCT116-2 cells. Points represent the mean of 3 – 5 experiments ± SEM.

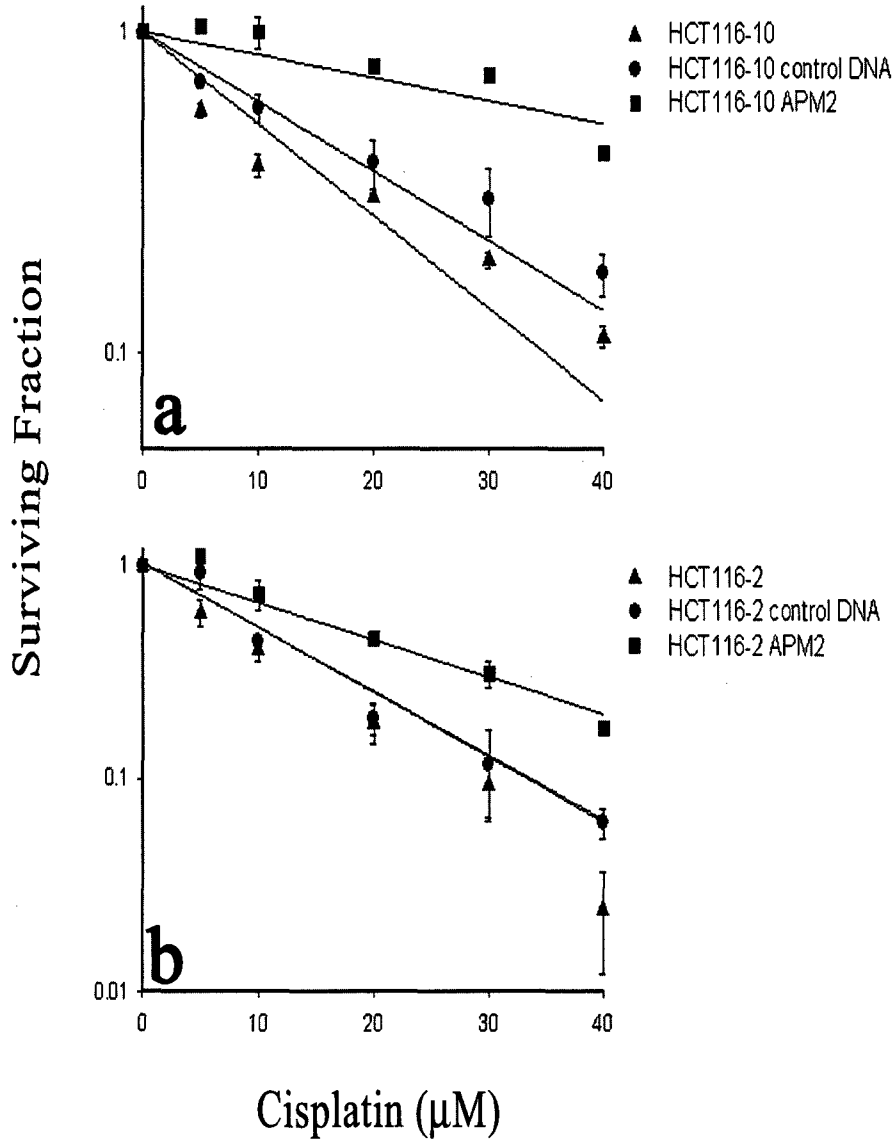

Figure 19: APM2 overexpression does affect the response of HCT116-10 and HCT116-2 to cisplatin. a) Survival of HCT116-10 cells transfected with an APM2 expression vector and subjected to treatment with cisplatin (0-40 μM). b) Same as in a) except HCT116-2 cells were used. Points represent the mean of 3 – 5 experiments ± SEM. Where error bars are not visible they are smaller than point symbols.

radiation. However, both cell lines, when overexpressing APM2, were significantly more resistant to cisplatin then control cells. The mean ID50 of HCT116-10 was 2.4 times greater when expressing APM2, while APM2-expressing HCT116-2 had a mean ID50 that was increased by a factor of 2.2. Table 4 lists the *p*-value associated with these differences.

3.2.3. - Silencing APM2 results in sensitization of the resistant HCT116-K to cisplatin

Since overexpression of APM2 resulted in increased cisplatin resistance, in relatively sensitive HCT116 derived cell lines, decreasing the expression of APM2 was investigated as a method of enhancing cisplatin sensitivity. HCT116-K is relatively resistant to cisplatin and expresses high levels of APM2; it was used as a model to investigate whether decreasing APM2 expression by RNA interference could potentially enhance cisplatin response. Figure 20 shows the effectiveness of several unique siRNA duplexes (siAPM2-duplex1 (siAPM2-D1), duplex2, duplex3, duplex4 (siAPM2-D4)) at silencing APM2 at the RNA level in HCT116-K. SiAPM2-D1 and –D4 were both very effective silencers of APM2 at concentrations of 50 and 100 nM. Silencing with siAPM2-duplex3 was also effective, but APM2 silencing was not as great as that observed with siAPM2-D1 or -D4. Silencing with siAPM2-duplex 2 was ineffective. To assess the efficiency of our transfection method, both HCT116 and HCT116-K were transfected with a Cy3 labelled siRNA. Fluorescence microscopy indicated that siRNA was delivered to cells in a highly efficient manner with >90% of cells fluorescing 24 h after transfection (Figure 21).

Clonogenic assays were carried out to assess the survival of APM2 depleted cells versus untransfected cells and cells transfected with a Non-targeting siRNA (siNT). As can be seen in Figure 22a, b, transfection of HCT116-K with siAPM2-D1 or siAPM2-D4

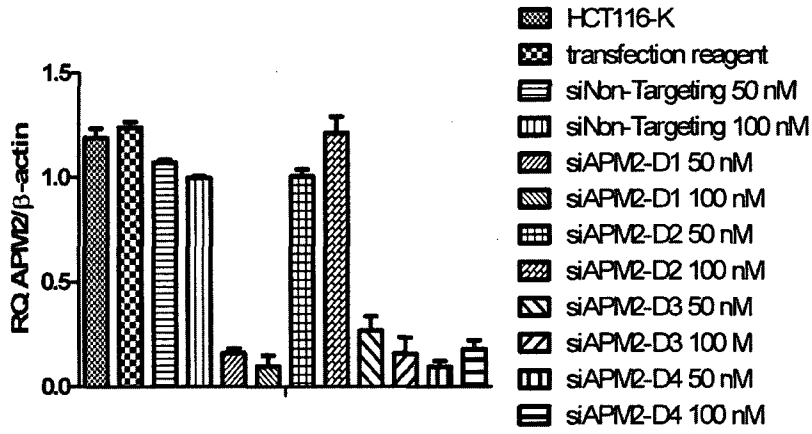

Figure 20: Transfection of HCT116-K with siRNAs against APM2 resulted in decreased levels of APM2 mRNA. HCT116-K cells were transfected with 50 or 100 nM siRNA. After 48 h total RNA was collected and APM2 expression was measured by real-time RT-PCR. 3 of the 4 siAPM2 duplexes were effective silencers of APM2 expression. Points represent the mean of 3 experiments ± SEM.

Figure 21: Transfection of HCT116-K cells with Cy3 labelled siRNA demonstrated high transfection efficiency. a) HCT116- K cells were transfected with 100 nM Cy3 labelled RNA. Cells were stained with DAPI and visualized by fluorescence microscopy. Cells were observed and photographed under the 40X objective. Magnification is 400X.

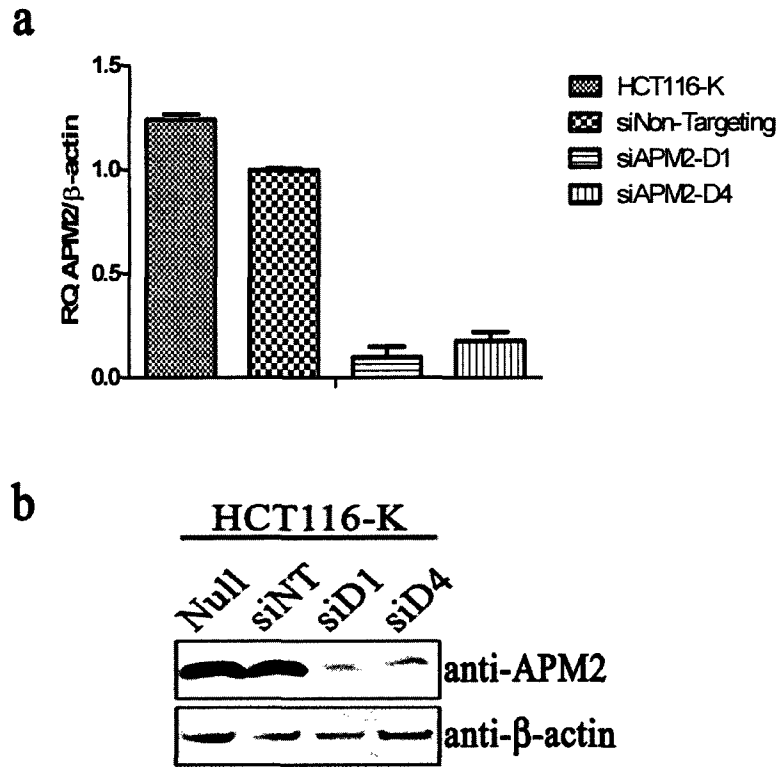

Figure 22: Transfection of HCT116-K with siAPM2 duplex1 or siAPM2 duplex2 reduced APM2 mRNA and protein levels. a) mRNA was collected from HCT116-K cells transfected with one of two siRNAs against APM2 or control siRNAs. Real time RT-PCR was used to assess the level of knockdown achieved with each duplex. Bars represent the mean of 3 experiments ± SEM. b) Immunoblotting was performed to assess the level of APM2 knockdown at the protein level. mRNA and protein were collected 48 h after transfection.

Table 4: Cytotoxicity of Chemotherapeutics and X-radiation on Mock transfected HCT116 clones or HCT116 clones Overexpressing APM2

Clone	Treatment	ID_{50} control transfected*†	ID_{50} *APM2* transfected*	*p-value*
HCT116-10	Etoposide	17.4 +/- 3.3 μM	14.8 +/- 4.9 μM	N.S.‡
	Topotecan	4.4 +/- 0.4 μM	3.7 +/- 0.3 μM	N.S.
	X-radiation	1.9 +/- 0.4 Gy	1.5 +/- 0.06 Gy	N.S.
	Cisplatin	14.9 +/- 2.2 μM	35.85 +/- 1.7 μM	0.000537
HCT116-2	Etoposide	12.2 +/- 2.6 μM	12.7 +/- 3.6 μM	N.S.
	Topotecan	3.7 +/- 0.3 μM	3.3 +/- 0.3 μM	N.S.
	X-radiation	2.3 +/-0.2 Gy	1.8 +/- 0.2 Gy	N.S.
	Cisplatin	8.8 +/- 0.2 μM	19.1 +/- 0.3 μM	0.001532

** ID_{50} refers to the concentration in μM for drugs, or Gy for radiation, necessary to inhibit colony formation by 50%.*

† Control transfected cells were transfected with the same DNA as APM2 transfected cells minus the APM2 insert.

‡ N.S – not significant by student's t-test ($p > 0.05$).

resulted in a robust decrease in the levels of both APM2 mRNA and APM2 protein after 48 h. The effect of APM2 depletion on cisplatin sensitivity is illustrated in Figure 23. Silencing of APM2, by siAPM2-D1, effectively sensitized HCT116-K to cisplatin-induced death. The statistical significance of the differences in sensitivity observed at CDDP concentrations 10, 20, and 40 μM are displayed in Table 6 along with the surviving fractions of the different expression profiles.

3.2.4. - Silencing APM2 results in sensitization of a variety of cancer cell types regardless of MMR and/or p53 status

As MMR and p53 status are major factors controlling cisplatin response [Fan et al., 1994; Lin and Howell, 2006; Segal-Bendirdjian et al., 1998; Siddik, 2003; Siddik et al., 1998; Vaisman et al., 1998], we investigated whether APM2 depletion could effectively sensitize cell lines, regardless of these two factors. Cell lines covering the full range of p53 and MMR proficient and deficient combinations were chosen for APM2 depletion and cisplatin treatment. The cell lines are listed in Table 5 and include HCT116 (p53+/MMR-) [Lin et al., 2000; Robinson et al., 2003], SW620 (p53-/MMR+)[Flanagan et al., 2008; Vegesna et al., 1999], MCF-7 (p53+/MMR+) [Schwarz et al., 2002; Vegesna et al., 1999], PC-3 (p53-/MMR-) [Chen et al., 2001; Zhang et al., 2003], and OV2008 (p53+/MMR+) [Aebi et al., 1996; Fraser et al., 2003]. Figures 24a,b, 25a,b, 26,ab 27a, and 28a show the effectiveness of APM2 siRNAs at silencing cellular APM2 in each of the above mentioned cell lines. APM2 knockdown was found to be greater than 75% in all cell lines. Sensitivity to cisplatin for each cell line was determined by clonogenic assay performed 48hrs after transfection with siRNAs. Figures 24c, 25c, 26c, 27b, and 28b illustrate the sensitization of each cell line to cisplatin by APM2 siRNA, regardless of p53 or MMR status. To confirm

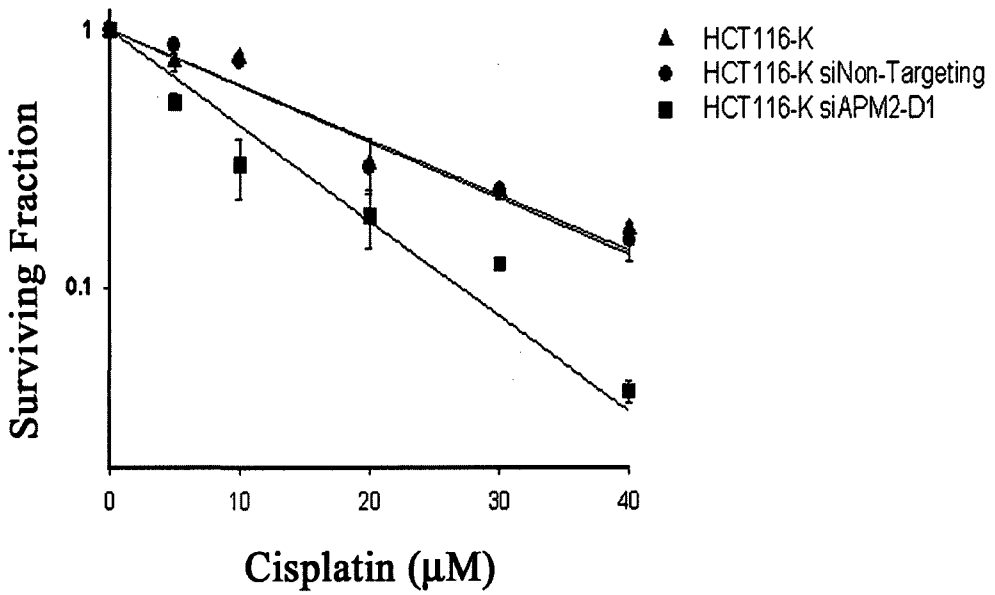

Figure 23: Silencing APM2 sensitized HCT116-K cells to cisplatin. a) SiAPM2 duplex1 was used to silence APM2 expression. The survival of cells with silenced APM2 was compared to that of cells transfected with a non-targeting siRNA and untransfected cells. Points represent the mean of 3 – 5 experiments ± SEM.

Table 5: p53 and MMR status of Cell lines used to assess cisplatin sensitivity after APM2 silencing

Cell Line	*Cell Type*	*p53 Status*	*MMR status*
HCT116	Colorectal carcinoma	+	-
SW620	Colorectal carcinoma	-	+
MCF-7	Breast carcinoma	+	+
PC-3	Prostate carcinoma	-	-
OV2008	Ovarian carcinoma	+	+

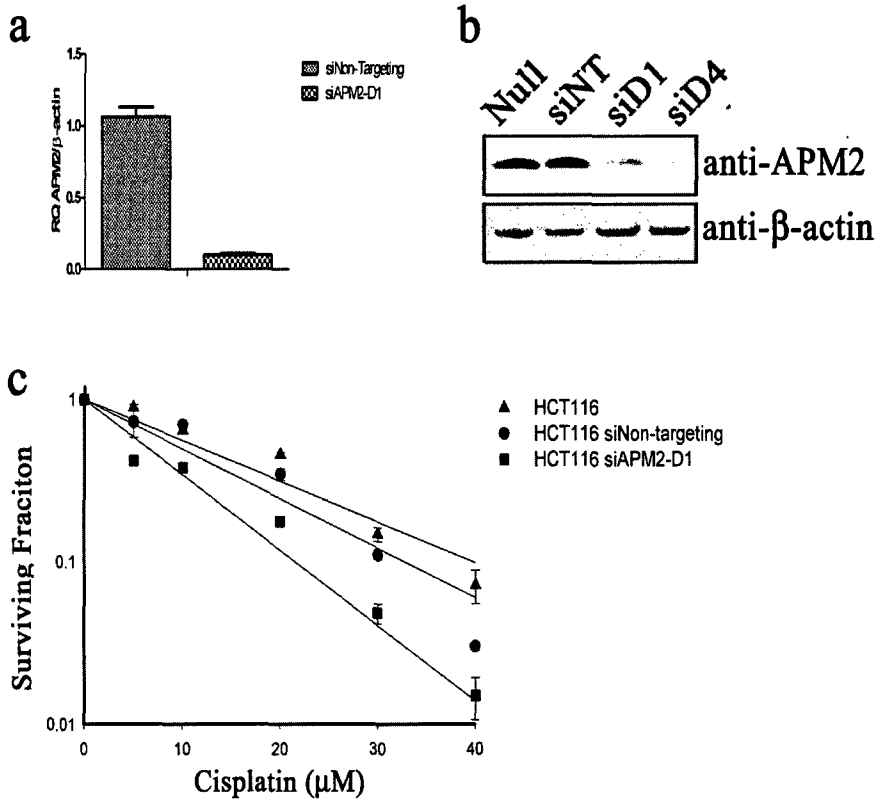

Figure 24: Silencing APM2 sensitizes HCT116 cells to the effects of cisplatin. a) Real time RT-PCR was used to assess APM2 knockdown at the mRNA level in HCT116 cells transfected with one of two siRNAs against APM2. b) Immunoblotting was used to assess the level of APM2 silencing achieved in HCT116 cells after transfection with one of two siRNAs against APM2 c) Survival of HCT116 cells with silenced APM2 was compared to the survival of non-targeting siRNA transfected and untransfected HCT116 cells. Points represent the mean of 3 – 5 experiments ± SEM.

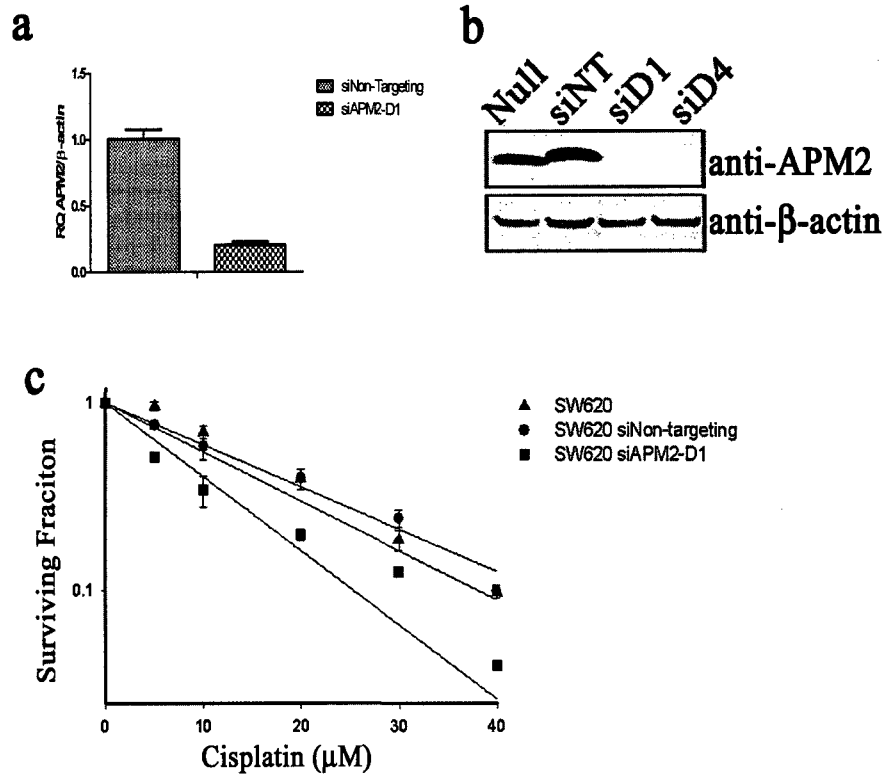

Figure 25: Silencing APM2 sensitized SW620 cells to the effects of cisplatin. a) Real time RT-PCR was used to assess APM2 knockdown at the mRNA level in SW620 cells transfected with one of two siRNAs against APM2. b) Immunoblotting was used to assess the level of APM2 silencing achieved in SW620 cells after transfection with one of two siRNAs against APM2 c) Survival of SW620 cells with silenced APM2 was compared to the survival of non-targeting siRNA transfected and untransfected SW620 cells. Points represent the mean of 3 – 5 experiments ± SEM.

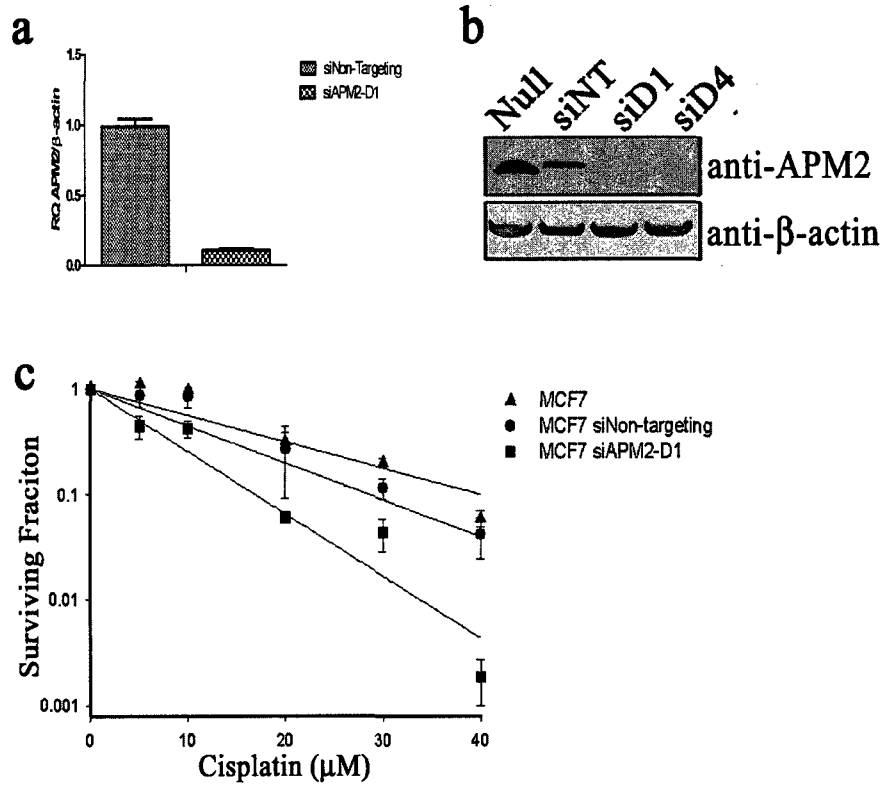

Figure 26: Silencing APM2 sensitized MCF7 cells to the effects of cisplatin. a) Real time RT-PCR was used to assess APM2 knockdown at the mRNA level in MCF7 cells transfected with one of two siRNAs against APM2. b) Immunoblotting was used to assess the level of APM2 silencing achieved in MCF7 cells after transfection with one of two siRNAs against APM2 c) Survival of MCF7 cells with silenced APM2 was compared to the survival of non-targeting siRNA transfected and untransfected MCF7 cells. Points represent the mean of 3 – 5 experiments ± SEM.

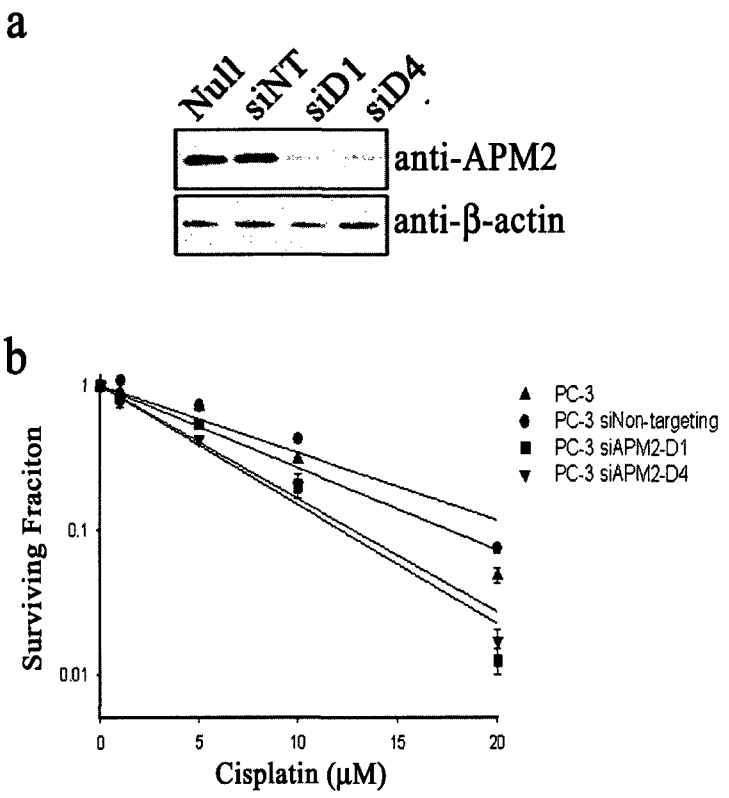

Figure 27: Silencing APM2 sensitized PC-3 cells to the effects of cisplatin. a) Immunoblotting was used to assess the level of APM2 silencing achieved in PC-3 cells after transfection with one of two siRNAs against APM2 b) Survival of PC-3 cells with silenced APM2 was compared to the survival of non-targeting siRNA transfected and untransfected PC-3 cells. Points represent the mean of 3 – 5 experiments ± SEM.

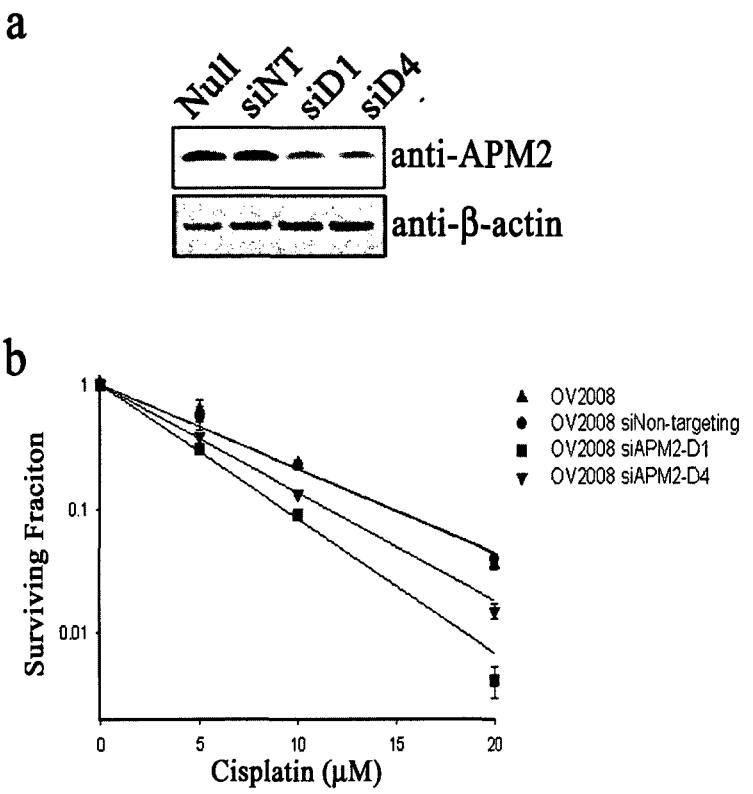

Figure 28: Silencing APM2 sensitized OV2008 cells to the effects of cisplatin. a) Immunoblotting was used to assess the level of APM2 silencing achieved in OV2008 cells after transfection with one of two siRNAs against APM2 b) Survival of OV2008 cells with silenced APM2 was compared to the survival of non-targeting siRNA transfected and untransfected OV2008 cells. Points represent the mean of 3 experiments ± SEM.

that this effect was not siRNA specific, survival assays were carried out on PC3 and OV2008 using a second siRNA (siAPM2-D4) targeting a unique region of the APM2 mRNA. Significant sensitization of theses cell lines was observed regardless of whether siAPM2-D1 or siAPM2-D4 was used to silence APM2, strengthening the argument that sensitization is not due to off-target effects of the siRNA duplexes. Student's t-tests were carried out for several doses along each curve comparing the mean surviving fraction (SF) of APM2 silenced cells to the mean SF of siNT transfected cells. The results of these tests are listed in Table 5.

3.2.5. - Clones stably expressing shRNAs against APM2 are sensitized to cisplatin

The role of APM2 levels in the response to cisplatin was further investigated by transfecting HCT116 and HCT116-K with pSil-shNT, pSil-shAPM2-1, or pSil-shAPM2-4. Clones stably expressing each construct were isolated by puromycin selection and passaged normally. The cisplatin response of each clone stably expressing shNT (HNT-clone#, KNT-clone#), shAPM2-1 (HD1-clone#, KD1-clone#), or shAPM2-4 (HD4-clone#, KD4-clone#) was measured by clonogenic assay. Immunoblotting confirmed whether or not transfection and selection resulted in blocked APM2 expression (Figures 29a, 30a). Figures 29b and 30b exhibit the responses of clones expressing shNT, shD1, or shD4, to cisplatin. Almost all clones expressing shRNAs against APM2 were significantly more sensitive to cisplatin than their shNT-expressing counterparts. Interestingly, one clone, HD4-3, survived puromycin selection yet still expressed APM2. When the clonogenic capacity of HF4-3 was measured, it was found to be as resistant to cisplatin as the parental cell line or clones expressing shNT. HCT116-10 was added to Figure 30b to show that HCT116-K has

Table 6: Surviving fractions of cell lines transfected with Non-targeting siRNA or siAPM2 and treated with cisplatin and their associated p-values.

Cell line	*siRNA**	*SF† 10uM CDDP*	*p-value‡*	*SF 20 uM CDDP*	*p-value*	*SF 40 uM*	*p-value*
HCT116-K	siNon-targeting	0.758 ± 0.0104	-	0.291 ± 0.0881	-	0.153 ± 0.0269	
	siAPM2 duplex1	0.299 ± 0.0783	0.0202	0.190 ± 0.0488	N.S.	0.0397 ± 0.00408	0.014
HCT116	siNon-targeting	0.698 ± 0.0171	-	0.346 ± 0.0277	-	0.0302 ± 0.00156	
	siAPM2 duplex1	0.380 ± 0.00633	<0.0001	0.177 ± 0.00167	0.00365	0.0149 ± 0.00435	0.0299
SW620	siNon-targeting	0.586 ± 0.0906	-	0.395 ± 0.00193	-	0.0989 ± 0.00760	
	siAPM2 duplex1	0.342 ± 0.0649	N.S.	0.197 ± 0.0135	0.00149	0.0396 ± 0.00248	0.00279
MCF-7	siNon-targeting	0.842 ± 0.174	-	0.268 ± 0.175	-	0.0421 ± 0.0176	
	siAPM2 duplex1	0.423 ± 0.0733	0.04	0.0611 ± 0.00547	N.S.	0.00186 ± 0.000862	<0.0001
	siNon-targeting	0.396 ± 0.0289	-	0.0740 ± 0.00422	-	NA	NA
PC-3	siAPM2 duplex1	0.202 ± 0.0235	0.0032	0.0125 ± 0.00250	0.0001	NA	NA
	siAPM2 duplex4	0.204 ± 0.0375	0.0065	0.0167 ± 0.00362	<0.0001	NA	NA
	siNon-targeting	0.224 ± 0.0173	-	0.0389 ± 0.00441	-	NA	NA
OV2008	siAPM2 duplex1	0.0891 ± 0.00565	0.0005	0.00414 ± 0.00118	0.0006	NA	NA
	siAPM2 duplex4	0.134 ± 0.00582	0.005	0.0149 ± 0.00207	0.0038	NA	NA

* Cell lines were transfected with siNon-targeting, siAPM2 duplex1, or siAPM2 duplex4 48 h before treatment with cisplatin. 98

† SF = Surviving fraction, assessed by clonogenic assay. SF's are the mean of 3-5 experiments. Results expressed as mean ± SEM.

‡ *p*-value = siNon-targeting vs. siAPM2 duplex1 or siAPM2 duplex4, calculated by student's *t-test.* $p<0.05$ was considered significant (N.S. – not significant).

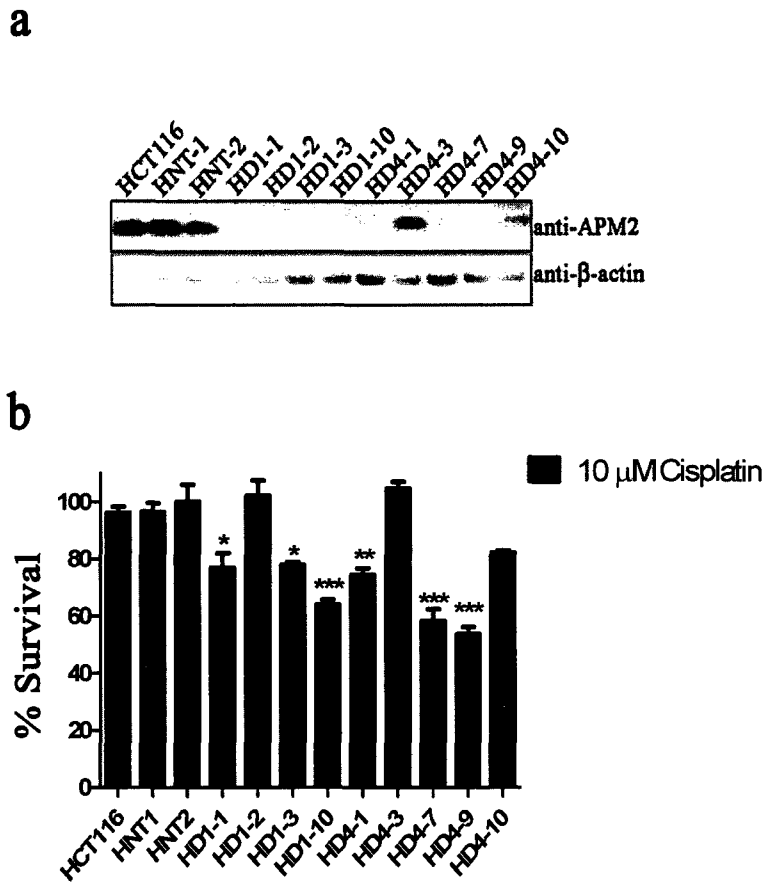

Figure 29: HCT116 clones with stably silenced APM2 were sensitized to cisplatin. a) Immunoblotting was used to confirm a reduction in APM2 at the protein level in clones stably transfected with either shAPM2-D1 or shAPM2-D4. b) Each clone's survival was measured after treatment with cisplatin (10 μM). Significant differences in survival were assessed by one-way ANOVA and Dunnett's post-hoc testing using HNT-1 as the control clone. * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$. Bars represent the mean of 3 experiments ± SEM.

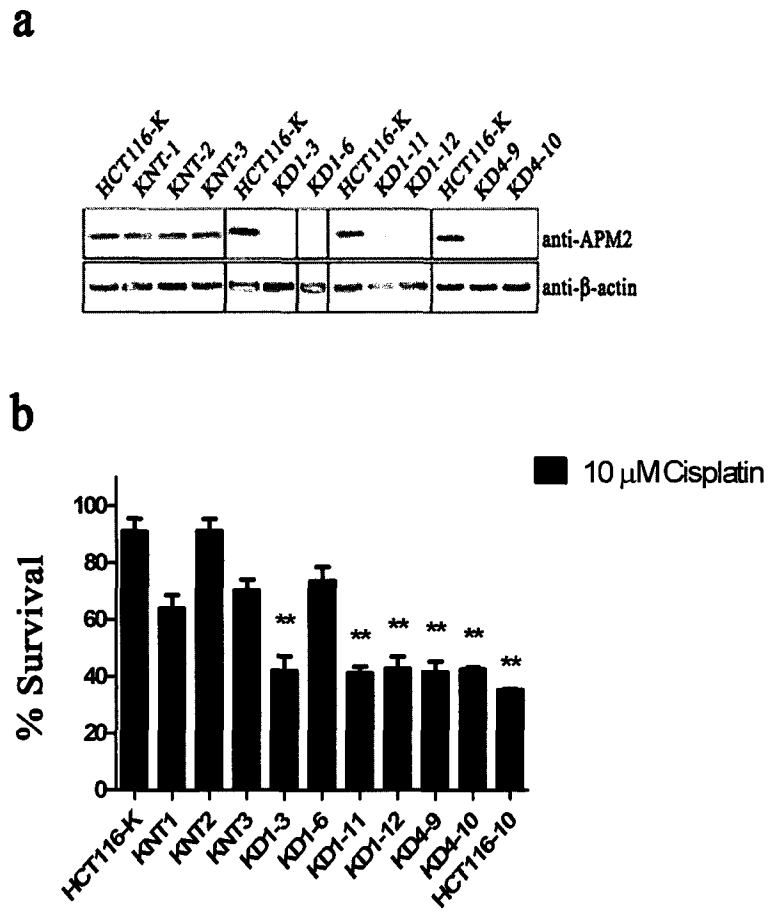

Figure 30: HCT116-K clones with stably silenced APM2 were sensitized to cisplatin. a) Immunoblotting was used to confirm a reduction in APM2 at the protein level in clones stably transfected with either shAPM2-D1 or shAPM2-D4. b) Each clone's survival was measured after treatment with cisplatin (10 μM). Significant differences in survival were assessed by one-way ANOVA and Dunnett's post-hoc testing using KNT-3 as the control clone. * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$. Bars represent the mean of 3 experiments ± SEM.

maintained its cisplatin resistant phenotype in comparison to our other HCT116 derived cell lines.

3.3. – EXAMINING THE EFFECT OF APM2 SUPPRESSION, IN VIVO, ON TUMOUR GROWTH USING A HUMAN XENOGRAFT MODEL OF CANCER

3.3.1. - HCT116-K sublines expressing shRNAs against APM2 have greatly reduced levels of APM2

Reducing the expression of APM2, *in vitro*, effectively sensitizes a number of cancer cell types to cisplatin. However, whether this method of cisplatin sensitization is effective *in vivo* is unclear. To investigate this, clones of HCT116-K were established that expressed non-targeting shRNA (HCT116-K shNT), or shRNAs against APM2 (HCT116-K shAPM2-D1, HCT116-K shAPM2-D4) (Figure 31). Figure 31a demonstrates, by immunoblotting, that the HCT116-K shAPM2-D1/D4 clones expressed greatly reduced levels of APM2, on the day of tumour cell injection, relative to either the parental or HCT116-K shNT cell lines. Densitometry (Figure 31b) was used to quantify the relative protein levels for this blot. APM2 protein levels were reduced by >85% in HCT116-K shAPM2-D1 and by greater than >90% in HCT116-K shAPM2-D4 when compared to the parental HCT116-K.

3.3.2. - Silencing of APM2 does not affect tumour growth in vivo, but enhances growth inhibition in response to cisplatin treatment

HCT116-K shNT, HCT116-K shAPM2-D1, and HCT116-K shAPM2-D4 were injected subcutaneously onto the backs of CD-1 nude mice. No significant differences in growth were observed by tumours that were allowed to grow without undergoing any form of cancer therapy, regardless of APM2 status (Figure 32). However, when mice were

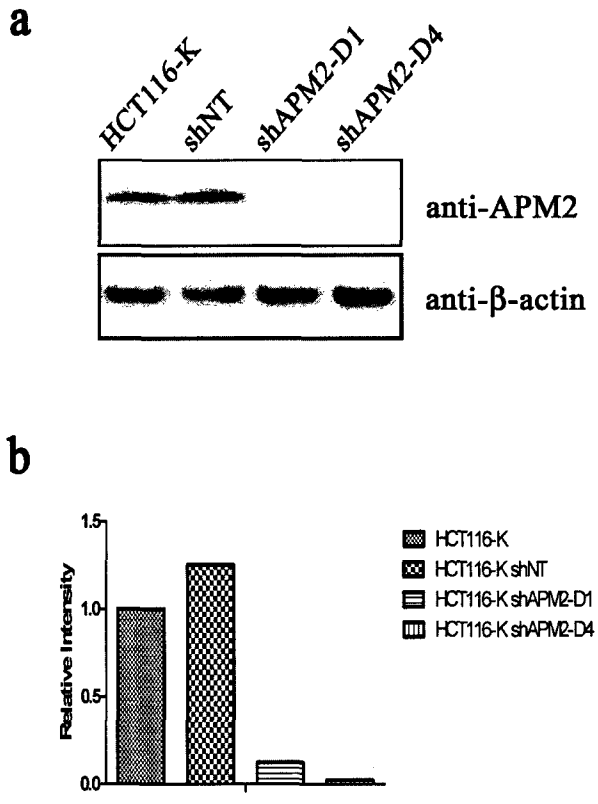

Figure 31: Expression of APM2 in shNT and shAPM2 HCT116-K clones used for seeding tumours in vivo. a, b) Immunoblot performed on shRNA expressing HCT116-K whole cell lysates and corresponding densitometry. Whole cell lysates were collected at the time of xenograft seeding.

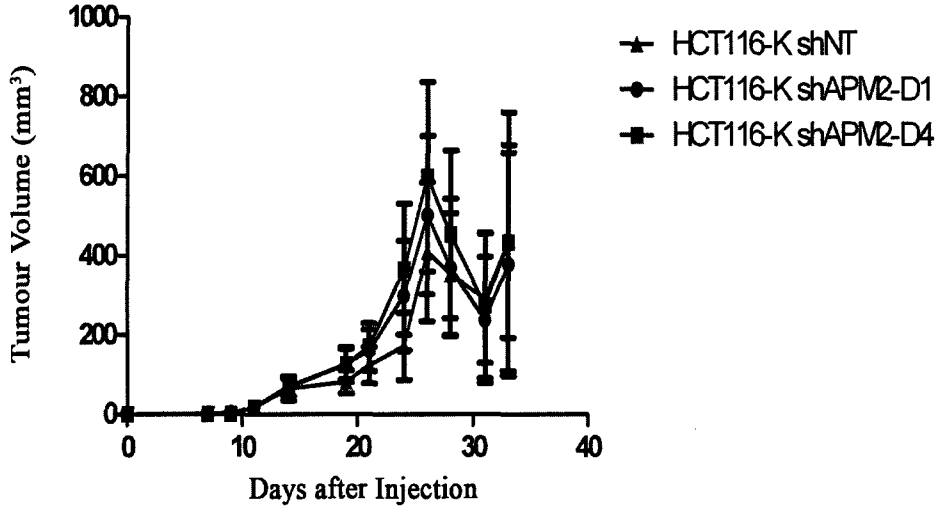

Figure 32: APM2 expression does not affect the growth of untreated HCT116-K xenografts in vivo. HCT116-K clones expressing shNT, shAPM2-D1, or shAPM2-D4 were injected onto the backs of CD-1 nude mice and their growth was measured over a period of 5 weeks. The sudden drop in tumour sizes at day 30 is the result of ethical endpoints relating to tumour size. Points represent the mean of 12-16 mice ± SEM.

treated with cisplatin (3 x 4 mg/Kg), APM2 silenced tumours were significantly more growth inhibited than tumours expressing a non-targeting shRNA (RANOVA, $p<0.0001$) (Figure 33). In addition, Tukey-Kramer post-hoc testing confirmed that cisplatin treated HCT116-K-shAPM2-D1, and HCT116-K-shAPM2-D4, tumours were significantly smaller than cisplatin treated HCT116-shNT tumours ($p<0.001$) but not significantly different from each other ($p>0.05$) [Gillespie et al., 2007]. This indicates that silencing with either shRNA resulted in the same level of growth inhibition when mice received cisplatin treatment.

3.3.3. - OV2008 clones expressing shAPM2-D1 or shAPM2-D4 have reduced levels of APM2

To confirm our findings that APM2 silencing is capable of enhancing cisplatin response, *in vivo*, similar experiments were performed using a second cell line. Since ovarian cancers are frequently treated with platinum, OV2008 cells were chosen for these experiments. Obtaining OV2008 clones that stably expressed shNT, shAPM2-D1, or shAPM2-D4 proved to be more difficult than with other cell lines. OV2008 was highly sensitive to puromycin selection. While HCT116 and HCT116-K required doses of 2μg/ml for selection, most OV2008 cells died when treated with as little as 0.5 μg/ml. However, clones expressing shNT, shAPM2-D1, or shAPM2-D4 were generated. While the silencing of APM2 by shAPM2-D1 was approximately 90% relative to OV2008 cells expressing the non-targeting shNT, silencing by shAPM2-D4 was not particularly effective and reduced APM2 protein levels by <40% (Figure 34a, b).

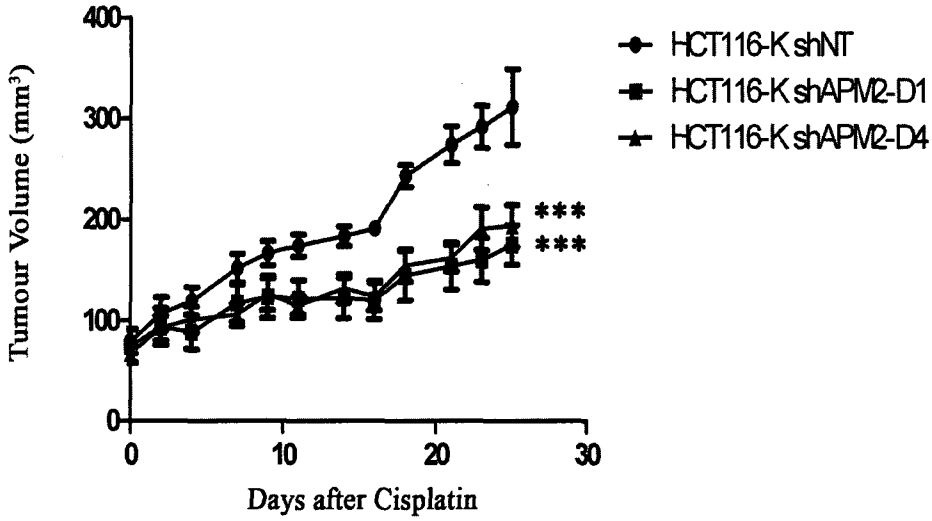

Figure 33: Silencing APM2 in HCT116-K tumour xenografts enhances the effects of cisplatin treatment. a) HCT116-K clones expressing shNT, shAPM2-D1, or shAPM2-D4 were injected onto the backs of CD-1 nude mice. When tumours reached the appropriate size mice received 4 mg/Kg cisplatin thrice over one week. Tumour growth was monitored for several weeks after treatment. Significant differences were assessed by repeated measures ANOVA followed by Tukey-Kramer post-hoc testing to assess differences between groups. *- $p<0.05$, **- $p<0.01$, ***- $p<0.001$. p-values on graph represent significance of each group versus the control, shNT, group. Points represent the mean of 6-12 mice ± SEM

3.3.4. - Reducing APM2 expression enhances the response of ovarian carcinoma derived tumour xenografts to cisplatin

Contrary to what was observed in HCT116-K, OV2008 derived xenografts expressing shAPM2-D1 grew significantly faster than tumours expressing shAPM2-D4 or shNT when left untreated (Figure 35). It should be noted that siAPM2-D1 OV2008 tumours had greatly reduced APM2 expression in comparison with OV2008-shAPM2-D4 or –shNT. However, when treated with cisplatin (3 X 4 mg/Kg) tumours stably expressing shAPM2-D1 were significantly more sensitive than tumours expressing the non-targeting shNT (Figure 36). Conversely, tumours bearing the shAPM2-D4 construct, in which APM2 silencing was not particularly effective, were not sensitized to cisplatin to any significant degree. To determine whether the lack of sensitization was a consequence of *in vivo* growth, sensitivity of the OV2008 clones was assessed *in vitro* by clonogenic assay. Exposure of the OV2008 clones to 10 μM cisplatin revealed that OV2008 shAPM2-D1 was more sensitive than both the parental and control cell lines (Figure 37). Similar to what was observed *in vivo*, OV2008 shAPM2-D4 was not more senstitive to cisplatin than either the parental or control cells.

3.4. – APM2 LOCALIZATION

3.4.1. - Cellular fractionation and immunofluorescence demonstrate that APM2 localizes to cytoplasmic and membrane fractions in HCT116 cells

HCT116 cells were used to study the localization of APM2 protein within the cell. Cellular fractionation was used to separate the cytoplasmic, nuclear, membranous and cytoskelteal compartments both before and after a 4 h treatment with 100 μM cisplatin. Immunoblotting of the fractions showed that APM2 was present in the cytoplasm and associated with membranes in these cells (Figure 38). Treatment with cisplatin did not

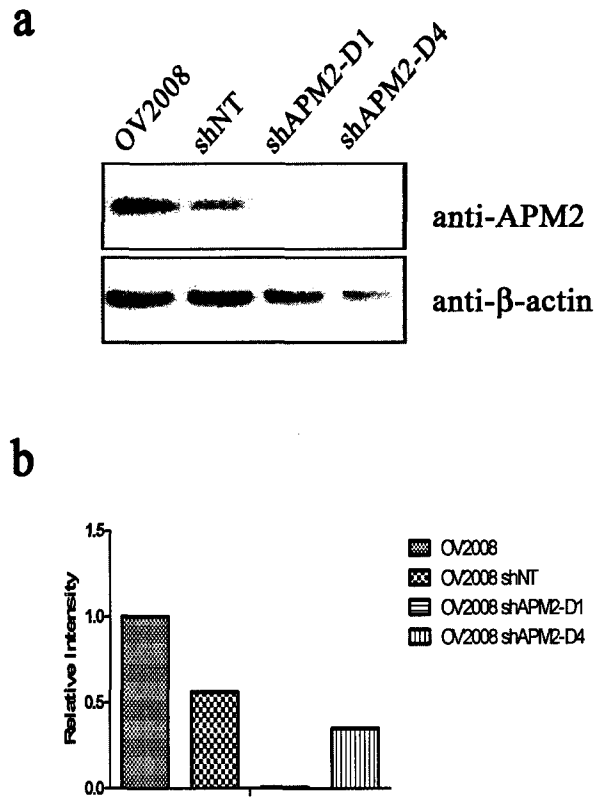

Figure 34: Expression of APM2 in shNT and shAPM2 OV2008 clones used for seeding tumours in vivo. a, b) Immunoblot performed on shRNA expressing OV2008 whole cell lysates and corresponding densitometry. Whole cell lysates were collected at the time of xenograft seeding.

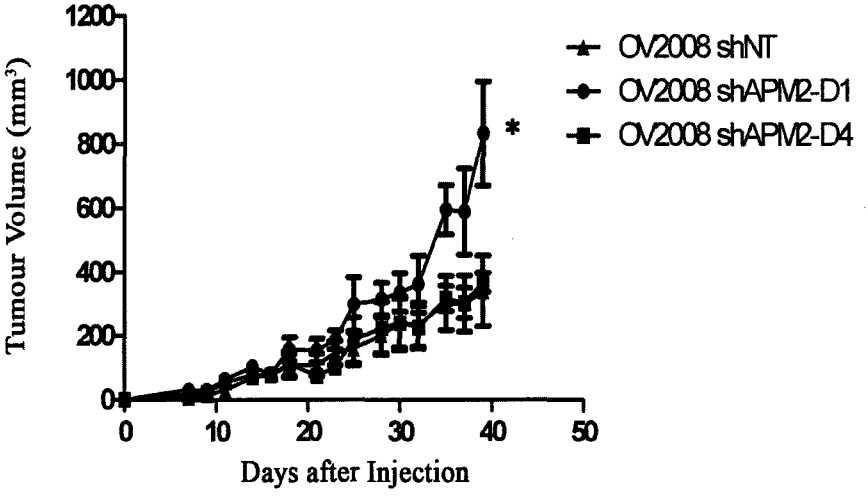

Figure 35: OV2008 shAPM2-D1 xenografts displayed an increased growth rate in vivo. OV2008 clones expressing shNT, shAPM2-D1, or shAPM2-D4 were injected onto the backs of CD-1 nude mice and their growth was measured over a period of 5 weeks. Points represent the mean of 11-12 mice ± SEM. * - $p<0.001$, by repeated-measures ANOVA and Tukey's post test.

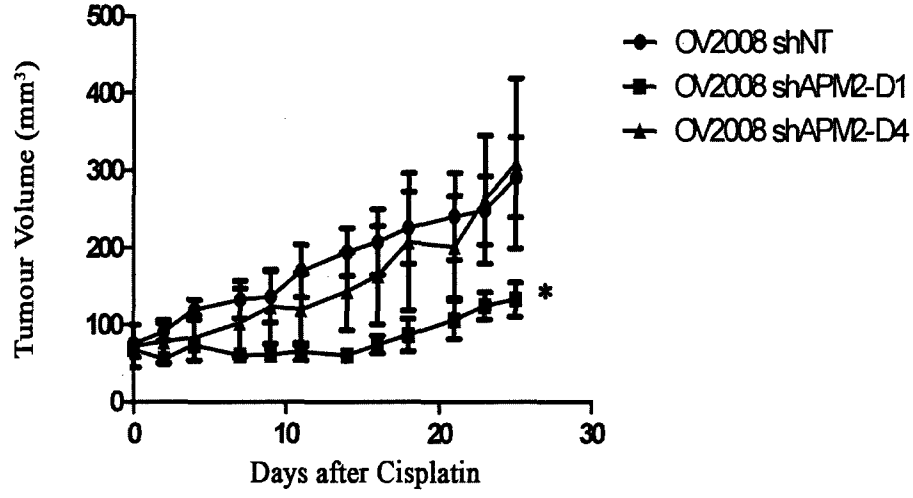

Figure 36: Silencing APM2 in OV2008 tumour xenografts enhances the effects of cisplatin treatment. a) OV2008 clones expressing shNT, shAPM2-D1, or shAPM2-D4 were injected onto the backs of CD-1 nude mice. When tumours reached the appropriate size mice received 4 mg/Kg cisplatin thrice over one week. Tumour growth was monitored for several weeks after treatment. Significant differences were assessed by repeated measures ANOVA followed by Tukey-Kramer post-hoc testing to assess differences between groups. *- $p<0.001$. Points represent the mean of 5-7 mice ± SEM.

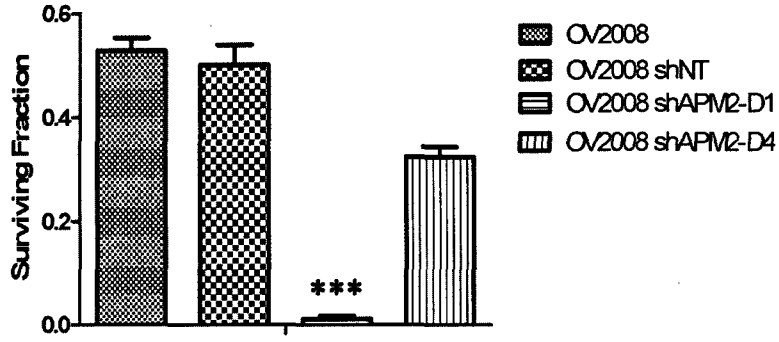

Figure 37: OV2008 shAPM2-D1 is more sensitive to cisplatin in vitro than OV2008 shNT. shAPM2-D4 is not. Each clone's survival was measured after treatment with cis-platin (10 μM). Significant differences in survival were assessed by one-way ANOVA and Dunnett's post-hoc testing using shNT as the control. * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$. Bars represent the mean of 3 experiements ± SEM.

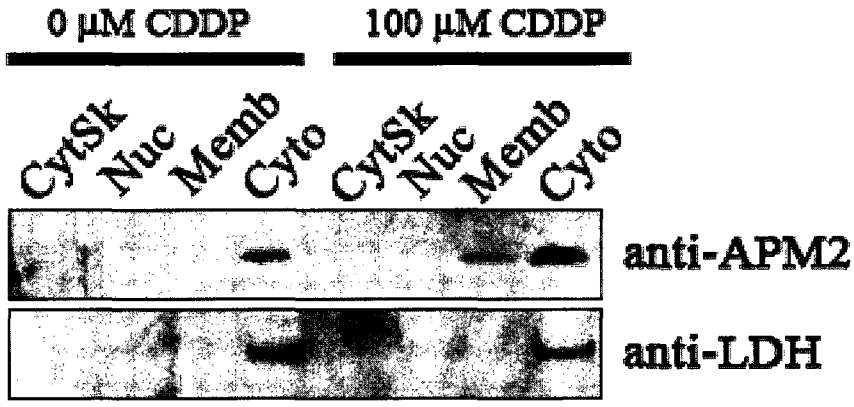

Figure 38: APM2 localizes to the cytoplasmic and membrane fractions in HCT116 cells. HCT116 cells were fractionated into cytoplasmic, nuclear, membrane, and cytoskeltal compartments. Each compartment was analyzed, by immunoblot, for the presence of APM2. Treatment of cells with cisplatin (100 µM, 4 h) did not affect the distribution of APM2.

affect the distribution of APM2 between these compartments. Immunoblots were also probed with anti-LDH to show that the membrane, nuclear, and cytoskeletal compartments were not contaminated by cytoplasmic proteins. Immunofluorescence performed using the same cell line confirmed that APM2 resides within the cytoplasm of HCT116 cells. Furthermore, vesicular staining was apparent within the cell, indicating that APM2 is associated with membranous structures. Interestingly, fluorescence was also observed outside the cell (Figure 39). Extracellular, fluorescence was not observed on negative control slides (2° antibody only or blocked APM2 antibody) leading us to hypothesize that APM2 may be a secreted protein.

3.4.2. - APM2 is secreted by HCT116 cells

To verify that APM2 is secreted by HCT116 cells, immunoprecipitation was performed on HCT116 conditioned media and unconditioned media. Immunoblotting was performed on proteins eluted from immunoprecipitations (Figure 40). APM2 protein is clearly visible in the lane loaded with protein immunoprecipitated from HCT116 conditioned media. APM2 was not detectable in proteins immunoprecipitated from unconditioned media. HCT116 whole cell lysate was run in lane 3 as a positive control.

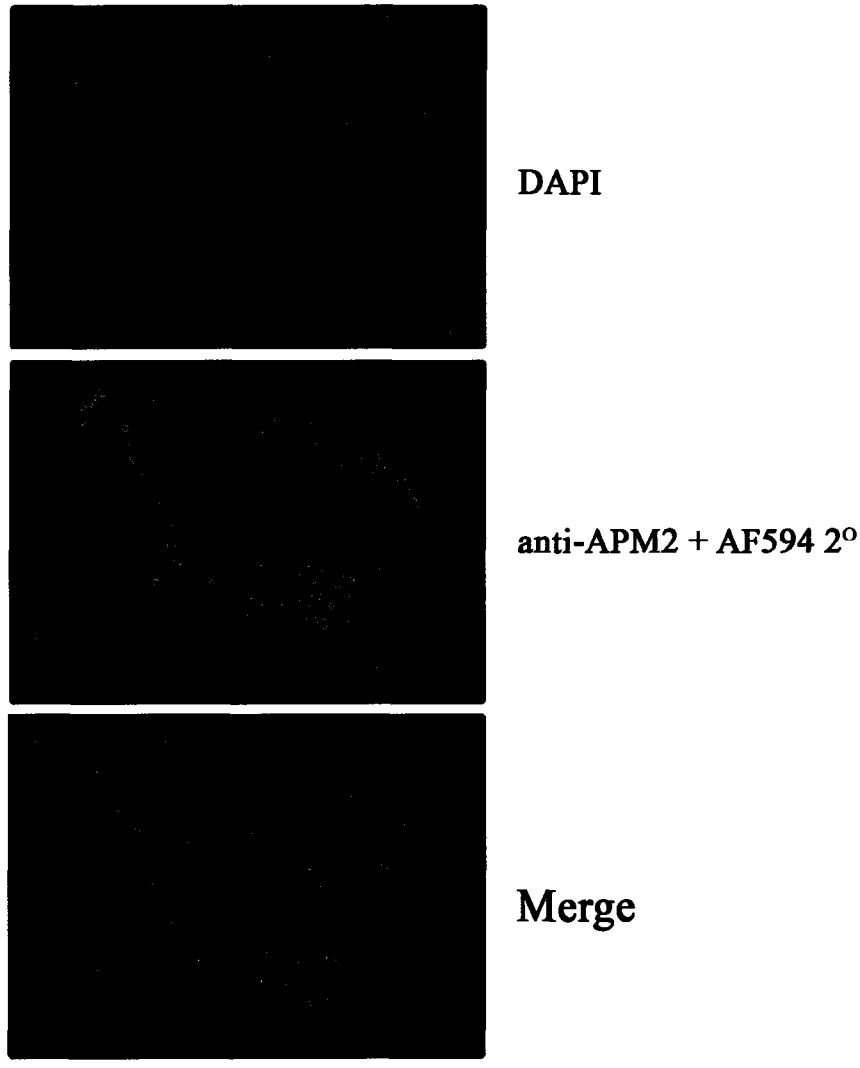

Figure 39: Immunofluorescence supports cell fractionation results. APM2 appears to be localized to the cytoplasm and associated with vesicular structures. APM2 localization was assessed by immunofluorescence in HCT116 cells using our custom polyclonal APM2 antibody. Fluorescence was not observed when: i) cells were incubated with a peptide blocked primary antibody + secondary antibody or ii) cells were incubated with secondary antibody only. Note the presence of fluorescence in the extracellular space. Cells were viewed under the 63X oil immersion lense. Magnification is 630X.

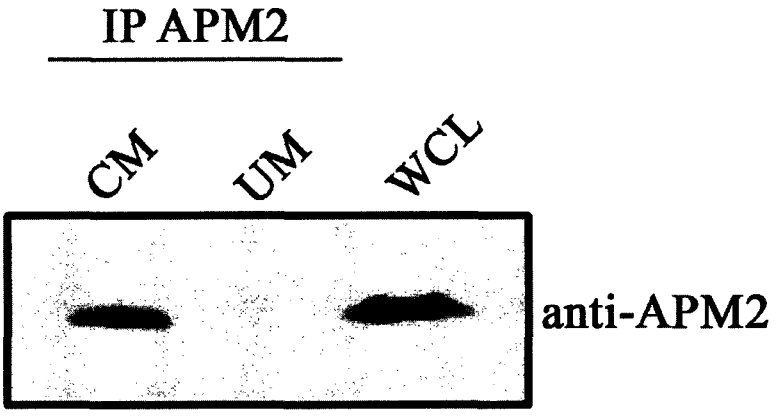

Figure 40: APM2 protein is secreted from HCT116 cells. APM2 immunoprecipitations were carried out on HCT116 conditioned and uncondititoned media. Immunoprecipitations were subjected to SDS-PAGE and immunoblotting with anti-APM2 antibody. Note the presence of APM2 in the HCT116 conditioned media lane. CM – conditioned media, UM – unconditioned media, WCL – whole cell lysate (+ ctrl). conditioned media. APM2 was not detectable in proteins immunoprecipitated from unconditioned media. HCT116 whole cell lysate was run in lane 3 as a positive control.

CHAPTER 4

DISCUSSION

The work presented details the search for, and the identification and characterization of genes involved in the cellular response to X-radiation or chemotherapeutics or a combination of the two. Specifically, one gene, APM2, is characterized as being involved in the response of cells to cisplatin, a major chemotherapeutic currently being used. The work is unique in that it utilizes untreated clones of the human colorectal tumour cell line, HCT116 [Qutob et al., 2004]. These clones display differing sensitivities to various cancer treatments and, therefore, are suited to identifying genes whose expressions are regulated differently in clones with different drug/XR response phenotypes. In this chapter, the results will be discussed in relation to the current body of knowledge pertaining to XR/chemotherapeutic resistance and, specifically, cisplatin resistance.

4.1. – IDENTIFICATION OF GENES WITH POTENTIAL INVOLVEMENT IN THE RESPONSES TO XR/CHEMOTHERAPEUTICS.

The comparison of the transcriptomes of HCT116-K and HCT116-2 cells to that of HCT116-10 identified a small set of genes that were overexpressed or underexpressed in either clone. This observation is likely due to the high similarity of these clones. HCT116-K, -2, and -10 were never exposed to drug or XR treatment before their isolation, although the patient they were isolated from may haved received some form of treatment. Once the clones were isolated and expanded their sensitivities to various insults were assessed. Therefore, any differences in drug/XR sensitivity were solely due to inherent differences, within the clonal populations, in relation to the cells to which they were compared. In contrast, other studies have attempted to identify genes that play a role in the response to chemotherapeutics/XR by establishing treatment resistant sublines [Gosepath et al., 2008; Kang et al., 2004; Young Min Chung, 2005]. This method tends to lead to the identification of large numbers of differentially regulated genes. For example, the study by Young Min Chung et Al, comparing gene expression between cells with acquired radiation resistance versus a parent cell line, identified over 300 genes whose expression was changed by more than 2-fold. Kang et Al, who compared the transcriptomes of cells with acquired doxorubicin, 5-FU, or cisplatin resistance found that over 200, 38, and 27 genes, respectively, were significantly different among cells with acquired resistance compared to their parental counterparts. It is well known that cells regulate gene expression in response to treatment with XR/chemotherapeutics, and therefore, it is not surprising that an increased number of genes are identified using the "induced resistance" type of model [DeHaan et al., 2001; Sherman et al., 1990; Yamaguchi et al., 2008]. Treatment induced gene regulation is

exemplified by cells that regulate *c-jun* and *c-fos* expression in response to treatment with XR. Upon XR treatment *c-jun/c-fos* mRNA expression is increased substantially [Sherman et al., 1990]. *C-jun* and *c-fos* code for proteins that interact to form the AP-1, "immediate early gene" transcription factor. AP-1 is involved in the transcriptional regulation of many genes and, therefore, its regulation results in the subsequent regulation of numerous genes in response to XR treatment [Milde-Langosch, 2005]. The fact that so few genes were identified by our study is probably due to the method by which our models of treatment resistance were isolated. One potential drawback to the method that we have chosen to uncover genes involved with treatment response is that we may only be identifying genes involved in the inherent or intrinsic response to various cancer treatments. However, our model may be more realistic than models based on cell lines with induced resistance which, often display levels of resistance far greater than what is observed clinically [Marij J.P. Welters, 1997]. A Two-fold increase in cisplatin resistance, as observed in this thesis, is relevant clinically, and several studies have shown that responsiveness to the drug can be achieved by doubling the normal clinical dose of cisplatin, indicating that clinical resistance to cisplatin is, indeed, in the area of two-fold [Ozols et al., 1984; Schilder and Ozols, 1992]. *In vitro* models of cisplatin resistance, in which resistance has been induced, often display >20 fold resistance [Marij J.P. Welters, 1997; Siddik, 2003]. Our model of resistance to cisplatin is more in line with what is observed clinically and, therefore, may be more indicative of what occurs in patients presenting with tumours that are resistant to cisplatin.

Comparing gene expression between HCT116-K and HCT116-10 revealed that several genes were upregulated in the XR sensitive, chemotherapeutic resistant clone. Verification by real time RT-PCR narrowed this list. *APM2, SSAT, GSK-3α* and *ALDH6* were upregulated by HCT116-K compared to the "normal" HCT116-10, while *CYP24A1*

was found to be downregulated by HCT116-K. Comparison of the XR resistant, cisplatin sensitive, HCT116-2, by cDNA microarray and real-time RT-PCR, resulted in the identification of one gene, *XRRA1* as underexpressed when compared to HCT116-10. Cell populations have been shown to have heterogeneous gene expression, that is, single cells within a cell population express genes at varying levels [Bengtsson et al., 2005]. To limit the possibility that the aforementioned genes were identified solely due to the normal variation within clones of the same cell line, the expression of each gene was measured in a panel of 9 HCT116 clones and in the parental cell line. One-way ANOVA and Dunnett's post-hoc testing was performed to determine whether the expression of a gene was outside of the normal variation observed within the panel. By testing the expression of each of the above mentioned genes we were able to further shorten our list of potential drug/X-radiation response genes.

After testing for significance it was found that *GSK-3α* and *ALDH6* were not expressed significantly higher in HCT116-K than in the rest of the panel of HCT116 clones. Therefore, these genes were not pursued as factors involved in drug/XR resistance.

CYP24A1 expression was significantly lower in HCT116-K than in 6 of the other 8 clones tested. *CYP24A1* appeared interesting to us as many cytochrome p450's are involved in the detoxification of drugs [Guengerich, 2008]. This particular CYP is involved in the metabolism of 1,25-dihydroxyvitamin-D3 (vitamin D) and, as such, plays a role in regulating calcium homeostasis within the cell [Okuda et al., 1995]. However, we did not investigate *CYP24A1* further because HCT116-K and HCT116-2, which have opposite treatment response phenotypes, expressed *CYP24A1* at very similar levels. In hindsight, *CYP24A1* may have been an interesting gene to investigate for its role in cisplatin resistance. A recent report demonstrated that 1,25-dihyroxyvitamin-D3 potentiates the

antitumor activity of cisplatin against head and neck cancers by inducing expression of p73 [Ma et al., 2008]. Since CYP24A1 is responsible for the degradation of 1,25-dihridroxyvitamin-D3, reduced CYP24A1 expression would promote vitamin-D3's antitumor effects. Although, these findings may support a role for CYP24A1 in cisplatin resistance it would not account for the resistance in HCT116-K as HCT116-K is resistant to cisplatin, not sensitive as would be expected in response to decreased CYP24A1 expression. However, it should be noted that HCT116-2 (cisplatin sensitive) also displayed greatly reduced CYP24A1 levels in comparison to our panel of HCT116 clones and the parental cell line and, therefore, CYP24A1 suppression may be partly responsible for the sensitivity of these cells to cisplatin.

XRRA1 was initially identified as underexpressed in HCT116-2 versus HCT116-10. However, further studies showed that this may not have been accurate or that the expression of *XRRA1* is not stable in HCT116-2 (Figure 6). In addition, real time RT-PCR demonstrated that *XRRA1* was not significantly downregulated in HCT116-2 compared to the other HCT116 clones in our panel.

The expressions of both *SSAT* and *APM2* were significantly higher in HCT116-K than in all other clones tested. Therefore, these genes were selected for further study. The role of SSAT in radioresistance was investigated by another member of our lab and the results were positive in that high SSAT levels did appear to affect the response of HCT116 cells to XR [Qutob et al., 2005]. SSAT plays an important role in the catabolism of polyamines [Fogel-Petrovic et al., 1996]. Furthermore, polyamines have been suggested to protect cells from XR induced death [Snyder and Schroeder, 1994; Thomas and Thomas, 2001]. Therefore, it was not completely surprising that increasing the expression of SSAT sensitized cells to the effects of XR treatment. The role of SSAT in cisplatin resistance,

however, was not assessed in our laboratory due to time constraints. Interestingly, another group has since shown that overexpression or induction of SSAT restores sensitivity to the, cisplatin resistant, ovarian cancer cell line, C13* [Marverti et al., 2005].

APM2 appeared to be a good candidate for involvement in drug/radiation response for several reasons. First, it was the most highly overexpressed gene identified in the XR sensitive/drug resistant HCT116-K. Second, APM2 expression was significantly higher in HCT116-K then in any of the other HCT116 clones included in our panel giving us confidence that its identification was not simply a product of clonal variation. Third, Spearman's correlation analysis of the ID50s of HCT116-K, HCT116-10, HCT116-2, and HCT116, plotted against relative APM2 expression, demonstrated a strong correlation between APM2 expression and sensitivity to cisplatin (Figure 13). In addition to our own experimental evidence, APM2 was also identified in an independent microarray study as overexpressed in gastric cancer cells that were resistant to cisplatin [Kang et al., 2004]. Since HCT116-K cells are also resistant to cisplatin, it appeared worthwhile to investigate the possible role of APM2 as a factor in cisplatin resistance.

4.2. – INVESTIGATING THE ROLE OF APM2 IN DRUG/XR RESISTANCE IN VITRO

HCT116-K is cross-resistant to etoposide and topotecan in addition to cisplatin but is sensitive to XR [Qutob et al., 2004]. Hence, APM2 levels were examined as a factor in resistance, or conversely sensitivity, to these agents. Overexpression of APM2 in HCT116-2 or HCT116-10 had no significant effect on the efficacy of either of these drugs, or to XR, but caused a significant increase in cisplatin resistance in both clones, indicating that

APM2 expression is specifically associated with response to cisplatin and not other drugs. Supporting these findings is the study by Kang et al in which APM2 was found to be overexpressed in cisplatin resistant gastric cancer cells but not in gastric cancer cells resistant to 5-FU or doxorubicin [Kang et al., 2004]. It is possible that APM2 expression also plays a role in the response to other platinating agents; however, this has not yet been tested. If, for example, APM2 depletion was found to be an effective method of sensitizing cells to oxaliplatin or carboplatin it would become a more attractive target for the treatment of a wider variety of cancers as oxaliplatin and carboplatin are indicated as first line treatment for colorectal [Graham et al., 2004], ovarian, and lung cancer [Lafay-Cousin et al., 2008]. It has been demonstrated that cell lines bearing resistance to cisplatin are often cross-resistant to carboplatin [Rixe et al., 1996; Stewart, 2007] and, therefore, it could be assumed that if reducing APM2 expression is capable of sensitizing cells to cisplatin it is likely capable of sensitizing cells to carboplatin as well. Oxaliplatin, however, does not exhibit the same level of cross-resistance with cisplatin that carboplatin does and, therefore, would not necessarily be more effective in the presence of reduced APM2 levels [Rixe et al., 1996].

This study made use of a variety of cancer cell lines with different genetic backgrounds with respect to p53 and the MMR pathway. The tumour suppressor, p53, has been shown to be an important factor related to cisplatin response. In ovarian cancer, p53 can interact directly with proteins involved in cisplatin resistance such as the Flice like inhibitory proteins (FLIP), targeting these proteins for proteasomal degradation, and thus increasing cellular sensitivity to cisplatin [Abedini et al., 2008]. In addition, p53 has been linked to the induction of apoptosis through a number of mechanisms in response to cisplatin. These include: upregulation of p53 in response to PTEN overexpression, where

the absence of p53 abrogated PTEN dependant increases in the apoptotic response to cisplatin [Yan et al., 2006], and decreased phosphorylation of p53 in the presence of active Akt leading to a decrease in cisplatin induced cell death [Fraser et al., 2008]. These studies have clearly established p53 as an important mediator of cisplatin resistance, at least in the response of ovarian cancer cells to cisplatin.

MMR is another important factor regarding cisplatin response. In cells with an intact MMR system, DNA damaged by cisplatin is recognized by the MMR complex [Aebi et al., 1996; Plumb et al., 2000]. However, the MMR complex is unable to repair the damage due to interference by the platinum adduct. When the MMR system is unable to repair the detected damage the apoptotic program is initiated leading to cell death. In cells that are deficient in MMR, damage caused by cisplatin is not recognized, replication is allowed to continue, and apoptosis is not initiated. This results in cisplatin resistance [Martin et al., 2008]. Because p53 and MMR are often lost in cancers treated with cisplatin, or their status are simply unknown, we reasoned that strategies that can sensitize tumours to cisplatin regardless of these factors could be powerful tools in the clinic. In addition, at least one study has demonstrated that patients treated with cisplatin actually lose expression of both hMLH1 and hMSH2, proteins necessary for the formation of the MMR complex [Samimi et al., 2000], further demonstrating the need for methods to sensitize these cancers to cisplatin. Here, we have shown that siRNAs against APM2 can sensitize a variety of cancer cell types regardless of their p53 status or ability to perform MMR. These findings suggest that the mechanism by which APM2 affects the response to cisplatin is independent of these two important mediators of cell survival. This, in fact, makes APM2 a more attractive target than some other proteins that have been shown to influence the response to cisplatin. For example, upregulation of PTEN has been shown to enhance the apoptotic

response of ovarian carcinoma cells to cisplatin [Yan et al., 2006]. This consequence was dependent upon a functional p53. Given the fact that at least 50% of all cancers are p53 deficient [Nguyen et al., 2005], a therapy that relied on those findings would be severely limited in its application.

We have shown that by depleting APM2 levels in cells, using siRNA, it is possible to sensitize them to cisplatin no matter what their background with respect to p53 or MMR. Although the level of sensitization is not breathtakingly large ($\approx$ 2 fold), it has been suggested that two-fold increases in resistance to cisplatin are large enough to cause treatment failure [Aebi et al., 1996; Andrews et al., 1990], and therefore a two-fold increase in sensitivity could be clinically significant to patients requiring cisplatin therapy. Furthermore, any amount of increased sensitization by APM2 to cisplatin could potentially interact synergistically with XR in chemoradiation treatments involving cisplatin. Additionally, because chemotherapeutic treatments often comprise a cocktail of drugs, it is conceivable that modulation of APM2 could potentially provide a means to modulate response to cisplatin specifically (e.g. allowing the amount of cisplatin to be reduced if undesired side effects on normal tissues are observed).

4.3. – THE EFFECT OF REDUCED APM2 EXPRESSION ON TUMOUR GROWTH, IN VITRO

In addition to showing that APM2 depletion in cells leads to an increase in cisplatin sensitivity, we have extended these findings to demonstrate that silencing of APM2 also effectively increases the response of HCT116-K and OV2008 tumour xenografts to

cisplatin. HCT116-K is a cisplatin resistant variant of the parental HCT116 cell line, which is known to be deficient in MMR due to loss of the hMLH1 gene while OV2008 cells are proficient in performing MMR [Aebi et al., 1996]. Both cell lines retain wild-type p53 function. However, Righetti et al. previously demonstrated that ovarian cancer cells may lose p53 function during cisplatin treatment and furthermore, that continued treatment with cisplatin preferentially selects for those cells carrying inactivating p53 mutations [Righetti et al., 1999]. Hence, it is possible that during treatment cells become deficient in functional p53. HCT116-K tumour xenografts stably expressing shAPM2-D1 and shAPM2-D4 both showed significant improvement in their response to cisplatin compared to shNT expressing HCT116-K xenografts. OV2008 tumours stably expressing shAPM2-D1 also showed dramatically increased sensitivity to cisplatin when compared to tumours expressing the non-targeting control shRNA. Surprisingly, OV2008-shAPM2-D4 cells were not sensitized to cisplatin. However, this is likely due to the incomplete depletion of APM2 in this line compared to the knockdown in the other lines used for the *in vivo* studies (Figure 34). OV2008 cells have also been demonstrated to acquire resistance during treatment with cisplatin and, in fact, several cisplatin resistant cell lines have been derived from the OV2008 cell line [Aebi et al., 1996]. It is possible that this could have occurred during treatment of our xenograft models with cisplatin, causing our OV2008-shAPM2-D4 clone to become more resistant to cisplatin than other clones with depleted APM2. Numerous factors contribute to cisplatin resistance, and selection of APM2 suppressed clones may have led to the isolation of clones with changes in one or more of these factors in addition to decreased APM2 expression. Thus, it is also possible that during selection we have isolated a clone that is intrinsically more resistant to cisplatin than other clones with depleted APM2.

As already mentioned, numerous proteins and pathways play roles in cisplatin resistance. How to translate these findings into clinically relevant discoveries is a difficult challenge. Nonetheless, where protein or mRNA depletion is the goal, therapeutic siRNAs may hold promise. Advances have been made in improving the stability of siRNA *in vivo*, and also in delivering siRNAs to the appropriate targets [Ikeda and Taira, 2006; Wolfrum et al., 2007]. For example, Wolfrum et al. have demonstrated that conjugation of siRNAs to bile acids or long-chain fatty acids and cholesterol mediates efficient delivery of siRNAs into organs such as the liver, gut, kidney, and steroidogenic organs [Wolfrum et al., 2007]. Taken together with our findings, therapeutic siRNAs may be useful in enhancing patient response to traditional chemotherapeutics such as cisplatin.

While delivery of synthetic siRNAs *in vivo* does appear to hold some promise, the cost of synthesizing siRNAs on a scale large enough for treatment purposes would likely be prohibitive. However, viral delivery of vectors coding for the production of siRNAs inside the cell may overcome this obstacle. Adenoviral delivery of siRNA coding vectors is an effective method of transiently silencing target genes. In fact, using adenoviral delivery of siRNA coding vectors has been shown to effectively silence p53 and caspase-8 in several cell types including MCF-7 (breast carcinoma), A549 (lung carcinoma), and Hela S3 (cervical carcinoma) [Shen et al., 2003; Tomar et al., ; Zhao et al., 2003]. The studies mentioned only demonstrate adenoviral delivery of siRNAs *in vitro*. However, more recent experimentation demonstrated that it is possible to deliver adenoviral vectors *in vivo* and achieve silencing of desired target genes. For example, intratracheal injection of siRNA coding adenoviral vectors into mice resulted in the reduction of C5aR protein expression [Sun et al., 2006]. Interesting, and in support of the argument that adenoviral shRNA delivery may become an important method of alternative gene therapy, is a report by Chu et

Al. In it the authors describe the construction of an adenoviral vector for the targeted silencing of Apollon, a protein known for its involvement in the inhibition of apoptosis. The adenoviral vector was delivered both *in vitro*, to several cancer cell lines, and *in vivo,* to athymic nude mice. In both cases the adenoviral shRNA successfully reduced the amount of Apollon protein detectable. More importantly, reducing the amount of Apollon significantly delayed tumour growth, *in vivo*. In addition, silencing the expression of Apollon enhanced the effects of 5-FU and almost completely eradicated established tumours [Chu et al., 2008]. Another recent report has shown that adenoviral mediated silencing of APE1, a protein involved in DNA repair and redox signalling, results in enhanced radiosensitivity in a mouse model of colorectal cancer [Xiang et al., 2008]. Studies such as these provide the proof-in-principle that siRNAs can be delivered *in vivo*, effectively, silencing their targets. The silencing of such targets can enhance the effects of various cancer therapies. An important next step in investigating APM2 silencing as an enhancer of cisplatin sensitivity would be to develop adenoviral vectors expressing the short-hairpin sequences described in this thesis. The viral particles could then be delivered, in combination with cisplatin, to mice with established tumours of various types. An experiment of this type would provide a good indication of whether transient silencing of APM2 would be capable of enhancing cisplatin's effects *in vivo*.

Our *in vivo* experiments demonstrate a novel method of sensitizing tumours to cisplatin. However, APM2 silencing in our model is stable. This, however, is likely not what would be achieved in a clinical setting. More likely, a transient method of APM2 silencing would need to be applied. Those methods discussed above are proof that transient gene silencing is achievable *in vivo* and is capable of enhancing the response of cancers to

chemotherapeutics and radiation. It is possible that by applying similar methods of gene silencing *in vivo*, we could enhance the effects of cisplatin by silencing APM2.

4.4. – APM2 LOCALIZATION

Little is known about the actual cellular functions that APM2 performs. Knowing the cellular localization of proteins can often help one to infer possible processes and functions that involve the protein of interest. To determine the cellular localization of APM2, cellular fractionation was performed. Western blotting, performed on each fraction illustrated that APM2 was present in the cytoplasm and in the membrane fraction but was not associated with nuclear or cytoskeletal fractions. To verify these findings immunofluorescence was carried out on HCT116 cells with a specific APM2 antibody. The immunofluorescence confirmed the cell fractionation studies. Given that resistance to cisplatin occurs by several mechanisms that take place in certain cellular compartments, these findings allow for the elimination of APM2s involvement in at least one. One of the major mechanisms that accounts for the development of resistance to cisplatin is an increased rate of DNA repair (see for example: [Chang et al., 2005b; Furuta et al., 2002; Petersen et al., 1996; Rosell et al., 2003; Welsh et al., 2004; Yang et al., 2000; Youn et al., 2004]). Since APM2 is not localized to the nucleus it is unlikely that it is directly involved in any DNA repair mechanism, though it could take part in signalling pathways that affect the rate of DNA repair.

Interestingly, APM2 immunofluorescence, performed on HCT116 cells, displayed fluorescence outside of the cell. This did not occur on slides incubated solely with the

secondary antibody or on slides treated with a peptide-blocked APM2 antibody. The presence of fluorescence outside the cell led us to believe that APM2 may be a secretory protein. APM2 does not contain a classical signal peptide; however, immunoprecipitation and western blotting, performed on HCT116 conditioned media, showed that APM2 was present in the media of these cells. Other modifications that can target proteins for secretion include glycosylation or the presence of internal signal sequences [Lodish, 2003] and APM2 may be targeted through one of these alternative methods. An *in* silico analysis of APM2 uncovered several interesting functional sites that are frequently associated with secretory proteins. First, two possible glycosaminoglycan sites were identified. Secondly, a c-terminal amidation site was found. Both of these modifications are typical of proteins that are exported to the extracellular matrix [Puntervoll et al., 2003]. The discovery that APM2 is secreted is interesting in light of its role in cisplatin resistance. We showed that increasing the expression of APM2 resulted in a corresponding increase in resistance to cisplatin. It has been well documented that increased efflux or decreased accumulation of cisplatin are important mechanisms that contribute to cisplatin resistance. In addition, molecules such as metallothioneins and glutathiones are capable of binding directly to cisplatin, through sulphur containing amino acids, and inactivating the drug [Cullen et al., 2003; Hagrman et al., 2003; Surowiak et al., 2005; Surowiak et al., 2007]. Thus, one proposed mechanism by which APM2 could contribute to cisplatin resistance may be the direct binding of cisplatin, by APM2, in the cytoplasm, followed by APM2 secretion into the extracellular space. If APM2 does in fact bind directly to cisplatin, its secretion from the cell would reduce the intracellular accumulation of the drug thereby reducing its cytotoxic effect (Figure 41). Interesting future experiments to test this hypothesis would be to investigate the interaction, if any exists, between APM2 and cisplatin and to measure the

amount of cisplatin in cells with "normal" APM2 levels and cells with increased levels of APM2 by inductively-coupled-plasma-mass-spectrophotometry (ICP-MS). This is only one possible mechanism by which APM2 could be responsible for resistance to cisplatin. Indeed, further investigation is warranted to elucidate the specific mechanisms that are responsible for APM2 mediated cisplatin resistance. An understanding of how APM2 regulates the response of cells to cisplatin may lead to the identification of new targets useful in enhancing the effects of cisplatin on cancer cells.

4.5. – CONCLUSIONS

Presently, despite intensive research over the past decade, classical methods of treating cancer such as surgery, radiation, and chemotherapy with broad based agents like X-radiation and the conventional chemotherapeutic drugs (e.g. cisplatin, carboplatin, topoisomerase poisons – etoposide, camptothecin, analogs) are still the most effective clinically [Argiris and Schiller, 2004; Pisters, 2005]. While targeted therapies could become more useful, it is already quite clear that an approach that combines such newer targeted drugs with traditional treatment methods is potentially more useful than applying targeted therapies alone [Johnson, 2006; Rodriguez et al., 2007]. Thus it is clear that any method that optimizes the response to conventional chemotherapeutics remains very important. Unfortunately, cellular resistance to traditional chemotherapeutics, such as cisplatin, can render them ineffective [Siddik, 2003; Stewart, 2007]. Hence, uncovering the factors responsible for treatment resistance is imperative if we are to develop strategies to combat it. Here we have described the identification, by cDNA microarray, of a

gene/protein, APM2, whose level of expression can influence the response of a wide variety of cancer cell types to cisplatin. In addition, APM2 expression is able to modulate the response to cisplatin regardless of whether cells express normal p53 or are able to perform MMR, two key factors in cisplatin resistance. *In vivo* studies confirmed that reducing APM2 expression enhances the effects of cisplatin on tumour growth, significantly delaying the growth of tumours when compared to tumours expressing high levels of APM2. While the exact mechanism of APM2 induced cisplatin resistance is unclear, it is clear that modulating its expression is capable of affecting the response of cells to cisplatin. In the future it may be possible to deliver siRNA sequences, targeting APM2, to tumours to make them more sensitive to cisplatin. This would benefit the thousands of patients that undergo platinum treatment each year, as many of them are likely to develop resistance to the drug. However, care should be taken with the application of such a treatment. It is not guaranteed that all patients would even express APM2 within their particular cancer and, therefore, silencing APM2 would not be useful in such a case. To prevent such cases, pre-screening for APM2 expression by immunohistological techniques may help to identify patients for whom such a therapy would be useful.

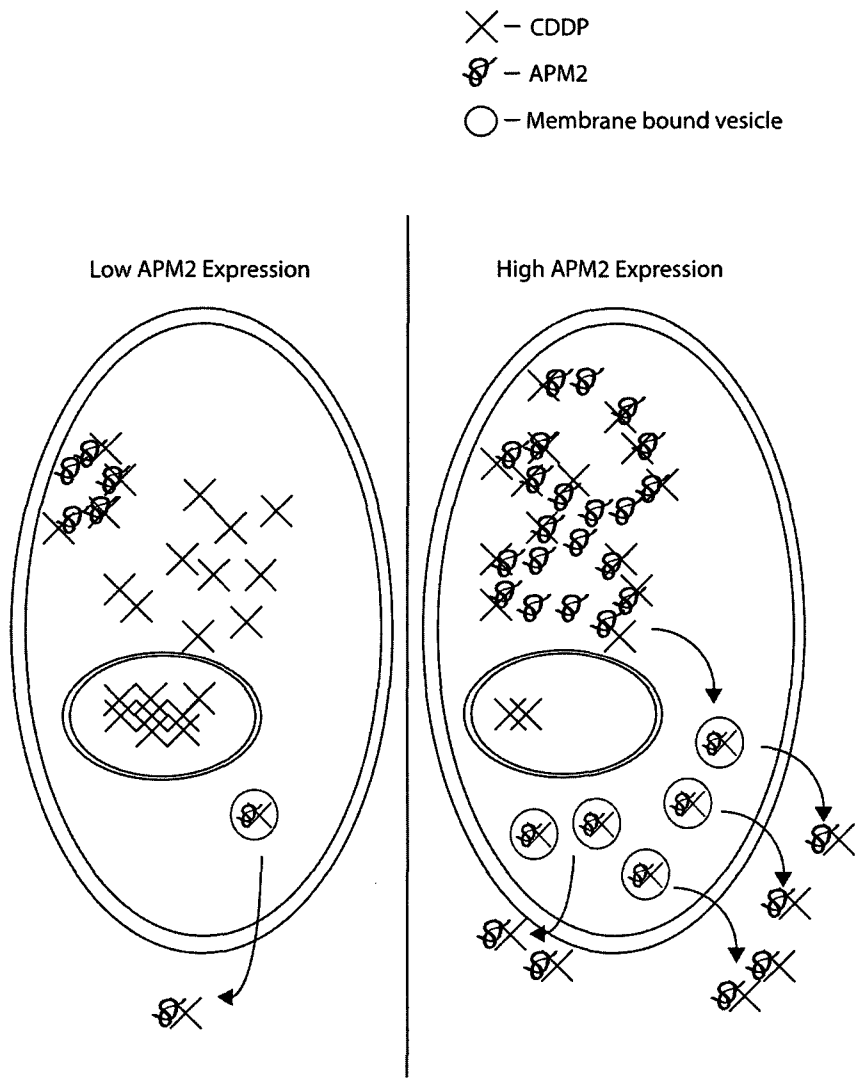

Figure 41: A Hypothetical Model of APM2 mediated cisplatin resistance. APM2 may bind directly to cisplatin through reaction with amino acid side chains containing reactive sulfur, nitrogen or oxygen species. APM2 export may then result in cellular efflux of cisplatin. This mechanism would decrease the amount of cisplatin available to react with DNA inside the nucleus, thereby, reducing its cytotoxic effects.

REFERENCES

Abedini MR, Muller EJ, Brun J, Bergeron R, Gray DA, Tsang BK (2008): Cisplatin Induces p53-Dependent FLICE-Like Inhibitory Protein Ubiquitination in Ovarian Cancer Cells. Cancer Res 68:4511-4517.

Abraham RT (2001): Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 15:2177-96.

Aebi S, Kurdi-Haidar B, Gordon R, Cenni B, Zheng H, Fink D, Christen RD, Boland CR, Koi M, Fishel R, Howell SB (1996): Loss of DNA Mismatch Repair in Acquired Resistance to Cisplatin. Cancer Res 56:3087-3090.

Alsbeih G, Torres M, Al-Harbi N, Alsubael M (2004): Loss of wild-type Trp53 protein in mouse fibroblasts leads to increased radioresistance with consequent decrease in repair of potentially lethal damage. Radiat Res 161:185-92.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997): Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-402.

Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schaffer AA, Yu YK (2005): Protein database searches using compositionally adjusted substitution matrices. Febs J 272:5101-9.

American Cancer Society (2008): Radiation Therapy Principles American Cancer Society.

Andrews PA, Jones JA, Varki NM, Howell SB (1990): Rapid emergence of acquired cis-diamminedichloroplatinum(II) resistance in an in vivo model of human ovarian carcinoma. Cancer Commun 2:93-100.

Argiris A, Jayaram P, Pichardo D (2005): Revisiting induction chemotherapy for head and neck cancer. Oncology (Williston Park) 19:759-70.

Argiris A, Schiller JH (2004): Can Current Treatments for Advanced Non-Small-Cell Lung Cancer Be Improved? JAMA 292:499-500.

Asselin E, Mills GB, Tsang BK (2001): XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. Cancer Res 61:1862-8.

Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN (2006): Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444:756-60.

Beier D, Rohrl S, Pillai DR, Schwarz S, Kunz-Schughart LA, Leukel P, Proescholdt M, Brawanski A, Bogdahn U, Trampe-Kieslich A, Giebel B, Wischhusen J, Reifenberger G, Hau P, Beier CP (2008): Temozolomide Preferentially Depletes Cancer Stem Cells in Glioblastoma. Cancer Res 68:5706-5715.

Bengtsson M, Stahlberg A, Rorsman P, Kubista M (2005): Gene expression profiling in single cells from the pancreatic islets of Langerhans reveals lognormal distribution of mRNA levels. Genome Research 15:1388-1392.

Bhoola S, Hoskins WJ (2006): Diagnosis and management of epithelial ovarian cancer. Obstet Gynecol 107:1399-410.

Binks SP, Dobrota M (1990): Kinetics and mechanism of uptake of platinum-based pharmaceuticals by the rat small intestine. Biochem Pharmacol 40:1329-36.

Bond GL, Hu W, Bond EE, Robins H, Lutzker SG, Arva NC, Bargonetti J, Bartel F, Taubert H, Wuerl P, Onel K, Yip L, Hwang SJ, Strong LC, Lozano G, Levine AJ (2004): A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell 119:591-602.

Borgmann K, Roper B, El-Awady R, Brackrock S, Bigalke M, Dork T, Alberti W, Dikomey E, Dahm-Daphi J (2002): Indicators of late normal tissue response after radiotherapy for head and neck cancer: fibroblasts, lymphocytes, genetics, DNA repair, and chromosome aberrations. Radiother Oncol 64:141-52.

Brem R, Certa U, Neeb M, Nair AP, Moroni C (2001): Global analysis of differential gene expression after transformation with the v-H-ras oncogene in a murine tumor model. Oncogene 20:2854-8.

Bristow RG, Hill RP (1990): Comparison between in vitro radiosensitivity and in vivo radioresponse in murine tumor cell lines. II: In vivo radioresponse following fractionated treatment and in vitro/in vivo correlations. Int J Radiat Oncol Biol Phys 18:331-45.

Brognard J, Clark AS, Ni Y, Dennis PA (2001): Akt/Protein Kinase B Is Constitutively Active in Non-Small Cell Lung Cancer Cells and Promotes Cellular Survival and Resistance to Chemotherapy and Radiation. Cancer Res 61:3986-3997.

Brown KD, Rathi A, Kamath R, Beardsley DI, Zhan Q, Mannino JL, Baskaran R (2003): The mismatch repair system is required for S-phase checkpoint activation. Nat Genet 33:80-4.

Bryant PE, Liu N (1994): Responses of radiosensitive repair-proficient cell lines to restriction endonucleases. Int J Radiat Biol 66:597-601.

Buchholz M, Kestler H, Holzmann K, Ellenrieder V, Schneiderhan W, Siech M, Adler G, Bachem M, Gress T (2005): Transcriptome analysis of human hepatic and pancreatic stellate cells: organ-specific variations of a common transcriptional phenotype. Journal of Molecular Medicine 83:795-805.

Cabelguenne A, Loriot MA, Stucker I, Blons H, Koum-Besson E, Brasnu D, Beaune P, Laccourreye O, Laurent-Puig P, De Waziers I (2001): Glutathione-associated enzymes in head and neck squamous cell carcinoma and response to cisplatin-based neoadjuvant chemotherapy. Int J Cancer 93:725-30.

Canadian Cancer Society NCIoC (2008): Canadian Cancer Statistics, 2008 Toronto, Canada.

Cao C, Mu Y, Hallahan DE, Lu B (2004): XIAP and survivin as therapeutic targets for radiation sensitization in preclinical models of lung cancer. Oncogene 23:7047-52.

Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G (2004a): Cell death by mitotic catastrophe: a molecular definition. Oncogene 23:2825-37.

Castedo M, Perfettini JL, Roumier T, Valent A, Raslova H, Yakushijin K, Horne D, Feunteun J, Lenoir G, Medema R, Vainchenker W, Kroemer G (2004b): Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. Oncogene 23:4362-70.

Chakravarti A, Zhai GG, Zhang M, Malhotra R, Latham DE, Delaney MA, Robe P, Nestler U, Song Q, Loeffler J (2004): Survivin enhances radiation resistance in primary human glioblastoma cells via caspase-independent mechanisms. Oncogene 23:7494-506.

Chang I-Y, Youn C-K, Kim H-B, Kim M-H, Cho H-J, Yoon Y, Lee Y-S, Chung M-H, You HJ (2005a): Oncogenic H-Ras Up-regulates Expression of Ku80 to Protect Cells from {gamma}-Ray Irradiation in NIH3T3 Cells. Cancer Res 65:6811-6819.

Chang IY, Kim MH, Kim HB, Lee do Y, Kim SH, Kim HY, You HJ (2005b): Small interfering RNA-induced suppression of ERCC1 enhances sensitivity of human cancer cells to cisplatin. Biochem Biophys Res Commun 327:225-33.

Chen AY, Okunieff P, Pommier Y, Mitchell JB (1997): Mammalian DNA topoisomerase I mediates the enhancement of radiation cytotoxicity by camptothecin derivatives. Cancer Res 57:1529-36.

Chen Y, Wang J, Fraig MM, Metcalf J, Turner WR, Bissada NK, Watson DK, Schweinfest CW (2001): Defects of DNA Mismatch Repair in Human Prostate Cancer. Cancer Res 61:4112-4121.

Cho HJ, Kim JK, Kim KD, Yoon HK, Cho MY, Park YP, Jeon JH, Lee ES, Byun SS, Lim HM, Song EY, Lim JS, Yoon DY, Lee HG, Choe YK (2005): Upregulation of Bcl-2 is associated with cisplatin-resistance via inhibition of Bax translocation in human bladder cancer cells. Cancer Lett.

Chu L, Gu J, Sun L, Qian Q, Qian C, Liu X (2008): Oncolytic adenovirus-mediated shRNA against Apollon inhibits tumor cell growth and enhances antitumor effect of 5-fluorouracil. Gene Ther 15:484-94.

Covell DG, Wallqvist A, Rabow AA, Thanki N (2003): Molecular Classification of Cancer: Unsupervised Self-Organizing Map Analysis of Gene Expression Microarray Data. Mol Cancer Ther 2:317-332.

Crompton NE, Shi YQ, Emery GC, Wisser L, Blattmann H, Maier A, Li L, Schindler D, Ozsahin H, Ozsahin M (2001): Sources of variation in patient response to radiation treatment. Int J Radiat Oncol Biol Phys 49:547-54.

Cullen KJ, Newkirk KA, Schumaker LM, Aldosari N, Rone JD, Haddad BR (2003): Glutathione S-transferase pi amplification is associated with cisplatin resistance in head and neck squamous cell carcinoma cell lines and primary tumors. Cancer Res 63:8097-102.

de Gramont A (2005): Rapid evolution in colorectal cancer: therapy now and over the next five years. Oncologist 10 Suppl 2:4-8.

DeHaan RD, Yazlovitskaya EM, Persons DL (2001): Regulation of p53 target gene expression by cisplatin-induced extracellular signal-regulated kinase. Cancer Chemother Pharmacol 48:383-8.

Deng HB, Adikari M, Parekh HK, Simpkins H (2004): Ubiquitous induction of resistance to platinum drugs in human ovarian, cervical, germ-cell and lung carcinoma tumor cells overexpressing isoforms 1 and 2 of dihydrodiol dehydrogenase. Cancer Chemother Pharmacol 54:301-7.

di Pietro A, Vries EG, Gietema JA, Spierings DC, de Jong S (2005): Testicular germ cell tumours: the paradigm of chemo-sensitive solid tumours. Int J Biochem Cell Biol 37:2437-56.

Douglas KT (1987): Mechanism of action of glutathione-dependent enzymes. Adv Enzymol Relat Areas Mol Biol 59:103-67.

Du Bois A, Pfisterer J (2005): Future options for first-line therapy of advanced ovarian cancer. Int J Gynecol Cancer 15 Suppl 1:42-50.

Dynlacht JR, Xu M, Pandita RK, Wetzel EA, Roti Roti JL (2004): Effects of heat shock on the Mre11/Rad50/Nbs1 complex in irradiated or unirradiated cells. Int J Hyperthermia 20:144-56.

Elshaikh M, Ljungman M, Ten Haken R, Lichter AS (2005): Advances in Radiation Oncology. Annu Rev Med.

Fan S, el-Deiry WS, Bae I, Freeman J, Jondle D, Bhatia K, Fornace AJ, Jr., Magrath I, Kohn KW, O'Connor PM (1994): p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. Cancer Res 54:5824-30.

Fedier A, Fink D (2004): Mutations in DNA mismatch repair genes: implications for DNA damage signaling and drug sensitivity (review). Int J Oncol 24:1039-47.

Ferry KV, Hamilton TC, Johnson SW (2000): Increased nucleotide excision repair in cisplatin-resistant ovarian cancer cells: role of ERCC1-XPF. Biochem Pharmacol 60:1305-13.

Fink D, Nebel S, Norris PS, Baergen RN, Wilczynski SP, Costa MJ, Haas M, Cannistra SA, Howell SB (1998): Enrichment for DNA mismatch repair-deficient cells during treatment with cisplatin. Int J Cancer 77:741-6.

Fink D, Zheng H, Nebel S, Norris PS, Aebi S, Lin TP, Nehme A, Christen RD, Haas M, MacLeod CL, Howell SB (1997): In vitro and in vivo resistance to cisplatin in cells that have lost DNA mismatch repair. Cancer Res 57:1841-5.

Flanagan SA, Krokosky CM, Mannava S, Nikiforov MA, Shewach DS (2008): MLH1 Deficiency Enhances Radiosensitization with 5-Fluorodeoxyuridine by Increasing DNA Mismatches. Mol Pharmacol 74:863-871.

Fogel-Petrovic M, Vujcic S, Brown PJ, Haddox MK, Porter CW (1996): Effects of Polyamines, Polyamine Analogs, and Inhibitors of Protein Synthesis on Spermidine-Spermine N1-Acetyltransferase Gene Expression. Biochemistry 35:14436-14444.

Fortugno P, Wall NR, Giodini A, O'Connor DS, Plescia J, Padgett KM, Tognin S, Marchisio PC, Altieri DC (2002): Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtubule function. J Cell Sci 115:575-85.

Fraser M, Bai T, Tsang BK (2008): Akt promotes cisplatin resistance in human ovarian cancer cells through inhibition of p53 phosphorylation and nuclear function. Int J Cancer 122:534-46.

Fraser M, Leung BM, Yan X, Dan HC, Cheng JQ, Tsang BK (2003): p53 Is a Determinant of X-Linked Inhibitor of Apoptosis Protein/Akt-Mediated Chemoresistance in Human Ovarian Cancer Cells. Cancer Res 63:7081-7088.

Friedberg EC (2001): How nucleotide excision repair protects against cancer. Nat Rev Cancer 1:22-33.

Friesen C, Glatting G, Debatin K-M, Reske S (2007): Radioresistance to yttrium-90 and gamma-irradiation depends on DNA-double-strand-break repair. J NUCL MED MEETING ABSTRACTS 48:345P-.

Friesner JD, Liu B, Culligan K, Britt AB (2005): Ionizing radiation-dependent gamma-H2AX focus formation requires ataxia telangiectasia mutated and ataxia telangiectasia mutated and Rad3-related. Mol Biol Cell 16:2566-76.

Frit P, Canitrot Y, Muller C, Foray N, Calsou P, Marangoni E, Bourhis J, Salles B (1999): Cross-resistance to ionizing radiation in a murine leukemic cell line resistant to cis-dichlorodiammineplatinum(II): role of Ku autoantigen. Mol Pharmacol 56:141-6.

Furuta T, Ueda T, Aune G, Sarasin A, Kraemer KH, Pommier Y (2002): Transcription-coupled nucleotide excision repair as a determinant of cisplatin sensitivity of human cells. Cancer Res 62:4899-902.

Gillespie DL, Whang K, Ragel BT, Flynn JR, Kelly DA, Jensen RL (2007): Silencing of Hypoxia Inducible Factor-1{alpha} by RNA Interference Attenuates Human Glioma Cell Growth In vivo. Clin Cancer Res 13:2441-2448.

Gius D, Cui H, Bradbury CM, Cook J, Smart DK, Zhao S, Young L, Brandenburg SA, Hu Y, Bisht KS, Ho AS, Mattson D, Sun L, Munson PJ, Chuang EY, Mitchell JB, Feinberg AP (2004): Distinct effects on gene expression of chemical and genetic manipulation of the cancer epigenome revealed by a multimodality approach. Cancer Cell 6:361-71.

Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC, Anderson ME (1992): High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proceedings of the National Academy of Sciences of the United States of America 89:3070-3074.

Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, Findlay BP, Pitot HC, Alberts SR (2004): A randomized controlled trial of fluorouracil plus

leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 22:23-30.

Gonzalez VM, Fuertes MA, Alonso C, Perez JM (2001): Is cisplatin-induced cell death always produced by apoptosis? Mol Pharmacol 59:657-63.

Gosepath EM, Eckstein N, Hamacher A, Servan K, von Jonquieres G, Lage H, Gyorffy B, Royer HD, Kassack MU (2008): Acquired cisplatin resistance in the head-neck cancer cell line Cal27 is associated with decreased DKK1 expression and can partially be reversed by overexpression of DKK1. Int J Cancer 123:2013-9.

Graham J, Muhsin M, Kirkpatrick P (2004): Oxaliplatin. Nat Rev Drug Discov 3:11-12.

Gudkov AV, Komarova EA (2003): The role of p53 in determining sensitivity to radiotherapy. Nat Rev Cancer 3:117-29.

Guengerich FP (2008): Cytochrome p450 and chemical toxicology. Chem Res Toxicol 21:70-83.

Hagrman D, Goodisman J, Dabrowiak JC, Souid AK (2003): Kinetic study on the reaction of cisplatin with metallothionein. Drug Metab Dispos 31:916-23.

Hansen LT, Lundin C, Helleday T, Poulsen HS, Sorensen CS, Petersen LN, Spang-Thomsen M (2003): DNA repair rate and etoposide (VP16) resistance of tumor cell subpopulations derived from a single human small cell lung cancer. Lung Cancer 40:157-64.

Hawkins RB (2005): The influence of concentration of DNA on the radiosensitivity of mammalian cells. Int J Radiat Oncol Biol Phys 63:529-35.

Hefferin ML, Tomkinson AE (2005): Mechanism of DNA double-strand break repair by non-homologous end joining. DNA Repair (Amst) 4:639-48.

Hill RP, Rodemann HP, Hendry JH, Roberts SA, Anscher MS (2001): Normal tissue radiobiology: from the laboratory to the clinic. Int J Radiat Oncol Biol Phys 49:353-65.

Holcik M, Yeh C, Korneluk RG, Chow T (2000): Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. Oncogene 19:4174-7.

Holzer AK, Katano K, Klomp LW, Howell SB (2004a): Cisplatin rapidly down-regulates its own influx transporter hCTR1 in cultured human ovarian carcinoma cells. Clin Cancer Res 10:6744-9.

Holzer AK, Katano K, Klomp LWJ, Howell SB (2004b): Cisplatin Rapidly Down-regulates Its Own Influx Transporter hCTR1 in Cultured Human Ovarian Carcinoma Cells. Clin Cancer Res 10:6744-6749.

Holzer AK, Manorek GH, Howell SB (2006): Contribution of the major copper influx transporter CTR1 to the cellular accumulation of cisplatin, carboplatin, and oxaliplatin. Mol Pharmacol 70:1390-4.

Hovinga KE, Stalpers LJ, van Bree C, Donker M, Verhoeff JJ, Rodermond HM, Bosch DA, van Furth WR (2005): Radiation-enhanced vascular endothelial growth factor (VEGF) secretion in glioblastoma multiforme cell lines - a clue to radioresistance? J Neurooncol 74:99-103.

Hsieh P, Yamane K (2008): DNA mismatch repair: molecular mechanism, cancer, and ageing. Mech Ageing Dev 129:391-407.

Huang Z, Lei X, Zhong M, Zhu B, Tang S, Liao D (2007): Bcl-2 small interfering RNA sensitizes cisplatin-resistant human lung adenocarcinoma A549/DDP cell to cisplatin and diallyl disulfide. Acta Biochim Biophys Sin (Shanghai) 39:835-43.

Ikeda Y, Taira K (2006): Ligand-targeted delivery of therapeutic siRNA. Pharm Res 23:1631-40.

Imai K, Yamamoto H (2008): Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. Carcinogenesis 29:673-80.

Ishida S, Lee J, Thiele DJ, Herskowitz I (2002): Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. Proc Natl Acad Sci U S A 99:14298-302.

Ismail SM, Buchholz TA, Story M, Brock WA, Stevens CW (2004): Radiosensitivity is predicted by DNA end-binding complex density, but not by nuclear levels of band components. Radiother Oncol 72:325-32.

Jameel JK, Rao VS, Cawkwell L, Drew PJ (2004): Radioresistance in carcinoma of the breast. Breast 13:452-60.

Jamieson ER, Lippard SJ (1999): Structure, Recognition, and Processing of Cisplatin-DNA Adducts. Chem Rev 99:2467-98.

Johnson DH (2006): Targeted Therapies in Combination with Chemotherapy in Non-Small Cell Lung Cancer. Clin Cancer Res 12:4451s-4457.

Jones KR, Gewirtz DA, Yannone SM, Zhou S, Schatz DG, Valerie K, Povirk LF (2005): Radiosensitization of MDA-MB-231 breast tumor cells by adenovirus-mediated overexpression of a fragment of the XRCC4 protein. Mol Cancer Ther 4:1541-7.

Jones NA, Turner J, McIlwrath AJ, Brown R, Dive C (1998): Cisplatin- and paclitaxel-induced apoptosis of ovarian carcinoma cells and the relationship between bax and bak up-regulation and the functional status of p53. Mol Pharmacol 53:819-26.

Kalayda GV, Wagner CH, Buss I, Reedijk J, Jaehde U (2008): Altered localisation of the copper efflux transporters ATP7A and ATP7B associated with cisplatin resistance in human ovarian carcinoma cells. BMC Cancer 8:175.

Kang HC, Kim IJ, Park JH, Shin Y, Ku JL, Jung MS, Yoo BC, Kim HK, Park JG (2004): Identification of genes with differential expression in acquired drug-resistant gastric cancer cells using high-density oligonucleotide microarrays. Clin Cancer Res 10:272-84.

Kang M-K, Kang S-K (2008): Pharmacologic blockade of chloride channel synergistically enhances apoptosis of chemotherapeutic drug-resistant cancer stem cells. Biochemical and Biophysical Research Communications 373:539-544.

Kase H, Kodama S, Nagai E, Tanaka K (1998): Glutathione S-transferase pi immunostaining of cisplatin-resistant ovarian cancer cells in ascites. Acta Cytol 42:1397-402.

Kawasaki S, Kawamoto S, Yokoi N, Connon C, Minesaki Y, Kinoshita S, Okubo K (2003): Up-regulated gene expression in the conjunctival epithelium of patients with Sjögren's syndrome. Experimental Eye Research 77:17-26.

Kelland L (2007): The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer 7:573-84.

Kienker LJ, Shin EK, Meek K (2000): Both V(D)J recombination and radioresistance require DNA-PK kinase activity, though minimal levels suffice for V(D)J recombination. Nucleic Acids Res 28:2752-61.

Kim JJ, Tannock IF (2005): Repopulation of cancer cells during therapy: an important cause of treatment failure. Nat Rev Cancer 5:516-25.

Kitahara O, Katagiri T, Tsunoda T, Harima Y, Nakamura Y (2002): Classification of sensitivity or resistance of cervical cancers to ionizing radiation according to expression profiles of 62 genes selected by cDNA microarray analysis. Neoplasia 4:295-303.

Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB (1992): Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc Natl Acad Sci U S A 89:7491-5.

Lafay-Cousin L, Sung L, Carret AS, Hukin J, Wilson B, Johnston DL, Zelcer S, Silva M, Odame I, Mpofu C, Strother D, Bouffet E (2008): Carboplatin hypersensitivity reaction in pediatric patients with low-grade glioma: a Canadian Pediatric Brain Tumor Consortium experience. Cancer 112:892-9.

Lambin P, Malaise EP, Joiner MC (1996): Might intrinsic radioresistance of human tumour cells be induced by radiation? Int J Radiat Biol 69:279-90.

Landis SH, Murray T, Bolden S, Wingo PA (1999): Cancer statistics, 1999. CA Cancer J Clin 49:8-31, 1.

Lee EJ, Kong G, Lee SH, Rho SB, Park CS, Kim BG, Bae DS, Kavanagh JJ, Lee JH (2005): Profiling of differentially expressed genes in human uterine leiomyomas. Int J Gynecol Cancer 15:146-54.

Lee KB, Parker RJ, Bohr V, Cornelison T, Reed E (1993): Cisplatin sensitivity/resistance in UV repair-deficient Chinese hamster ovary cells of complementation groups 1 and 3. Carcinogenesis 14:2177-80.

Leith JT, Michelson S (1990): Tumor radiocurability: relationship to intrinsic tumor heterogeneity and to the tumor bed effect. Invasion Metastasis 10:329-51.

Levin WP, Kooy H, Loeffler JS, Delaney TF (2005): Proton beam therapy. Br J Cancer 93:849-54.

Levy D, Adamovich Y, Reuven N, Shaul Y (2008): Yap1 Phosphorylation by c-Abl Is a Critical Step in Selective Activation of Proapoptotic Genes in Response to DNA Damage. 29:350-361.

Li GC, He F, Shao X, Urano M, Shen L, Kim D, Borrelli M, Leibel SA, Gutin PH, Ling CC (2003): Adenovirus-mediated heat-activated antisense Ku70 expression radiosensitizes tumor cells in vitro and in vivo. Cancer Res 63:3268-74.

Li L, Story M, Legerski RJ (2001): Cellular responses to ionizing radiation damage. Int J Radiat Oncol Biol Phys 49:1157-62.

Li YZ, Lu DY, Tan WQ, Wang JX, Li PF (2008): p53 initiates apoptosis by transcriptionally targeting the antiapoptotic protein ARC. Mol Cell Biol 28:564-74.

Liang X-J, Mukherjee S, Shen D-W, Maxfield FR, Gottesman MM (2006): Endocytic Recycling Compartments Altered in Cisplatin-Resistant Cancer Cells. Cancer Res 66:2346-2353.

Liang XJ, Yin JJ, Zhou JW, Wang PC, Taylor B, Cardarelli C, Kozar M, Forte R, Aszalos A, Gottesman MM (2004): Changes in biophysical parameters of plasma membranes influence cisplatin resistance of sensitive and resistant epidermal carcinoma cells. Exp Cell Res 293:283-91.

Lin X, Howell SB (2006): DNA mismatch repair and p53 function are major determinants of the rate of development of cisplatin resistance. Mol Cancer Ther 5:1239-47.

Lin X, Ramamurthi K, Mishima M, Kondo A, Howell SB (2000): p53 Interacts with the DNA Mismatch Repair System to Modulate the Cytotoxicity and Mutagenicity of Hydrogen Peroxide. Mol Pharmacol 58:1222-1229.

Lodish B, Matsudaira, Kaiser, Krieger, Scott, Zipursky, Darnell (2003): "Molecular Cell Biology 5th Ed." W.H. Freeman.

Luo Y, Lin FT, Lin WC (2004): ATM-mediated stabilization of hMutL DNA mismatch repair proteins augments p53 activation during DNA damage. Mol Cell Biol 24:6430-44.

Ma Y, Yu WD, Hershberger PA, Flynn G, Kong RX, Trump DL, Johnson CS (2008): 1alpha,25-Dihydroxyvitamin D3 potentiates cisplatin antitumor activity by p73 induction in a squamous cell carcinoma model. Mol Cancer Ther 7:3047-55.

Mackay HJ, Cameron D, Rahilly M, Mackean MJ, Paul J, Kaye SB, Brown R (2000): Reduced MLH1 expression in breast tumors after primary chemotherapy predicts disease-free survival. J Clin Oncol 18:87-93.

Marchenko ND, Zaika A, Moll UM (2000): Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. J Biol Chem 275:16202-12.

Marchetti P, Cannita K, Ricevuto E, De Galitiis F, Di Rocco ZC, Tessitore A, Bisegna R, Porzio G, De Rubeis GP, Ventura T, Martinotti S, Ficorella C (2003): Prognostic value of p53 molecular status in high-risk primary breast cancer. Ann Oncol 14:704-8.

Marij J.P. Welters AMJF-SRABMAJAHWJFvdVJCBJ (1997): Relationship between the parameters cellular differentiation, doubling time and platinum accumulation and cisplatin sensitivity in a panel of head and neck cancer cell lines. International Journal of Cancer 71:410-415.

Martin LP, Hamilton TC, Schilder RJ (2008): Platinum Resistance: The Role of DNA Repair Pathways. Clin Cancer Res 14:1291-1295.

Marverti G, Monti MG, Pegg AE, McCloskey DE, Bettuzzi S, Ligabue A, Caporali A, D'Arca D, Moruzzi MS (2005): Spermidine/spermine N1-acetyltransferase transient overexpression restores sensitivity of resistant human ovarian cancer cells to N1,N12-bis(ethyl)spermine and to cisplatin. Carcinogenesis 26:1677-1686.

Matsumoto S, Tanaka T, Kurokawa H, Matsuno K, Hayashida Y, Takahashi T (2007): Effect of copper and role of the copper transporters ATP7A and CTR1 in intracellular accumulation of cisplatin. Anticancer Res 27:2209-16.

Mattern MR, Hofmann GA, McCabe FL, Johnson RK (1991): Synergistic cell killing by ionizing radiation and topoisomerase I inhibitor topotecan (SK&F 104864). Cancer Res 51:5813-6.

Maxwell JA, Johnson SP, McLendon RE, Lister DW, Horne KS, Rasheed A, Quinn JA, Ali-Osman F, Friedman AH, Modrich PL, Bigner DD, Friedman HS (2008): Mismatch Repair Deficiency Does Not Mediate Clinical Resistance to Temozolomide in Malignant Glioma. Clin Cancer Res 14:4859-4868.

McKinnon PJ (2004): ATM and ataxia telangiectasia. EMBO Rep 5:772-6.

McMillan TJ, Tobi S, Mateos S, Lemon C (2001): The use of DNA double-strand break quantification in radiotherapy. Int J Radiat Oncol Biol Phys 49:373-7.

Miao Y, Hylarides M, Fisher DR, Shelton T, Moore H, Wester DW, Fritzberg AR, Winkelmann CT, Hoffman T, Quinn TP (2005): Melanoma Therapy via Peptide-Targeted {alpha}-Radiation. Clin Cancer Res 11:5616-5621.

Milde-Langosch K (2005): The Fos family of transcription factors and their role in tumourigenesis. Eur J Cancer 41:2449-61.

Miralbell R, Lomax A, Cella L, Schneider U (2002): Potential reduction of the incidence of radiation-induced second cancers by using proton beams in the treatment of pediatric tumors. Int J Radiat Oncol Biol Phys 54:824-9.

Miyashita T, Reed JC (1995): Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80:293-9.

Miyoshi E, Haruma K, Hiyama T, Tanaka S, Yoshihara M, Shimamoto F, Chayama K (2001): Microsatellite instability is a genetic marker for the development of multiple gastric cancers. Int J Cancer 95:350-3.

Nakano K, Vousden KH (2001): PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 7:683-94.

Nehme A, Baskaran R, Aebi S, Fink D, Nebel S, Cenni B, Wang JYJ, Howell SB, Christen RD (1997): Differential Induction of c-Jun NH2-Terminal Kinase and c-Abl Kinase in DNA Mismatch Repair-proficient and -deficient Cells Exposed to Cisplatin. Cancer Res 57:3253-3257.

Ng CE, Liu QY, Qutob SS, Scott BJ, Tan WL, Mesak FM (2007): Transcriptomic profiling of radiation-resistant or -sensitive human colorectal cancer cells: acute effects of a single X-radiation dose. Int J Oncol 30:1369-80.

Nguyen KH, Hachem P, Khor LY, Salem N, Hunt KK, Calkins PR, Pollack A (2005): Adenoviral-E2F-1 radiosensitizes p53wild-type and p53null human prostate cancer cells. Int J Radiat Oncol Biol Phys 63:238-46.

Nix P, Cawkwell L, Patmore H, Greenman J, Stafford N (2005): Bcl-2 expression predicts radiotherapy failure in laryngeal cancer. Br J Cancer 92:2185-9.

Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y, Taya Y (2000): p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 102:849-62.

Ohmichi M, Hayakawa J, Tasaka K, Kurachi H, Murata Y (2005): Mechanisms of platinum drug resistance. Trends Pharmacol Sci 26:113-6.

Okuda K, Usui E, Ohyama Y (1995): Recent progress in enzymology and molecular biology of enzymes involved in vitamin D metabolism. J Lipid Res 36:1641-52.

Okutsu J, Tsunoda T, Kaneta Y, Katagiri T, Kitahara O, Zembutsu H, Yanagawa R, Miyawaki S, Kuriyama K, Kubota N, Kimura Y, Kubo K, Yagasaki F, Higa T, Taguchi H, Tobita T, Akiyama H, Takeshita A, Wang YH, Motoji T, Ohno R, Nakamura Y (2002): Prediction of chemosensitivity for patients with acute myeloid leukemia, according to expression levels of 28 genes selected by genome-wide complementary DNA microarray analysis. Mol Cancer Ther 1:1035-42.

Olivieri G, Bodycote J, Wolff S (1984): Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. Science 223:594-7.

Omori S, Takiguchi Y, Suda A, Sugimoto T, Miyazawa H, Takiguchi Y, Tanabe N, Tatsumi K, Kimura H, Pardington PE, Chen F, Chen DJ, Kuriyama T (2002): Suppression of a DNA double-strand break repair gene, Ku70, increases radio- and chemosensitivity in a human lung carcinoma cell line. DNA Repair (Amst) 1:299-310.

Ozols RF, Corden BJ, Jacob J, Wesley MN, Ostchega Y, Young RC (1984): High-dose cisplatin in hypertonic saline. Ann Intern Med 100:19-24.

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M (2004): EGFR Mutations in Lung Cancer: Correlation with Clinical Response to Gefitinib Therapy. Science 304:1497-1500.

Pal T, Permuth-Wey J, Kumar A, Sellers TA (2008): Systematic Review and Meta-analysis of Ovarian Cancers: Estimation of Microsatellite-High Frequency and Characterization of Mismatch Repair Deficient Tumor Histology. Clin Cancer Res 14:6847-54.

Pal T, Sutphen R, Sellers T (2004): Mismatch repair gene expression defects contribute to microsatellite instability in ovarian carcinoma. Cancer 100:2485-6; author reply 2486.

Pardo FS, Su M, Borek C, Preffer F, Dombkowski D, Gerweck L, Schmidt EV (1994): Transfection of Rat Embryo Cells with Mutant p53 Increases the Intrinsic Radiation Resistance. Radiation Research 140:180-185.

Pearce AG, Segura TM, Rintala AC, Rintala-Maki ND, Lee H (2001): The generation and characterization of a radiation-resistant model system to study radioresistance in human breast cancer cells. Radiat Res 156:739-50.

Pennati M, Binda M, Colella G, Folini M, Citti L, Villa R, Daidone MG, Zaffaroni N (2003): Radiosensitization of human melanoma cells by ribozyme-mediated inhibition of survivin expression. J Invest Dermatol 120:648-54.

Petersen LN, Mamenta EL, Stevnsner T, Chaney SG, Bohr VA (1996): Increased gene specific repair of cisplatin induced interstrand crosslinks in cisplatin resistant cell lines, and studies on carrier ligand specificity. Carcinogenesis 17:2597-602.

Pisters KM, Le Chevalier T (2005): Adjuvant chemotherapy in completely resected non-small-cell lung cancer. J Clin Oncol 23:3270-8.

Pisters KMW (2005): Adjuvant Chemotherapy for Non-Small-Cell Lung Cancer -- The Smoke Clears. N Engl J Med 352:2640-2642.

Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R (2000): Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. Cancer Res 60:6039-44.

Puntervoll P, Linding R, Gemund C, Chabanis-Davidson S, Mattingsdal M, Cameron S, Martin DM, Ausiello G, Brannetti B, Costantini A, Ferre F, Maselli V, Via A, Cesareni G,

Diella F, Superti-Furga G, Wyrwicz L, Ramu C, McGuigan C, Gudavalli R, Letunic I, Bork P, Rychlewski L, Kuster B, Helmer-Citterich M, Hunter WN, Aasland R, Gibson TJ (2003): ELM server: A new resource for investigating short functional sites in modular eukaryotic proteins. Nucleic Acids Res 31:3625-30.

Pyo H, Choy H, Amorino GP, Kim JS, Cao Q, Hercules SK, DuBois RN (2001): A selective cyclooxygenase-2 inhibitor, NS-398, enhances the effect of radiation in vitro and in vivo preferentially on the cells that express cyclooxygenase-2. Clin Cancer Res 7:2998-3005.

Qutob SS, Multani AS, Pathak S, Feng Y, Kendal WS, Ng CE (2004): Comparison of the X-radiation, drug and ultraviolet-radiation responses of clones isolated from a human colorectal tumor cell line. Radiat Res 161:326-34.

Qutob SS, Proulx D, Mesak FM, Ng CE (2005): Effects of N1, N13-diethylnorspermine (DENSPM) and X-radiation treatment on human colorectal tumor clones with varying X-radiation and drug responses. Radiat Res 163:357-63.

Raaphorst GP, Wilkins DE, Mao JP, Miao JC, Ng CE (1999): Evaluation of cross-resistance between responses to cisplatin, hyperthermia, and radiation in human glioma cells and eight clones selected for cisplatin resistance. Radiat Oncol Investig 7:153-7.

Reyes Lopez V, Navalpotro Yague B (2005): Adjuvant treatment of locally-advanced head and neck tumours. Clin Transl Oncol 7:183-8.

Richardson DL, Backes FJ, Seamon LG, Zanagnolo V, O'Malley DM, Cohn DE, Fowler JM, Copeland LJ (2008): Combination gemcitabine, platinum, and bevacizumab for the treatment of recurrent ovarian cancer. Gynecol Oncol 111:461-6.

Righetti SC, Perego P, Corna E, Pierotti MA, Zunino F (1999): Emergence of p53 Mutant Cisplatin-resistant Ovarian Carcinoma Cells following Drug Exposure:Spontaneously Mutant Selection. Cell Growth Differ 10:473-478.

Rixe O, Ortuzar W, Alvarez M, Parker R, Reed E, Paull K, Fojo T (1996): Oxaliplatin, tetraplatin, cisplatin, and carboplatin: spectrum of activity in drug-resistant cell lines and in the cell lines of the National Cancer Institute's Anticancer Drug Screen panel. Biochem Pharmacol 52:1855-65.

Robinson BW, Im MM, Ljungman M, Praz F, Shewach DS (2003): Enhanced Radiosensitization with Gemcitabine in Mismatch Repair-Deficient HCT116 Cells. Cancer Res 63:6935-6941.

Rodriguez J, Zarate R, Bandres E, Viudez A, Chopitea A, Garcia-Foncillas J, Gil-Bazo I (2007): Combining chemotherapy and targeted therapies in metastatic colorectal cancer. World J Gastroenterol 13:5867-76.

Rogers PM, Beale PJ, Al-Moundhri M, Boxall F, Patterson L, Valenti M, Raynaud F, Hobbs S, Johnston S, Kelland LR (2002): Overexpression of BclXL in a human ovarian carcinoma cell line: paradoxic effects on chemosensitivity in vitro versus in vivo. Int J Cancer 97:858-63.

Rosell R, Taron M, Barnadas A, Scagliotti G, Sarries C, Roig B (2003): Nucleotide excision repair pathways involved in Cisplatin resistance in non-small-cell lung cancer. Cancer Control 10:297-305.

Rusch V, Baselga J, Cordon-Cardo C, Orazem J, Zaman M, Hoda S, McIntosh J, Kurie J, Dmitrovsky E (1993): Differential expression of the epidermal growth factor receptor and its ligands in primary non-small cell lung cancers and adjacent benign lung. Cancer Res 53:2379-85.

Russell NS, Begg AC (2002): Editorial radiotherapy and oncology 2002: predictive assays for normal tissue damage. Radiother Oncol 64:125-9.

Safaei R (2005): Role of copper transporters in the uptake and efflux of platinum containing drugs. Cancer Lett.

Safaei R, Howell SB (2005): Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs. Crit Rev Oncol Hematol 53:13-23.

Safaei R, Katano K, Larson BJ, Samimi G, Holzer AK, Naerdemann W, Tomioka M, Goodman M, Howell SB (2005): Intracellular localization and trafficking of fluorescein-labeled cisplatin in human ovarian carcinoma cells. Clin Cancer Res 11:756-67.

Sakakura C, Sweeney EA, Shirahama T, Igarashi Y, Hakomori S, Tsujimoto H, Imanishi T, Ogaki M, Ohyama T, Yamazaki J, Hagiwara A, Yamaguchi T, Sawai K, Takahashi T (1997): Overexpression of bax sensitizes breast cancer MCF-7 cells to cisplatin and etoposide. Surg Today 27:676-9.

Sakamoto M, Kondo A, Kawasaki K, Goto T, Sakamoto H, Miyake K, Koyamatsu Y, Akiya T, Iwabuchi H, Muroya T, Ochiai K, Tanaka T, Kikuchi Y, Tenjin Y (2001): Analysis of gene expression profiles associated with cisplatin resistance in human ovarian cancer cell lines and tissues using cDNA microarray. Hum Cell 14:305-15.

Samimi G, Fink D, Varki NM, Husain A, Hoskins WJ, Alberts DS, Howell SB (2000): Analysis of MLH1 and MSH2 expression in ovarian cancer before and after platinum drug-based chemotherapy. Clin Cancer Res 6:1415-21.

Samimi G, Katano K, Holzer AK, Safaei R, Howell SB (2004a): Modulation of the cellular pharmacology of cisplatin and its analogs by the copper exporters ATP7A and ATP7B. Mol Pharmacol 66:25-32.

Samimi G, Safaei R, Katano K, Holzer AK, Rochdi M, Tomioka M, Goodman M, Howell SB (2004b): Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin, and oxaliplatin in ovarian cancer cells. Clin Cancer Res 10:4661-9.

Samimi G, Varki NM, Wilczynski S, Safaei R, Alberts DS, Howell SB (2003): Increase in expression of the copper transporter ATP7A during platinum drug-based treatment is associated with poor survival in ovarian cancer patients. Clin Cancer Res 9:5853-9.

Sancar A (1994): Mechanisms of DNA excision repair. Science 266:1954-6.

Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S (2004): Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 73:39-85.

Sanchez-Perez I, Martinez-Gomariz M, Williams D, Keyse SM, Perona R (2000): CL100/MKP-1 modulates JNK activation and apoptosis in response to cisplatin. Oncogene 19:5142-52.

Sasaki H, Sheng Y, Kotsuji F, Tsang BK (2000): Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. Cancer Res 60:5659-66.

Sasaki MS, Ejima Y, Tachibana A, Yamada T, Ishizaki K, Shimizu T, Nomura T (2002): DNA damage response pathway in radioadaptive response. Mutat Res 504:101-18.

Schilder RJ, Ozols RF (1992): New therapies for ovarian cancer. Cancer Invest 10:307-15.

Schwarz VA, Hornung R, Fedier A, Fehr MK, Walt H, Haller U, Fink D (2002): Photodynamic therapy of DNA mismatch repair-deficient and -proficient tumour cells. Br J Cancer 86:1130-1135.

Segal-Bendirdjian E, Mannone L, Jacquemin-Sablon A (1998): Alteration in p53 pathway and defect in apoptosis contribute independently to cisplatin-resistance. Cell Death Differ 5:390-400.

Seno JD, Dynlacht JR (2004): Intracellular redistribution and modification of proteins of the Mre11/Rad50/Nbs1 DNA repair complex following irradiation and heat-shock. J Cell Physiol 199:157-70.

Sharma H, Sen S, Muzio LL, Mariggio A, Singh N (2005): Antisense-mediated downregulation of anti-apoptotic proteins induces apoptosis and sensitizes head and neck squamous cell carcinoma cells to chemotherapy. Cancer Biol Ther 4:720-7.

Shaw P, Bovey R, Tardy S, Sahli R, Sordat B, Costa J (1992): Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc Natl Acad Sci U S A 89:4495-9.

Shen C, Buck AK, Liu X, Winkler M, Reske SN (2003): Gene silencing by adenovirus-delivered siRNA. FEBS Letters 539:111-114.

Sherman ML, Datta R, Hallahan DE, Weichselbaum RR, Kufe DW (1990): Ionizing radiation regulates expression of the c-jun protooncogene. Proceedings of the National Academy of Sciences of the United States of America 87:5663-5666.

Shiloh Y (2001): ATM and ATR: networking cellular responses to DNA damage. Current Opinion in Genetics & Development 11:71-77.

Siddik ZH (2003): Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 22:7265-79.

Siddik ZH, Mims B, Lozano G, Thai G (1998): Independent pathways of p53 induction by cisplatin and X-rays in a cisplatin-resistant ovarian tumor cell line. Cancer Res 58:698-703.

Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rasoul-Rockenschaub S, Bodingbauer M, Crevenna R, Monia BP, Peck-Radosavljevic M, Wacheck V (2006): Mcl-1 overexpression in hepatocellular carcinoma: A potential target for antisense therapy. J Hepatol 44:151-157.

Sjoholm K, Palming J, Olofsson LE, Gummesson A, Svensson P-A, Lystig TC, Jennische E, Brandberg J, Torgerson JS, Carlsson B, Carlsson LMS (2005): A Microarray Search for Genes Predominantly Expressed in Human Omental Adipocytes: Adipose Tissue as a Major Production Site of Serum Amyloid A. J Clin Endocrinol Metab 90:2233-2239.

Sklar MD (1988): The ras oncogenes increase the intrinsic resistance of NIH 3T3 cells to ionizing radiation. Science 239:645-647.

Skvara H, Thallinger C, Wacheck V, Monia BP, Pehamberger H, Jansen B, Selzer E (2005): Mcl-1 blocks radiation-induced apoptosis and inhibits clonogenic cell death. Anticancer Res 25:2697-703.

Smith ML, Chen IT, Zhan Q, O'Connor PM, Fornace AJ, Jr. (1995): Involvement of the p53 tumor suppressor in repair of u.v.-type DNA damage. Oncogene 10:1053-9.

Snyder RD, Schroeder KK (1994): Radiosensitivity of polyamine-depleted HeLa cells and modulation by the aminothiol WR-1065. Radiat Res 137:67-75.

Song IS, Savaraj N, Siddik ZH, Liu P, Wei Y, Wu CJ, Kuo MT (2004): Role of human copper transporter Ctr1 in the transport of platinum-based antitumor agents in cisplatin-sensitive and cisplatin-resistant cells. Mol Cancer Ther 3:1543-9.

Sordella R, Bell DW, Haber DA, Settleman J (2004): Gefitinib-Sensitizing EGFR Mutations in Lung Cancer Activate Anti-Apoptotic Pathways. Science 305:1163-1167.

Stears RL, Martinsky T, Schena M (2003): Trends in microarray analysis. Nat Med 9:140-5.

Stewart DJ (2007): Mechanisms of resistance to cisplatin and carboplatin. Crit Rev Oncol Hematol 63:12-31.

Strathdee G, MacKean MJ, Illand M, Brown R (1999): A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. Oncogene 18:2335-41.

Sun L, Gao H, Sarma VJ, Guo RF, Ward PA (2006): Adenovirus-Mediated In Vivo Silencing of Anaphylatoxin Receptor C5aR. J Biomed Biotechnol 2006:28945.

Surowiak P, Materna V, Kaplenko I, Spaczynski M, Dietel M, Lage H, Zabel M (2005): Augmented expression of metallothionein and glutathione S-transferase pi as unfavourable prognostic factors in cisplatin-treated ovarian cancer patients. Virchows Arch 447:626-33.

Surowiak P, Materna V, Maciejczyk A, Pudelko M, Markwitz E, Spaczynski M, Dietel M, Zabel M, Lage H (2007): Nuclear metallothionein expression correlates with cisplatin resistance of ovarian cancer cells and poor clinical outcome. Virchows Arch 450:279-85.

Thomas T, Thomas TJ (2001): Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cell Mol Life Sci 58:244-58.

Tomar RS, Matta H, Chaudhary PM Use of adeno-associated viral vector for delivery of small interfering RNA. Oncogene 22:5712-5715.

Tong QS, Zheng LD, Wang L, Zeng FQ, Chen FM, Dong JH, Lu GC (2005): Downregulation of XIAP expression induces apoptosis and enhances chemotherapeutic sensitivity in human gastric cancer cells. Cancer Gene Ther 12:509-14.

Vaisman A, Varchenko M, Umar A, Kunkel TA, Risinger JI, Barrett JC, Hamilton TC, Chaney SG (1998): The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and

oxaliplatin resistance: correlation with replicative bypass of platinum-DNA adducts. Cancer Res 58:3579-85.

van de Donk NW, Lokhorst HM, Bloem AC (2005): Growth factors and antiapoptotic signaling pathways in multiple myeloma. Leukemia.

Varma RR, Hector SM, Clark K, Greco WR, Hawthorn L, Pendyala L (2005): Gene expression profiling of a clonal isolate of oxaliplatin-resistant ovarian carcinoma cell line A2780/C10. Oncol Rep 14:925-32.

Vegesna R, Ch S, Ghanshyam S (1999): Induction of p53 dependent apoptosis upon overexpression of a nuclear protein tyrosine phosphatase. FEBS letters 453:308-312.

Virrey JJ, Dong D, Stiles C, Patterson JB, Pen L, Ni M, Schonthal AH, Chen TC, Hofman FM, Lee AS (2008): Stress Chaperone GRP78/BiP Confers Chemoresistance to Tumor-Associated Endothelial Cells. Mol Cancer Res 6:1268-1275.

Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, Liu YP, van Duijse J, Drost J, Griekspoor A, Zlotorynski E, Yabuta N, De Vita G, Nojima H, Looijenga LH, Agami R (2006): A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell 124:1169-81.

Vrána O, Masek V, Drazan V, Brabec V (2007): Raman spectroscopy of DNA modified by intrastrand cross-links of antitumor cisplatin. Journal of Structural Biology 159:1-8.

Wang D, Lippard SJ (2005): Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov 4:307-20.

Wang H, Wang H, Powell SN, Iliakis G, Wang Y (2004a): ATR affecting cell radiosensitivity is dependent on homologous recombination repair but independent of nonhomologous end joining. Cancer Res 64:7139-43.

Wang W, Ke S, Chen G, Gao Q, Wu S, Wang S, Zhou J, Yang X, Lu Y, Ma D (2004b): Effect of lung resistance-related protein on the resistance to cisplatin in human ovarian cancer cell lines. Oncol Rep 12:1365-70.

Welsh C, Day R, McGurk C, Masters JR, Wood RD, Koberle B (2004): Reduced levels of XPA, ERCC1 and XPF DNA repair proteins in testis tumor cell lines. Int J Cancer 110:352-61.

Whiteside MA, Chen DT, Desmond RA, Abdulkadir SA, Johanning GL (2004): A novel time-course cDNA microarray analysis method identifies genes associated with the development of cisplatin resistance. Oncogene 23:744-52.

Wolff S (1998): The adaptive response in radiobiology: evolving insights and implications. Environ Health Perspect 106 Suppl 1:277-83.

Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, Rajeev KG, Nakayama T, Charrise K, Ndungo EM, Zimmermann T, Koteliansky V, Manoharan M, Stoffel M (2007): Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. Nat Biotechnol 25:1149-57.

Wong E, Giandomenico CM (1999): Current status of platinum-based antitumor drugs. Chem Rev 99:2451-66.

Wood RD, Araujo SJ, Ariza RR, Batty DP, Biggerstaff M, Evans E, Gaillard PH, Gunz D, Koberle B, Kuraoka I, Moggs JG, Sandall JK, Shivji MK (2000): DNA damage recognition and nucleotide excision repair in mammalian cells. Cold Spring Harb Symp Quant Biol 65:173-82.

Wulfing C, van Ahlen H, Eltze E, Piechota H, Hertle L, Schmid KW (2007): Metallothionein in bladder cancer: correlation of overexpression with poor outcome after chemotherapy. World J Urol 25:199-205.

Xiang DB, Chen ZT, Wang D, Li MX, Xie JY, Zhang YS, Qing Y, Li ZP, Xie J (2008): Chimeric adenoviral vector Ad5//F35-mediated APE1 siRNA enhances sensitivity of human colorectal cancer cells to radiotherapy in vitro and in vivo. Cancer Gene Ther 15:625-635.

Yaes RJ (1989): Tumor heterogeneity, tumor size, and radioresistance. Int J Radiat Oncol Biol Phys 17:993-1005.

Yamaguchi K, Ishikawa T, Kondo Y, Fujisawa M (2008): Cisplatin regulates Sertoli cell expression of transferrin and interleukins. Mol Cell Endocrinol 283:68-75.

Yan X, Fraser M, Qiu Q, Tsang BK (2006): Over-expression of PTEN sensitizes human ovarian cancer cells to cisplatin-induced apoptosis in a p53-dependent manner. Gynecol Oncol 102:348-55.

Yang LY, Li L, Jiang H, Shen Y, Plunkett W (2000): Expression of ERCC1 antisense RNA abrogates gemicitabine-mediated cytotoxic synergism with cisplatin in human colon tumor cells defective in mismatch repair but proficient in nucleotide excision repair. Clin Cancer Res 6:773-81.

Yoshimasa Nobeyama EO-T, Junichi Furuta, Yohei Miyagi, Kanako Kikuchi, Akifumi Yamamoto, Yukihiro Nakanishi, Hidemi Nakagawa, Toshikazu Ushijima (2007): Silencing of tissue factor pathway inhibitor-2 gene in malignant melanomas. International Journal of Cancer 121:301-307.

Yoshizawa K, Nozaki S, Kitahara H, Ohara T, Kato K, Kawashiri S, Yamamoto E (2007): Copper efflux transporter (ATP7B) contributes to the acquisition of cisplatin-resistance in human oral squamous cell lines. Oncol Rep 18:987-91.

Youn CK, Kim MH, Cho HJ, Kim HB, Chang IY, Chung MH, You HJ (2004): Oncogenic H-Ras up-regulates expression of ERCC1 to protect cells from platinum-based anticancer agents. Cancer Res 64:4849-57.

Young Min Chung B-GK, Chang-Soo Park, Seung Jae Huh, Jhingook Kim, Jong Kuk, Park Sun Mi, Cho Byung Soo, Kim Jun Suk, Kim Young Do, Yoo Duk-Soo Bae. (2005): Increased expression of ICAM-3 is associated with radiation resistance in cervical cancer. International Journal of Cancer 117:194-201.

Zamble DB, Lippard SJ (1995): Cisplatin and DNA repair in cancer chemotherapy. Trends Biochem Sci 20:435-9.

Zembutsu H, Ohnishi Y, Tsunoda T, Furukawa Y, Katagiri T, Ueyama Y, Tamaoki N, Nomura T, Kitahara O, Yanagawa R, Hirata K, Nakamura Y (2002): Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. Cancer Res 62:518-27.

Zhang Z, Li M, Wang H, Agrawal S, Zhang R (2003): Antisense therapy targeting MDM2 oncogene in prostate cancer: Effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. Proc Natl Acad Sci U S A 100:11636-41.

Zhao L-J, Jian H, Zhu H (2003): Specific gene inhibition by adenovirus-mediated expression of small interfering RNA. Gene 316:137-141.

Zhivotovsky B, Joseph B, Orrenius S (1999): Tumor radiosensitivity and apoptosis. Exp Cell Res 248:10-7.

Ziegler DS, Wright RD, Kesari S, Lemieux ME, Tran MA, Jain M, Zawel L, Kung AL (2008): Resistance of human glioblastoma multiforme cells to growth factor inhibitors is overcome by blockade of inhibitor of apoptosis proteins. J Clin Invest 118:3109-22.

APPENDIX

FORMULAE:

1. **Calculation of the Surving Fraction from Clonogenic Assays.**

$$SF = \frac{\text{Colonies observed}}{\text{(PE x cells plated)}}$$

Where: SF = Surviving Fraction

$$\text{PE (plating efficiency)} = \frac{\text{colonies on control}}{100}$$

2. **Calculation of the tumour volume from mouse xenograft studies.**

$$\textit{Tumour Volume} = l \times w \times d\ (\pi/6)$$

Where: l = length

w = width

d = depth

PRELIMINARY DATA:

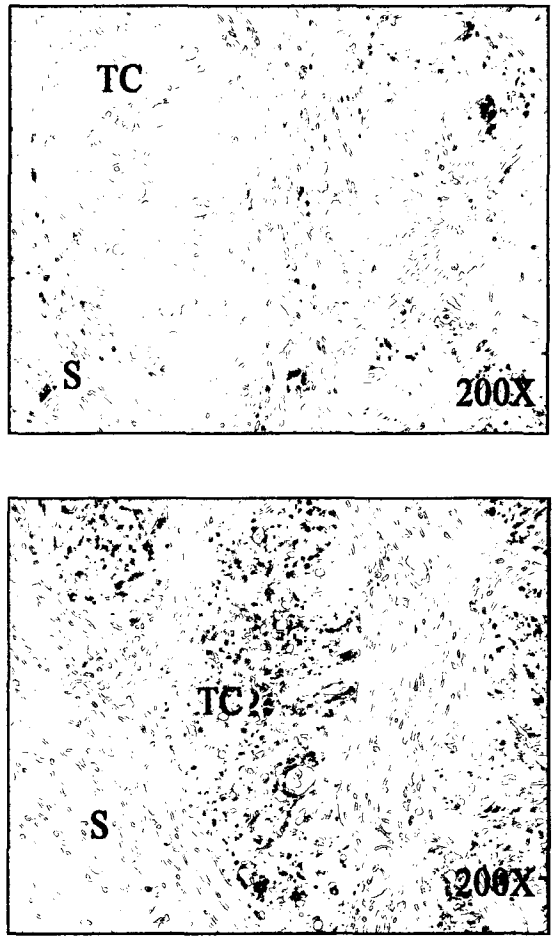

Figure 42: APM2 immunohistochemistry in ovarian carcinoma. APM2 staining in cancerous tissue removed from a patient with recurrent, stage 3, grade 3, and predominantly serous carcinoma with an endometrioid component. Sectioning and immunohistochemistry was performed with the assistance of Ms. Colleen Crane of the Vanderhyden laboratory at the Ottawa Regioanl Cancer Centre. Strong staining of APM2 is seen in the cytoplasm of tumour cells (TC) but not in the stroma (S). No staining was observed when the primary antibody was not applied. Top panel – secondary antibody only, bottom panel – APM2 antibody + secondary antibody. Both panels counterstained with hematoxylin. Magnification 200X.