8-11-2015

Combating the Epigenome: Elucidation of Mechanisms Underlying Chemoresistance and Enhancing Tumor Immunogenicity

Ercan Cacan

Georgia State University

Follow this and additional works at: https://scholarworks.gsu.edu/biology_diss

Recommended Citation

https://scholarworks.gsu.edu/biology_diss/156

This Dissertation is brought to you for free and open access by the Department of Biology at ScholarWorks @ Georgia State University. It has been accepted for inclusion in Biology Dissertations by an authorized administrator of ScholarWorks @ Georgia State University. For more information, please contact scholarworks@gsu.edu.
COMBATING THE EPIGENOME: ELUCIDATION OF MECHANISMS
UNDERLYING CHEMORESISTANCE AND ENHANCING TUMOR IMMUNOGENICITY

by

ERCAN CACAN

Under the Direction of Susanna F. Greer, PhD

ABSTRACT

Chemotherapy and radiation therapy remain the backbone of cancer treatments, and now cancer immunotherapy offers promising new approaches for the treatment of malignancies. One of the major obstacles for chemo-based therapies is acquired chemoresistance. We find Regulator of G-protein signaling protein (RGS10) is an important regulator of cell survival and chemoresistance in ovarian cancer. Our findings further indicate RGS10 transcript expression is suppressed by DNA hypermethylation and histone deacetylation during acquired chemoresistance in ovarian cancer. We identify two important epigenetic regulators, HDAC1 and DNMT1, which exhibit aberrant association with RGS10 promoters in chemoresistant
ovarian cancer cells. Inhibition of DNMT1 or HDAC1 significantly increases RGS10 expression and cisplatin-mediated cell death.

We further focus on modulation of death receptors in advanced colorectal cancer (CRC) cells. We use a combination treatment of irradiation and proteasome inhibition to further induce activation of tumor-specific immune responses. We investigate the effect of the 26S proteasome inhibitor bortezomib alone or in combination with radiotherapy, on the expression of death receptors in normal colon and in colorectal cancer cell lines. Our results indicate a combination of 26S proteasome inhibition and sub-lethal radiation significantly increases the sensitivity of carcinoma cells to apoptosis. Combination treatment up-regulates cell surface expression of DR4, DR5 and Fas by increasing their transcriptional activation. Thus, the combination treatment enhanced sensitivity to killing through FAS and TRAIL receptors by CD8+ T cells. We further characterized the mechanisms by which radiation controls CRC expression of death receptors. We have shown that sub-lethal irradiation increases expression of our target molecules by enhancing histone acetylation at promoter regions through decreasing binding of HDAC2 and HDAC3, and by DNA hypomethylation, via decreasing binding of DNMT1. In sum, our studies provide insight into the alteration of molecular pathways involved in cancer cell death and survival.

INDEX WORDS: Chemoresistance, Immunotherapy, Epigenetics, Radiation, Proteasome
COMBATING THE EPIGENOME: ELUCIDATION OF MECHANISMS UNDERLYING CHEMORESISTANCE AND ENHANCING TUMOR IMMUNOGENICITY

by

ERCAN CACAN

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences Georgia State University 2015
COMBINATING THE EPIGENOME: ELUCIDATION OF MECHANISMS
UNDERLYING CHEMORESISTANCE AND ENHANCING TUMOR IMMUNOGENICITY

by

ERCAN CACAN

Committee Chair: Susanna F. Greer

Committee: Timothy L. Denning
Charlie Garnett-Benson

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
August 2015
DEDICATION

This dissertation is dedicated to my both daughters Hazal and Hilal, and my wife Hatun. This work would not have been possible without their perpetual love, faith, support, patience and encouragement.
ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor, Dr. Susanna F. Greer for guiding, supporting and encouraging me throughout my PhD study. I would like to thank her for her unconditional availability and scientific inputs for every step in the research and providing an excellent laboratory atmosphere that is positive and very supportive, where an independent thought is encouraged but guidance is always available. Greer lab has been definitely a place that I have matured as a scientist and had a chance to interact with a wonderful group of people.

My thesis committee has been very helpful and always accessible. I would like to thank Dr. Charlie Benson for her enormous contributions, experimental helps and availability and Dr. Timothy Denning for his encouragement, feedback and suggestions on my research.

I would like to extend my thanks to all my labmates for their encouragement, collegiality and for being such a huge family. I would like to thank the departmental and university staff for rendering services cordially and efficiently

I owe a huge debt of gratitude to my family for their constant love and support that allow me to take every challenge with confidence.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................................. v

LIST OF FIGURES ........................................................................ xi

1 INTRODUCTION ............................................................................. 1

1.1 Ovarian Cancer and Chemoresistance ........................................ 2

1.2 Regulators of G-protein Signaling (RGS) Proteins and their Regulation in Cancer ........................................................................ 4

1.3 RGS10 and its Regulation in Cancer ............................................ 6

1.4 Chromatin Organization and Accessibility ................................. 6

1.5 Histone Acetylation .................................................................. 7

1.6 DNA Methylation ...................................................................... 8

1.7 The Immune System ............................................................... 9

1.8 Tumor Immunosurveillance and Immunoediting ......................... 11

1.9 Roles of CTLs and NK Cells in Antitumor Immunity .................. 11

1.10 Apoptosis and Death Receptors .............................................. 12

1.11 Radiation-Induced Gene Expression ........................................ 14

1.12 The 26S Proteasome ............................................................. 15

1.13 Summary .............................................................................. 16

2 EPIGENETIC REGULATION OF REGULATOR-G PROTEIN SIGNALING 10 (RGS10) PROTEIN AND OVARIAN CANCER CHEMORESISTANCE .............. 26
2.1 RESULTS........................................................................................................................................... 28

2.1.1 RGS10 Expression is Suppressed in Ovarian Cancer Cells ................... 28

2.1.2 Histone Modifications at RGS10 Promoters in Ovarian Cancer Cells ..... 28

2.1.3 HDAC1 and DNMT1 Suppress RGS10 Expression in Chemoresistant Ovarian Cancer Cells .................................................................................................................................... 31

2.1.4 Inhibition of HDAC and DNMT Activity Enhances RGS10 Expression and Decreases Ovarian Cancer Cell Viability .................................................................................................................................. 32

2.1.5 Knocking Down HDAC1 Enhances Cisplatin-Stimulated Apoptosis in Chemoresistant Cells ........................................................................................................................................... 34

2.1.6 DNMT1 Knock Down Decreases HDAC1 Binding to the RGS10 Promoter in Chemoresistant Ovarian Cancer Cells ......................................................................................................................... 35

2.2 DISCUSSION .................................................................................................................................... 36

2.3 MATERIALS AND METHODS ........................................................................................................ 40

2.3.1 Cell Lines and Reagents ................................................................................................. 40

2.3.2 siRNA Constructs and Transient Transfection ......................................................................... 41

2.3.3 RNA Expression and Quantitative Real-Time PCR ................................................................ 41

2.3.4 Chromatin Immunoprecipitation (ChIP) Assay ..................................................................... 42

2.3.5 Chromatin Immunoprecipitation Assay in siRNA Treated Cells .................................. 43

2.3.6 RNA Expression in siRNA Treated Cells ............................................................................. 44

2.3.7 Apoptosis Assay ..................................................................................................................... 44
3 COMBINATION TREATMENT WITH SUB-LEthal IOnIZING RADIATION AND THE PROTEASOME INHIBITOR, BORTEZOMIB, ENHANCES TUMOR IMMUNOGENICITY AND ANTI-TUMOR IMMUNE ATTACK....................... 60

3.1 RESULTS.......................................................................................................................... 62

3.1.1 Effects of Combination Treatment on Colorectal Cancer Cell Viability.... 62

3.1.2 Combined Treatment does not Inhibit the Initial DNA Repair Response.. 63

3.1.3 Inhibition of the 26S Proteasome Further Enhances Transcript Expression of DR4, DR5 and Fas Over Radiation Alone Treated Carcinoma Cells................................. 65

3.1.4 Combination Treatment with Bortezomib and Radiation Up-regulates Cell Surface Protein Expression of Death Receptors Over Either Modality Alone ............. 66

3.1.5 Proteasome Inhibition can Further Increase Radiation-Induced Sensitivity to Killing Through FasL and TRAIL Receptors.............................................................. 67

3.1.6 Proteasome Inhibition can Further Increase Radiation-Induced Sensitivity to Killing of CRC CELLS by CD8\(^+\) T Cells................................................................. 68

3.2 DISCUSSION ..................................................................................................................... 69

3.3 MATERIAL AND METHODS.......................................................................................... 73

3.3.1 Reagents and Cell Lines.............................................................................................. 73

3.3.2 Irradiation.................................................................................................................... 73

3.3.3 Apoptosis Assay.......................................................................................................... 73

3.3.4 Comet Assay............................................................................................................... 74

3.3.5 RNA Expression and Quantitative Real-Time PCR................................................. 75
3.3.6 Cell Surface Staining and Flow Cytometry Analysis ........................................ 76
3.3.7 Functional Death Receptor Assay ..................................................................... 76
3.3.8 CTL Killing Assay .......................................................................................... 77
3.3.9 Statistical Analyses ....................................................................................... 78

4 RADIATION ENHANCES EXPRESSION OF DEATH RECEPTORS
THROUGH EPigenETIC MECHANISMS ..................................................................... 88

4.1 RESULTS .......................................................................................................... 91

4.1.1 Surface expression of death receptors increase when DNMTs and HDACs are inhibited ............................................................. 91

4.1.2 Radiation increased histone acetylation at Fas and DR5 promoters .......... 92

4.1.3 Radiation treatment decreased binding of HDAC2 and HDAC3 to Fas promoter ........................................................................................................... 93

4.1.4 Radiation treatment decreased DNMT1 binding to Fas promoter .......... 94

4.2 DISCUSSION ..................................................................................................... 94

4.3 MATERIAL AND METHODS .......................................................................... 98

4.3.1 Reagents and Cell Lines ............................................................................... 98

4.3.2 Irradiation ..................................................................................................... 98

4.3.3 Cell Surface Staining and Flow Cytometry Analysis .................................. 99

4.3.4 Western Blot Analyses ................................................................................ 99

4.3.5 Chromatin Immunoprecipitation (ChIP) Assay .......................................... 99
4.3.6 Statistics ........................................................................................................ 100

5 CONCLUSIONS ........................................................................................................ 105

6 REFERENCES ........................................................................................................ 115
LIST OF FIGURES

Figure 1.1 GPCR-mediated cell survival and acquired chemoresistance.................. 19
Figure 1.2 Chromatin organization and accessibility.................................................. 20
Figure 1.3 Histone Acetylation.................................................................................. 21
Figure 1.4 DNA Methylation...................................................................................... 22
Figure 1.5 Cancer immunotherapy .......................................................................... 23
Figure 1.6 Activation of death receptor mediated apoptosis. ..................................... 24
Figure 1.7 Structure of the 26S proteasome................................................................. 25
Figure 2.1 Loss of RGS10 transcript expression in ovarian cancer cells..................... 45
Figure 2.2 Histone acetylation at RGS10 promoters in parental A2780 cells and
chemoresistant A2780-AD cells. .............................................................................. 46
Figure 2.3 HDAC binding at RGS10 promoters in chemoresistant A2780-AD cells
and parental A2780 cells. ..................................................................................... 47
Figure 2.4 Histone H3 and acetylation levels at RGS10 promoters in chemoresistant
C13 cells and parental OV2008 cells ....................................................................... 48
Figure 2.5 Histone acetylation and HDAC binding at RGS10 promoters in IOSE-80
and CAOV-3 ovarian cells. ..................................................................................... 49
Figure 2.6 HDAC1 knockdown significantly increases RGS10 expression while
HDAC1 overexpression decreases RGS10 expression in chemoresistant ovarian cancer
cells. .......................................................................................................................... 50
Figure 2.7 DNMT1 binding is increased at RGS10 promoter and DNMT1 knock
down enhances the RGS10 transcript expression in chemoresistant ovarian cancer cells.. 52
Figure 2.8 Effects of HDAC inhibitor trichostatin A (TSA) and DNMT inhibitor 5-Aza-2′-deoxycytidine (5-Aza-dC) on RGS10 transcript expression ................................................................. 54

Figure 2.9 Effects of combination treatment of TSA and 5-Aza-dC on RGS10 transcript expression and cell viabilities ......................................................... 55

Figure 2.10 HDAC1 knockdown increases cisplatin stimulated apoptosis in chemoresistant cells. ......................................................................................... 56

Figure 2.11 DNMT1 knock down decreases HDAC1 binding to RGS10 promoters in chemoresistant ovarian cancer cells. ......................................................... 58

Figure 3.1 Tumor cells remain viable after a combination treatment of sub-lethal irradiation and proteasome inhibitor ................................................................ 79

Figure 3.2 Initial DNA damage response is not inhibited by 26S proteasome inhibition ........................................................................................................ 80

Figure 3.3 Inhibition of the 26S proteasome enhances transcript expression of death receptors with combination of radiation in tumor cells. ......................... 81

Figure 3.4 A combination treatment of sub-lethal dose of radiation and proteasome inhibition up-regulates cell surface expression of death receptors. ..................... 82

Figure 3.5 Inhibition of the 26S proteasome and sub-lethal irradiation can enhance sensitivity to killing through FAS in HCT116 cells, but not in CCD-18Co cells ............... 83

Figure 3.6 Sub-lethal irradiation and inhibition of the 26S proteasome can enhance sensitivity to killing through TRAIL receptors in both HCT116 and SW620 colorectal cancer cell lines, but not in normal CCD-18Co colon cells. ........................................ 85

Figure 3.7 Combination of sub-lethal irradiation and bortezomib or irradiation alone enhances the killing of SW620 mediated by CTLs. ........................................ 86
Figure 4.1 Cell surface expression of death receptors is regulated by chromatin remodeling enzymes in HCT116 cells. ................................................................. 101

Figure 4.2 IR, TSA or 5-Aza-dC up-regulates the cell surface expression of death receptors in SW620 cells................................................................. 102

Figure 4.3 Histone acetylation at DR5 and FAS promoters in non-radiated and irradiated cells................................................................. 103

Figure 4.4 Histone deacetylases binding to Fas promoters in non-radiated and irradiated cells................................................................. 104

Figure 4.5 DNA methyltransferases binding to Fas promoters in non-radiated and irradiated cells................................................................. Error! Bookmark not defined.
1 INTRODUCTION

Cancer is characterized by uncontrolled cell growth, increased cell proliferation and migration, and decreased apoptosis (Hanahan and Weinberg 2000). How cancer is managed clinically depends on the type and stage of the disease with surgery, chemotherapy, radiotherapy and immunotherapy being the most common cancer treatment modalities (Joensuu 2000). Surgery can help to prevent cancer recurrence, but only in the unlikely scenario that the tumor has not spread to other locations within the body. Chemotherapy is a major therapeutic strategy which uses a variety of toxic drugs to interfere with the basic replication machinery of tumor cells; however, cancer cells can develop resistance to chemotherapeutic drugs (McGuire 2003). Accumulating evidences suggest that one of the mechanisms behind chemoresistance is genetic and epigenetic alternations of crucial genes in cancer cells during the recurring treatment of chemotherapy (Brown, Curry et al. 2014). These alterations can cause the dysregulation of tumor suppressor or oncogenes in cancer cells and gradually induce tumor cells to acquire chemoresistance against chemotherapeutic agents (Wang and Zhong 2015). Radiotherapy is widely used as the standard of care for multiple cancers due to its ability to kill cancer cells directly through via cytotoxic effects. Recent findings suggest that irradiation also modifies the tumor microenvironment, leading to the recruitment of immune cells which can further target tumors for immune regulated killing (Kwilas, Donahue et al. 2012, Kumari, Cacan et al. 2013, Garnett-Benson, Hodge et al. 2015). Cancer immunotherapy approaches are now increasingly being investigated for the treatment of malignancy. Although the immune system has an intrinsic ability to recognize and eliminate tumor cells, tumors frequently interfere with the development and function of anti-tumor immune responses (Murphy 2010, Vitale, Cantoni et al. 2014). Despite many challenges in the field, immunotherapy approaches are promising for cancer
treatment especially for advanced stage cancer (Schweizer and Drake 2014, Simeone, Gentilcore et al. 2014).

The focus of this dissertation will be elucidation of potential mechanisms for ovarian cancer chemoresistance, particularly epigenetic regulation of Regulators of G-protein Signaling 10 (RGS10) protein in chemoresistant ovarian cancer cells, and enhancing tumor immunogenicity against immune responses by using a combination treatment of sub-lethal ionizing radiation and proteasome inhibitor. Our particular interest will be on death receptors. Finally, we will focus on potential mechanisms that sub-lethal radiation use to enhance expression of death receptors.

1.1 Ovarian Cancer and Chemoresistance

Ovarian cancer is one the most deadly gynecological cancer worldwide (Leung, Diamandis et al. 2014). Majority of ovarian cancer patients are diagnosed at advanced stages where the tumor start to spread to other locations, which makes adjuvant chemotherapy necessary for the clinical management of patients (Colombo, Labaki et al. 2014). Platinum based combination chemotherapy is the standard treatment strategy for ovarian cancer patients (Bookman 2012, Luvero, Milani et al. 2014). First line therapeutic drugs such as cisplatin are often initially quite effective and the majority of patients with ovarian cancer respond to the drug, however more than 60% of patients whose tumors initially respond to cisplatin relapse within two-three years with drug-resistant, terminal disease (Galluzzi, Senovilla et al. 2012). Thus, chemoresistance is the major clinical obstacle for the treatment of ovarian cancer patients.

The platinum compound cisplatin is a cytotoxic anti-tumor drug used as first-line chemotherapy in multiple cancers including ovarian (McGuire 2003). Cisplatin reacts
preferentially with the N7 position of guanine base and form variety of monofunctional and bifunctional adducts which cause crosslinks and prevents normal DNA function (Siddik 2003). Damage in DNA blocks replication machinery. This initiates a DNA damage response, leading to activation of p53 tumor suppressor and subsequent G2/M cell cycle arrest and activation of caspase-dependent apoptosis (Dasari and Tchounwou 2014). Cisplatin resistance can be due to limitations in the formation of cytotoxic platinum-DNA adducts and DNA damage tolerance by cellular proteins (Helm and States 2009). DNA adducts activate several signal transduction pathways, including p53 apoptotic pathway. However, mechanisms that inhibit DNA damage signal to apoptosis such as activation of phosphatidylinositol 3-kinase/protein kinase B (also known PI3-K/Akt) pathway interfere with caspase activation (Siddik 2003). Thus, the development of resistance to cisplatin, in part, reflects changes in the signaling pathways that link DNA damage to cell cycle arrest and initiation of apoptosis.

Accumulating evidences suggest that some cancer cells are resistant to chemotherapeutic drugs from the very beginning of anti-cancer drug treatment (Gottesman 2002). These types of cancer cells have the capacity of drug-resistance such as limiting drug uptake and enhancing efflux (Vinogradov and Wei 2012). On the other hand, genetic and epigenetic alternations of crucial genes in cancer cells can also acquire chemoresistance during the recurring treatment of chemotherapy (Brown, Curry et al. 2014, Wang and Zhong 2015). The genetic and epigenetic alterations result in the dysregulation of tumor suppressor or oncogenes in ovarian cells and gradually induce tumor cells to acquire chemoresistance against chemotherapeutic agents. These changes are one mechanism by which ovarian cancer cells undergo the ability to survive in the presence of chemotherapeutic drugs (Berry and Bapat 2008, Karaca, Atmaca et al. 2013).

Although the precise mechanisms for acquired chemoresistance in ovarian cancer
remains unclear, studies suggest that activation of Akt has been strongly linked to cisplatin resistance in ovarian cancer (Yang, Fraser et al. 2008, Zhang, Zhang et al. 2009, Hooks, Callihan et al. 2010, Peng, Wang et al. 2010). Akt (also known as protein kinase B) is a master regulator of multiple cell signaling pathways that involved in cell survival, proliferation and death (Cecconi, Mauro et al. 2012). Akt activation is a potential mechanism for enhanced survival and acquired chemoresistance (Ali, Farrand et al. 2012, Singh, Chaudhry et al. 2013). The Akt signaling pathway is activated by G-protein coupled receptor (GPCR) mediated cell survival signals (Radeff-Huang, Seasholtz et al. 2004) in response to growth factor such as lysophosphatidic acid (LPA).

1.2 Regulators of G-protein Signaling (RGS) Proteins and their Regulation in Cancer

GPCRs are a diverse family of cell surface signaling receptors that regulate a wide variety of cellular functions, including cell proliferation, migration, survival and the development of cancer (Lappano and Maggiolini 2011). Ligand binding to GPCR results in conformational changes in G-protein, which promotes the exchange guanosine triphosphate (GTP) binding to the G-proteins lead to activation of these proteins by splitting into the active Go and Gβγ subunits (Tuteja 2009). Active G-proteins can activate downstream effector proteins PI3K and Akt (Cheaib, Auguste et al. 2015).

Signaling of G-proteins in the cell is terminated by hydrolysis of GTP to guanosine diphosphate (GDP); however, the hydrolysis of GTP to GDP is very slow in the basal state. RGS proteins have profound physiological effects on GPCR signaling (Neitzel and Hepler 2006) and the hydrolysis of GTP to GDP is dramatically accelerated by RGS proteins through their GTPase activating protein (GAP) activity (Hooks, Martemyanov et al. 2008). Thus, alterations in the
expression and activity of RGS proteins can result in profound effects on the G-proteins mediated cellular signaling pathways.

There are over 30 members of the mammalian RGS protein family (Traynor 2010). RGS proteins are composed of variety of sizes and domain, but they share a conserved 120 amino acid, which is known as the RGS domain (RGS box) (Kimple, Bosch et al. 2011). This RGS domain contains the canonical GAP activity of RGS proteins and accelerates the hydrolysis of GTP into GDP up to 1000-fold (Popov, Yu et al. 1997); thus rapid hydrolysis of GTP results in G-protein down-regulating GPCR mediated signaling.

The RGS protein family is classified into eight subfamilies based on their domain structure and sequence homology (Wieland and Mittmann 2003). Thus, this diverse protein family has been associated with different types of cancer. The ability of RGS proteins to attenuate GPCR mediated signaling leads to inhibition of tumor cell migration, proliferation and survival (Hurst, Henkel et al. 2008). Among members of the RGS protein family, RGS2 shows tumor suppressor like activity, particularly in prostate cancer cells (Cao, Qin et al. 2006). RGS5 plays roles in vessel remodeling during carcinogenesis (Holobotovskyy, Manzur et al. 2013). RGS5-deficient mice display vessels morphological changes and improved blood flow (Manzur, Hamzah et al. 2009). RGS16 suppresses the activity of PI3K by sequestering its p85alpha subunit, thus attenuating PI3K-mediated growth signaling pathways in breast cancer cells (Liang, Bansal et al. 2009). RGS10 has been identified as an inhibitor of GPCR mediated cell survival and proliferation signaling in ovarian cancer cells (Hooks, Callihan et al. 2010, Ali, Cacan et al. 2013). It has further been shown that RGS2 is a Gq selective GAP, however; RGS10 and RGS17 specifically display GAP activity against Gi family G-proteins (Hurst and Hooks 2009). In contrast, some RGS proteins have independent of G proteins and GAP activity, for example
RGS13 and RGS16 directly bind and inhibit PI3K (Sethakorn and Dulin 2013). Despite these highly diverse structures and functions in different cancer models, the main role of RGS proteins remains to regulate the duration and amplitude of G-protein signaling through their GAP activity.

1.3 RGS10 and its Regulation in Cancer

It has recently shown however that RGS10 function as tumor suppressor gene that down-regulates LPA stimulated cell survival signaling pathways (Hooks, Callihan et al. 2010). In our study, we will focus on RGS10, which is a relatively small protein and lacks the multiple regulatory domains found in other RGS proteins. Our data demonstrates that RGS10 expression significantly is suppressed in chemoresistant ovarian cancer cells. We further demonstrate that suppressed-RGS10 expression in chemoresistant cells indirectly amplifies receptor-stimulated cell survival signaling to allow cancer cells to escape chemotherapeutic drug-stimulated cell death (Figure 1.1) (Ali, Cacan et al. 2013, Cacan, Ali et al. 2014).

1.4 Chromatin Organization and Accessibility

Eukaryotic cellular DNA is tightly packed in a highly organized form; differential packaging of DNA with histone and non-histone proteins into chromatin determines DNA accessibility during transcription (Andersson, Bjorkroth et al. 1984, Yaniv 2014). The fundamental particle of chromatin is the nucleosome. Histones are integral components of nucleosomes structures as they provide a scaffold for double stranded DNA to wrap around. H2A, H2B, H3 and H4 are four histone proteins, two copies of which form the octameric nucleosome structure along with the linker histone, H1 (Figure 1.2) (Ramakrishnan 1997, Yaniv
Histone tails are subject to several modifications that influence the ability of nucleosomes to form stable higher chromatin structures (Kouzarides 2007).

Compacted chromatin structures are generally repressive to DNA interactions which prevent the movement of RNA polymerases as well as the binding of transcription factors (Venters and Pugh 2009). DNA is negatively charged while histones are positively charged, and thus double stranded DNA is wrapped tightly around histones due to charge differences (Morales, Giamarchi et al. 2001). Histone tails are subject to several modifications that alter N-terminal tails of histones and create the epigenetic code, which influences the accessibility of chromatin (Morales and Richard-Foy 2000). Epigenetic mechanisms cause heritable changes in gene expression without altering the primary DNA sequence (Ptashne 2007). Along with histone modifications, DNA methylation also regulates gene expression. DNA methylation occurs by covalent modification of cytosines to CpG dinucleotides (Strathdee and Brown 2002). DNA methylation and histone acetylation have been heavily studied due to their significant impact on gene expression.

1.5 Histone Acetylation

Histone acetylation generally facilitates gene expression whereas histone deacetylases (HDACs) return DNA to a less accessible conformation by removing acetyl groups from histones (Figure 1.3.) (Kuo and Allis 1998). Histone acetylation is carried out by a group of enzymes called histone acetyltransferases (HATs), which catalyze the transfer of an acetyl group from acetyl-CoA to the amino groups of lysine residues on the N-terminal tails of histones (Hodawadekar and Marmorstein 2007). Acetylation of positively charged lysine residues in...
histone tails influences the ability of histones to neutralize the charge on DNA and thus reduces the stability of chromatin structures (Zhang and Reinberg 2001).

HDACs are a class of enzymes that remove acetyl groups from lysine residues on histones, allowing histones to wrap DNA more tightly (Secrist, Zhou et al. 2003). HDACs are classified into four groups. Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) are found primarily in the nucleus, while HDAC3 is able to shuttle between the nucleus and the cytoplasm (Nakagawa, Oda et al. 2007). Therefore, members of class I HDACs are mainly responsible for regulating gene expression. Pharmacological HDAC inhibitors are widely used to enhance histone acetylation and increase gene expression. For example, Trichostatin A (TSA) selectively inhibits class I and class II HDACs. HDAC inhibitors bind the active site of HDACs to inhibit their function, and have been used for cancer therapy in the last decade (Delcuve, Khan et al. 2013). For instance, the FDA has approved two HDAC inhibitors, Vorinostat and Romidepsin, for the treatment of cutaneous T cell lymphoma and peripheral T-cell lymphoma (Rangwala, Zhang et al. 2012).

Besides the roles of histone acetylation and deacetylation in terms of promoter accessibility, it is also suggested that acetylated or deacetylated histone tails could serve as signals for the binding of other proteins (Braunstein, Sobel et al. 1996). In this case, histone acetylation or deacetylation is targeted by proteins that could lead to alteration in chromatin structures.

1.6 DNA Methylation

DNA methylation is the most studied epigenetic mechanism associated with gene expression. DNA methylation mainly occurs on the cytosine residues of CpG dinucleotides
(Strathdee and Brown 2002) and is carried out by DNA methyltransferases (DNMTs) by catalyzing transfer of the methyl groups to cytosine residues in DNA form methyl cytosine by using S-adenosylmethionine (SAMe) as a methyl donor (Rhee, Jair et al. 2000). In mammalian cells, three active DNMTs have been identified, DNMT1, DNMT3a, and DNMT3b. DNMT3a and DNMT3b facilitate formation of de novo DNA methylation patterns while DNMT1 is required for maintenance of the established patterns of DNA methylation (Jin, Li et al. 2011).

Methylated promoters preferentially bind to methyl CpG binding proteins, which inhibits their recognition by transcription factors and RNA polymerase. Accumulating evidence has shown DNA methylation to play significant roles in cancer by activating oncogenes and silencing tumor suppressors (Wajed, Laird et al. 2001). DNA hypermethylation of CpG dinucleotides frequently contributes to loss of tumor suppressor genes by accumulating in their promoter regions (Figure 1.4.) (Esteller 2002), and aberrant DNA methylation is now associated with drug resistance in cancer (Maeda, Ando et al. 2014). DNA methylation is reversible, and thus, DNA demethylating agents are in clinical trials for several cancer types, with the supposition that DNA demethylation may reactivate tumor suppressor genes (Seidel, Florean et al. 2012, Glasspool, Brown et al. 2014, Navada, Steinmann et al. 2014). Two cytosine analogs, 5-azacytidine and 5-aza-2'-deoxycytidine, have been approved by the FDA for the treatment of myelodysplastic syndrome, a heterogeneous bone marrow disorder, and acute myeloid leukemia (Muller, Ruter et al. 2006).

1.7 The Immune System

The immune system is a complex network of molecules, cells, tissues and organs that work together to protect the body from non-self and also has the ability to recognize growing
tumors via the recognition of cancer specific tumor antigens. The immune system is highly organized into multiple levels of defense. The skin and mucosal surfaces represent the first defense level. If this first level of defense is broken, our body use two equally important types of defense against infections; innate and adaptive immunity.

Innate immunity is fast and broadly effective. Any pathogen that able to penetrate an epithelial surface is immediately encountered by the effector cells and molecules of the innate immune response. Following the infections, resident macrophages phagocytize the pathogen and release inflammatory cytokines that recruit other component of innate immunity such as neutrophils and natural killer (NK) cells to the infected area to terminate the infection (Diefenbach and Raulet 2002, Kobayashi, Voyich et al. 2005, Kumar, Kawai et al. 2009). While a majority of infections are cleared by innate immunity, some pathogens escape from innate immune responses. In this case, the pathogen faces as combination of innate and adaptive immunity.

Adaptive immunity is the third line immune defense of the body and adds specificity to the immune response. Adaptive immunity has two essential components, B and T lymphocytes. B and T cells use highly diverse surface receptors to recognize a vast variety of antigens. B cells can intact pathogens while T cells recognizes only antigens that are presented by major histocompatibility complex (MHC) class I or II molecules (Klug, Miller et al. 2009). CD4+ T cells recognize peptides that are presented by MHC class II and help in the activation of antigen presenting cells (macrophages, dendritic and B cells) (Steinitz, van Helden et al. 2012). CD8+ cytotoxic T cells recognize peptides that are presented by MHC class I and kill infected cells and tumor cells (Foss 2002). Two major mechanisms of CD8+ cytotoxic T cells killing will be discussed below.
1.8 Tumor Immunosurveillance and Immunoediting

Tumor immunosurveillance is the process of identification and elimination of tumor cells by the immune system. Immune responses to cancer cells are similar have to those of virally infected cells (Figure 1.5). Cancer immunosurveillance is an important host protection process that inhibits carcinogenesis and maintains cellular homeostasis (Smyth, Dunn et al. 2006). The immune system is often able to recognize and eliminate cancer cells (Corthay 2014). However, despite tumor immune surveillance, tumors often develop and escape immune recognition. This concept is called immunoediting, which has three main phases; elimination, equilibrium and escape (Dunn, Old et al. 2004). In the elimination phase, the immune system detects and eliminates tumor cells. If the immune system fails to completely eliminate tumor cells, tumor cells either remain dormant or continue to evolve. This phase is known the equilibrium phase where tumor cells undergo additional changes such as modulating the tumor-specific antigens and cell surface receptors. Eventually, the immune system may fail to control tumor growth and development, leading to the escape phase. In this phase, tumor cells continue to grow and expand in an uncontrolled manner and may eventually lead to metastatic malignancies (Dunn, Bruce et al. 2002).

1.9 Roles of CTLs and NK Cells in Antitumor Immunity

The immune system plays an important role in controlling malignancy and tumor elimination (Aptsiauri, Cabrera et al. 2007, Roberti, Mordoh et al. 2012). Tumor specific cytotoxic T lymphocytes (CTLs) and activated NK cells directly contact and kill target tumor cells through two common mechanisms: cytoplasmic granule (perforin/granzyme) release (de
Poot and Bovenschen 2014) and ligating death receptor-induced apoptosis (Chen 2009). Perforins and granzymes protein families can activate cell-death pathways through activation or absence of caspases (Ewen, Kane et al. 2012). In the second pathway, the engagement of target-cell death receptors by their cognate ligands on CTL or NK cells results in caspase dependent apoptosis (Wajant 2014). The death receptor mediated apoptotic pathway is active against a variety of tumor cells. However, the tumor micro-environment subverts the function of CTLs and NK cells by impairing ligation through down-regulating expression of surface receptors on tumor cells (Purdy and Campbell 2009, Qiao and Wong 2009). As self-tissue, tumors are often weakly immunogenic and evoke immune tolerance instead of immunity (Lavoue, Thedrez et al. 2013). Immune tolerance is controlled by various mechanisms of immune suppression and, as a result, anti-tumor CTLs can be rendered ineffective. Common causes for ineffective anti-tumor CTL responses include improper co-stimulation within the tumor site, and down-regulation of cell surface molecules on tumor cells such as death receptors (McDonnell, Robinson et al. 2010, Leone, Shin et al. 2013, Cullen and Martin 2015).

1.10 Apoptosis and Death Receptors

Cell death can be characterized based on its morphological appearance, enzymological criteria, or immunological characteristics (Kroemer, Galluzzi et al. 2009). Apoptosis is characterized in two distinct pathways; the death-receptor-mediated extrinsic path and the Bcl-2-regulated intrinsic path (Hassan, Watari et al. 2014). The extrinsic pathway is initiated by ligation of an appropriate ligand to a subset of TNF receptor superfamily (TNFRSF) members. Following ligation, a series of biochemical and morphological changes occur, which are the result of activation of a subset of the caspases including caspase-3, -6, -7, -8 and -9 (Mcllwain,
Berger et al. 2013). Death receptors are members of the TNFRSF, which include DR4 (TNFRSF10A/TRAIL-R1), DR5 (TNFRSF10B/TRAIL-R2), Fas (CD95/Apo-1), and TNFR1 (Guicciardi and Gores 2009). Ligation of death receptors with cognate death ligands from anti-tumor immune cells induces apoptotic signals in tumor cells (Grimm, Kim et al. 2010). Death receptors contain a death domain which recruits the intracellular adaptor molecule Fas-associated protein with death domain (FADD) and procaspase-8 into the death-inducing signaling complex (DISC) to initiate apoptosis (Figure 1.6). This pathway is negatively regulated by cellular FLICE-like inhibitory protein (cFLIP). Activated caspase-8 can directly activate the extrinsic cell death pathway through activation of caspases-3, -6 and -7 and/or cleave BH3-interacting domain death agonist (Bid) to trigger mitochondrial intrinsic pathway. Cleavage of Bid by caspase-8 results in mitochondria outer membrane permeabilization, release of cytochrome c, formation of the apoptosome, and in caspase-9 activation. Both caspase-8 and 9 cleavage cause downstream activation of the executioner caspases such as caspase-3, -6 and -7 (Scaffidi, Fulda et al. 1998, MacFarlane 2003).

In our study, the focus is on Fas, DR4, and DR5, which are among the most common death receptors that CTLs use to kill tumor cells. Fas is expressed in a variety of cell types including antigen presenting and tumor cells, and is the complementary receptor for Fas-ligand (FasL), which is expressed on tumor cells, CTLs and NK cells (Ramaswamy, Cleland et al. 2009). The interaction of Fas and FasL plays an essential role in triggering apoptosis (Wajant 2014). During cancer progression, the interaction between Fas and FasL is largely impaired due to suppression of Fas expression on tumor cells (Zhu, Liu et al. 2005, Pryczynicz, Guzinska-Ustymowicz et al. 2010). DR4 and DR5 are also essential for driving apoptosis in many types of tumor cells (Koornstra, Kleibeuker et al. 2003), and are receptors for the TNF-related apoptosis-
inducing ligand (TRAIL), which is highly expressed in NK cells and CD8+ T cells (Mirandola, Ponti et al. 2004). TRAIL is part of a natural mechanism to kill tumor cells by the immune system and selectively induces apoptosis in cancer cells with less toxicity towards healthy/non-cancerous cells (Allen and El-Deiry 2012); however, suppression of DR4/5 by tumor cells leads to the development of resistance to immune mediated cell killing (Perraud, Akil et al. 2011). CD8+ T cells and activated NK cells express both TRAIL and its receptor DR5, but both cell types are resistant to TRAIL-mediated cytotoxicity because these cells express high levels of c-FLIP and thus physiologically protect NK and CD8+ T cells from apoptotic action (Mirandola, Ponti et al. 2004).

1.11 Radiation-Induced Gene Expression

Radiation therapy (RT) is commonly used for local tumor control due to its ability to directly kill tumor cells. RT also induces expression of functionally important molecules in tumor cells, and thus enhances the ability of immune cells to recognize and eliminate tumor cells (Ifeadi and Garnett-Benson 2012, Kumari, Cacan et al. 2013). It has also been reported that sub-lethal doses of irradiation results in upregulation of expression of surface molecules on tumor cells, which further influences the processes of co-stimulation, adhesion and transvascular migration of immune cells (Iarilin 1999, Kumari, Cacan et al. 2013, Bernstein, Garnett et al. 2014). Modulation of tumor phenotype by RT enhances anti-tumor T-cell activity in both in vitro and in vivo models (Bernstein, Garnett et al. 2014, Gameiro, Jammeh et al. 2014). These findings indicate that RT not only causes DNA damage, but also upregulates expression of functionally important immunogenic genes that enhance immune responses against tumor cells.
1.12 The 26S Proteasome

The 26S proteasome is a 2.5 MDa multi-protein complex formed by the 19S regulatory and 20S core subcomponents (Figure 1.7), and it is found in the nucleus and cytoplasm of eukaryotic cells (Bedford, Paine et al. 2010). The 26S proteasome is the main non-lysosomal protein degradation machinery and inhibition of the 26S proteasome alters protein turnover and impacts cellular homeostasis (Chen and Dou 2010). Inhibition of the 26S proteasome also alters expression of numerous target genes at the transcriptional level by increasing the stability of transcription factors and/or by regulating epigenetic modifiers (Kinyamu, Jefferson et al. 2008, Bhat and Greer 2011). It has also been reported that critical components of the 19S particle interact with chromatin remodeling enzymes and facilitate their recruitment to promoter regions of some target genes such as MHC-II and class II transactivator (CIITA) promoters, which leads to activation of histone modifications and an opening of chromatin structure. (Koues, Dudley et al. 2008, Koues, Dudley et al. 2009). Roles of the 26S proteasome in regulation has been observed for several transcription factors (Fuchs 2013). For example, inhibition of the 26S proteasome enhances recruitment of p53 to p21waf1 responsive promoters and some components of 19S subunit also physically interact with p53 to facilitate its recruitment to p21waf1 responsive promoters (Zhu, Wani et al. 2007). These findings further suggest that the 26S proteasome can regulate both transcription factors and histone modifying enzymes through its proteolytic and non-proteolytic functions.

Despite advances in understanding the involvement of 26S proteasome on transcription regulation of target genes, it is not well understood how proteasome inhibition alters gene expression. It remains interesting that inhibition of the 26S proteasome promotes its disassociation into 19S and 20S subunits (Aiken, Kaake et al. 2011). This inhibition suggests that
the 26S proteasome potentially regulates transcript regulation of target genes in two ways; first, by stabilizing expression of transcription factors and histone modifying enzymes, and second, by promoting disassociation of the 19S subunit which then directly interacts with transcription factors and histone modifying enzymes and recruits them to promoter regions of target genes. Most of the commercially available proteasome inhibitors directly bind to the 20S catalytic unit where protein degradation takes place (Shen, Schmitt et al. 2013). Bortezomib inhibits the chymotrypsin-like activity of the 26S proteasome (Niewerth, Dingjan et al. 2013), is the first FDA approved 26S proteasome inhibitor, and is being used for the treatment of multiple myeloma and mantle cell lymphoma (Bross, Kane et al. 2004, Chen, Frezza et al. 2011).

1.13 Summary

The dysregulation of gene expression is a hallmark of cancer (Shay and Roninson 2004, Sharma, Kelly et al. 2010, Haria and Naora 2013). Dysregulated gene expression has profound effects on cellular function and is a cause of a majority of human diseases. It is well known that tumor cells aberrantly activate oncogenic pathways and inhibit or down regulate tumor suppressor pathways. Cancer arises not only from defects in genetic events but also from dysregulated epigenetics modifications. Disruption in the balance of DNA methylation or histone acetylation at specific promoters could lead to aberrant expression of oncogenes or suppression of tumor suppressor genes. The contribution of histone acetylation and DNA methylation in dysregulation of gene expression during carcinogenesis is well known, however; involvement of these epigenetic mechanisms at specific promoters is still quite limited. RGS proteins have ability to regulate GPCR signaling, thus alteration in the expression of RGS proteins may dramatically alter the strength of GPCR signaling. Therefore, it is important to understand the
regulation RGS proteins in cancer progression. It has been shown that some of the members of RGS family are dynamically regulated in ovarian cancer cells (Hurst and Hooks 2009, Hooks, Callihan et al. 2010, Ali, Cacan et al. 2013). A recent study reported that RGS2 expression is silenced by DNA methylation, which promotes prostate cancer cell growth (Wolff, Xie et al. 2012). These studies suggest that suppression of some RGS proteins could be due to epigenetic mechanisms. In chapter 2, we show that RGS10 expression is suppressed during ovarian cancer progression and the suppression of RGS10 is linked to aberrant accumulation of DNMTs and HDACs. We find that HDAC1 and DNMT1 synergistically suppress RGS10 expression in chemoresistant ovarian cancer cells. Understanding the regulatory mechanisms that dictate RGS10 gene expression during ovarian cancer progression is an important step towards developing better and more specific adjuvants for ovarian cancer.

Of cancers, the most lethal are those that have gained metastatic ability. Metastasized cancers often down regulate expression of various genes to avoid recognition and response by the immune system. It is appreciated that epigenetic alterations play critical roles in aberrant expression of genes which contribute to tumor cell metastasis. Recent evidence indicates one mechanism utilized by tumor cells to escape recognition and elimination by the immune system is suppression of cell surface expression of immunogenic genes.

Radiotherapy has been extensively used for cancer therapies including colorectal cancer. Sub-lethal doses of radiation can modulate gene expression, making tumor cells more susceptible to T-cell-mediated immune attack (Ifeadi and Garnett-Benson 2012, Kumari, Cacan et al. 2013). Previous studies and clinical trials have shown that combining radiation with other treatments is more effective then radiation treatment alone (Finkelstein and Fishman 2012, Finkelstein, Salenius et al. 2014). In chapter 3, we use a combination treatment of irradiation and proteasome
inhibition to further induce activation of tumor-specific immune responses. We investigate the effects of the 26S proteasome inhibitor, bortezomib, alone or in combination with radiotherapy, on the expression of immunogenic genes in normal colon and in colorectal cancer cell lines. We examine normal colon and colorectal cancer cell lines for changes in expression of multiple death receptors (DR4, DR5 and Fas). Our results indicate a combination of 26S proteasome inhibition and sub-lethal radiation increases the sensitivity of carcinoma cells to apoptosis. Combination treatment up-regulates cell surface expression of death receptors by increasing their transcriptional activation. Thus, the combination treatment enhanced sensitivity to killing through FAS and TRAIL receptors by CD8+ T cells.

In order to better understand radiation induced gene expression, we have investigated novel regulatory mechanisms of death receptors by sub-lethal radiation in chapter 4. As we describe above, expression of mammalian genes is regulated at multiple levels, including transcriptional control. Chapters 4 of this dissertation focuses on the novel roles played by radiation in regulating transcript expression of death receptors. In this chapter we present novel ways of regulating transcription of death receptors, where histone acetylation and DNA methylation play important roles in the expression of death receptors.
Figure 1.1 GPCR-mediated cell survival and acquired chemoresistance.

Agonist binding to GPCR activates G-proteins, which mediate downstream signaling pathways. Akt activation is a potential mechanism for enhanced survival and acquired chemoresistance. RGS proteins inhibit receptor-stimulated Akt activity, and thus suppressed RGS expression in chemoresistant cells indirectly amplifies receptor-stimulated Akt survival signaling to allow cells to escape cisplatin-stimulated cell death.
Figure 1.2 Chromatin organization and accessibility.

DNA is packed with histone and non-histone proteins and differential packaging of DNA with these proteins into chromatin determines DNA accessibility during transcription. H2A, H2B, H3 and H4 are four histone proteins, two copies of which form the octameric nucleosome structure along with the linker histone, H1. Histone tails are subject to several modifications that influence the ability of nucleosomes to form stable higher chromatin structures.
Histone acetylation facilitates gene expression whereas histone deacetylases (HDACs) remove acetyl groups from histones. Acetylation of positively charged lysine residues in histone tails by histone acetyltransferase (HAT) activity influence the ability of histones to neutralize the charge on DNA and thus, reduce the stability of chromatin structure.
Figure 1.4 DNA Methylation

DNA methylation suppresses gene expression and is carried out by DNA methyltransferases (DNMTs) by catalyzing transfer of the methyl groups to cytosine residues in DNA. In mammalian cells, three active DNMTs have been identified. DNMT3a and DNMT3b facilitate formation of de novo DNA methylation patterns while DNMT1 is mainly required for maintenance of the established patterns of DNA methylation.
CD8$^+$ T cells can recognize and eliminate tumor cells. However, dysregulation of surface receptors on tumor cells results in CD8$^-$ T cell to become anergic and tumor cells continue to grow. To elicit an effective immune response against tumors, T cells need to recognize tumor antigens presented by MHC molecules in conjunction with appropriate co-stimulation.

Upregulation of surface proteins on tumor cells leads to activation and proliferation of CD8$^+$ T cells, and activated tumor specific CD8$^+$ T cells can directly contact kill the tumor cells.
Figure 1.6 Activation of death receptor mediated apoptosis.

DR4, DR5 and Fas are death receptors and ligation of death receptors with cognate death ligands from anti-tumor immune cells induces apoptotic signals in tumor cells. Activated caspase-8 can directly activate the extrinsic cell death pathway through activation of caspases-3, -6 and -7 and/or cleave Bid to trigger mitochondrial intrinsic pathway.
Figure 1.7 Structure of the 26S proteasome

The 26S proteasome comprised of core 20S catalytic complex and 19S regulatory complex. The 20S proteasome core has caspase-like, trypsin-like and chymotrypsin-like activities that are associated with three distinct units: β1, β2, β5, respectively. Proteasome inhibitor bortezomib (Brt) specifically inhibit the chymotrypsin-like activity at the β5 subunit.
Ovarian cancer is one of the deadliest gynecological cancers, with a 60% mortality rate in patients and a 5-year survival rate of lower than 30% in advanced stage disease (Siegel, Naishadham et al. 2013). The high mortality rate is due in large part to the development of resistance to chemotherapeutic drugs (Hooks, Callihan et al. 2010, Liu, Nash et al. 2010). Thus, understanding the molecular and genetic mechanisms that drive the development of acquired chemoresistance will enable us to improve current therapeutic agents for ovarian cancer treatment. G-protein coupled receptors (GPCRs) initiate multiple oncogenic signaling pathways in cancer cells by activating their associated G-proteins (Cai and Xu 2013, O’Hayre, Vazquez-Prado et al. 2013). Activation of GPCRs by growth factors such as Lysophosphatidic acid (LPA) triggers survival signaling pathways that drive resistance to chemotherapeutic drugs such as cisplatin and taxane (Hurst and Hooks 2009). GPCR activation of G-proteins is opposed by the activity of regulator of G-protein signaling (RGS) proteins. RGS proteins inhibit G-protein signaling pathways by directly binding to the activated Gα subunit of G-proteins to accelerate hydrolysis of GTP into GDP, which returns G-proteins to an inactive state (Berman and Gilman 1998, Zhong and Neubig 2001, Shi, Harrison et al. 2004, Hurst and Hooks 2009). Relevant to our studies, recent reports indicate that RGS proteins inhibit breast, lung, prostate, and ovarian cancer cell growth through inhibition of GPCRs signaling pathways (Cao, Qin et al. 2006, Liang, Bansal et al. 2009, Xie, Wolff et al. 2009, Hooks, Callihan et al. 2010, Ali, Cacan et al. 2013, Bodle, Mackie et al. 2013).
RGS10 is among the smallest of the RGS proteins and is highly expressed in a broad range of cell types (Lu, Gosslau et al. 2008, Garcia-Bernal, Dios-Espomera et al. 2011, Lee, Chung et al. 2012, Rivero, Gabilondo et al. 2013). RGS10 is an important regulator of cell survival and chemoresistance (Hooks, Callihan et al. 2010), and RGS10 transcript expression is significantly suppressed in multiple ovarian cancer cell lines (Ali, Cacan et al. 2013). Thus, the suppression of RGS10 proteins may contribute to chemoresistance by amplifying GPCR-mediated cell growth and survival signaling pathways. We have recently shown that suppression of RGS10 is due in part to DNA hypermethylation and to histone deacetylation, two important gene-silencing mechanisms which contribute to the progression of many cancers. DNA methylation is maintained by DNA methyl transferases (DNMTs) (Rhee, Jair et al. 2000) and histone deacetylation is maintained by histone deacetylases (HDACs) (Ito, P et al. 2000). Often, these two enzymes coordinately suppress transcriptional activity of genes (Ghoshal, Datta et al. 2002, Cai, Geutjes et al. 2013). Fuks et al. have reported that DNMT1 is associated with histone deacetylase activity and has the ability to bind HDAC1 (Fuks, Burgers et al. 2000). However, the molecular mechanisms by which DNA hypermethylation and histone deacetylation suppress RGS10 and the contribution of these enzymes to acquired chemoresistance remains unknown.

We investigate here the molecular mechanisms of epigenetic regulation of RGS10 expression in ovarian cancer cells and focus on chemosensitive parental A2780 cells and their derivative cell line, chemoresistant A2780-AD. We identify two important epigenetic regulators, HDAC1 and DNMT1, which are highly associated with the RGS10 promoter in chemoresistant ovarian cancer cells. HDAC1 and DNMT1 knock down significantly increases RGS10 expression and cisplatin-stimulated cell death. Our results suggest that HDAC1 and DNMT1 contribute to the suppression of RGS10 during acquired chemoresistance and support growing
evidence that inhibition of HDAC1/DNMT1 represent novel therapeutic approaches to overcoming ovarian cancer chemoresistance.

2.1 RESULTS

2.1.1 RGS10 Expression is Suppressed in Ovarian Cancer Cells

Previous studies have shown that downregulation of RGS10 expression in ovarian cancer cell lines with acquired chemoresistance (Hooks, Callihan et al. 2010). To determine if RGS10 transcript expression is also downregulated in ovarian cancer cell lines we compared RGS10 expression in benign IOSE-80 ovarian cells and the ovarian cancer cell line CAOV-3 (Fig 2.1A). RGS10 transcript expression was significantly lower in CAOV-3 cells compared to IOSE control cells. Next, we compared RGS10 transcript expression chemosensitive parental A2780 cells to their derivative chemoresistant A2780-AD cells. Again, we have seen a significant decrease in RGS10 mRNA expression in chemoresistant cells as compare to parental chemosensitive cells (Fig 2.1B). Taken together, RGS10 transcript expression is reduced in primary ovarian cancer cells and the CAOV-3 cancer cell line relative to immortalized ovarian epithelial cells, and in A2780-AD cells relative to parental cells. These data suggest that RGS10 transcript expression is decreased as ovarian cancer progress.

2.1.2 Histone Modifications at RGS10 Promoters in Ovarian Cancer Cells

Next, we sought to explore potential mechanisms of regulation of RGS10 expression, we assessed histone modifications at RGS10 promoters using ChIP experiments. We compared
acetylation at histones associated with the RGS10 promoter in A2780 and A2780-AD cells, using the GAPDH promoter as a control. Total H3 histone binding was similar at RGS10 and GAPDH promoters (data not shown). In contrast, acetylated H3 histone levels were significantly lower at RGS10 promoters in the chemoresistant A2780-AD cells, while similar levels of acetylated histone H3 were associated with the GAPDH promoter in both cell types (Fig 2.2A-B). Reduced acetylation at Lysine residue 18 in histone 3 (H3K18) is associated with cancer recurrence and poorer clinical outcome in lung, kidney, and breast cancer patients (Seligson, Horvath et al. 2005, Seligson, Horvath et al. 2009). To determine if loss of acetylation of this residue contributed to the loss of histone acetylation in RGS10 promoters in chemoresistant cells, we performed ChIP assays with H3K18-specific antibodies. We observed a slight but significant decrease in H3K18 association with the RGS10 promoter in chemoresistant cells as compared to A2780 parental cells (Fig 2.2C), with no change at the GAPDH control promoter (Fig 2.2D). Histone acetylation is dynamically regulated in cells by the opposing actions of HATs that add the acetyl functional group to histones, and HDACs that remove them. Class I HDACs are over expressed in ovarian cancer tissues and are thought to play a significant role in gene silencing during ovarian cancer progression (Jin, Pak et al. 2008). We observed a striking increase in HDAC1 association with RGS10 promoters in A2780-AD cells as compared to parental A2780 cells (Fig 2.3A). This increase reflects a specific recruitment to the RGS10 promoter, as HDAC1 association with GAPDH promoters was unchanged between cell lines (Fig 2.3B), and total HDAC1 expression levels were not higher in A2780-AD cells (Fig 2.3C).

To confirm these findings in additional cell lines, ChIP assays were carried out in the chemosensitive ovarian cancer cell line OV2008 and in chemoresistant C13 daughter cells. While total levels of histone H3 are similar at both RGS10 and GAPDH promoters in
chemosensitive and chemoresistant cells (Fig 2.4A and 2.4D), levels of acetylated histone H3 are significantly lower at RGS10 promoters in the chemoresistant C13 ovarian cancer cells as compared to chemosensitive OV2008 cells (Fig 2.4B).

We next performed ChIP assays to determine if loss of H3K18 contributes to the loss of global histone acetylation at RGS10 promoters in chemoresistant C13 cells. A significant decrease in H3K18 acetylation at RGS10 promoters was observed in chemoresistant C13 cells (Fig 2.4C), while H3K18 acetylation at the GAPDH promoters in OV2008 and C13 cells remained unchanged (Fig 2.4F). Together these data suggest the loss of acetylation at RGS10 promoters contributes to the loss of RGS10 expression in two independent cell models of chemoresistant ovarian cancer.

To determine if histone modifications at RGS10 promoters may account for the difference in expression in IOSE-80 and CAOV-3 cells, we performed ChIP assays to compare histone acetylation. Again, the level of acetylated histone H3 levels at the GAPDH promoter were unchanged between the cell lines (Fig 2.5B), while the level of acetylated histone H3 associated with the RGS10 promoter in CAOV-3 cancer cells was half that observed in IOSE-80 normal ovarian epithelial cells (Fig 2.5A). We also compared the association of HDAC1 with RGS10 promoters in IOSE-80 and CAOV-3 cells. The level of HDAC1 associated with the control promoter GAPDH was unchanged between cell lines (Fig 2.5D), but was more than doubled at RGS10 promoters in the cancer cell line, compared to IOSE-80 cells (Fig 2.5C). These data show that decreased RGS10 expression in CAOV-3 ovarian cancer cells correlates with enhanced HADC1 binding and loss of histone acetylation at the RGS10 promoter as compared to IOSE-80 cells.
2.1.3 **HDAC1 and DNMT1 Suppress RGS10 Expression in Chemoresistant Ovarian Cancer Cells**

We previously demonstrated that HDAC1 proteins bind with significantly increased frequency to the RGS10 promoter in chemoresistant A2780-AD cells compared to parental chemosensitive A2780 cells (Ali, Cacan et al. 2013). To investigate molecular roles for HDAC1 in regulating RGS10 expression, a siRNA duplex was utilized to specifically knock down endogenous HDAC1 expression in A2780-AD cells. siRNA-mediated knockdown of HDAC1 resulted in a more than 3-fold increase in endogenous RGS10 expression as compared to control siRNA (Fig. 2.6A), suggesting that HDAC1 plays a critical role in regulating RGS10 transcription. In a similar experiment, A2780-AD cells were transfected with HDAC1 to determine the effects of ectopic expression of HDAC1. Overexpression of HDAC1 dramatically reduced RGS10 expression in chemoresistant A2780-AD cells (Fig 2.6B). HDAC1 knockdown increased RGS10 protein expression as well (Fig 2.6C) while HDAC1 overexpression decreased cellular RGS10 protein level in chemoresistant ovarian cancer cells (Fig 2.6D). Together, these data indicate that HDAC1 accumulation at RGS10 promoters contributes to suppression of RGS10 in chemoresistant ovarian cancer cells.

The RGS10 promoter contains a high concentration of CpG dinucleotides making it a potential target for DNMT maintenance methylation during ovarian cancer progression. We first showed by western blot analysis that DNMT1 expression levels are similar in both A2780 and A2780-AD cell lines (Fig 2.7C). To further explore the relevance of DNMT1 in the specific suppression of RGS10 expression, ChIP assays were carried out in A2780 parental and their derivative resistant A2780-AD cells. Lysates were immunoprecipitated with control or anti-DNMT1 antibody and associated DNA was analyzed via quantitative real-time PCR (qRT-PCR).
using specific primers and probes spanning the RGS10 and GAPDH promoters. In contrast to total protein abundance, ChIP assays reveal that binding of DNMT1 is significantly increased at the RGS10 promoter in chemoresistant cells as compared to chemosensitive ovarian cancer cells (Fig 2.7A). Together these data indicate that accumulation of DNMT1 at the RGS10 promoter likely contributes to suppression of RGS10 during ovarian cancer chemoresistance.

Accumulation of DNMT1 at the RGS10 promoter led us to determine if that accumulation affects RGS10 transcript expression level in chemoresistant cells. For this purpose, A2780-AD cells were transfected with DNMT1 siRNA or control siRNA. RNA was extracted and generated cDNA was quantified using qRT-PCR with primers and probes specific for the RGS10 coding region and normalized to housekeeping gene GAPDH expression. The data reveals that knocking down DNMT1 significantly increases endogenous RGS10 transcript (Fig 2.7B) and protein expression (Fig 2.7D) in A2780-AD cells. This increase suggests that DNMT1 is another important player and functions with HDAC1 to regulate the suppression of RGS10 transcription in chemoresistant A2780-AD cells.

2.1.4 **Inhibition of HDAC and DNMT Activity Enhances RGS10 Expression and Decreases Ovarian Cancer Cell Viability**

We next sought to determine if pharmacologic inhibitors of histone deacetylation and DNA methylation can alter the expression of RGS10 in chemoresistant ovarian cancer cells. The HDAC inhibitor TSA and DNMT inhibitor 5-Aza-dC were used to inhibit HDACs and DNMTs, respectively. A2780-AD cells were treated with 500 nM TSA and were incubated for 2 days or were treated with 20µM 5-Aza-dC and incubated for 3, 5 and 7 days. Total RNA was isolated from untreated control cells, TSA, and 5-Aza-dC treated cells. The relative expression of RGS10
transcript expression was quantified by qRT-PCR and was normalized to GAPDH transcript expression. Consistent with observations from HDAC1 and DNMT1 knock down experiments, TSA and 5-Aza-dC treatments both significantly enhanced RGS10 transcript expression in chemoresistant A2780-AD cells (Fig 2.8A and 2.8B).

To explore potential synergistic roles for HDAC1 and DNMT1 in regulating RGS10 expression, combination studies were performed using TSA and 5-Aza-dC in chemoresistant ovarian cancer cells. Again, TSA or 5-Aza-dC alone enhance RGS10 expression in A2780-AD cells, and the combination of these two drugs results in a fold increase in RGS10 expression greater than the sum of the individual effect, suggesting a potential cooperative effect (Fig 2.9A). To investigate cooperative roles for HDAC1 and DNMT1 in cell growth and chemoresistance, A2780-AD cells were treated with TSA and/or 5-Aza-dC in the presence of cisplatin and cell viability assays were performed. 5-Aza-dC alone reduced cell growth by approximately 40%, while TSA alone had a modest but significant effect on cell viability; however, the combination of TSA and 5-Aza-dC inhibited cell viability by 90%. As expected, A2780-AD cells were resistant to cisplatin toxicity, but either TSA or 5-Aza-dC partially re-sensitized the cells to cisplatin-mediated cytotoxicity (Fig 2.9B). To determine if RGS10 upregulation by TSA/5-Aza-dC combined treatment could fully account for loss of cell viability, we attempted to rescue cell viability in the presence of 5-Aza-dC and TSA with RGS10 siRNA. Not surprisingly, knock-down of RGS10 alone did not rescue cell viability, consistent with the broad range of HDAC and DNMT target genes in cancer cells (Fig 2.9C). Thus, RGS10 coordinately regulates cell viability and chemosensitivity with additional HDAC and DNMT target genes.
2.1.5 **Knocking Down HDAC1 Enhances Cisplatin-Stimulated Apoptosis in Chemoresistant Cells**

Our previous work suggests that suppression of RGS10 expression contributes to the development of chemoresistance during ovarian cancer progression through amplification of endogenous survival signaling pathways (Hooks, Callihan et al. 2010, Ali, Cacan et al. 2013), and our results presented here suggest that HDAC1 contributes to the loss of RGS10 expression in chemoresistant ovarian cancer cells. To determine HDAC1-mediated changes in cell survival of chemoresistant ovarian cancer cells, cisplatin resistant A2780-AD cells were transfected with HDAC1 siRNA. Following transfection, cells were incubated with 50µM cisplatin and apoptosis was analyzed using an Annexin V:PE apoptosis detection kit (BD Pharmingen). Annexin V binds phosphatidylserine, which is exposed only in apoptotic cells, while the membrane impermeant DNA label 7-Aminoactinomycin D (7-AAD) selectively binds to GC regions of the DNA only in late apoptotic or dead cells with compromised membranes (Modest and Sengupta 1974, Zwaal and Schroit 1997, van Engeland, Nieland et al. 1998). Thus, early apoptotic cells are stained with only annexin V-PE, while late apoptotic and dead cells are stained with both annexin V-PE and 7-AAD. Flow cytometric analysis was used to distinguish between populations of unlabeled and singly- or doubly-labeled cells. HDAC1 knock down significantly increased the population of cisplatin-stimulated cells from 12.9% to 32.1% that are positive for both annexin V-PE and 7-AAD (late apoptotic or dead cells) (Fig 2.10A). The results are confirmed by three independent experiments (Fig 2.10B) and knock down efficiency of HDAC1 and RGS10 protein expression following the HDAC1 knock down was confirmed in A2780-AD cells by western blot analysis (Fig 2.10C). Together these data suggest that HDAC1 mediated
reduction of RGS10 expression blunts the ability of cisplatin to induce cell death in A2780/AD ovarian cancer cells.

2.1.6 **DNMT1 Knock Down Decreases HDAC1 Binding to the RGS10 Promoter in Chemoresistant Ovarian Cancer Cells**

HDAC1 and DNMT1 contribute to gene silencing through recruiting transcriptional repressors to promoter regions (Robertson, Ait-Si-Ali et al. 2000, Ballestar and Esteller 2002, Di Croce, Raker et al. 2002) and work together to suppress gene expression (Fuks, Burgers et al. 2000, Fuks, Burgers et al. 2001). Our data suggest that HDAC and DNMT activities cooperatively silence RGS10. To investigate crosstalk between these two epigenetic regulators, A2780-AD cells were transfected with DNMT1 siRNA or control siRNA and were incubated for 72 hours. HDAC1 binding to the RGS10 promoter was examined by ChIP assays in DNMT1 siRNA or control siRNA treated A2780-AD cells. DNMT1 knock down significantly decreased binding of HDAC1 to the RGS10 promoter (Fig 2.11A) and western blot analysis (Fig 2.11B) demonstrated successful and specific knockdown of DNMT1. The converse experiment was also performed where A2780-AD cells were transfected with HDAC1 siRNA. Western blot analysis demonstrated knockdown of HDAC1 resulted in suppression of DNMT1 protein expression (data not shown); an observation seen by others as well (Zhou, Agoston et al. 2008). These data suggest that HDAC1 is recruited to the RGS10 promoter via DNMT1 and methyl-CpG binding protein 2 (MeCP2) dependent mechanisms (Fig 2.12). MeCP2 directly interacts with DNMT1 and possible MeCP2-DNMT1 complex recruits HDAC1 to RGS10 promoter via binding Sin3 (Jones, Veenstra et al. 1998, Nan, Ng et al. 1998, Jones, Wade et al. 2001, Vaissiere, Sawan et al. 2008) or NuRD complex (Cai, Geutjes et al. 2013). In this manner, DNMT1 and HDAC1
synergistically contribute suppression of RGS10 transcription expression as ovarian cancer progresses.

2.2 DISCUSSION

GPCRs have been recognized as important drug targets for treatment of multiple cancers (Hurst and Hooks 2009, Carrieri, Perez-Nuerno et al. 2013, Xie, Abel et al. 2013). The strength of signaling pathways initiated by GPCRs is attenuated by members of the RGS protein family, including RGS10 (Hunt, Fields et al. 1996, Popov, Yu et al. 1997, Gold, Heifets et al. 2002, Shi, Harrison et al. 2004). DNA methylation and histone deacetylation are often associated with transcriptional repression of gene expression (Fuks 2005, Robertson 2005) and with decreased responsiveness to chemotherapy (Esteller, Garcia-Foncillas et al. 2000, Giacinti, Vici et al. 2008). We recently linked the suppression of RGS10 expression to ovarian cancer cell survival and chemoresistance, and further showed RGS10 knock down to increase cell growth and survival (Hooks, Callihan et al. 2010, Ali, Cacan et al. 2013). DNA hypermethylation and histone deacetylation contribute to ovarian cancer chemoresistance through amplification of master regulators of multiple cell survival proteins (Terasawa, Sagae et al. 2004, Stronach, Alfraidi et al. 2011, Zeller, Dai et al. 2012, Ali, Cacan et al. 2013). Recent findings suggest epigenetic control mechanisms for cisplatin resistance in ovarian cancer, and multiple genes targets that may be subjected to epigenetic control (Terasawa, Sagae et al. 2004). In our current study, we demonstrate specific contribution of two important epigenetic regulators, HDAC1 and DNMT1, to the suppression of RGS10 expression in chemoresistant ovarian cancer cells.

HDACs remove acetyl groups from substrates, including the histones of chromatin. Conversely, HDAC inhibitors preserve the acetylation status of proteins and induce growth arrest
and apoptosis of cancer cells (Hassig and Schreiber 1997, Kruhlak, Hendzel et al. 2001, Barneda-Zahonero and Parra 2012). Clinical trials show HDAC inhibitors to be effective anti-tumor drugs (Marks, Miller et al. 2003) and HDAC inhibitors have recently shown great therapeutic promise against ovarian cancer (Arts, Angibaud et al. 2007, Witt, Deubzer et al. 2009, Wang, Henkes et al. 2011). Our data suggest that reversal of HDAC1-mediated silencing of RGS10 likely contributes to the growth arrest initiated by HDAC inhibitors.

Multiple epigenetic modifications are commonly disrupted during carcinogenesis. Oncogenes undergo hypomethylation on DNA and acetylation and hypermethylation on histones in order to drive enhanced expression (Jin, Yao et al. 2009, Muller, Jana et al. 2013). Conversely, DNA hypermethylation and histone deacetylation commonly occur on tumor suppressor genes (Nguyen, Gonzales et al. 2001, Herman and Baylin 2003, Esteller 2007). In combination with histone deacetylase inhibition, the addition of a DNA methylation inhibitor has shown robust re-expression of silenced genes in tumor cells (Cameron, Bachman et al. 1999). The data presented here further indicate DNMT1 accumulation at the RGS10 promoter suppresses RGS10 transcription in chemoresistant cells.

We previously observed that RGS10 suppressed activation of the survival factor Akt while RGS10 knock down increased activation of Akt; and thus established a causative relationship between suppression of RGS10 and reduced susceptibility to chemotherapeutic cytotoxicity (Hooks, Callihan et al. 2010). Thus, RGS10 functions as a tumor suppressor by blunting endogenous survival pathways, with the level of expression of endogenous RGS10 playing a critical role in the determination of apoptosis or survival. Hence, we focused here on therapeutic approaches to decrease chemoresistance by enhancing RGS10 expression in chemoresistant ovarian cancer cells. HDAC1 knock down enhanced cisplatin-stimulated
apoptosis in chemoresistant cells, suggesting that knocking down HDAC1 increases RGS10 expression and thus inhibits GPCR-stimulated survival signaling pathways, resulting in an increase of apoptotic cells by the chemotherapeutic drug cisplatin.

DNA methylation and histone deacetylation act synergistically to silence cancer-associated genes in ovarian cancer (Fuks, Burgers et al. 2000, Ghoshal, Datta et al. 2002, Meng, Su et al. 2011, Cai, Geutjes et al. 2013). Ovarian cancer is associated with elevated expression of DNMTs and HDACs and high-level expression of DNMT1 and HDAC1 is prominent in high-grade ovarian tumors (Gu, Yang et al. 2013). HDAC1 binds the N-terminus of DNMT1 to form a transcriptional repression complex and the MeCP2 directly interacts with DNMT1 to maintain DNA methylation (Kimura and Shiota 2003). To explore interactions between DNMT1 and HDAC1 at RGS10 genes, DNMT1 was knocked down and the level of HDAC1 binding to RGS10 promoters was determined by ChIP. DNMT1 knock down significantly decreased HDAC1 binding to the RGS10 promoter in chemoresistant ovarian cancer cells, indicating HDAC1 is recruited to the RGS10 promoter via DNMT1 and MeCP2 dependent mechanisms. Recent reports suggest DNMT1 and HDAC1 expression increases with ovarian cancer stage (Gu, Yang et al. 2013). Our results further imply that the increased expression of DNMT1 and HDAC1 results in the generation of repressive complexes which target RGS10 promoters and cooperate in regulating ovarian cancer progression.

The gold standard of care for ovarian cancer is currently a combination of platinum and taxane chemotherapy which, along with second or third line chemotherapy regimens, often does not provide sufficient results (Dear, Gao et al. 2010, Herzog, Sill et al. 2011). New agents, or new agents in combination with current chemotherapeutic agents, are therefore urgently needed to overcome the drug resistance phenomenon. Gene silencing epigenetic modifications are
reversible (Zhu and Otterson 2003, Espino, Drobic et al. 2005, Carafa, Nebbioso et al. 2011), thus inhibition of DNA hypermethylation and histone deacetylation can be considered as an adjuvant therapeutic approach for ovarian cancer treatment.

HDAC and DNMT1 inhibitors induce a potent anticancer response by inhibiting histone deacetylation and DNA hypermethylation (Johnstone 2002, Takai, Desmond et al. 2004, Lyko and Brown 2005). We have shown that inhibition of DNMTs and HDACs by 5-Aza-dC and TSA induced significant RGS10 transcript expression in chemoresistant cells. Of note, the increase in RGS10 transcript expression was similar in HDAC1 and DNMT1 knock down experiments as in experiments with the general inhibitors TSA (HDACs) or 5-Aza-dC (DNMTs), suggesting that HDAC1 and DNMT1 are the primary enzyme subtypes responsible for suppression of RGS10 genes.

Recent reports indicate tumorigenicity and metastasis of ovarian cancer cells is significantly suppressed by the combination of HDAC inhibitor TSA and 5-aza-2′-deoxycytidine in xenograft mouse models (Meng, Sun et al. 2013). Thus, we determined the effects of a combination of TSA and 5-Aza-dC treatments on RGS10 expression and cell viability in chemoresistant ovarian cancer cells. The combination of these two drugs synergistically enhanced RGS10 transcript expression and chemoresistant cell viability. Further, we show that HDAC1 binding to RGS10 promoters in chemoresistant cells is dependent on DNMT activity. A likely mechanism for this suppression of RGS10 is that the DNMT1 repression complex recruits HDAC1 to RGS10 promoters.

A recent phase I clinical trial was the first to attempt to reverse platinum resistance in ovarian cancer with a combination of methylation and histone deacetylase inhibition (Carafa, Nebbioso et al. 2011, Falchook, Fu et al. 2013). These results show that changing methylation
and acetylation results in changes in clinical outcomes. Further, preselecting patients based on known methylation status may optimize treatment responses (Falchook, Fu et al. 2013). Our study supports RGS10 genes as targets for demethylating and acetylating therapy and identifies RGS10 de-suppression as a likely contributing mechanism for the clinical efficacy of DNMT1 and HDAC1 inhibitors in the treatment of chemoresistant ovarian cancer.

2.3 MATERIALS AND METHODS

2.3.1 Cell Lines and Reagents

The chemosensitive A2780 parental cell line and their derivative chemoresistant A2780-AD cells (derived as described (Louie, Behrens et al. 1985)) were generously provided by Dr. Bob Brown, Imperial College London. These cells were maintained in RPMI 1640 medium (Mediatech Inc.) supplemented with 10% FBS and 5 mM L-glutamine. Chemoresistant cells were further maintained in 3 µM cisplatin. All cells were grown in 5 mM penicillin-streptomycin at 37°C with 5% CO₂. OV2008 and C13 cells were generously provided by Dr. Patricia Kruk, University of South Florida.

5-Aza-2’-deoxycytidine (5-Aza-dC), Trichostatin A (TSA), and cisplatin were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies recognizing HDAC1, goat-anti-rat IgG-HRP (Horseradish peroxidase), and HRP conjugated rabbit antibodies were obtained from Santa Cruz (Santa Cruz, CA). Antibodies recognizing DNMT1 were obtained from Abcam (Cambridge, MA). HRP conjugated mouse antibodies were purchased from Promega (Madison, WI). Antibodies recognizing β-Actin were obtained from Cell Signaling (Beverly, MA).
2.3.2 *siRNA Constructs and Transient Transfection*

Short interfering RNA (siRNA) pre-designed for HDAC1 (Qiagen) and DNMT1 (Santa Cruz) were used to knock down expression of HDAC1 or DNMT1. Scrambled All Star Control siRNA (Qiagen) was used as a control. A2780-AD cells were transfected with 10nM of HDAC1 or DNMT1 specific siRNA or All Star scrambled control siRNA using HiPerfect transfection reagent (Qiagen) according to the manufacturer’s instruction. Following indicated incubation time, cells were harvested and analyzed in western blot, RNA expression, or chromatin immunoprecipitation experiments.

2.3.3 *RNA Expression and Quantitative Real-Time PCR*

mRNA was isolated using Qiazol RNA extraction reagent (Qiagen) as described in the manufacturer’s protocol. Briefly, cells were lysed in Qiazol and agitated on a 3D rotator for 5 minutes. 200µl of chloroform was added and was incubated for three minutes at room temperature. Samples were centrifuged and the aqueous phase (400µl) was transferred to an eppendorf tube. 500µl of isopropanol was added and was incubated for 10 minutes at room temperature. Following centrifugation, pellets were washed with 1mL of cold 75% ethanol, centrifuged and resuspended in 50µl of RNAse free water. RNA was quantified and cDNA was generated from 1µg of total extracted RNA using an Omniscript Reverse Transcription Kit (Qiagen). Following cDNA synthesis, quantitative real-time polymerase chain reaction was performed using TaqMan Universal PCR Master Mix (Roche) and specific primers and probes targeting RGS10 or GAPDH coding regions. Transcript expression was assessed using an ABI prism 7900HT Real-Time PCR System (Applied Biosystems). Reactions were normalized against GAPDH expression and calculations were performed using standard curves generated.
Primers used were: RGS10 Forward: 5'-GAC CCA AGA AGG CGT GAA AAG A-3', RGS10 Reverse: 5'-GCT GGA CAG AAA GGT CAT GTA GA-3', RGS10 probe: 5'-AGA TAA GAC GCA GAT GCA GGA AAA GGC-3', GAPDH Forward: 5'-GGA AGC TCA CTG GCA TGG C-3', GAPDH Reverse: 5'-TAG ACG GCA GGT CAG GTC CA-3' and GAPDH probe: 5'-CCC CAC TGC CAA CGT GTC AGT G-3'.

To determine the effect of 5-Aza-dC and TSA exposure on RGS10 transcript expression, 1 x 10^6 A2780-AD cells were plated per 10cm^2 tissue culture plate and were incubated overnight. Cells were treated with 20µM 5-Aza-dC or with 500ng TSA dissolved in DMSO. TSA treated cells were incubated for 48 hours and 5-Aza-dC treated cells were incubated for three, five, or seven days, with media aspirated and replaced daily. RNA isolation and DNA synthesis were performed as described above.

2.3.4 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as previously described (Ali, Cacan et al. 2013). Briefly, cells were plated at a density of 2 x 10^6 in 10cm^2 plates. Three million cells were crosslinked with 1% formaldehyde for eight minutes at room temperature. Crosslinking reactions were stopped by the addition of 0.125M glycine. Cell nuclei were isolated and were concentrated by lysis in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0) plus protease inhibitors for 30 minutes on ice followed by flash freezing in liquid nitrogen. Nuclei were sonicated using a Bioruptor water bath sonicator (Diagenode) to generate an average of 500bp of sheared DNA which was confirmed by agarose gel electrophoresis. Sonicated lysates were precleared with salmon-sperm/agarose beads (Millipore), 5% of the total lysate was stored as input for normalization. Half of the remaining lysate was immunoprecipitated with 5µg of indicated antibody overnight at 4°C and the other half of the lysate was immunoprecipitated with a control
antibody. Following an additional two hour immunoprecipitation with salmon-sperm coated agarose beads, all samples were washed with each of the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl), LiCl (0.25M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris pH 8.0), and 1xTE (Tris-EDTA); DNA was then eluted with SDS elution buffer (1% SDS, 0.1M NaHCO3). Following elution, cross-links were reversed overnight with 5M NaCl at 65°C and the immunoprecipitated DNA was isolated using phenol:chloroform:isopropanol mix (Invitrogen) as per the manufacturer’s instructions. Isolated DNA was quantified by real time PCR on an ABI prism 7900HT (Applied Biosystems, Foster City, CA) using specific primers and probes targeting RGS10 and GAPDH promoters region. Values generated from real time PCR reactions were calculated based on standard curves generated, were run in triplicate reactions, and were analyzed using the SDS 2.0 program (Applied Biosystems).

### 2.3.5 Chromatin Immunoprecipitation Assay in siRNA Treated Cells

Chemoresistant A2780-AD cells were plated at a density of 1.2 x 10^6 cells per 10cm² tissue culture plates. Cells were treated with siRNA constructs as described above and were incubated for 72 hours. Cells were harvested and 1/10 of the cell volume was removed for analysis of knockdown efficiency by western blot analysis. ChIP assays were carried out as described above on the remaining harvested cells.
2.3.6 **RNA Expression in siRNA Treated Cells**

Cells were plated at a density of 1 x 10^6 cells per 10cm² tissue culture plate and were incubated overnight. The cells were then transfected with the indicated siRNA construct as described above and incubated for 72 hours, and an RNA extraction was performed as described above.

2.3.7 **Apoptosis Assay**

Apoptosis of A2780-AD cells was assessed using the Annexin V: PE Apoptosis Detection Kit I (BD Pharmingen). 1 x 10^6 A2780-AD cells were plated on a 10cm² tissue culture plate and were incubated for 24 hours at 37°C. The cells were treated with HDAC1 siRNA or with control siRNA using the HiPerfect transfection reagent. Following 48 hours incubation, 50µM of cisplatin was added to both HDAC1 siRNA and control siRNA treated cells and the cells were incubated for an additional 48 hours. A2780-AD cells were briefly trypsinized and were harvested. A fraction of the cell volume was removed for western blot analysis and the remaining fraction of cells was washed with cold PBS twice and was resuspended in Annexin V binding buffer at a concentration of 1 x 10^6 cells/ml. The cells were then transferred to 5mL culture tubes containing 5µl of Annexin V-PE and/or 5µl of 7-Aminoactinomycin D (7-AAD). The samples were gently mixed and were incubated for 20 minutes at room temperature. Following the addition of 400µl of Annexin V binding buffer to each tube, samples were analyzed and quantified by flow cytometry and resulting data were analyzed using FlowJo software.
Figure 2.1 Loss of RGS10 transcript expression in ovarian cancer cells.

A) RGS10 transcript expression levels were compared in CAOV-3 ovarian cancer cell lines and IOSE-80 benign ovarian epithelial cells using qRT-PCR. B) RGS10 transcript levels were compared in chemoresistant A2780-AD cells relative to their parental chemosensitive cell line A2780. **: p<0.01, ***: p<0.0001
ChIP assays were carried out in A2780 parental cells and multi-drug resistant A2780-AD. Lysates were immunoprecipitated with control, anti-acetyl histone H3 or anti-acetyl H3K18 antibody. Associated DNA was isolated and analyzed via real time PCR using primers spanning the RGS10 and GAPDH promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). Input values represent 5% of the total cell lysate. * P<0.05. A. Global levels of Histone H3 acetylation associated with RGS10 and B. GAPDH promoters in A2780 and A2780-AD ovarian cancer cells. Values represent mean ± SEM of four independent experiments. C. Levels of histone H3 acetylated at lysine 18 associated with RGS10 and D. GAPDH promoters in A2780 and A2780-AD ovarian cancer cells. Values represent mean ± SEM of four independent experiments.

Figure 2.2 Histone acetylation at RGS10 promoters in parental A2780 cells and chemoresistant A2780-AD cells.
ChIP assays were carried out in A2780 parental cells and multi-drug resistant A2780-AD. Lysates were immunoprecipitated with control or anti-HDAC1 antibody. Associated DNA was isolated and analyzed via real-time PCR using primers spanning the RGS10 and GAPDH promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). Input values represent 5% of the total cell lysate. * P<0.05. A. Levels of HDAC1 associated with RGS10 and B. GAPDH promoters in A2780 and A2780-AD ovarian cancer cells. Values represent mean ± SEM of three independent experiments. C. Western blot analysis of global HDAC1 levels in A2780 and A2780-AD cells.
Figure 2.4 Histone H3 and acetylation levels at RGS10 promoters in chemoresistant C13 cells and parental OV2008 cells

ChIP assays were carried out in OV2008 parental cells and drug resistant C13 cells. Lysates were immunoprecipitated with control, anti-histone H3, anti-acetyl histone H3, or anti-acetyl H3K18 antibodies. Associated DNA was isolated and analyzed via real time PCR using primers spanning the RGS10 and GAPDH promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). Input values represent 5% of the total cell lysate. **p<0.005. A) Global histone H3 levels associated with RGS10 promoter. B) Global levels of histone H3 acetylation associated with RGS10 promoter. C) Levels of histone H3 acetylated at lysine 18 associated with RGS10 promoter. D) Global histone H3 levels associated with GAPDH promoter. E) Global levels of histone H3 acetylation associated with GAPDH promoter. F) Levels of histone H3 acetylated at lysine 18 associated with GAPDH promoter.
Figure 2.5 Histone acetylation and HDAC binding at RGS10 promoters in IOSE-80 and CAOV-3 ovarian cells.

ChIP assays were carried out in normal ovarian IOSE-80 cells and in CAOV-3 ovarian cancer cells. Lysates were immunoprecipitated with control antibody, anti-acetyl histone H3 antibody, or with anti-HDAC1 antibody. Associated DNA was isolated and quantified via real time PCR using primers spanning the RGS10 and GAPDH promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). Input values represent 5% of the total cell lysate. * P<0.05  
A) Global levels of Histone H3 acetylation associated with RGS10 and B) GAPDH promoters in normal and chemosensitive ovarian cancer cells. Values for histone H3 acetylation represent mean ± SEM of two independent experiments. C) HDAC1 levels associated with RGS10 and D) GAPDH promoters in normal and chemosensitive ovarian cancer cells. Values for HDAC1 binding are representative data. Error bars show deviation between technical errors.
**Figure 2.6** HDAC1 knockdown significantly increases RGS10 expression while HDAC1 overexpression decreases RGS10 expression in chemoresistant ovarian cancer cells.

**A)** Knockdown of HDAC1 increases RGS10 mRNA transcription. A2780-AD cells were transfected with HDAC1 siRNA or control siRNA and incubated for 72 hours. RNA was extracted and cDNA was generated by using a reverse primer targeting for RGS10 or GAPDH coding region. Data was quantified using qPCR with primers and probes specific for RGS10 and
GAPDH coding regions. Graph data shows the average of three independent experiments, with error bars denoting standard error of the mean (SEM). Significance was calculated with a Student’s t test **p<0.005. B) HDAC1 overexpression decreases RGS10 expression in chemoresistant cells. A2780/AD cells were plated in 24-well plate and allowed to attach overnight. Cells were transfected with 500 ng HDAC1 or empty vector using FuGene 6 reagent (Promega) according to the manufacturer's protocol. Following 48 hours incubation, cells were harvested in TRIzol (Invitrogen) and the expression of RGS10 and HDAC1 genes was assessed using RT-PCR as described, and normalized to actin gene expression. Values represent mean ± SEM of four independent experiments ***p<0.0005. C) Western blot analysis demonstrating efficiency of HDAC1 knockdown and RGS10 protein expression following HDAC1 knock down with Beta-Actin controls. D) Western blot analysis demonstrating RGS10 protein expression following HDAC1 overexpression with Beta-Actin controls.
Figure 2.7 DNMT1 binding is increased at RGS10 promoter and DNMT1 knock down enhances the RGS10 transcript expression in chemoresistant ovarian cancer cells.

A) Levels of DNMT1 associated with RGS10 promoters in A2780 and A2780-AD ovarian cancer cells. ChIP assays were carried out in A2780 parental and their derivative resistant A2780-AD cells. Lysates were immunoprecipitated with control or anti-DNMT1 antibody. Associated DNA was isolated and analyzed via real time PCR using specific primers and probes spanning the RGS10 and GAPDH promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). Values were normalized to GAPDH and represent mean ± SEM of three independent experiments **p<0.005. B) A2780-AD cells were transfected with DNMT1 siRNA or with control siRNA and were incubated for 72 hours. RNA was extracted and cDNA was generated by using reverse primers targeting RGS10 and GAPDH.
coding regions. Data generated was quantified using qRT-PCR with primers and probes specific for the RGS10 coding region. Values represent mean ± SEM of four independent experiments **p<0.005. C) Western blot analysis of global DNMT1 levels in A2780 and A2780-AD cells. Cells were harvested and lysates were used for immunoprecipitation. Antibody-conjugated agarose beads (IP) for DNMT1 were incubated with rotation overnight. Beads were washed, eluted and subjected to western blot with respective antibodies. D) Western blot analysis for efficiency of knocking down DNMT1 and for RGS10 protein expression following DNMT1 knock down.
Figure 2.8 Effects of HDAC inhibitor trichostatin A (TSA) and DNMT inhibitor 5-Aza-2′-deoxycytidine (5-Aza-dC) on RGS10 transcript expression.

Total RNA was isolated from untreated control cells and TSA or 5-Aza-dC treated cells. The relative expression of RGS10 mRNA was quantified by qRT-PCR and normalized to GAPDH transcript expression * p<0.05; ** p<0.005. A) HDAC inhibition increases RGS10 expression in chemoresistant A2780-AD cells. The cells were plated in a 10cm2 plate and incubated for 24 hours. The following day, cells were treated with 500 nM TSA and were incubated for an additional 48 hours. B) DNMT inhibitor 5-Aza-dC enhances RGS10 expression in chemoresistant cells. Two million A2780-AD cells were seeded in 10 cm2 plates and were incubated for 24 hours. The following day, cells were treated with 20 µM 5-Aza-dC. Media and drug were refreshed every 24 hours. After indicated incubation time (3, 5, and 7 days), cells were harvested, mRNA was isolated, and cDNA was generated and quantified using qRT-PCR with specific primers and probe.
A) A2780-AD cells were plated in 96-well plates and treated with 5 μM 5-Aza-dC for 5 days, 500 nM TSA for 36 hours, a combination of 5 μM 5-Aza-dC for 5 days and 500 nM TSA for the final 36 hours or DMSO. Gene expression was assessed using qRT-PCR as described, and normalized to RPL13A gene expression. The arrow indicates the expression level predicted by an additive effect of TSA and 5-Aza-dC. B) In a parallel experiment, A2780-AD cells were treated under the same conditions as 4C with or without 30 μM cisplatin for the final 12 hours. Cell survival was assessed using CellTiter-Blue fluorimetric viability assays. ***: p<0.001 comparing epigenetic drug to DMSO control in the absence of cisplatin. ###: p<0.001 comparing vehicle versus cisplatin treatment within epigenetic drug treatment groups. The dotted box indicates the cell viability predicted by an additive effect of TSA and 5-Aza-dC. C) A2780/AD cells (5000 cells/well) were plated in 96-well plate and transfected with negative control or RGS10 siRNA duplexes (Ambion Grand Island, NY) as per the manufacturer's protocol using Dharmafect1 transfection reagent (Dharmacon). Cells were dosed with a combination of 5 μM 5-Aza-dC for 3 days and 500 nM TSA for the last 36 h or DMSO. 30 μM cisplatin or vehicle was added for the last 12 h. Cell survival was assessed using CellTiter-Blue fluorimetric viability assays.
Figure 2.10 HDAC1 knockdown increases cisplatin stimulated apoptosis in chemoresistant cells.

ChIP A2780-AD cells were treated with HDAC1 siRNA or control siRNA and were incubated for 48 hours. Following incubation, cells were treated with 50 µM cisplatin and
incubated for addition 48 hours in RPMI 1640 media. An Annexin V: PE Apoptosis Detection Kit I (BD Pharmingen) was used for staining; results were quantified by Flow cytometry analysis and were analyzed using FlowJo software. Viable cells were negative for both annexin V-PE and 7-AAD; early apoptotic cells were positive for annexin V-PE and negative for 7-AAD, whereas late apoptotic dead cells were positive for both annexin V-PE and 7-AAD labeling. A) The relative increase of apoptotic cells in HDAC1- siRNA transfected A2780-AD cells which incorporated the Annexin V-PE and 7-AAD stains. B) Graph represent average of three independent experiments, with error bars denoting SEM *p<0.05. C) Western blot analysis represents efficiency of HDAC1 knockdown and RGS10 protein expression following HDAC1 knock down.
A2780-AD cells were transfected with HDAC1 siRNA or with control siRNA and were incubated for 72 hours. HDAC1 binding to RGS10 promoters was explored with ChIP assays in HDAC1 siRNA or in control siRNA treated A2780-AD cells. Lysates were immunoprecipitated with control or anti-HDAC1 antibodies. Associated DNA was isolated and analyzed via real time PCR using primers spanning the RGS10 and GAPDH promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). Input values represent 5% of the total cell lysate ** p<0.005. A) Levels of HDAC1 associated with RGS10 promoters following DNMT1 knock-down in A2780-AD ovarian cancer cells. Data were normalized to GAPDH and values represent mean ± SEM of three independent experiments. B) Western blot analysis shows specificity and efficiency of DNMT1 knock down in A2780-AD cells.
Figure 2.12 Model for mechanism by which RGS10 is suppressed in chemoresistant A2780-AD cells.

Model for mechanism by which RGS10 is suppressed in chemoresistant A2780-AD cells. MeCP2 binds DNA region, containing methylated CpG dinucleotides. MeCP2 directly interacts with DNMT1 to maintain DNA methylation. MeCP2-DNMT1 complex also recruits HDAC1 to RGS10 promoter via binding Sin3 complex.
3 COMBINATION TREATMENT WITH SUB-LETHAL IONIZING RADIATION AND THE PROTEASOME INHIBITOR, BORTEZOMIB, ENHANCES TUMOR IMMUNOGENICITY AND ANTI-TUMOR IMMUNE ATTACK

Colorectal cancer (CRC) is the third most common cancer type and the 5 year survival rate is less than 30% for advanced colorectal cancer (Siegel, Desantis et al. 2014). Immunotherapies offer a promising modality for the treatment of advanced cancers because the immune system is systemic and thus able to attack metastatic disease (Takeda, Akita et al. 2013, Ahmed, Guha et al. 2014). Tumor-specific cytotoxic T lymphocytes (CTLs) and activated natural killer (NK) cells play particularly important roles in cancer cell killing and are the basis of many immunotherapies (Casati, Varghaei-Nahvi et al. 2013, Cheng, Chen et al. 2013).

One way to improve tumor cell killing by CTLs or NK cells is to enhance expression of death receptors on tumor cells. DR4 (TRAIL-R1), DR5 (TRAIL-R2) and FAS (CD95/Apo-1) are members of the tumor necrosis factor receptor superfamily (TNFRSF), and ligation of death receptors by binding with cognate death ligands from anti-tumor immune cells induces apoptotic signals into tumor cells (Grimm, Kim et al. 2010). FAS is the complementary receptor for FAS-ligand and this interaction plays an important role in triggering apoptosis. During cancer progression, the interaction between FAS and FASL is largely impaired due to suppression of FAS expression on tumor cells (Zhu, Liu et al. 2005, Pryczynicz, Guzinska-Ustymowicz et al. 2010). DR4 and DR5 are receptors for the tumor necrosis factors-related apoptosis-inducing ligand (TRAIL) and they are also essential for driving apoptosis in many types of tumor cells (Koornstra, Kleibeuker et al. 2003). TRAIL is highly expressed in NK cells and CD8+ T cells.
(Mirandola, Ponti et al. 2004), and it is part of a natural mechanism to kill tumor cells by the immune system and selectively induces apoptosis in cancer cells with less toxicity towards healthy/non-cancerous cells (Allen and El-Deiry 2012). However, tumor cells often down-regulate cell surface expression of death receptors in order to avoid elimination by immune cells (Perraud, Akil et al. 2011, Kykalos, Mathaiou et al. 2012). Thus, enhancing the expression of these death receptors on cancer cells could increase tumor cell sensitivity to CTL-mediated killing.

We have shown that sub-lethal doses of radiation can modulate gene expression, making tumor cells more susceptible to immune responses including enhancing T-cell-mediated immune attack (Garnett, Palena et al. 2004, Ifeadi and Garnett-Benson 2012, Kumari, Cacan et al. 2013). While radiation is a useful tool to make tumor cells more susceptible to immune cells (Agassi, Myslicki et al. 2014), effective immunotherapy approaches need to be developed for the treatment of multiple advanced cancer types. The 26S proteasome is a large protein complex formed by 19S regulatory and 20S core subcomponents, and found in the nucleus and cytoplasm of eukaryotic cells (Bedford, Paine et al. 2010). The 26S proteasome is the main non-lysosomal protein degradation machinery and inhibition of the 26S alters protein turnover and impacts cellular homeostasis (Chen and Dou 2010). Inhibition of the 26S also alters expression of numerous target genes at the transcriptional level by increasing the stability of transcription factors and/or epigenetic modifiers (Kinyamu, Jefferson et al. 2008, Bhat and Greer 2011). Bortezomib is the first FDA approved 26S proteasome inhibitor and is currently used for the treatment of multiple myeloma and mantle cell lymphoma (Bross, Kane et al. 2004). Bortezomib specifically inhibits the chymotrypsin-like activity of the 26S (Niewerth, Dingjan et al. 2013).
Recent clinical trials demonstrate the feasibility of using bortezomib concurrently with carboplatin/paclitaxel and radiation in non-small cell lung cancer (Zhao, Foster et al. 2014), and the combination of histone deacetylase and proteasome inhibitors was shown to enhance CD8$^+$ T cell responses in a preclinical cervical cancer model (Huang, Peng et al. 2015). However, it remains unclear if the combination of radiation and proteasome inhibition alters immune responses against tumors. Here we hypothesize that a combination treatment of low dose radiation and bortezomib will increase expression of death receptors in CRC cells, which will make these tumor cells more susceptible to death-receptor mediated cell killing, and will enhance the CTL-mediated anti-tumor immune attack. Our specific goal is to increase expression of death receptors in CRC cells by a combination of low dose radiation and inhibition of the 26S proteasome to enhance CTL-mediated tumor killing. Our data demonstrate that a combination of 26S proteasome inhibition and sub-lethal radiation significantly increases the sensitivity of carcinoma cells, but not normal non-malignant epithelial cells, to apoptosis. Combination treatment upregulates cell surface expression of multiple death receptors by increasing transcriptional activation of each gene. Our studies suggest that combining radiotherapy and proteasome inhibition may simultaneously enhance tumor immunogenicity and the induction of antitumor immunity by enhancing tumor-specific T-cell activity.

3.1 RESULTS

3.1.1 Effects of Combination Treatment on Colorectal Cancer Cell Viability

To investigate the effects of the 26S proteasome inhibitor bortezomib, in combination with radiotherapy, on tumor cell death we used two well characterized colorectal cancer cell
lines (SW620 and HCT116). The tumor cells were mock-irradiated (0 Gy) or irradiated with 5 Gy and were re-cultured for 24 hr. Following incubation, mock-irradiated or irradiated cells were treated with 10nM bortezomib and were incubated for an additional 24 hr. Cell viability was detected based on Annexin V and 7AAD staining (Fig 3.1). Flow cytometric analysis was used to distinguish between populations of live (Annexin V and 7AAD double negative), apoptotic (Annexin V single positive) and dead (Annexin V and 7AAD double positive) cells.

Greater than 90% of the cells remained viable after treatment with IR alone, as previously reported, and greater than 80% remained viable following bortezomib treatment. Interestingly, the combination treatment markedly increased the population of cells that are positive for both annexin V-PE and 7-AAD (late apoptotic and dead cells). The observed values for dead cells went from 0.86% (untreated) to 9.97% (combination treated) of SW620 cells (Fig 3.1A), and from 1.1% (untreated) to 14.0% (combination treated) of HTC116 cells (Fig 3.1B). However, approximately 80% of SW620 and 70% of HCT116 cells remained viable even after combination treatment with both treatments. Our data demonstrate that most tumor cells remain viable after a combination treatment of sub-lethal irradiation and proteasome inhibitor, however the combination treatment enhances tumor cell death as compared to control or individual treatments.

3.1.2 Combined Treatment does not Inhibit the Initial DNA Repair Response

With the observed increase in cellular apoptosis after combined treatment, single cell gel electrophoresis (Comet assays) was used to evaluate whether the combined treatment negatively impacts the DNA damage response. Comet assays allow for a direct visualization of the extent of DNA damage: the greater the damage, the larger the “tail” of the comet (Ostling and Johanson
1984). As cells repair DNA damage, the extent of the comet tail will diminish. Thus, a comparison of results at equal time-points will give insight into differences in the DNA damage repair response following different treatment conditions. To probe for bortezomib’s potential interference in the DNA repair process, cells were pretreated with bortezomib prior to low dose radiation treatment and then assayed at early time-points in order to evaluate any changes in the initial DNA damage repair response. SW620 cells were either untreated or treated with 10nM bortezomib and allowed to incubate for 24 hours. After incubation, the cells were harvested and either mock-irradiated (0 Gy) or irradiated with 10 Gy and then immediately placed on ice or allowed to incubate at room temperature for 20 min followed by ice for 10 min prior to preparation for comet assays under alkaline conditions. The latter incubation conditions allow for approximately 50% DNA damage repair to occur in untreated irradiated cells.

As anticipated, non-irradiated cells (both untreated and treated with 10nM bortezomib) have a near zero Olive tail moment due to a lack of induced DNA damage. Irradiated cells that were not incubated at room temperature exhibit the maximum tail moment due to a lack of a DNA damage repair response; for these assays, there was no difference in the Olive moments between bortezomib treated cells versus untreated cells (Fig 3.2; 0 Gy & 0 min). Cells that were allowed to incubate for 20 min at room temperature and 10 min on ice allowed for approximately 50% DNA repair as seen in the Olive moment; again for these assays, there was no difference in the Olive moment between the bortezomib treated cells versus the untreated cells. All results shown are representative of duplicate experiments; more than 75 measurements were taken for each condition. These data establish that the observed slight increase in apoptosis is not a result of impaired response to initial DNA damage. DNA damage repair occurs rapidly, within the first 2 hour of damage (Calini, Urani et al. 2002). As such, colorectal cancer cells treated with low
dose irradiation followed by bortezomib treatment resulted in cells with no DNA damage after 24 hour incubation (data not shown).

3.1.3  **Inhibition of the 26S Proteasome Further Enhances Transcript Expression of DR4, DR5 and Fas Over Radiation Alone Treated Carcinoma Cells**

The role of proteasome inhibition in the expression of death receptors in response to radiation has not been investigated. We began our investigation by treating cells with either 5 Gy radiation, 10nM bortezomib, or the combination in order to detect altered transcript expression of death receptors. We further included a normal human cell line into our investigation to compare alterations in expression of death receptors between non-malignant and malignant human cells. The non-malignant human cell line, CCD-18Co, and two malignant carcinoma cell lines, SW620 and HCT116, were irradiated and were then treated with bortezomib for 24 hr. DR4, DR5 and FAS mRNA expression was then quantified by qRT-PCR. No alteration in the transcript expression of death receptors was observed in the normal colon CCD-18Co cell line (Fig 3.3A) following any of the individual treatments nor the combination. In contrast, bortezomib upregulated the transcript expression of DR4, DR5 and FAS by 2.5, 2 and 5 fold in SW620 cells (Fig 3.3B). While radiation only increased DR5 transcript expression by 2 fold in SW620 cells, the combination treatment of radiation and bortezomib significantly increased the transcript expression of DR5 by 5.9 fold. DR4 and FAS expression were increased by 4.6 and 7.2 fold after combination treatment.

To determine if increase in the expression of these genes is a common mechanism in carcinoma cells, we evaluated a second human CRC cell line, HCT116. 5 Gy radiation increased transcript expression of death receptors up to 5-fold and bortezomib treatment upregulated the
transcript expression of DR4, DR5 and FAS by 4.5, 3.6 and 2.4 fold (Fig 3.3C). The combination treatment of radiation and bortezomib significantly increased the transcript expression of DR4, DR5 and FAS by 6.8, 5.2 and 12.1 fold (black bars). Overall, HCT116 cells were more responsive to these treatments than SW620 cells. However, the highest quantities of death receptor mRNA for both cells lines were detected following treatment of CRC cells with combination of radiation and bortezomib.

3.1.4 Combination Treatment with Bortezomib and Radiation Up-regulates Cell Surface Protein Expression of Death Receptors Over Either Modality Alone

Next, to determine if combination treatment of proteasome inhibition and low dose radiation could synergize to alter protein expression of DR4, DR5 and FAS, we investigated cell surface expression of these death receptors following treatment of non-malignant and malignant cells. Normal CCD-18Co colon fibroblast cells expressed low level of DR4 without treatment and the surface expression slightly increased with IR treatment but was never detected in greater than 20% of the normal cells. Untreated CCD-18Co colon cells expressed high levels of DR5 and FAS on the surface, but neither radiation nor bortezomib treatment significantly altered DR5 or FAS surface expression on CCD-18Co cells (Fig 3.4A). In contrast to CCD-18 Co cells, both individual treatments and the combination treatment significantly increased cell surface protein expression of DR4, DR5 and FAS in both colorectal carcinoma cell lines (Fig 3.4B-C). High levels of DR5 and FAS surface expression on untreated HCT116 cells make it difficult to see changes in the frequency of cells expressing these receptors. However, changes in the median fluorescence intensity (MFI) values reveal a substantial change in the expression (density) levels of both DR5 and FAS following radiation, bortezomib or combination treatment (Fig 3.4; inset).
Consistent with mRNA expression data, the combination treatment had a considerable impact on protein expression of death receptors in both tumor cells. In contrast, irradiation and inhibition of the 26S proteasome did not impact the near normal cell line. Overall, these data suggest that the combination treatment had little impact on the expression of death receptors in normal colon cell line but significant effect on expression of these proteins in colorectal cancer cells.

3.1.5 Proteasome Inhibition can Further Increase Radiation-Induced Sensitivity to Killing Through FasL and TRAIL Receptors

To investigate if enhanced expression of FAS on colorectal cancer cells by radiation and bortezomib treatment is functional, cells were mock-irradiated (0 Gy) or were irradiated with 5 Gy and re-cultured for 24 hr. Following incubation, mock-irradiated or irradiated cells were treated with 10nM bortezomib and were incubated for an addition 24 hr. Cells were then incubated for 3 hr with of agonistic anti-Fas antibody. Both a low concentration (0.1ug/ml) and a high concentration (1ug/ml) were evaluated and activated caspase-3 was used to determine the percentage of apoptotic cells by flow cytometry. Bortezomib and sub-lethal irradiation did not sensitize near normal colon CCD-18Co cells to killing by anti-Fas (Fig 3.5A). SW620 cells are known to be insensitive to Fas-mediated cell death (Huerta, Heinzerling et al. 2007, Ifeadi and Garnett-Benson 2012), and were used as negative control. As expected, neither radiation nor bortezomib treatment sensitized these cells to killing by the anti-Fas antibody (Fig 3.5B) and only background killing by radiation, bortezomib, and the combination treatment was detectable in these cells. Conversely, irradiation alone or in combination with bortezomib significantly sensitized HCT116 cells to killing by anti-Fas (Fig 3.5C). 5 Gy radiation, 10nM bortezomib or the combination treatment enhanced the percentage of active caspase-3 by more than 2-fold as
compared to untreated cells following incubation with anti-Fas in HCT116 cells. We also found a significant increase with combination treatment as compare to irradiation or bortezomib treatment alone following high or low concentration of anti-Fas treatments in HCT116 cells.

To investigate TRAIL mediated apoptosis following irradiation or inhibition of the 26S proteasome, cells were either irradiated or bortezomib treated and then incubated with recombinant TRAIL protein for 3 hr and the level of activated caspase-3 was measure by flow cytometry. Similar to our observations with Fas-mediated killing, we saw that bortezomib and sub-lethal irradiation did not sensitize CCD-18Co cells to killing by TRAIL (Fig 3.6A). However, irradiation, bortezomib, or the combination treatment significantly enhanced the percentage of active caspase-3 in both colorectal cancer cell lines (Fig 3.6B-C). While SW620 cells are known to be resistant to FAS mediated killing, we observed cell death with TRAIL treatment. These data suggest that low dose radiation or proteasome inhibition upregulates cell surface expression of death receptors, which sensitizes tumor cells to killing by their ligands.

3.1.6 Proteasome Inhibition can Further Increase Radiation-Induced Sensitivity to Killing of CRC cells by CD8+ T Cells

To test if the combination treatment enhances colorectal cancer cell sensitivity to CTLs, SW620 cells were irradiated (5 Gy), treated with bortezomib alone, or treated with combination irradiation plus bortezomib prior to incubation with carcinoembryonic antigen (CEA)-specific CD8+ T cells. The level of active caspase-3 was evaluated in tumor cells by flow cytometry after co-culturing with CEA specific CTLs as a measure of cells undergoing caspase dependent cell death. Tumor cells displayed significantly increased levels of caspase-3 after irradiation (21.0%) and combination treatment with irradiation and bortezomib (37.5%), and a slight increase after
bortezomib treatment alone (11.4%) following incubation with tumor-specific T cells (Fig 3.7A). Moreover, the combination treatment resulted in higher killing than either treatment alone. In the absence of CEA specific T cells the caspase-3 expressions was low after each of the different treatments (control-4.66%, 5Gy-7.4%, bortezomid-10.3%, combination-15.8%; Fig 3.7B) similar to the low levels of cell death seen by Annexin-V and 7AAD viability analysis (Fig 1). Background subtracted values from the average of three independent experiments show that radiation (12.3%) and the combination (17.6%) treatment greatly increase tumor-specific T cell activity against tumor cells. Thus, these data suggest that colorectal carcinoma cells treated with combination irradiation and bortezomib are even more sensitive to killing mediated by tumor antigen specific T cells than tumor cells treated with either modality alone.

3.2 DISCUSSION

Proteasome inhibitor, bortezomib, stimulates multiple signaling cascades, primarily the NF-kB pathway, to induce apoptosis (Sunwoo, Chen et al. 2001, Jane, Premkumar et al. 2011, Yang, Jove et al. 2012). The combination treatment of bortezomib with other agents has been widely studied (Wright 2010). Most of these studies focus on additive or synergistic effect of a combination treatment to directly induce apoptosis (Chang, Huang et al. 2014, Kunami, Katsuya et al. 2014, Uziel, Cohen et al. 2014). Here we focused on the effect of bortezomib and radiation on gene expression that mediates immune mediated apoptosis. Our work significantly contributes to the cancer immunotherapy field by using combination of low dose radiation and proteasome inhibitor in controlling the expression of death receptors on tumor cells for sensitivity to cytolysis by CTLs. In this study, we show that the combination treatment of bortezomib and sub-
lethal radiation significantly increases the cell surface expression of multiple death receptors by increasing their transcriptional abundance and surface expression.

We started to investigate the effects of the 26S proteasome inhibitor, bortezomib, alone or in combination with radiotherapy, on direct induction of cell death in two colorectal cancer cell lines. We found that the level of radiation utilized in these experiments is sub-lethal and the bortezomib concentration is very lowly lethal in colorectal cancer cell lines. Despite an increase in cell death with the combination treatment, over 80% of tumor cells remain viable (Fig 3.1). We also evaluated the possibility of an impairment in the initial DNA damage response as a potential mechanism for the increase in apoptosis. No difference in the extent of DNA damage was observed after the addition of bortezomib to radiation (Fig 3.2) indicating that the DNA damage response is not inhibited by the combination treatment and therefore not likely to be the cause of the increased apoptosis.

It has been shown that ionizing radiation effects proteasome structures (Pervan, Iwamoto et al. 2005) but the dynamics of the induction of proteasome inhibitor in irradiated cells is unclear, and the role of proteasome inhibition in expression of death receptors in response to radiation has not been investigated. Treatments significantly increased the transcript expression of DR4, DR5 and FAS in colorectal tumor cells (Fig 3.3). Interestingly, we did not see any alteration in the transcript expression of death receptor in normal colon CCD-18Co cell line. Consistent with mRNA data, individual treatments or the combination treatment significantly increase cell surface protein expression of DR4, DR5 and FAS in both colorectal carcinoma cell lines (Fig 3.4).

Activation of caspase-3 is known as the endpoint of the caspase cascade that facilitates apoptosis (Tawa, Tam et al. 2001). Thus, we measured the frequency of cells with active
caspase-3 to identify the number of apoptotic cells following treatment with anti-FAS or recombinant TRAIL protein to test if enhanced expression of death receptors by irradiation and bortezomib treatment would increase sensitivity to killing through FASL or TRAIL receptors in tumor cells. Our data indicate that bortezomib and sub-lethal irradiation did not sensitize normal colon CCD-18Co cells to killing by anti-Fas (Fig 3.5A). However, irradiation itself or with combination of bortezomib significantly sensitized HCT116 cells to killing by anti-Fas (Fig 3.5C). Consistent with previous data, none of the treatments sensitized SW620 cells to killing by anti-Fas (Huerta, Heinzerling et al. 2007, Ifeadi and Garnett-Benson 2012) (Fig 3.5B). SW620 cells have acquired genetic defects in apoptotic pathways and thus are resistant to FAS mediated apoptosis, which could be a potential mechanism of how some colon cancer cells escape the immune system.

TRAIL selectively induce apoptosis in tumor cells by binding both DR4 and DR5 death receptors (Schneider, Thome et al. 1997). Our results show that bortezomib and sub-lethal irradiation did not sensitize CCD-18Co cells to killing by TRAIL (Fig 3.6A). However, the treatments significantly enhanced the percentage of active caspase-3 in both colorectal cancer cell lines (Fig 3.6B & 3.6C). Despite having defects in FAS-mediated apoptosis, SW620 cells were sensitized to TRAIL-mediated apoptosis following radiation, bortezomib and the combination treatment.

One of the most effective cancer immunotherapy strategies is to generate tumor-associated antigen (TAA) specific CTLs that are capable of killing tumor cells (Blattman and Greenberg 2004, Dunn, Old et al. 2004, Rosenberg, Restifo et al. 2008). TAAs are derived from normal cellular proteins that have been mutated or are overexpressed by tumor cells (Zhang, Casiano et al. 2003). For example, carcinoembryonic antigen (CEA) peptide is highly expressed
in several cancer types, including CRC, and the immune system is not tolerant to these tumor-derived antigens (Tsang, Zaremba et al. 1995). The generation of CEA specific CTL responses in human has been under investigation in clinical trials for several cancer types (Gulley, Arlen et al. 2008, Lesterhuis, De Vries et al. 2010, Mohebtash, Tsang et al. 2011). Thus, enhancing tumor cell recognition by CTLs could increase tumor killing rate by TAA specific CTLs. Our results demonstrate that bortezomib and sub-lethal irradiation dramatically enhanced the percentage of SW620 colorectal cancer cells killed by CEA-specific T cells and the combination treatment further increased the percentage of apoptotic cells upon interaction with T cells (Fig 7). These data suggest that low dose radiation or proteasome inhibition sensitize tumor cells to death-receptor mediated apoptosis, possibly through upregulation of death receptors, which sensitizes tumor cells to CTL-mediated killing.

Consistent with previous studies, bortezomib treatment did not alter expression of death receptors in normal colon cells (Nawrocki, Carew et al. 2006), which suggest that bortezomib has tumor selectivity. It is unclear why normal cells show less response to bortezomib treatment, but this could be because tumor cells require more protein synthesis which increases their dependency on proteasomal degradation. Furthermore, we didn’t see much change in FAS and TRAIL induced apoptosis in normal cells following bortezomib or radiation treatment (Fig 3.5A & 3.6A). Conversely, tumor cells were much more sensitive than normal cells to proteasome inhibition and radiation treatment. This could be due to a loss of checkpoint mechanisms in cancer cells.

Overall, these findings indicate that combined treatment of bortezomib and radiation can be used as potential therapeutic regimen for the treatment of advanced colorectal cancer with limited toxicity to normal non-malignant cells.
3.3 MATERIAL AND METHODS

3.3.1 Reagents and Cell Lines

Bortezomib were purchased from LC Laboratories (Woburn, MA). Colorectal tumor cell line HCT116 cells were generously provided from the Laboratory of Tumor Immunology and Biology, NCI, NIH. Human colorectal carcinoma cell line SW620 and near normal CCD-18Co cells were purchased from ATCC. All cells were cultured in media designated by ATCC for propagation and maintenance. Cells were incubated at 37°C incubator with 5% CO₂ and tested to ensure absence of Mycoplasma.

3.3.2 Irradiation

Tumor and normal cells were irradiated by using a RS-2000 biological X-ray irradiator (Rad source technology, Suwanee, GA). Cells were irradiated at a dose rate of 2 Gy/min for 2.5 min by setting irradiator voltage and current at 160 kV and 25 mA. During irradiation, the cells were maintained in recommended media and kept on ice. Following irradiation, the culture media was replaced with the fresh media.

3.3.3 Apoptosis Assay

Apoptosis of tumor cells was assessed using the Annexin V- PE Apoptosis Detection Kit I (BD Pharmingen). Tumor cells were mock-irradiated (0 Gy) or irradiated with 5 Gy and re-
cultured for 24 hr. Following incubation, mock-irradiated or irradiated cells were treated with 10nM bortezomib and incubated for addition 24 hr. The tumor cells were briefly trypsinized and harvested. The cells were then washed with cold PBS twice and resuspended in Annexin V binding buffer at a concentration of 1 x 10⁶ cells/ml. Cells were then transferred to 5mL culture tubes containing 5µl of Annexin V-PE and/or 5µl of 7-Aminoactinomycin D (7-AAD). The samples were gently mixed and were incubated for 20 min at room temperature. Following the addition of 400µl of Annexin V binding buffer to each tube, samples were analyzed and quantified by flow cytometry and resulting data were analyzed using FlowJo software.

3.3.4 Comet Assay

The extent of DNA damage and resulting DNA damage repair was assessed using single cell gel electrophoresis (Comet assay) under alkaline conditions. Briefly, SW620 cells were plated and cultured for 24 hr, then treated or not treated with bortezomib and incubated for an additional 24 hr. The tumor cells were rinsed with PBS to remove dead cells, briefly trypsinized, harvested, and gently resuspended in fresh media. Tumor cells were then mock-irradiated (0 Gy) or irradiated with 10 Gy and immediately placed on ice or allowed to incubate at room temperature for 20 min and then placed on ice. Approximately 1 x 10⁴ cells were gently mixed with previously melted 0.5% low-melting agarose in PBS at 37 °C, applied to a slide previously coated with 1% normal-melting agarose, allowed to solidify in a cold box, and then immediately placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 0.02% Triton, pH 10). Following lysis, the slides were placed in an alkaline solution (300 mM NaOH, 1 mM EDTA) for 30 minutes and subjected to electrophoresis in alkaline solution for 30 min (33 V, 300 mA). Following electrophoresis, slides were washed 4 times with cold H₂O, DNA was precipitated
with cold 100% ethanol for 5 min, and slides were allowed to dry at room temperature overnight. The resulting gels were stained using Sybr Green I and visualized using an LSM 700 scanning confocal microscope. Comet images were analyzed with the OpenComet algorithm (v 1.3) (Gyori, Venkatachalam et al. 2014). Late apoptotic cells present a different profile in the comet assay due to the degradation of genomic DNA (a characteristic “hedgehog” shape with a small head and a large “fan like” tail); these profiles were not included in the assay. The Olive tail moment was used for analysis: Olive Tail moment = (Tail Mean – Head Mean) x % DNA in Tail / 100. Statistical analysis was performed using GraphPad.

3.3.5 RNA Expression and Quantitative Real-Time PCR

mRNA was isolated using QIAzol RNA extraction reagent (Qiagen) as described in Cacan et al. (Cacan, Ali et al. 2014). Briefly, cells were lysed in QIAzol and agitated on a 3D rotator for 5 min. 200µl of chloroform was added and was incubated for 3 min at room temperature. Samples were centrifuged and the aqueous phase was transferred to an eppendorf tube. 500µl of isopropanol was added and was incubated for 10 min at room temperature. Following centrifugation, pellets were washed with 75% cold ethanol, centrifuged and resuspended in RNAse free water. RNA was quantified and cDNA was generated from 1µg of total extracted RNA using an Omniscript Reverse Transcription Kit (Qiagen). Following cDNA synthesis, quantitative real-time polymerase chain reaction was performed using TaqMan Universal PCR Master Mix (Qiagen) and specific primers and probes targeting gene of interest (Applied Biosystems; FAS/CD95; Hs00163653_m1, DR4/TNFRSF10A; Hs00269492_m1, DR5/TNFRSF10B; Hs00366278_m1 and HPRT1; Hs99999909) according to manufacturer’s protocol. Transcript expression was assessed using an ABI prism 7900HT Real-Time PCR.
System (Applied Biosystems). Reactions were normalized against HPRT1 expression and calculations were performed using standard curves generated.

### 3.3.6 Cell Surface Staining and Flow Cytometry Analysis

Cell surface staining of tumor and normal cells were performed using the following primary labeled antibodies; FAS-PE, DR4-PE, DR5-APC and the appropriate isotype matched controls (BioLegend. San Diego, CA). Surface staining was performed in cell staining buffer for 45 min on ice. Stained cells were acquired on a BD Fortessa flow cytometer (BD PharMingen. San Diego, CA). Dead cells were excluded from the analysis based on scatter profile. Isotype control staining was less than 5% for all samples analyzed.

### 3.3.7 Functional Death Receptor Assay

Cells were mock-irradiated (0 Gy) or irradiated with 5 Gy and re-cultured for 24 hr. Following incubation, mock-irradiated or irradiated cells were treated with 10nM bortezomib and incubated for addition 24 hr. The cells were harvested and counted. Cells were then incubated for 3 hr with varying concentrations of agonistic anti-Fas antibody, clone CH11 (MBL. Watertown, MA) or recombinant TRAIL protein (Millipore. Billerica, MA). Control cells were incubated with IgM isotype control antibody (BD Biosciences. San Diego, CA). Cells were subsequently fixed and permeabilized before being stained for intracellular active caspase-3 with a PE-labeled monoclonal antibody (BD Biosciences. San Diego, CA). Stained cells were acquired on a BD Fortessa flow cytometer (BD PharMingen. San Diego, CA). The level of activated caspase-3 was quantified by flow cytometry, as described above.
### 3.3.8 CTL Killing Assay

Peripheral blood mononuclear cells (PBMCs) from HLA-A2\(^+\) donors were purchased from Hemacare (Van Nuys, CA) for generation of antigen specific CTLs. PBMCs were cultured in AIM-V media (Life Technologies) for 2 hr to allow them to adhere to the culture flask. Non-adherent cells were removed for lymphocyte isolation. Adherent cells were cultured for a week in the presence of 100ng/mL of human granulocyte-macrophage colony stimulating factor (GM-CSF) and 20ng/mL of IL-4 (Miltenyi Biotec, Inc. Auburn, CA) to induce dendritic cell differentiation. On day five, 500ng/mL of CD40L (Millipore) were added to mature the dendritic cells (DC). On day seven, DCs were harvested and 1 \( \times 10^5 \) of DCs were plated in a 12-well plate and then were pulsed with 40µg/mL of HLA-A\(^2\) binding CEA peptide (YLSGANLNL) for 4 hr in 37°C. CEA loaded DCs were irradiated with 50 Gy to inhibit DCs proliferation and processing of new antigens. CD8\(^+\) T cells were isolated from non-adherent PBMCs using immunomagnetic beads (Miltenyi Biotec Inc. Auburn, CA), as described by the manufacturer. Isolated CD8\(^+\) T cells were co-cultured with peptide loaded DCs in the presence of 10ng/mL of IL-7 and 30U/mL of IL-2 (Millipore. Temecula, CA) to promote T cells viability and clonal expansion. IL-7 and IL-2 were refreshed on the third day. T cells were re-stimulated every 7-days with freshly pulsed DCs and peptide as described above. After three in vitro stimulations T cells were isolated over ficoll and used for cytotoxic killing assays. SW620 cells were treated with IR (5Gy), bortezomib or combination of bortezomib and irradiation. After 24 hr bortezomib treatment and 48 hr irradiation, the tumor cells were harvested and co-cultured with CEA specific CTLs (E:T ratio 10:1) at 37°C for 3.5 hr. After co-incubation, tumor cells were harvested and stained with EpCAM (Epithelial cell adhesion molecule; Miltenyi Biotech) followed by intracellular staining with active caspase-3 (BD Pharmingen).
3.3.9  Statistical Analyses

Results were statistically evaluated using Student paired $t$ test. The $p$ values <0.05 are indicated by one asterisk (*). The $p$ values <0.005 are indicated by two asterisks (**). The $p$ values <0.0005 are indicated by three asterisks (***).
Figure 3.1 Tumor cells remain viable after a combination treatment of sub-lethal irradiation and proteasome inhibitor

Tumor cells were mock-irradiated (0 Gy) or irradiated with 5 Gy and cultured for 24 hr. Following incubation, mock-irradiated or irradiated cells were treated with 10nM bortezomib and incubated for addition 24 hr. An Annexin V: PE Apoptosis Detection Kit I (BD Pharmingen) was used for staining; results were quantified by Flow cytometry analysis and were analyzed using FlowJo software. Viable cells were negative for both annexin V-PE and 7-AAD; early apoptotic cells were positive for annexin V-PE and negative for 7-AAD, whereas late apoptotic and dead cells were positive for both annexin V-PE and 7-AAD labeling (upper right quadrant). Experiment was repeated three times with similar results. The relative increase of dead tumor cells in (A) SW620 (B) and HTC116 colorectal cancer cells.
Figure 3.2 Initial DNA damage response is not inhibited by 26S proteasome inhibition.

SW620 cells were untreated or treated with 10nM bortezomib, incubated for 24 hr, mock-irradiated (0 Gy) or irradiated with 10 Gy, and either immediately placed on ice and prepared for comet assays or incubated for 20 min at room temperature followed by 10 min on ice and prepared for comet assays. (A) Representative images for each treatment. (B) Olive tail moments for non-irradiated, irradiated with 10 Gy with no incubation, and irradiated with 10 Gy with incubation were compared for untreated (black) and bortezomib treated (gray) cells. Data for irradiated cells are the average of two independent experiments with error bars denoting standard deviation.
Figure 3.3 Inhibition of the 26S proteasome enhances transcript expression of death receptors with combination of radiation in tumor cells.

Following the treatments, adherent cells were harvested, RNA was extracted and cDNA was generated. Data was quantified using qRT-PCR with primers and probes specific for DR4, DR5 or FAS coding regions and the obtained data were normalized to housekeeping gene HPRT1 expression. Graphed data shows the average of three independent experiments, with error bars denoting SEM *p<0.05, **p<0.005. Relative mRNA expression of DR4, DR5 and FAS in (A) CCD-18Co, (B) SW620 (C) and HTC116 cells.
Figure 3.4 A combination treatment of sub-lethal dose of radiation and proteasome inhibition up-regulates cell surface expression of death receptors.

Following the treatments, cells were harvested and stained with PE-labeled antibody to human DR4, FAS or APC-labeled DR5. Cell surface protein expression was evaluated by flow cytometry. Isotype control stained cells were set to 5% positive. Graph represent average of three independent experiments, with error bars denoting SEM *p<0.05, **p<0.005, ***p<0.0005. Cells surface expression of DR4, DR5 and FAS in (A) CCD-18Co, (B) SW620 (C) and HTC116 cells.
Figure 3.5 Inhibition of the 26S proteasome and sub-lethal irradiation can enhance sensitivity to killing through FAS in HCT116 cells, but not in CCD-18Co cells.

Tumor cells were mock-irradiated (0 Gy) or irradiated with 5 Gy and cultured for 24 hr. Following incubation, mock-irradiated or irradiated cells were treated with 10nM bortezomib and incubated for addition 24 hr. The tumor cells were then incubated for 3 hr with low and high concentrations of agonistic anti-Fas antibody. Control cells were incubated with IgM isotype control antibody. Cells were subsequently fixed and permeabilized before being stained for intracellular active caspase-3 with a PE-labeled monoclonal antibody. The level of activated caspase-3 was evaluated by flow cytometry. Isotype control stained cells were analyzed for each treatment group individually and set to 5% positive. Graph shows average of three independent experiments, with error bars denoting SEM *p<0.05, **p<0.005, ***p<0.0005. Percentage of active caspase-3 in (A) CCD-18Co, (B) SW620 (C) and HTC116 cells.
**A)**

For CCD-18Co cells, the graph shows the percentage of active caspase-3 under different treatments:
- Control
- IR
- Brtz
- IR+Brtz

The bars represent untreated conditions and various TRAIL concentrations (25 ng/mL and 100 ng/mL) along with IgM.

**B)**

For SW620 cells, the graph shows the percentage of active caspase-3:
- Control
- IR
- Brtz
- IR+Brtz

The bars indicate the effects of untreated, TRAIL 25 ng/mL, TRAIL 100 ng/mL, and IgM.

**C)**

For HCT116 cells, the graph shows the percentage of active caspase-3:
- Control
- IR
- Brtz
- IR+Brtz

The bars reflect untreated conditions, TRAIL 25 ng/mL, TRAIL 100 ng/mL, and IgM. The significance is indicated by asterisks (*, **, ***).
Figure 3.6 Sub-lethal irradiation and inhibition of the 26S proteasome can enhance sensitivity to killing through TRAIL receptors in both HCT116 and SW620 colorectal cancer cell lines, but not in normal CCD-18Co colon cells.

Following radiation and bortezomib treatment, cells were then incubated for 3 hr with two different concentrations of recombinant TRAIL protein. Control cells were incubated with IgM isotype control antibody. Cells were subsequently fixed, permeabilized and stained PE-labeled active caspase-3 antibody. The level of activated caspase-3 was evaluated by flow cytometry. Graph shows average of three independent experiments, with error bars denoting SEM *p<0.05, **p<0.005, ***p<0.0005. Percentage of active caspase-3 in (A) CCD-18Co, (B) SW620 (C) and HTC116 cells.
Figure 3.7 Combination of sub-lethal irradiation and bortezomib or irradiation alone enhances the killing of SW620 mediated by CTLs.

A) SW620 cells were treated with 0 Gy (white bar), 5 Gy (gray bar), 10nM bortezomib (checked bar) and combination of radiation and bortezomib (black bar). Cells were harvested and co-incubated with human CEA specific CTLs (E: T ratio 10:1) for 3.5 hr at 37°C in a 96 well
The frequency of tumor cells expressing active caspase-3 was determined by flow cytometry and data was analyzed by flowjo software. 

**B)** As a negative control, SW620 cells were treated and incubated under similar condition as described above in the absence of CTLs. 

**C-D)** Bar graph showing the average of two additional replicate experiments. Error bars represent the SEM. * indicates P value <0.05. CEA= Carcino-embryonic antigen and E:T-effector cell to target cell ratio.
4 RADIATION ENHANCES EXPRESSION OF DEATH RECEPTORS THROUGH EPIGENETIC MECHANISMS

Ionizing radiation (IR) a pivotal treatment modality for several cancers and IR treatment leads to alteration of multiple signaling pathways that control cell survival or cell death (Szumiel 2014). While classical responses to DNA damage post-radiation are well described, recent biological discoveries suggest additional mechanisms of cellular responses to IR (Agassi, Myslicki et al. 2014, Finkelstein, Salenius et al. 2014, Garnett-Benson, Hodge et al. 2015). It is well known that ionizing radiation induces DNA damage in human cells and triggers expression of a number of genes (Ostling and Johanson 1984, Di Leonardo, Linke et al. 1994, d'Adda di Fagagna, Reaper et al. 2003). However, radiation can also alter expression of many genes that are not typically involved in DNA damage and repair pathways (Tusher, Tibshirani et al. 2001, Kumari, Cacan et al. 2013). For example, it has been shown that sub-lethal doses of IR can modulate expression of important immunogenic genes, making tumor cells more susceptible to immune responses (Garnett, Palena et al. 2004, Ifeadi and Garnett-Benson 2012, Kumari, Cacan et al. 2013, Gameiro, Jammeh et al. 2014). We have recently shown that sub-lethal radiation enhances expression of multiple death receptors (Ifeadi and Garnett-Benson 2012) and co-stimulatory molecules (Kumari, Cacan et al. 2013) in colorectal cancer (CRC) cells; however, it remains unclear how IR enhance expression of these molecules mechanistically.

Tumor cells escaping immune responses by down-regulating genes that essential for effective anti-tumor immunity (Kim, Emi et al. 2007), genes involved in presentation of antigens to T cells (Bubenik 2004), stimulation of T cells (Lee, Kim et al. 2010) and susceptibility to apoptotic signals (French and Tschopp 2002, Hopkins-Donaldson, Ziegler et al.
have been reported in cancer cells. Activation of T cells is a critical process in antitumor immunity and, following initial recognition of specific antigens in MHC-I, co-stimulation provides essential signal for T cell activation. Increasing the sensitivity of tumor cells to apoptotic signals sent by responding T cells represents another opportunity for eliminating resistant tumor cells (Driscoll 2014). DR4 (TRAIL-R1), DR5 (TRAIL-R2), and Fas (CD95/Apo-1) are the most common death receptors that cytotoxic T lymphocytes (CTLs) use to kill tumor cells (Guicciardi and Gores 2009). Fas is the complementary receptor for Fas-ligand (FasL) and; DR4 and DR5 are receptors for the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Interaction of these death receptors with their cognate ligands on anti-tumor immune cells are also essential for driving apoptosis in many types of tumor cells (Koornstra, Kleibeuker et al. 2003) and increased expression of death receptors could also enhance susceptibility of tumor cells to death signals from anti-tumor T cells. Thus, defining cancer therapies and mechanisms that can result in increased expression of increase susceptibility to death pathways, could greatly improve design of combination therapies involving immunotherapies.

subject to several modifications that influence the ability of nucleosomes to form stable higher chromatin structures (Kouzarides 2007). For example, histone acetylation facilitates gene expression whereas histone deacetylases (HDACs) return DNA to a less accessible conformation by removing acetyl groups from histones (Kuo and Allis 1998). A cancer cell increasing deacetylation and silencing genes that contributes to its development. Class I histone deacetylases (HDAC1, HDAC2 and HDAC3) are recruited into specific transcriptional repression complexes that target HDAC activity to chromatin resulting in chromatin condensation and transcriptional silencing (Millard, Watson et al. 2013). HDACs 1-3 are highly expressed in many cancers, including colorectal cancer (Stypula-Cyrus, Damania et al. 2013). DNA hypermethylation of CpG dinucleotides frequently contributes to loss of tumor suppressor genes by accumulating in their promoter regions (Esteller 2002). DNA methylation is carried out by DNA methyltransferases (DNMTs) via catalyzing transfer of the methyl groups to cytosine residues in DNA (Rhee, Jair et al. 2000). In mammalian cells, three active DNMTs have been identified; DNMT3a and DNMT3b facilitate formation of de novo DNA methylation patterns while DNMT1 is mainly required for maintenance of the established patterns of DNA methylation (Jin, Li et al. 2011). It has been reported that Fas, DR4 and DR5 expression are regulated by DNA methylation and histone acetylation in several cancer types (Maecker, Yun et al. 2002, Gazin, Wajapeyee et al. 2007, Jazirehi and Arle 2013, Venza, Visalli et al. 2013), however the precise molecular mechanism remains unknown.

It has been shown that radiation downregulates the expression of class I HDACs (Mueller, Yang et al. 2011) and decreases the activity of DNA methyltransferases (Antwih, Gabbara et al. 2013, Szumiel 2014). Radiation has also been reported to increase expression of Fas, DR4 and DR5 (Ifeadi and Garnett-Benson 2012), and we have previously reported increased
histone acetylation at the promoter for 4-1BBL, an important co-stimulatory molecule that plays role in T cell activation (Kumari, Cacan et al. 2013). These findings suggest that low dose radiation treatment could up-regulate transcript expression of death receptor genes, through modulation of epigenetic enzymes. However, the roles and molecular mechanisms of DNA methylation and histone acetylation in the expression of death receptors in response to radiation have not been investigated. It is important to determine if low dose radiation enhances the expression of these molecules via epigenetic modulation to extend the usage of radiation, including improving cancer immunotherapy strategies. In the current study, we hypothesize that sub-lethal dose radiation of CRC cells enhances expression of death receptors, in part, through modulation of epigenetic enzyme activity at specific genes. We have shown that enhancement in the gene expression following radiation treatment is due in part to changes in DNA methylation and histone acetylation. Sub-lethal IR increased expression of death receptors by decreasing binding of DNMT1 and HDAC2 to promoter regions of these molecules, resulting in modulation DNA methylation and in histone acetylation at the promoter regions of these genes.

4.1 RESULTS

4.1.1 Expression of death receptors increase when DNMTs and HDACs are inhibited

Epigenetic regulation and suppression of death receptors have been previously reported in other tumor cell lines (Hopkins-Donaldson, Ziegler et al. 2003, Aguilera, Das et al. 2009, Watson, O’Kane et al. 2012). We first sought to determine if pharmacologic inhibitors of histone deacetylation and DNA methylation could alter the expression of death receptors in colorectal cancer cells. The HDAC inhibitor, TSA, and DNMT inhibitor, 5-Aza-dC, were used to inhibit HDACs and DNMTs activity, respectively. HCT116 cells were irradiated (5Gy) or treated with
125 nM TSA and were incubated for 2 days, or were treated with 20µM 5-Aza-dC and incubated for 3 days. The cell surface protein expression of DR4, DR5 and Fas was quantified by flow cytometry. IR, TSA or 5-Aza-dC treatment increased cell surface protein expression of DR4 in HCT116 colorectal carcinoma cell line (Fig 4.1A). HCT116 cells uniformly express DR5 (>98% positive) and Fas (100% positive) on their surface (Fig 4.1B-C) and there was no significant decrease in the frequency of these cells expressing DR5 and Fas. However, changes in the median fluorescence intensity (MFI) values show a significant change in the expression (density) levels of both DR5 and Fas following radiation, TSA or 5-Aza-dC treatment (Fig 4.1D-E). Interestingly, radiation increased both Fas and DR5 MFI more robustly than TSA or 5Aza-dC and all three genes were not modulated the same by three diverse treatments. 5-Aza-dC treatment robustly increased expression of Fas and DR4, but modestly increased expression of DR5. TSA treatment significantly increased expression of Fas, DR5, but not DR4. These data suggest that the weight of DNA methylation and histone acetylation on regulation of each death receptors is different. Consistent with previous observations radiation significantly increased expression of death receptors (Ifeadi and Garnett-Benson 2012). Here we observed that TSA or 5-Aza-dC treatments were also able to considerably enhance expression of these molecules.

Overall, these data indicate that expression of DR4, DR5 and Fas are regulated and by histone acetylation and DNA methylation, and radiation increases expression of these genes in CRC cell lines.

4.1.2 Radiation increased histone acetylation at Fas and DR5 promoters

Our data indicates that DR4, DR5 and Fas expression can be influenced by drugs that inhibit epigenetic activities in the cell and that radiation also increases expression of these
genes in CRC cell lines. We observed a significant increase in the expression of DR5 and Fas in colorectal cancer cells following IR. To explore whether histone modifications are also regulated in part by radiation, we first assessed the levels of histone acetylation at the promoters of DR5 and Fas by ChIP assays in both untreated and irradiated HCT116 cells. Histone H3 acetylation levels significantly increased at the DR5 and Fas promoters following 5Gy radiation as compared to untreated control cells (Fig 4.2A-B). In contrast, we saw no change in the overall levels of H3 and total H3 histone binding was similar at DR5 and Fas promoters (Fig 4.2C-D). These data suggest that the increased acetylation at these promoters is not simply due to increased overall histone H3 levels in the cells. These data suggest that radiation increases the expression of death receptors by increasing promoter histone acetylation, which facilitates transcription initiation by loosening interactions between the histones and DNA.

4.1.3 **Radiation treatment decreased binding of HDAC2 to Fas promoter**

Histone acetylation is dynamically regulated in cells by the opposing actions of histone acetyltransferases (that add the acetyl functional group to histones), and HDACs (that remove them). We have observed an increase in histone acetylation levels at promoters of Fas and DR5 following radiation treatment of HCT116 colorectal cancer cells. To determine the potential mechanism that could be responsible for the enhancement of histone acetylation following radiation treatment we sought to evaluate the binding of HDACs to the promoter region of our genes of interest. To narrow down our study, HCT116 cells were irradiated (5Gy) and ChIP assay was performed to determine binding of class I HDACs to the promoter region of Fas. We observed that HDAC2 and HDAC3 proteins bind with decreased frequency to the Fas promoter in radiation treated cells compared to untreated cells (Fig 4.3B-C) and the change in
HDAC2 binding was more significant than that observed with HDAC3. We did not observe any alterations in the binding of HDAC1 to the Fas promoter between control and irradiated cells (Fig 4.3A). These data suggest that HDAC2 plays a role in regulating Fas transcription following radiation of tumor cells and radiation increases expression of Fas, in part, by decreasing HDAC2 accumulation at the promoter region of this gene in colorectal cancer cells.

4.1.4 Radiation treatment decreased DNMT1 binding to Fas promoter

Recent studies suggest that radiation treatment alters expression of some target genes by changing DNA methylation status of these genes (Bae, Kim et al. 2015). However, the role of methylation in expression of death receptors in response to radiation has not been investigated. DNMTs are responsible for methylating DNA and silencing genes. To determine if radiation treatment causes changes in DNA methylation status of these genes by altering DNMTs binding, the human colorectal cell line HCT116 was irradiated and DNMTs binding to the Fas promoter were assessed by ChIP assays. We observed a similar binding of DNMT3a and DNMT3b to the Fas promoter following radiation treatment (Fig 4.4B-C). However, we detected a significant decrease in DNMT1 binding to the promoter region of Fas (Fig 4.4A). These data suggesting that radiation was reducing the binding of DNMT1 to the death receptors promoter and DNMT1 also plays an important role in regulating Fas transcription in colorectal cancer cells.

4.2 DISCUSSION

A better understanding of how radiation works to modulate the expression of death receptors will extend the clinical usage of radiation, including improving cancer immunotherapy
strategies. In this study, we investigate the molecular mechanisms of epigenetic regulation of gene expression in response to IR in colorectal cancer cells. We identify that radiation treatment decreases HDAC2 and DNMT1 binding to the Fas promoter in HCT116 colorectal cancer cell line.

HDACs and DNMTs inhibitors induce a potent anticancer response by inhibiting histone deacetylation and DNA hypermethylation (Johnstone 2002, Takai, Desmond et al. 2004, Lyko and Brown 2005). Our data indicate that inhibition of DNMTs and HDACs by 5-Aza-dC and TSA induce expression of death receptors in colorectal cancer cells. Of note, the increase in death receptors expression with radiation was similar in HDACs and DNMTs inhibitors, suggesting that the expression of these death receptors are regulated by epigenetic enzymes.

DNA methylation and histone acetylation play important roles in chromatin organization and gene regulation in eukaryotic cells. Multiple epigenetic modifications are commonly disrupted during carcinogenesis. Oncogenes undergo hypomethylation on DNA and acetylation and hypermethylation on histones in order to drive enhanced expression (Jin, Yao et al. 2009, Muller, Jana et al. 2013). Conversely, DNA hypermethylation and histone deacetylation commonly occur on tumor suppressor genes (Nguyen, Gonzales et al. 2001, Herman and Baylin 2003, Esteller 2007). The data presented here further indicate accumulation of DNMTs and HDACs at the Fas, DR5 and promoter suppresses their expression in colorectal cancer cells.

While several studies have reported that radiation can alter histone modifications (Yu, Teng et al. 2005, Pollack, Sapkota et al. 2009, Ji, Tian et al. 2014), the direct effect of radiation on histone acetylation and HDACs remain unclear. It has been reported that DR4 expression was downregulated in medulloblastoma tumor samples and increased histone H3 and H4 acetylation at the DR4 promoter sensitizes medulloblastoma cell lines to TRAIL (Aguilera, Das et al. 2009).
To our knowledge, this is the first report showing effect of IR on promoter acetylation of death receptors and alterations in direct binding of class I HDACs to the promoter region of these genes. Our data demonstrate that radiation treatment enhances histone acetylation by decreasing binding HDAC2 to the Fas promoter in HCT116 colorectal cancer cell line. Interestingly, HDAC1 was found to bind to the Fas promoter in T cells (Zimmerman, Singh et al. 2012), which suggest that different HDACs may play role in regulation of Fas in different cells or tissue. Pollack et al. had observed an increase at the level of histone H3 acetylation following UVR expose at some genes promoters, such as ATF3, COX2 and IL-8, while no increase at the promoter region of the MHC class II gene in HaCaT cells (Pollack, Sapkota et al. 2009). Yu et al. had observed that UV irradiation triggers genome-wide histone hyperacetylation at both histone H3 and H4 in yeast (Yu, Teng et al. 2005).

Fas downregulation by DNMT1 accumulation has observed in osteosarcoma and lung carcinoma cell lines (Thaler, Spitzer et al. 2013), and aberrant methylation of Fas promoter was observed in bladder carcinoma (Li, Xia et al. 2011, Watson, O'Kane et al. 2012). DNMT1 silencing upregulates DR5 expression and sensitizes human hepatoma cells to TRAIL-mediated apoptosis (Kurita, Higuchi et al. 2010) and hypermethylation of the DR4 promoter was noted in invasive gastric carcinoma (Lee, Lim et al. 2009). Furthermore, Fas and DR4 suppression was reported in in small cell lung carcinoma cell lines and 5-Aza-dC treatment reverse expression of these genes (Hopkins-Donaldson, Ziegler et al. 2003). A number of studies have suggested that radiation-induced genomic instability is maintained by epigenetic changes (Tamminga and Kovalchuk 2011, Mothersill and Seymour 2012, Szumiel 2015). Some studies have suggested that radiation induces global DNA hypomethylation (Chaudhry and Omaruddin 2012) while others observed both DNA hypomethylation and hypermethylation (Aypar, Morgan et al. 2011).
These findings suggest that different tissues or cells show different biological responses to radiation. We began our investigation by treating cells with 5-Aza-dC to inhibit DNA methylation in order to determine if this would alter expression of some death receptors in human CRC cells. In our data, we observed a decrease in the binding of DNMT1 to the Fas promoter, but no change in global DNA methylation in HCT116 colorectal cancer cells. DNMT1 is mainly required for maintenance of the established patterns of DNA methylation and it is highly associated with tumor progression. DNMT1 is recruited to the promoter region of target genes via multiple steps and DNMT1 associates with methyl-CpG binding protein (MeCP2) in order to perform maintenance methylation (Kimura and Shiota 2003). A recent report indicates that radiation treatment decreases level of proteins that function DNA methylation such as MeCP2 (Bae, Kim et al. 2015). These data suggest that radiation treatment may not directly regulate DNMT1 expression, but alters DNMT1 recruitment to target gene promoters. As we observe in our data, IR decreases DNMT1 binding to Fas promoter. Interestingly, it has been reported that immunoprecipitated MeCP2 complexes show DNA methyltransferase activity to hemimethylated DNA (Kimura and Shiota 2003). This could possible explain why we didn’t observe any alterations in the binding of DNMT3a and DNMT3b to the promoter region of Fas.

In summary, a full understanding of how IR modulates the expression of death receptors will extend the usage of radiation treatment, specifically to improve cancer immunotherapy strategies by enhancing ability of T cells or activated Natural killer cells to eliminate tumor cells. DNA methylation and histone deacetylation are often associated with transcriptional repression of gene expression (Fuks 2005, Robertson 2005) and with decreased responsiveness to chemotherapy (Esteller, Garcia-Foncillas et al. 2000, Giacinti, Vici et al. 2008). DNA hypermethylation and histone deacetylation contribute to cancer progression and
chemoresistance through amplification of master regulators of multiple cell survival (Gan, Chen et al. 2011, Lee, Cheong et al. 2011, Lin, Lin et al. 2014, Ng and Yu 2015, Vaish, Khare et al. 2015). Clinical trials show HDAC inhibitors to be effective anti-tumor drugs (Marks, Miller et al. 2003) and HDAC inhibitors have recently shown great therapeutic promise against colorectal cancer (Tampakis, Tampaki et al. 2014). Overall, our study suggests that sub-lethal radiation can be used to enhance immunogenicity of tumors through epigenetic modulation of death receptors.

4.3 MATERIAL AND METHODS

4.3.1 Reagents and Cell Lines

5-Aza-2'-deoxycytidine (5-Aza-dC) and Trichostatin A (TSA) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies recognizing histone H3 and acetylated histone H3 were from Millipore (Lake Placid, NY). Antibody recognizing HDAC1 were from Santa Cruz. Antibodies recognizing HDAC2, HDAC3, DNMT1, DNMT3a and DNMT3b were from Abcam. Colorectal tumor cell line HCT116 cells were generously provided from the Laboratory of Tumor Immunology and Biology, NCI, NIH. Cells were cultured in media designated by ATCC for propagation and maintenance. Cells were incubated at 37°C incubator with 5% CO₂ and tested to ensure absence of Mycoplasma.

4.3.2 Irradiation

Tumor cells were irradiated by using a RS-2000 biological X-ray irradiator (Rad source technology, Suwanee, GA). Cells were irradiated at a dose rate of 2 Gy/min for 2.5 min by setting irradiator voltage and current at 160 kV and 25 mA. During irradiation, the cells were
maintained in recommended media and kept on ice. Following irradiation, the culture media was replaced with the fresh media.

4.3.3 Cell Surface Staining and Flow Cytometry Analysis

HCT116 cells were irradiated (5Gy), TSA (125 nM) or 5-AZA-dC (20 uM) treated. Cell surface staining of tumor and normal cells were performed using the following primary labeled antibodies; Fas-PE, DR4-PE, and DR5-APC and the appropriate isotype matched controls (BioLegend. San Diego, CA). Surface staining was performed in cell staining buffer for 1 hr on ice. Stained cells were acquired on a BD Fortessa flow cytometer (BD PharMingen. San Diego, CA). Dead cells were excluded from the analysis based on scatter profile. Isotype control staining was less than 5% for all samples analyzed.

4.3.4 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as previously described in Cacan et al. (Cacan, Ali et al. 2014). Briefly, HCT116 cells were irradiated (5Gy) or TSA (125 nM) treated. Cells were then crosslinked with 1% formaldehyde and cell nuclei were isolated and concentrated. Cell nuclei were sonicated using a Bioruptor to generate an average of 500 to 700 bp of sheared DNA. Sonicated lysates were then precleared with salmon-sperm/agarose beads (Upstate) and 5% of the total lysate was stored as input for normalization. Half of the remaining lysate was immunoprecipitated with control antibody, and the other half was immunoprecipitated with 5 µg of indicated antibody overnight at 4°C. Following an additional two hour immunoprecipitation with salmon-sperm/agarose beads, all samples were washed and DNA was isolated. Isolated DNA was quantified by real time PCR on an ABI prism 7900 (Applied Biosystems, Foster City, CA) using the following primers and probe for CD95/Fas: forward, 5’-TCG AGG TCC TCA CCT GAA G-3’, reverse, 5’-TGC ACA AAT GGG CAT TCC T-3’ and probe, 5’-CCA GCC
ACT GCA GGA ACG CC-3’; for DR5: forward, 5’-CCC AAG TGC CTC CCT CAA-3’, reverse, 5’-CCG GGC TGT GGT TTG TTT C-3’ and probe, 5’- CCC CAA GTT TCG GTG CCT GTC CT-3’; and for GAPDH: forward, 5’-AAT GAA TGG GCA GCC GTT A-3’, reverse, 5’-TAG CCT CGC TCC ACC TGA CT-3’ and probe, 5’-CCT GCC GGT GAC TAA CCC TGC GCT CCT-3’. Values generated from real time PCR reactions were calculated based on standard curves generated, were run in triplicate reactions and were analyzed using the SDS 2.0 program.

4.3.5 Statistics

Statistical differences between groups were calculated using student t-test and calculated at 95% confidence. Values represent mean ± SEM of three independent experiments. The $p$ values <0.05 are indicated by one asterisk (*). The $p$ values <0.005 are indicated by two asterisks (**). The $p$ values <0.0005 are indicated by three asterisks (***).
Figure 4.1 Expression of death receptors is regulated by chromatin remodeling enzymes in HCT116 cells.

A) DR4, B) DR5 and C) Fas surface expression level was quantified using flow. D) Mean fluorescence intensity of DR5 and E) Fas surface expression. Cells were plated and treated with 5-Aza-dC (20 uM), TSA (125 nM) or 5Gy radiation. Adherent cells were harvested and stained with PE-labeled antibody to human DR4 and Fas or APC-labeled antibody to human DR5. Isotype control stained cells were analyzed for each treatment group individually and set to 5% positive. The mean of data is graphed with SEM and the p-values are based on technical replicates. Experiments were repeated at least three times with similar results. Percent positive values were compared to the level of gene expression see in untreated control samples. * indicates p value of <0.05, ** indicates p value of <0.005 and *** indicates p value of <0.0005.
Figure 4.2 Histone acetylation at DR5 and FAS promoters in non-radiated and irradiated cells.

ChIP assays were carried out in non-irradiated and irradiated (5Gy) HCT116 cells. Lysates were immunoprecipitated with control antibody, histone H3 or with anti-acetyl histone H3. Associated DNA was isolated and analyzed via real time PCR using primers spanning the DR5, Fas and GAPDH promoters. Input values represent 5% of the total cell lysate. Values represent mean ± SEM of three independent experiments and normalized to GAPDH levels. *p<0.05, **p<0.005. A-B) Global levels of Histone H3 acetylation associated with the DR5 and Fas promoters. C-D) Global levels of Histone H3 associated with DR5 and Fas promoters.
Figure 4.3 Histone deacetylases binding to Fas promoters in non-radiated and irradiated cells.

ChIP assays were carried out in non-irradiated and irradiated (5Gy) HCT116 cells. Following 48 hr post irradiation, lysates were immunoprecipitated with control, HDAC1, HDAC2 or HDAC3 antibody. Associated DNA was isolated and analyzed via real time PCR using primers spanning the Fas and GAPDH promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). Input values represent 5% of the total cell lysate. Values represent mean ± SEM of three independent experiments and values were normalized to GAPDH. *p<0.05, **p<0.005. A-C) HDAC1, HDAC2 and HDAC3 level associated with the Fas promoters.
Figure 4.4 DNA methyltransferases binding to Fas promoters in non-radiated and irradiated cells.

ChIP assays were carried out in non-irradiated and irradiated (5Gy) HCT116 cells. Lysates were immunoprecipitated with control, DNMT1, DNMT3a or DNMT3b antibody. Associated DNA was isolated and analyzed via real time PCR using primers spanning the Fas and GAPDH promoters. Input values represent 5% of the total cell lysate. Values represent mean ± SEM of three independent experiments and normalized to GAPDH, **p<0.005. A-C) DNMT1, DNMT3a and DNMT3b level associated with the Fas promoter.
5 CONCLUSIONS

Cancer treatment approaches have evolved from relatively nonspecific agents to selective specific agents during recent decades (Yaswen, MacKenzie et al. 2015). The main goal of chemotherapeutic approaches is to kill rapidly dividing tumor cells. Despite challenges and limitations in the field, chemotherapeutic drugs remain the backbone of cancer treatments. As more research is done in the understanding of cancer pathogenesis, new treatment options arise, including targeted agents and cancer immunotherapy. Chemotherapeutic and cytotoxic agents can also modulate immune responses by modulating tumor microenvironment (Kershaw, Devaud et al. 2013). These suggest the possibility that combinations of these agents with immunotherapy may improve clinical outcomes.

One of the major obstacles for chemo-based therapy is acquired chemoresistance (McGuire 2003). Accumulating evidence suggests that one of the mechanisms behind chemoresistance is genetic and epigenetic alternations of crucial genes in cancer cells during the recurring treatment of chemotherapy (Brown, Curry et al. 2014). These alterations can cause the dysregulation of tumor suppressor or oncogenes in cancer cells and gradually induce tumor cells to acquire chemoresistance against chemotherapeutic agents (Wang and Zhong 2015). Epigenetic modifications are frequently disrupted during carcinogenesis. Oncogenes may undergo DNA hypomethylation and histone hyperacetylation to drive their enhanced expression (Jin, Yao et al. 2009, Muller, Jana et al. 2013). Conversely, DNA hypermethylation and histone deacetylation commonly occur on tumor suppressor genes (Nguyen, Gonzales et al. 2001, Herman and Baylin 2003, Esteller 2007). Further, DNA methylation and histone deacetylation are often associated
with transcriptional repression of gene expression (Fuks 2005, Robertson 2005) and with decreased responsiveness to chemotherapy (Esteller, Garcia-Foncillas et al. 2000, Giacinti, Vici et al. 2008).

In the second chapter of this dissertation, we focused on one of the cancer models, ovarian cancer, which is acquired chemoresistance. The standard of care for ovarian cancer is surgery followed by a combination of platinum and taxane chemotherapy which, along with second or third line chemotherapy regimens, often does not provide sufficient results (Dear, Gao et al. 2010, Herzog, Sill et al. 2011). New agents, or new agents in combination with current chemotherapeutic agents, are therefore urgently needed to overcome the drug resistance phenomenon (Yin, Liu et al. 2013). While the precise mechanisms for acquired chemoresistance in ovarian cancer is not completely understood, studies suggest that GPCR-mediated activation of cell survival signaling has been strongly linked to platinum and taxane resistance in ovarian cancer (Ali, Farrand et al. 2012, Singh, Chaudhry et al. 2013). The GPCR-mediated cell signaling pathway is downregulated by RGS proteins. Among RGS protein family, RGS10 is found an important regulator of cell survival and chemoresistance in ovarian cancer (Hooks, Callihan et al. 2010, Ali, Cacan et al. 2013). Previous observations establish a causative relationship between suppression of RGS10 and a reduced susceptibility to chemotherapeutic cytotoxicity (Hooks, Callihan et al. 2010) and suggest RGS10 functions as a tumor suppressor by blunting endogenous survival pathways. Recent observations suggest RGS10 expression is suppressed in chemoresistant ovarian cancer cells (Hurst and Hooks 2009, Hooks, Callihan et al. 2010). Hence, we focused here on therapeutic approaches to decrease chemoresistance by enhancing RGS10 expression in chemoresistant ovarian cancer.
Our findings indicate RGS10 transcript expression is suppressed during acquired chemoresistance in ovarian cancer. We find the suppression of RGS10 is due to DNA hypermethylation and histone deacetylation. In chapter two, we fully investigate the molecular mechanisms of epigenetic silencing of RGS10 expression in chemoresistant A2780-AD ovarian cancer cells. We identify two important epigenetic regulators, HDAC1 and DNMT1, which exhibit aberrant association with RGS10 promoters in chemoresistant ovarian cancer cells. Inhibition of DNMT1 or HDAC1 significantly increases RGS10 expression and cisplatin-mediated cell death. Our data suggest that inhibition of HDAC1 and DNMT1 increases RGS10 expression and thus contributes to inhibition of GPCR-stimulated survival signaling pathways, resulting in an increase of apoptotic cells by the chemotherapeutic drug cisplatin (Ali, Cacan et al. 2013, Cacan, Ali et al. 2014).

Previous studies have report that complex epigenetic patterns have associated with ovarian cancer progression and drug resistance (Nguyen, Tian et al. 2014, Yamaguchi, Matsumura et al. 2014). Transcriptional silencing of genes by aberrant methylation is frequently seen in ovarian tumors and is seen more often in cells that acquire drug resistance following chronic exposure to cisplatin (Chang, Monitto et al. 2010). Gene silencing epigenetic modifications are reversible (Zhu and Otterson 2003, Espino, Drobic et al. 2005, Carafa, Nebbioso et al. 2011), thus inhibition of DNA hypermethylation and histone deacetylation may provide valuable prognostic information for ovarian cancer. Several HDAC and DNMT inhibitors are in clinical use or under trial. For example, the DNMT inhibitor, azacitidine, increases sensitivity of chemoresistant ovarian cancer cells to platinum based drugs (Li, Hu et al. 2009). However, a recent randomized, phase II trial of the DNMT inhibitor, 5-aza-2'-deoxycytidine, in combination with carboplatin showed poor clinical outcome for ovarian cancer
patients (Glasspool, Brown et al. 2014). In this study, poor clinical outcome may be due to delivery of 5-aza-2'-deoxycytidine in combination with carboplatin. Enhancing drug delivery and optimizing scheduling may induce a more marked and persistent demethylation.

HDAC inhibitors, vorinostat and belinostat, have been reported to have clinical activity against ovarian cancers that are generally refractory to chemotherapy (Modesitt, Sill et al. 2008, Mackay, Hirte et al. 2010). Interestingly, several preclinical studies suggest that HDAC inhibitors show enhanced anti-cancer activity in combination with demethylating agents (Cameron, Bachman et al. 1999, Matei and Nephew 2010, Capobianco, Mora et al. 2014). Further studies are necessary to elucidate whether the combinations producing the best treatment outcomes in clinical settings.

Epigenetic inhibitors may cause a global change in gene regulation in cancer. Inhibition of HDACs or DNMTs activity may also alter expression of oncogenes. Thus, focusing on individualized medicine may have more benefits than global implementation of anti-cancer agents for ovarian cancer patients. For example, defects in the expression of genes that function in cell survival and death pathways may influence the efficiency of anti-cancer agents. For instance, if we knew a patient had an RGS10 mutation, epigenetic therapy may not be a good option for the patient. RGS10 is an important regulator of GPCR mediated cell survival signaling pathway. Hence, focusing on enhancing expression of RGS10 may be a new avenue to overcome chemoresistance.

A main principle in cancer therapy is the selective killing of tumor cells while limiting toxicity to normal cells; however, a majority of chemotherapeutics agents trigger apoptotic pathways in tumor cells. Thus, defects in the programmed cell death limit the efficiency of potential therapeutic approaches. Hence, we further focused on modulation of death
receptors in advanced stage colorectal cancer cells. Colorectal cancer is the 3rd most common cancer worldwide and still negatively impact public health. Current standard therapies are often unsuccessful for the treatment of advanced stage colorectal cancer and new immune based therapies are under development. The immune system is a critical component of the tumor microenvironment and plays an integral role in preventing and promoting the development of cancer. Novel approaches to manipulate tumor itself and its environment will accelerate the development of immune-based therapeutics. In chapter 3, our research characterizes the use of sub-lethal radiation in combination with the proteasome inhibitor for the goal of increasing the expression of death receptors on CRC cells in an effort to make these tumor cells more susceptible to immune mediated cell killing and to enhance the cytotoxic T lymphocyte (CTL)-mediated anti-tumor immune attack.

Immunotherapy approaches for cancer are promising and provide durable cure with less toxicity. However, cancer cells are capable of directly inhibiting anti-tumor immune attack by a variety of immunosuppressive signaling or become resistant to death signaling pathways, which leads to tumor escape from immune elimination (Helmy, Patel et al. 2013). Thus, characterizing cell signaling pathways and molecules responsible for this enhanced killing by anti-tumor immune effector cells is essential to optimizing combination cancer therapies that include immunotherapy strategies.

Radiation treatment is a pivotal therapy for several cancer types, including colorectal cancer. Recent studies have shown that sub-lethal doses of radiation can modulate gene expression, making tumor cells more susceptible to T-cell-mediated immune attack (Ifeadi and Garnett-Benson 2012, Kumari, Cacan et al. 2013, Gameiro, Jammeh et al. 2014). Previous studies and clinical trials have shown that combining radiation with other treatments is more
effective then radiation treatment alone (Finkelstein and Fishman 2012, Finkelstein, Salenius et al. 2014). Proteasome inhibitors demonstrate broad anti-tumor activity in clinical and pre-clinical cancer models (Chen, Frezza et al. 2011, Crawford, Walker et al. 2011). Bortezomib, a proteasome inhibitor, has been recognized as potent chemotherapeutic drug and is FDA-approved for the treatment of cutaneous T cell lymphoma and multiple myeloma (Chen, Frezza et al. 2011). Recently it has been shown that treatment with bortezomib and a HDAC inhibitor induced tumor-specific immunity by rendering tumor cells more susceptible to killing by antigen-specific CD8\(^+\) T cells (Jagannath, Dimopoulos et al. 2010, Choi and Reddy 2014). It remains unclear how bortezomib contributes to the increased immune attack or if bortezomib could have similar activities in combination with other standard cancer therapies such as radiation.

In chapter 3, we use a combination treatment of proteasome inhibition and irradiation to further induce immunomodulation of tumor cells that could enhance tumor-specific immune responses. We investigate the effects of the 26S proteasome inhibitor, bortezomib, alone or in combination with radiotherapy, on the expression of immunogenic genes in normal colon and colorectal cancer cell lines. We examined cells for changes in the expression of several death receptors, DR4, DR5 and Fas, commonly used by T cells for lysis of target cells. Ligation of death receptors by binding with cognate death ligands from anti-tumor immune cells induces apoptotic signals into tumor cells (Grimm, Kim et al. 2010). Fas is the complementary receptor for FAS-ligand and this interaction plays an important role in triggering apoptosis, however; the interaction between Fas and FasL is largely impaired due to suppression of Fas expression on tumor cells during cancer progression (Zhu, Liu et al. 2005, Pryczynicz, Guzinska-Ustymowicz et al. 2010). DR4 and DR5 are receptors for the tumor necrosis factors-related apoptosis-
inducing ligand (TRAIL) and they are also essential for driving apoptosis in many types of tumor cells (Koornstra, Kleibeuker et al. 2003). However, tumor cells often down-regulate cell surface expression of death receptors in order to avoid elimination by immune cells (Perraud, Akil et al. 2011, Kykalos, Mathaiou et al. 2012). Thus, enhancing the expression of these death receptors on cancer cells could increase tumor cell sensitivity to CTL-mediated killing.

Our results indicate that most cells remain viable following 26S proteasome inhibition and sub-lethal radiation. The combination treatment resulted in increased cell surface expression of death receptors by increasing their transcript levels. Our results indicate that the combination of 26S proteasome inhibition and sub-lethal radiation also increases the sensitivity of carcinoma cells to apoptosis through FAS and TRAIL receptors but does not change the sensitivity of normal non-malignant epithelial cells. Furthermore, the combination treatment significantly enhances tumor cell killing by tumor specific CD8$^+$ T cells. This study suggests that combining radiotherapy and proteasome inhibition may simultaneously enhance tumor immunogenicity and the induction of antitumor immunity by enhancing tumor-specific T-cell activity. This study describes a novel basic cancer research discovery and provides a potential therapeutic approach for advanced colorectal cancer. Thus it would be of broad interest to the field. While both sub-lethal doses of radiation and proteasome inhibition have been described to alter gene expression in tumor cells, our is the first study that investigated the impact of combination therapies of human tumor cells, and normal non-malignant cells, on modulation of immune relevant genes specifically. It will be interesting to see the effect of the combination treatment on the expression of RGS10 and other pro-apoptotic molecules in chemoresistant ovarian cancer cells as well. Combination treatment of sub-lethal radiation and botezomib may
enhance expression of RGS10 as well as sensitize chemoresistant ovarian cancer cells to CTL or NK mediated immune responses.

It is important to identify and characterize the mechanisms by which radiation control CRC expression of DR4, DR5, and Fas genes. In chapter 4, we elucidate the molecular mechanisms by which radiation control CRC expression of these molecules. Here we report that, that enhancement in expression of these genes following radiation treatment of CRC is due, in part, to changes in DNA methylation and histone acetylation, two important gene-silencing mechanisms which contribute to the progression of many cancers. We observed that radiation (5Gy) significantly increased histone acetylation at the promoter regions of Fas and DR5. However, radiation did not induce changes in the global levels of acetylated histone 3 suggesting specificity in IR-induced changes. Furthermore, evaluation of epigenetic controlling enzymes revealed that IR did not alter overall cellular levels of HDACs (HDAC1, HDAC2 or HDAC3) or DNMTs (DNMT1, DNMT3a, or DNMT3b). Instead, radiation decreased binding of HDAC2 and HDAC3 at the promoter regions of Fas. Radiation also resulted in reduced DNMT1 at the Fas promoter regions but not a control gene. Our data suggest that radiation can influence the expression of immune response relevant genes in colorectal tumor cells by altered binding of epigenetic enzymes, and modulated histone acetylation, at specific gene promoters.

There is intense research ongoing in the field of cancer immunotherapy. The goal of immunotherapy is to trigger the immune system to respond to tumor-specific antigens and attack tumor cells (Koido, Ohkusa et al. 2013). Immunotherapy approaches include engineered dendritic cells, viral vector-based cancer vaccines, the use of peptides derived from tumor-associated antigens (TAAs), or whole tumor cells. Chapter 3 and 4 of this dissertation will significantly contribute to the cancer immunotherapy field by using a novel combination of low
dose radiation and proteasome inhibitor in controlling the expression of death receptors on tumor cells for recruitment of CTLs and NK cells. Enhancing expression of death receptors will sensitize tumor cells to immune mediated tumor killing. The results of these studies will increase scientific knowledge and open new insight into cancer immunotherapy strategies for the treatment of advanced CRC by introducing a novel potential therapeutic approach and understanding the contribution of death receptors to CTLs mediated tumor killing. Thus, defining cancer therapies and mechanisms that can result in increased expression of molecules that increase susceptibility to death pathways, could greatly improve design of combination therapies involving immunotherapies. Results of these studies will be interesting to scientists in the field of basic cancer biology and tumor immunology as well as to clinicians involved in treating cancer patients with combination radiation therapy, proteasome inhibition and immunotherapy strategies. Radiation and the proteasome inhibitor, bortezomib, are both clinically used for cancer treatment, though not currently in combination therapies. A better understanding of how radiation and the proteasome work together to modulate the expression of death receptors will allow us to improve cancer immunotherapy strategies by using these two treatments in novel combinations.

In sum, our studies provide insight into the alteration of molecular pathways involved in cancer cell death or survival. RGS10 is an important regulator of cell survival and death receptors are essential to induce apoptotic signal in cancer cells. Insights from our study will provide novel approaches for effective cancer treatment strategies. A full understanding of how radiation modulates the expression of death receptors will extend the usage of radiation treatment, specifically to improve cancer immunotherapy strategies. Clinical trials show HDAC inhibitors to be effective anti-tumor drugs (Marks, Miller et al. 2003) and HDAC inhibitors have
recently shown great therapeutic promise against colorectal cancer (Tampakis, Tampaki et al. 2014). Our study in chapter 4 suggests that sub-lethal radiation can be used to enhance immunogenicity of tumors through epigenetic modulation of death receptors molecules. Taken together, our studies evaluate the involvement of epigenetic mechanisms in tumor progression and will improve cancer-immunotherapy strategies by determining molecular mechanisms resulting from radiation to enhance anti-tumor immune responses.
REFERENCES


antitumoral activity against solid and haematological malignancies." Br J Cancer 97(10): 1344-1353.


carboplatin alone in patients with recurrent, partially platinum-sensitive ovarian cancer."


