EXPRESSION AND FUNCTION OF REGULATOR OF G-PROTEIN SIGNALING 10 (RGS10) IN OVARIAN CANCER AND MICROGLIA

by

MOURAD WAGDY AHMED ALI

(Under the Direction of Shelley B. Hooks)

ABSTRACT

G-Protein coupled receptors (GPCRs) mediate a wide array of cellular functions, such as cell proliferation, migration, and survival. Regulators of G-protein signaling (RGS) proteins are a diverse family of proteins that regulate signaling pathways downstream of GPCRs by acting on G-proteins. The focus of this dissertation is on the regulation of G-protein pathways in cancer and inflammation by RGS proteins, particularly by RGS10. We focused on signaling initiated by two related receptor families, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) receptors, which are implicated in ovarian cancer and neuroinflammation, respectively. Aberrant expression and mutations in RGS proteins have been implicated in diseases such as cancer and autoimmune disorders. The aim of this study was to define the function and the expression of RGS proteins, particularly RGS10, in ovarian cancer and microglia.

LPA is the predominant growth factor in ovarian cancer, promoting proliferation, migration, and survival. RGS proteins negatively regulate LPA-mediated effects in ovarian cancer. We determined that RGS proteins, RGS10 and RGS17 regulate LPA-mediated survival in ovarian cancer cells. Specifically, our data demonstrate that RGS10

and RGS17 negatively regulate LPA-mediated AKT survival pathway in ovarian cancer cells. Further, we show that RGS10 and RGS17 are down-regulated in chemoresistant ovarian cancer cells, and our results show that RGS10 is epigenetically silenced in chemoresistant ovarian cancer cells via increased DNA methylation and decreased histone acetylation of the RGS10 promoter by DNA methyltransferase 1 (DNMT1), and histone deacetylase 1 (HDAC1), respectively.

In addition to its role in chemoresistant ovarian cancer cells, RGS10 has been shown to exert an anti-inflammatory effect in microglia, the brain's innate immune cells, via blunting pro-inflammatory cytokines signaling, and RGS10 is suppressed in activated microglia. We investigated the mechanism by which RGS10 is down-regulated in activated microglia, as well as the mechanism by which RGS10 regulates signaling pathways in microglia. Our results indicate that RGS10 is epigenetically suppressed via decreased histone acetylation of its promoter in activated microglia. Our results also suggest that RGS10 negatively regulates protein kinase A (PKA) and glycogen synthase kinase-3 beta (GSK-3β) downstream of lipopolysaccharide (LPS) and S1P, which may account for its regulation of pro-inflammatory cytokine signaling in activated microglia.

INDEX WORDS: G-protein coupled receptors, regulator of G-protein signaling proteins, lysophosphatidic acid, sphingosine-1-phosphate, epigenetics, ovarian cancer, microglia

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MOURAD WAGDY AHMED ALI

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MOURAD WAGDY AHMED ALI

Major Professor:	Shelley B. Hooks
Committee:	James L. Franklin
	Mandi M. Murph
	Rajgopal Govindarajan
	Xiaoqin Ye

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

I would like to dedicate this work to my family, friends, and teachers. I could not have earned this degree without your help.

To my father, Wagdy Salem, you have always been my role model and my inspiration. To my mother, Nabila, I could not have achieved anything in my life without your support and guidance. To my sister, Radwa, you have always believed in me and supported me throughout the years. Thank you for everything.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW: REGULATION OF G-PROTEIN SIGNALING IN OVARIAN CANCER AND MICROGLIA

1.1. G-Protein Coupled Receptors (GPCRs)

G-protein coupled receptors (GPCRs) are a diverse family of receptors that regulate a wide variety of cellular functions. Binding of an extracellular ligand to these receptors induces conformational changes in the receptor structure resulting in the activation of G-proteins, which in turn tightly regulate cellular signaling pathways. G-proteins were first discovered in the early 1980s by Alfred G. Gilman and Martin Rodbel, who were awarded the Nobel Prize in 1994 for this discovery. Following the identification of G-proteins, extensive research to understand the physiological and pathological roles of these proteins and associated receptors ensued, culminating in the awarding of the Nobel Prize in Chemistry in 2012 to Robert Lefkowitz and Brian Kobilka for their work on the structure and activity of GPCRs.

GPCRs regulate the functions of many systems in the human body including cardiovascular, endocrine, immune, and nervous systems. Aberrations in GPCR signaling have also been implicated in a multitude of diseases, such as cancer, immunological disorders, cardiovascular and nervous system disorders. Almost half of all marketed drugs target GPCRs or GPCR-mediated pathways (Pierce et al., 2002, Dorsam and Gutkind, 2007). Examples of such drugs include the anti-allergic drug loratidine (Claritin®), the anti-migraine drug sumatriptan (Imitrex ®), the anti-hypertensive drug

metoprolol (Lopressor ®), and ranitidine hydrochloride (Zantac ®) which is used to treat ulcers and gastro-esophageal reflux.

GPCRs are activated by a diverse array of ligands, ranging from photons to peptides. GPCR ligands also include biogenic amines such as dopamine, amino acids such as glutamate (Heng et al., 2013), and cations that activate GPCRs in the taste buds of the tongue (Foster et al., 2013).

The GPCR superfamily is further divided into 5 subfamilies based on their functionality and their sequence homology: The largest subfamily is the Rhodopsin Receptors subfamily, which constitutes about 85% of GPCRs genes and includes receptors for hormones, such as angiotensin and neurotransmitters, such as dopamine, as well as light receptors; Secretin Receptors subfamily, which includes receptors for secretin, calcitonin, and parathyroid hormone/parathyroid hormone-related peptides; Adhesion Receptors subfamily whose members play important roles in regulating the immune system functions and neuronal development; Glutamate Receptors subfamily, which is important for learning, memory, and pain perception; and Frizzled/TAS2 Receptors subfamily, which has been implicated in regulating cell polarity, embryonic development, and neural synapsis formation (Fredriksson et al., 2003).

Despite their diverse functions and expression patterns, all GPCRs share common structural features: an extracellular N-terminus, connected by seven α-helical transmembrane domains to an intracellular C-terminus. GPCRs couple to heterotrimeric G-proteins through their C-terminus and the intracellular loops of the transmembrane domains. Heterotrimeric G-proteins consist of a guanine nucleotide-binding Gα subunit and Gβγ dimer (Venkatakrishnan et al., 2013).

Under resting conditions, the Gα subunit is bound to guanosine diphosphate (GDP). Ligand binding at the extracellular domain of the receptor results in conformational changes that cause the exchange of GDP for guanosine triphosphate (GTP), which in turn results in the release of the active GTP-bound Gα subunit as well as the Gβγ dimer. Both can interact with effector molecules to mediate cellular responses to GPCR activation. The G-protein cycle is deactivated through the hydrolysis of GTP back into GDP by the Gα subunit, which then recombines with Gβγ dimer. The Gα subunit possesses an intrinsic GTPase activity; however, this ability to hydrolyze GTP is very slow (Khafizov et al., 2009) making the GTPase activity the rate-limiting step in the G-protein activation/deactivation cycle. Regulators of G-protein signaling (RGS) proteins are a family of proteins that act as GTPase-Activating Proteins (GAPs) in order to accelerate the deactivation of the G-protein cycle. G-proteins utilize guanine nucleotide exchange/GTP hydrolysis dynamics in order to alternate between "on/off" states (Figure 1.1). RGS proteins are discussed in detail below.

Following ligand binding to GPCRs, the active GTP-bound Gα subunit and Gβγ dimer are released, which activate several downstream signaling cascades. There are four different families of Gα subunits, and an individual GPCR can couple to more than one type of Gα subunit. Thus, ligand binding to a single GPCR can modulate several intracellular signaling pathways. The four Gα families are: Gαs, Gαi, Gαq, and Gα12 (Simon et al., 1991). Gαs activates adenylyl cyclase (AC) and increases the production of cyclic adenosine monophosphate (cAMP). On the other hand, Gαi inhibits AC, thereby decreasing the level of intracellular cAMP. Intracellular level of cAMP is important for the tight regulation of downstream signaling molecules such as protein kinase A (PKA),

which is involved in the phosphorylation and the activation/deactivation of effector enzymes and transcription factors. Gαq activates phospholipase C (PLC), which generates diacylglycerol (DAG) and inositol triphosphate (IP_3). This in turn activates protein kinase C (PKC) and numerous Ca^{2+}-dependent enzymes, which regulate the activity of further downstream signaling cascades. Gα12 family controls cellular morphology and the cellular cytoskeleton through activation and regulation of Rho-guanine nucleotide exchange factors (RhoGEF) proteins. Gβγ dimers are also capable of regulating cellular activities by interacting with effector molecules such as PLCβ, AC, and phosphotidylinositol-3 kinase (PI3K) or by gating ion channels directly (Smrcka, 2008). Signaling by Gβγ is most significant following activation of Gαi heterodimers.

1.2. Regulator of G-Protein Signaling (RGS) Proteins

The regulator of G-protein signaling (RGS) family of proteins is a highly diverse group of proteins that regulate signaling pathways downstream of GPCRs. The main role of RGS proteins is to regulate the duration and amplitude of G-protein signaling through their ability to function as GAPs of heterotrimeric Gα subunits. RGS proteins accelerate the deactivation of G-proteins through stabilizing the Gα-GTP transition state, hence increasing GTP hydrolysis up to 1000-fold (Posner et al., 1999). All RGS proteins contain an RGS domain that consists of approximately 120 amino acids and is responsible for the GAP activity. The RGS domain is sufficient for the RGS proteins' activity and interaction with Gα proteins (De Vries et al., 1995).

The RGS family is further divided into eight subfamilies according to their sequence homology and domain structures. The simplest subfamily is the R4/B subfamily. Members of this subfamily are composed of the RGS domain and an N-terminus α-helix

that binds phospholipids (Bansal et al., 2007). Members of the RZ/A subfamily are characterized by an N-terminus cysteine string motif which can be reversibly palmitoylated and is involved in membrane binding. Members of subfamilies R7/C, R12/D, RA/E, GEF/F, G/GRK, and H/SNX contain additional domains that regulate protein function and subcellular localization. For instance, the Dishevelled/eg1-10/Pleckstrin (DEP) domain helps direct members of R7 subfamily to the plasma membrane or nucleus (Takida and Wedegaertner, 2004). Other domains such as Rac binding domain, Dishevelled homology (DH), Pleckstrin homology (PH), and PSD-95/Discus-large/ZO-1 homology (PDZ) domains facilitate protein-protein interactions, which leads to the formation of signaling complexes and increases signaling specificity. In addition to these domains, post-translational modifications of RGS proteins, such as phosphorylation, palmitoylation, and sumoylaton can have profound effects on their subcellular localization, GAP activity, and protein-protein interactions. This makes RGS proteins complex signaling molecules that are involved in a wide variety of cellular functions and signaling pathways.

RGS proteins may also exert certain cellular effects through GAP-independent mechanisms. For example, the guanine nucleotide exchange factor (GEF) domain in the GEF subfamily activates the small G-protein Rho, thereby linking Gα12/13 activation upstream of RGS proteins to the activation of downstream Rho proteins. Another example is GoLoco domains, which are unique to the R12 subfamily and act as guanine nucleotide dissociation inhibitors, thus blocking the activation of heterotrimeric G-proteins (Kimple et al., 2001).

RGS proteins can be involved in the formation of cellular signaling complexes. Examples of RGS proteins that are involved in formation of such signaling complexes are RGS2, RGS14, and RGS16. RGS2, through its N-terminus domain, binds muscarinic receptors M1 to selectively regulate Gαq-mediated signaling triggered by this receptor (Bernstein et al., 2004). RGS14, by virtue of its GoLoco domain, can act as a scaffold to integrate G-proteins and Ras/Raf signaling pathway (Shu et al., 2010). RGS16 has been shown to suppress the activity of PI3K by sequestering its p85alpha subunit, hence mitigating PI3K-mediated growth signaling pathways in breast cancer cells (Liang et al., 2009).

In our studies we focused on RGS10 and RGS17 proteins. RGS10 belongs to the R12/D subfamily, but it lacks the multiple regulatory domains that are found in the other members of that subfamily. RGS10 contains phosphorylation and palmitoylation sites that are important for regulating its subcellular localization. RGS10 selectively deactivates the Gαi family of G-proteins (Hunt et al., 1996).

RGS17 belongs to the RZ/A RGS protein subfamily. Like RGS10, RGS17 selectively deactivates Gαi family of G-proteins (Mao et al., 2004, Nunn et al., 2006). The N-terminus of RGS17 contains a palmitoylation site that regulates its subcellular localization, hence affecting its G-protein and receptor selectivity.

1.3. G-Protein Signaling in Cancer

Cancer is characterized by uncontrolled cell growth, increased cell proliferation, decreased apoptosis, and enhanced cell migration. Oncogenesis requires genetic changes that results in the activation of proto-oncogenes and the inactivation of tumor suppressor genes, hence allowing cells to grow uncontrollably. GPCRs regulate these processes, and

deregulation of GPCR expression and/or activity has been implicated in cancer. This makes GPCRs and molecules that regulate GPCR activity, such as RGS proteins attractive targets for therapeutic drugs.

GPCRs are expressed in cancerous tissues, and activation of GPCRs by oncogenic factors such as lysophosphatidic acid (LPA), endothelin, prostaglandins, and thrombin stimulates proliferation, migration, survival, and metastasis of cancer cells (Dorsam and Gutkind, 2007, O'Hayre et al., 2014). These GPCR agonists are found in high concentrations in primary and metastatic cancerous tissues. Further, several GPCRs are over-expressed in cancer cells, such as LPA receptors in ovarian, breast, and hepatocellular carcinomas (Umezu-Goto et al., 2004, Horak et al., 2007, Sokolov et al., 2013); prostaglandin receptor EP4 in breast cancer (Kundu et al., 2014); endothelin receptor type A (ETAR) in papillary thyroid carcinoma (Irani et al., 2014); and endothelin receptor type B (ETBR) in esophageal squamous cell carcinoma (Tanaka et al., 2013).

In addition to changes in expression levels of GPCRs, altered expression and/or activity of heterotrimeric G-proteins have been associated with cancer initiation and progression (see review:(O'Hayre et al., 2014)). For example, constitutively active Gαi-proteins have been reported in human endocrine tumors (Lyons et al., 1990); activation of Gαs and Gα12 subunits results in activation of PI3K/AKT pathway and the transactivation of androgen receptors in prostate cancer (Liu et al., 2011a); and activation of Gαq subunit following ultraviolet radiation (UVR) exposure has been associated with increased skin pigmentation and increased risk of melanoma (Bellono et al., 2014).

1.4. RGS Proteins in Cancer

Activity of GPCRs and G-proteins is tightly regulated by multiple regulatory proteins. As described in the previous section, RGS proteins regulate the rate-limiting step in the G-protein activation/deactivation cycle (Figure 1.1). Changes in expression and/or activity of RGS proteins can result in profound effects on the cellular signaling pathways downstream of G-proteins. Altered expression levels of RGS proteins have been reported in different types of cancers. Several reports have shown that multiple RGS proteins are differentially expressed in different types of cancers including ovarian (Hurst et al., 2008), breast (Smalley et al., 2007, Wiechec et al., 2008), prostate (Sood et al., 2001, Silva et al., 2003), thyroid (Nikolova et al., 2008), and pancreatic cancers (Hamzah et al., 2008). For example, changes in RGS2 expression have been linked to ovarian (Hurst et al., 2008), breast (Smalley et al., 2007), prostate (Cao et al., 2006), and bladder cancers (Berman et al., 2004). RGS5 gene is found to be up-regulated in hepatocellular carcinoma (Chen et al., 2004, Furuya et al., 2004), and is thought to play a role in tumor neovascularization (Furuya et al., 2004). RGS5 has also been identified as a broadly expressed tumor antigen, suggesting roles in multiple types of cancer (Boss et al., 2007). GPCRs and their cognate ligands have been shown to play important roles in the initiation and progression of cancer, and consequently, it is likely that RGS proteins are also significant to the regulation of oncogenic pathways.

Ovarian cancer tumorigenesis and the development of chemoresistance will be discussed in more detail in the following chapters. In chapters 2 through 4, studies are focused on understanding the roles that RGS proteins play downstream of GPCRs in

chemoresistant ovarian cancer cells, as well as the factors that regulate the expression of these RGS genes.

1.5. Lysophosphatidic Acid (LPA) Receptors

LPA is a major growth factor in ovarian cancer that stimulates tumor progression (Mills et al., 1988, Mills et al., 1990, Moolenaar and van Corven, 1990). LPA mediates cell proliferation (Mills et al., 1988, van Corven et al., 1989), migration (Huang et al., 2002), metastasis, survival and apoptotic signal evasion (Frankel and Mills, 1996, Goetzl et al., 1999, Yu et al., 2008). LPA is enriched in the ascites fluid that accumulates in the peritoneal cavity of ovarian cancer patients (Mills et al., 1988, Mills et al., 1990, Xu et al., 1995a, Xu et al., 1995b). LPA effects are mediated by at least six well characterized cell surface GPCRs known as LPA receptors (LPA1-6). LPA receptors 1-3 belong to the endothelial differentiation gene (Edg) lysophospholipid family of GPCRs which also includes sphingosine-1-phosphate (S1P) receptors (S1P receptors will be discussed later in this chapter). LPA receptors 4-6 belong to the purinergic family of receptors.

LPA1-3 receptors represent the "classical" LPA receptors. LPA1/Edg2 was the first LPA receptor to be discovered in 1996 (Hecht et al., 1996). Characterization of LPA2/Edg4 followed in 1998 (An et al., 1998) and LPA3/Edg7 in 1999 (Bandoh et al., 1999). LPA4, LPA5, and LPA6 represent the "non-classical" receptors. These receptors belong to the purinergic GPCR family and share only 20-24% sequence homology with the classical LPA receptors. Noguchi et. al first described LPA4/GPR239 in 2003 (Noguchi et al., 2003), whereas LPA5/GPR92 was characterized by kotarshy et. al and Lee et. al. in 2006 (Kotarsky et al., 2006, Lee et al., 2006). LPA6/P2Y5, which was first

described in 2008 (Pasternack et al., 2008) has been designated as the sixth LPA receptor in 2010 (Chun et al., 2010).

LPA receptors show wide spectrum of expression in different tissues and are expressed at different levels during development. Originally, LPA1 was found to be highly expressed in embryonic brain ventricular zone, hence its original name; ventricular zone gene-1 (vzg-1) (Hecht et al., 1996). LPA1 mRNA is expressed at high levels in brain oligodendrocytes, heart, small intestine, placenta, ovary, and kidney (Weiner et al., 1998, Anliker and Chun, 2004). LPA2 expression is high in embryonic brain, but is expressed at a much lower level in adult brain (Contos and Chun, 2000). LPA2 is expressed at high levels in ovary (Anliker and Chun, 2004). LPA3 is highly expressed in heart, prostate, testes, and ovary (Bandoh et al., 1999), whereas LPA4 expression is mostly confined to the ovary (Noguchi et al., 2003). LPA5 is abundantly expressed in embryonic brain, but shows low expression in adult tissues (Lee et al., 2006). Initially identified as an autosomal dominant genetic factor for hypotrichosis simplex, a rare form of familial baldness in humans, LPA6 became the most recent member of the LPA receptors family. Unlike other LPA receptors, activation of LPA6 requires a much higher concentration of LPA (Yanagida et al., 2009).

A single LPA receptor can couple to different G-proteins, thereby regulating multiple signaling pathways. Figure 1.2 depicts the different types of LPA receptors and the G-proteins they couple to. LPA1 couples to Gαi, Gαq and Gαs; LPA2 and LPA3 couple to Gαi and Gαq; LPA4 and LPA6 couple to all four types of G-proteins, Gαi, Gαs, Gαq, and Gα12; and LPA5 couples to Gαi, Gαq and Gα12 (Anliker and Chun, 2004, Lee et al., 2006, Blaho and Hla, 2011, Kihara et al., 2014).

Gαi activation is particularly important in the studies discussed in this dissertation because its activation downstream of LPA receptors in ovarian cancer cells causes activation of signaling cascades such as MAP kinase and AKT pathways (Long et al., 2005, Hurst and Hooks, 2009a), which are key signaling pathways that regulate survival of ovarian cancer cells and the emergence of chemoresistance.

1.6. Epigenetic Regulation of RGS Proteins in Chemoresistant Ovarian Cancer Cells.

In chapter 3, we identify the epigenetic factors that drive the suppression of RGS10 in chemoresistant ovarian cancer cells. Epigenetics are defined as heritable changes in the genetic code without changes in the DNA sequence itself. This includes DNA methylation, histone modifications, microRNA regulation, and chromatin remodeling. Several recent reviews ((Choi and Lee, 2013, Plass et al., 2013, Rivera and Ren, 2013)) describe the different types of epigenetic changes that regulate gene expression under physiological and pathological conditions.

DNA methylation occurs by adding a methyl group to carbon atom number 5 of a cytosine in the context of cytosine-phosphate-guanine (CpG) dinucleotide in the DNA sequence. DNA methylation often occurs at, or close to, gene promoters and results in gene silencing due to steric hindrance which prevents transcription factors from accessing the DNA (Jaenisch and Bird, 2003). Further, growing evidence suggests DNA methylation at sites distal to promoters and in regions outside of CpG islands also regulate gene expression (Ushijima, 2005). DNA methyltransferases (DNMTs) are a group of enzymes that catalyze the addition of a methyl group from the methyl donor, S-adenosyl methionine (SAM) to a cytosine nucleotide. DNMT1 is mainly involved in

“maintenance” DNA methylation, where it recognizes already methylated DNA sites and adds methyl groups to the newly synthesized DNA during cell division. On the other hand, DNMT3a and DNMT3b are considered “de novo” DNMTs due to their ability to add methyl groups to previously unmethylated DNA. DNMTs involved in de novo DNA methylation are of particular importance in early developmental stages as they are involved in gene imprinting processes taking place during embryogenesis (Bojang and Ramos, 2014). Another member of DNMT family is DNMT3L, which lacks the catalytic activity of the other DNMTs; however, it is important in assisting the binding between de novo DNMTs and DNA (Goll et al., 2006). DNA demethylating agents such 5-azacytidine (Vidaza ®) and 5-aza-2’-deoxycitidne (decitabine-Dacogen ®) exert their effect through inhibition of DNMTs and are used to drive the expression of genes that are silenced by DNA methylation (Pinto and Zagonel, 1993). Demethylating agents, such as azacytidine and 5-aza-2’deoxycytidine have been approved by FDA for the treatment of disorders such as myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Kantarjian et al., 2003, Kantarjian et al., 2006).

Another important category of epigenetic mechanisms that regulates gene expression is histone modifications, which include histone acetylation, methylation, phosphorylation, sumoylation, and ubiquitination (Choi and Lee, 2013, Helin and Dhanak, 2013). Histone methylation occurs on lysine and/or arginine residues of histones by histone methyltransferases (HMTs). Histone methylation at residues H3K4 and H3K79 has been associated with gene activation, while histone methylation at H3K9 and H3K27 has been linked to gene suppression (Zhang and Reinberg, 2001). Histone phosphorylation, especially H2 phosphorylation, has been implicated in cellular responses to DNA injury.

Phosphorylation of other histone proteins is important for chromatin remodeling (Rossetto et al., 2012). Histone sumoylation can occur on all four histone proteins and appears to block histone acetylation through competing for lysine residues where acetylation takes place (Shiio and Eisenman, 2003, Nathan et al., 2006). Histone ubiquitination results in the addition of a large moiety to the histone structure. This could be associated with gene silencing or gene activation (Wang et al., 2004, Lee et al., 2007).

Our studies of histone modifications focused on histone acetylation. Histone acetyltransferases (HATs) catalyze the addition of acetyl groups on lysine residues of histone tails. Histone deacetylases (HDACs) on the other hand facilitate the removal of the acetyl groups. Histone acetylation results in a more relaxed chromatin structure due to the conversion of positively charged amine groups on lysine residues of histone proteins into neutral amide groups, thereby decreasing their interaction with the negatively charged DNA. The more relaxed chromatin structure allows transcription factors to access DNA more freely, hence increasing gene expression. Conversely, HDAC activity results in a more compact chromatin structure, therefore leading to gene silencing (Grunstein, 1997, Struhl, 1998, Turner, 2000). HDAC family of proteins includes five different subfamilies: HDACI, IIA, IIB, III, and IV, which differ in their cellular localization, tissue distribution and substrate specificity. For example HDAC1, which belongs to subfamily I is localized in the nucleus. It has a ubiquitous expression pattern and, besides histones, can deacetylate substrates such as androgen receptors and p53 protein (Dokmanovic et al., 2007).

Pharmacological HDAC inhibitors are widely used to enhance histone acetylation and increase gene expression. An example of such inhibitors is trichostatin A (TSA) which

selectively inhibits HDACI and II families (Vanhaecke et al., 2004). TSA has some potential as an anti-cancer drug; however, the exact mechanisms by which TSA exerts its anti-cancer effect is not fully understood (Drummond et al., 2005).

As cells progress from a non-cancerous to a cancerous state, several epigenetic changes occur. Oncogenes becomes transcriptionally enhanced through decreased DNA methylation and increased histone acetylation (Jin et al., 2009, Muller et al., 2013). On the other hand, transcriptional expression of tumor suppressor genes become suppressed by DNA hypermethylation and decreased histone acetylation (Nguyen et al., 2001, Herman and Baylin, 2003, Esteller, 2007). Our lab, as well as others, have reported synergistic activity between DNA methylation and histone deacetylation in order to silence tumor suppressor genes in cancers (Fuks et al., 2000, Ghoshal et al., 2002, Meng et al., 2011) including ovarian (Cacan et al., 2014), and ovarian cancer is usually associated with increased expression levels of DNMTs and HDACs (Gu et al., 2013). In chapters 3 and 4, our studies are focused on DNA methylation and histone acetylation of the RGS10 promoter.

1.7. The Innate Immune System of the Brain

Due to its immense importance and its involvement in regulating virtually every system in the body, the brain is privileged with a unique physical barrier; the blood brain barrier (BBB). The BBB is composed mainly of endothelial cells, connected through tight junctions, along with astrocytes (Arthur et al., 1987, Alvarez et al., 2011) and pericytes (Zlokovic, 2008, Abbott et al., 2010). Under physiological conditions, the BBB limits blood-borne macromolecules, cells, and pathogens such as bacteria, viruses, and fungi from crossing into the brain; however, under pathological conditions, pathogens may be

able to cross into the brain due to compromised BBB selectivity. Hence, the brain must possess a robust, rapid, and highly selective immune system that is capable of combating pathogens. The presence of the BBB means that the brain exists within a confined space; therefore the brain's immune system must also be capable of maintaining the brain's homeostasis, through clearing of cellular debris and apoptotic cells. In the following section, the innate immune system in the brain will be discussed, as well as its major functions, the most common pathologies arising from its disruptions, the major GPCRs involved in its regulations, and the role RGS10 play in regulating GPCR signaling in the brain's immune cells.

The majority of the innate immune responses within the brain are orchestrated by microglia, the brain's resident macrophages. Microglial cells were first characterized by Pio del Rio-Hortega (Río-Hortega, 1962) and are known to exist in various species from invertebrate leech to humans (Kettenmann et al., 2011). In humans, microglia represent approximately 16 % of total brain cell population and between 5% and 12% of total rodent brain cell population (Lawson et al., 1990, Mittelbronn et al., 2001). Microglial cells are the only cell type in the brain of hematopoietic origin (Soulet and Rivest, 2008), originating from two sources; an early source in the embryonic yolk sac, and a later source from myeloid progenitors that invade the CNS during embryonic and postnatal development (Ginhoux et al., 2010, Ginhoux et al., 2013). In the CNS microenvironment, microglia mature from an amoeboid to a ramified phenotype that possesses thin non-overlapping processes (Ransohoff and Cardona, 2010), and are capable of self-renewal in order to maintain the microlgial population throughout life (Ajami et al., 2007). In rodents, microglia distribute evenly between grey matter and white matter tracts

(Milligan et al., 1991), whereas in humans microglia are predominantly found in the grey matter, with highest concentrations in hippocampus, substantia nigra, basal ganglia, and olfactory cortex (Mittelbronn et al., 2001).

Infiltrating monocyte-derived macrophages can also participate in innate immune functions in the brain. Under healthy conditions, blood-derived macrophages are excluded from the brain; however, under pathological conditions, macrophages can be recruited to the site of brain injury through the choroid plexus in the blood-cerebrospinal fluid barrier (Kunis et al., 2013, Shechter et al., 2013), hence facilitating the resolution of local immune responses at the injury (Shechter et al., 2009).

Under normal conditions, microglial cells are found in a "resting" state, where they constantly survey their surrounding environment (Nimmerjahn et al., 2005). Microglia remain in that resting state under the "calming" effects of signals that are constitutively released by neurons into the microglial microenvironment. An example of such signals is fractalkine (CX3CL1), which is bound to neuronal membrane. Fractalkine is later cleaved and released into the microglial microenvironment, activating microglial CX3CR1 receptors (Bisogno and Di Marzo, 2010, Bajramovic, 2011). GPCRs play important roles in the microglial "calming" effect. For instance, microglia secrete endocannabinoids such as anadamide, which activate the GPCR subfamily of cannabinoid receptors, CB1 and CB2 in an autocrine and paracrine fashion. Activation of these receptors helps keep microglial cells in their resting state (Ribeiro et al., 2013).

Microglial cells are capable of responding to a wide array of stimuli (Morioka et al., 1991) in a matter of minutes (Kreutzberg, 1996), developing into an activated state. Activated microglial cells are highly plastic in nature, existing in multiple phenotypes,

and are capable of switching between multiple activation states based on the type of stimulus in their vicinity (Mosser and Edwards, 2008, Hanisch, 2013). Activated microglia can perform pro-inflammatory, anti-inflammatory, cytotoxic, immunoregulatory, and repair functions (Hanisch and Kettenmann, 2007, Ransohoff and Perry, 2009, Perry et al., 2010, Ransohoff and Cardona, 2010). Two activation states have been proposed for microglia; M1 and M2 states. The M1 activation state (also known as the classically activated or the pro-inflammatory state) is associated with a phagocytic phenotype and transcriptional activation of nuclear factor-kappa B (NF-κB), which leads to increased production and release of pro-inflammatory cytokines (such as tumor necrosis factor alpha (TNFα), interleukin-1beta (IL-1β), and interleukin-6 (IL-6)), as well as cytotoxic mediators such as nitric oxide (NO), and reactive oxygen species (ROS).

In cases of infections, traumas, and other pathological conditions, molecules that abnormally exist in the CNS microenvironment induce the microglia to switch from the resting to the M1 activated state. Examples of such molecules are pathogen-associated molecular patterns (PAMPs), which include bacterial membrane components such as lipopolysaccharide (LPS), and danger-associated molecular patterns (DAMPs). DAMPs include heat-shock proteins, adenosine tri-phosphate (ATP), urea, and nucleic acid patterns (Kumar et al., 2011). PAMPs and DAMPs serve as ligands to multiple receptors that are expressed by microglia such as toll-like receptors (TLRs), nod-like receptors (NLRs), RIG1-like receptors (RLRs), cytokine receptors such as TNF receptors, and purinergic receptors such as $P2X_4$ (Bajramovic, 2011), and activation of these receptors results in microglial activation and increased pro-inflammatory cytokine production. Another endogenous ligand that induces the pro-inflammatory activated state in microglia

is sphingosine-1-phosphate (S1P). S1P activates five GPCRs known as S1P receptors (S1P1-5). The implications of S1P and S1P receptors in microglial activation will be discussed later in this chapter.

On the other hand, the M2 activation state (also known as the alternatively activated or the anti-inflammatory state) is associated with increased production and release of anti-inflammatory cytokines (such as interleukin-4 (IL-4), interleukin-10 (IL-10), and tumor necrosis factor beta (TNFβ)), and increased release of extracellular matrix, which promote tissue repair processes. The M2 state is induced by apoptotic signals such as heat-shock protein 60 (Hsp60), or anti-inflammatory cytokines such as IL-4 (Mosser and Edwards, 2008). Activated microglia can start in the M1 pro-inflammatory state then switch to the M2 anti-inflammatory state as a part of phagocytosis-oriented or repair-oriented processes (Mandrekar-Colucci et al., 2012). Recent reviews ((Benarroch, 2013, Schwartz et al., 2013a, Zabel and Kirsch, 2013)) comprehensively detail the different inducers of the M1 and the M2 activation states, as well as the microglial receptors involved.

The traditional function of microglia is to serve as the brain's defense line against pathogens. More recently, microglia have been shown to mediate other non-traditional roles, such as maintaining brain homeostasis and support roles for neurons. In the following section, some of the non-traditional functions of microglia will be briefly discussed.

In the developing brain, microglia are important for the removal of apoptotic neurons as well as promoting programmed cell death (PCD) (Trang et al., 2011), and synaptic pruning. In adult brain, microglia are involved in the regulation of neurogenesis in areas

of active neuronal division such as the subgranular zone of the dentate gyrus of the hippocampus (Choi et al., 2008). Further, the ability of microglia to release growth factors has been reported (Montero et al., 2009). Under minor disturbances, microglia release growth factors and reduce risk of excitotoxicity (Montero et al., 2009). In another study, microglial depletion increased neurotoxicity in models of excitotoxicity (Vinet et al., 2012). Additionally, Microglia have been shown to release brain-derived neurotrophic factor (BDNF) in response to injury in models of neuropathic pain (Gomes et al., 2013).

The microglia-to-neuron interaction has been the focus of several research groups. It has been shown that microglia possess the ability to make direct contact with neuronal soma (Nimmerjahn et al., 2005) and synapses (Wake et al., 2009). Wake et al. reported that microglia-to-neuron contact increases after transient ischemia, suggesting that microglia monitors the functional state of neuronal synapses. Another important function of microglia is the pruning of dysfunctional synapses during development. Neurons can display "tags" such as complement protein C1q, which serves as a signal for microglia to recognize and phagocytose the "tagged" synapse (Stevens et al., 2007).

An increasing body of data suggests that microglia are involved in the initiation and/or the progression of several neuroinflammatory and neurodegenerative diseases such as multiple sclerosis (MS), Alzheimer's disease (AD), and Parkinson disease (PD). In the following section, the clinical correlations between microglial dysfunctions and some disease states are briefly discussed.

Multiple sclerosis (MS) is an inflammatory demyelinating autoimmune disorder of the CNS and one of the leading causes of neurological disabilities in young adults in North America. CNS damage results from attacks by infiltrating T- and B-lymphocytes,

and macrophages crossing the BBB into the CNS (Lassmann et al., 2007). Microglia play key roles in the initiation and the progression of MS. Microglia can induce neuronal damage in MS through activation of TLRs and the release of pro-inflammatory cytokines such as TNFα and IL-1β (Jack et al., 2005). Additionally, these cytokines can induce indirect neuronal damage via increased sensitization of neurons to glutamate excitotoxicity (Pitt et al., 2000). Reactive oxygen species (ROS) produced by microglia, such as nitric oxide (NO) can cause direct neuronal damage through induction of mitochondrial dysfunction (Nikic et al., 2011).

Alzheimer's disease (AD) is caused mainly by the accumulation of amyloid-β (Aβ) in the brain. Chronic exposure of microglia to Aβ results in uncontrolled inflammation and increased production of ROS (Uchihara et al., 1997, Cagnin et al., 2001). Whether activation of the innate immune system in the CNS is the cause or the consequence of AD is still debatable.

1.8. S1P Signaling in Microglia

Sphingolipid signaling, especially that of S1P is emerging as a critical regulator of microglial function. There is increasing evidence that S1P, via the activation of S1P receptors is involved in microglial activation, suggesting that targeting S1P receptors could be beneficial in neuroinflammatory and neurodegenerative disorders that are characterized by excessive microglial activation. In this section, the literature of S1P signaling in microglia is briefly reviewed.

The brain is one of the richest organs in lipid content. Sphingolipids represent an important category of brain lipids, and disturbances in sphingolipid metabolism have been reported in CNS disorders (Jana et al., 2009, Mencarelli and Martinez-Martinez,

2013), including neuroinflammatory diseases (Haughey, 2010, Davies et al., 2013, Mencarelli and Martinez-Martinez, 2013). Brain sphingolipids are involved in regulating key cell signaling pathways that mediate pro-inflammatory cytokine and eicosanoid production (Farooqui et al., 2007). Sphingolipids can also regulate astroglial activation, T-cell migration, and activation of several receptor-mediated pathways (Hannun and Obeid, 2008, Jana et al., 2009, Davies et al., 2013). In Alzheimer's disease, it has been hypothesized that microglia-mediated inflammatory responses in AD brains may be due to S1P (Nayak et al., 2010), and defective sphingolipid storage has been linked to AD pathogenesis (Tamboli et al., 2011). Further, altered sphingolipid metabolism has also been implicated in another neurodegenerative disorder, Huntington's disease (HD) (Piccinini et al., 2010).

S1P consists of an amide backbone, a phosphate group, and an 18 carbon unsaturated fatty acid. S1P is produced during sphingomyelin metabolism. Sphingomyelins are membrane sphingolipids that are mainly found in the brain and nervous system. Degradation of sphingomyelin produces ceramide, then sphingosine. The latter is further phosphorylated into sphingosine-1-phoshphate (S1P) by two isoenzymes, sphingosine kinases (Sphk) 1 and 2 (Kohama et al., 1998, Liu et al., 2000), whereas S1P is degraded by S1P lyase and lipid phosphate phosphatases (LPPs) (Mandala et al., 2000, Le Stunff et al., 2002, Ogawa et al., 2003). S1P is produced by many cell types including activated platelets (Igarashi and Yatomi, 1998, Yatomi et al., 2000), cells of the innate immune system (Goetzl and Rosen, 2004), endothelial cells, neurons, and astrocytes in the CNS (Anelli et al., 2005, Bassi et al., 2006, Kajimoto et al., 2007, Singleton et al., 2007).

S1P signaling is mediated by five GPCRs that belong to the endothelial differentiation gene (edg) family. The five receptors are S1P1/Edg1, S1P2/Edg5, S1P3/Edg3, S1P4/Edg6, and S1P5/Edg8. These receptors are expressed in multiple tissues and at different stages of development. S1P1 is mostly expressed in brain, liver, heart, and immune cells (Ishii et al., 2004, Ohuchi et al., 2008). S1P2 is expressed at high levels in embryonic brain, adult lung, heart, and kidney (Okazaki et al., 1993, Ishii et al., 2001, Ishii et al., 2002, McGiffert et al., 2002). S1P3 is predominately expressed in embryonic brain, adult pancreas, lung, heart, and kidney (Zhang et al., 1999, McGiffert et al., 2002). Expression of S1P4 is restricted to lymphoid cells and tissues (Graler et al., 1998, 1999), whereas S1P5 is expressed in brain, lung, spleen, and skin (Niedernberg et al., 2002, Toman and Spiegel, 2002, Ohuchi et al., 2008).

S1P1 couples only to Gαi family G-proteins (Okamoto et al., 1998), whereas S1P2 and S1P3 couple to Gαi, Gαq, and Gα12 family G-proteins (Okamoto et al., 1999, An et al., 2000, Okamoto et al., 2000, Meacci et al., 2002). S1P4 and S1P5 couple to Gαi and Gα12 family G-proteins (Im et al., 2000, Malek et al., 2001, Niedernberg et al., 2002, Graler et al., 2003). S1P can also act intracellularly as a second messenger to regulate calcium mobilization, proliferation, and survival (Payne et al., 2002). Schematic representation of S1P receptors and their downstream effectors is detailed in figure 1.3.

Microglial cells express all five S1P receptor subtypes (Tham et al., 2003, Nayak et al., 2010), and microglial activation, such as in case of LPS-induced activation significantly alters S1P receptor expression levels. For instance, Noda et. al reported that LPS-induced activation reduced S1P2, S1P4, and S1P5 receptor expression levels (Noda et al., 2013).

S1P has been implicated in immune cell function, and has emerged as a key regulator of lymphocyte egress from lymphoid tissue (Cyster and Schwab, 2012). An S1P gradient is required for lymphocyte recruitment to the CNS (Brinkmann et al., 2004). S1P may also promote immune cell survival and proliferation, and can interfere with immune cell activation (Nixon, 2009). LPS activates the Sphk1/S1P signaling axis in many cells, including microglia leading to translocation of sphk1 from cytosol to plasma membrane, where it converts sphingosine into S1P (Jolly et al., 2005, Wattenberg et al., 2006). Sphk1 is also involved in TNFα-mediated inflammatory responses in immune cells (Hammad et al., 2008, Melendez, 2008). Nayak et al. reported that sphk1 modulates the expression of pro-inflammatory cytokines and NO production in activated microglia (Nayak et al., 2010). In vitro cultured primary microglia modulate S1P receptor levels upon activation, and S1P alters microglial cytokine production (Tham et al., 2003). Activation of S1P1 receptor up-regulates the activity of NF-κB (Lee et al., 2010), which drives the expression of pro-inflammatory cytokines such as TNFα and IL-1β (Newton and Dixit, 2012). Interestingly, TNFα also stimulates the synthesis and production of S1P in T-lymphocytes (Takeshita et al., 2012), suggesting a possible feed-forward mechanism.

Another piece of evidence supporting the importance of S1P signaling in microglia is that targeting S1P receptors has been found to be beneficial in some neuroinflammatory and neurodegenerative disorders. A prominent example is fingolimod (also known as FTY720), the first drug to be approved for humans that targets S1P receptors. FTY720 is an S1P1 analogue, which is approved for the treatment of relapsing forms of multiple sclerosis (MS) (Cohen et al., 2010, Kappos et al., 2010). The beneficial effects of

FTY720 in MS have been attributed to its ability to reduce immune cell entry into the CNS by sequestrating T- and B-lymphocytes in the lymph nodes (Graler and Goetzl, 2004). FTY720 is rapidly phosphorylated to the active form, FTY720-P, which binds to receptors S1P1, 3, 4, and 5 (Brinkmann et al., 2002). Whereas S1P causes receptor recycling, FTY720 exposure results in receptor endocytosis and degradation (Jo et al., 2005, Gonzalez-Cabrera et al., 2007, Oo et al., 2007), thus, although being an acute agonist, FTY720 is a long term functional antagonist. Additionally, growing evidence suggests that FTY720 efficacy requires CNS targets, such as S1P receptors in microglia and astrocytes.

These data suggest that S1P signaling pathways are involved in the inflammatory responses of activated microglia in an autocrine/paracrine fashion, and that targeting S1P synthesis, S1P receptors, or regulators of S1P signaling pathways downstream of S1P receptors, such as RGS proteins could prove beneficial in disorders characterized by excessive microglial activation and pro-inflammatory cytokine production such as multiple sclerosis.

In recent years, Regulator of G-protein signaling 10 (RGS10) has emerged as an important neuroprotective protein. RGS10 is expressed at high levels in the brain regions of hippocampus, striatum, and dorsal raphe (Gold et al., 1997), which are involved in higher brain functions. RGS10 is abundantly expressed in immune tissues such as thymus and spleen, and RGS10 transcript expression level is increased upon T-cell activation (Haller et al., 2002). RGS10 is expressed in microglia and is localized in different subcellular compartments including the nucleus (Waugh et al., 2005). Lee et al. has shown that RGS10 normally suppresses cytokine production following microglial

activation, and that LPS-induced microglial activation suppressed RGS10 expression. Brains of RGS10 knockout mice exhibited increased microglial activation, increased cytokine production, and higher vulnerability toward neuroinflammation-induced neuronal cell death (Lee et al., 2008). These results suggest a mechanism by which RGS10 suppression in microglia enhances inflammatory neuronal cell death. However, the mechanisms of RGS10 suppression following LPS-induced microglial activation, the upstream receptor that is regulated by RGS10, as well as how RGS10 negatively regulates cytokine production are not fully understood. Evidence from the literature and from our data suggest that RGS10 may regulate the S1P1 receptor; S1P stimulates production of pro-inflammatory cytokines in microglia, whereas RGS10 blunts their production; RGS10 selectively deactivates Gαi-proteins, whereas S1P1 selectively activates Gαi-proteins; finally, nuclear localization is necessary for RGS10 to blunt inflammatory responses, and S1P1 has been shown to translocate into the nucleus. In chapter 5, the mechanism that drive RGS10 suppression in LPS-activated microglia, as well as the mechanism by which RGS10 regulates LPS- and S1P-induced signaling in microglia are investigated.

1.9. RGS and Epigenetics

Given the ability of RGS proteins to regulate GPCR signaling, changes in RGS expression levels may dramatically alter the strength of GPCR signaling. Thus, understanding the regulation of RGS expression is important to understanding their physiological roles. Our lab has shown that multiple RGS proteins are dynamically regulated during ovarian cancer progression (Hurst et al., 2009). Several studies report that DNA methylation and histone deacetylation synergistically silence gene expression

in many cancers including ovarian cancer (Esteller, 2007, Huang et al., 2007, Nephew et al., 2009, Seligson et al., 2009). Li et al., report that DNA methylation is implicated in ovarian cancer chemoresistance, as global DNA methylation and DNMT expression are increased in cisplatin-resistant A2780 ovarian cancer cells (Li et al., 2009b). Further, inhibitors of DNA methylation re-sensitize previously resistant ovarian cancer cells to cisplatin (Steele et al., 2009b). A recent study reported that RGS2 is epigenetically silenced via enhanced DNA methylation in prostate cancer (Wolff et al., 2011). Taken together, the epigenetic silencing of gene expression by DNA methylation and histone deacetylation in ovarian cancer, and the dynamic regulation of RGS proteins during ovarian cancer progression (Fong et al., 2000, Anderson et al., 2007, Bodenstein et al., 2007, Cheng et al., 2008) suggest that epigenetic silencing of RGS proteins may be contribute to their dynamic expression during ovarian cancer progression.

In chapter 3, we describe that the RGS10 gene is epigenetically silenced through decreased histone acetylation and HDAC1 recruitment, and increased DNA methylation in chemoresistant ovarian cancer cells (Ali et al., 2013). Further, in chapter 4 our data suggest that HDAC1 and DNMT1 contribute to RGS10 suppression during acquired chemoresistance and support the use of inhibitors of HDAC1 and DNMT1 as an adjuvant therapeutic approach to overcome ovarian cancer chemoresistance (Cacan et al., 2014).

Similarly, epigenetics have been shown to play roles in regulating microglial function and modulation of neuroinflammation. HDAC inhibitors have been shown to have potential anti-inflammatory effects by reducing the production of pro-inflammatory cytokines in activated microglia; however, the exact mechanism of such effect is not completely understood. The HDAC inhibitors, SAHA and ITF2357, reduced the

production of pro-inflammatory cytokine production in activated microglia (Faraco et al., 2009). Another HDAC inhibitor, trichostatin A (TSA) reduced brain injury in a model of LPS-sensitized neonatal hypoxic-ischemia by reducing pro-inflammatory cytokine production in microglia (Fleiss et al., 2012). Garden et. al have recently reviewed the implications of epigenetics in modulation of neuroinflammation, including neuroinflammatory reactions mediated by microglia (Garden, 2013).

In chapter 5, the mechanisms of RGS10 suppression in activated microglia is investigated. Our results suggest that histone deacetylation plays a role in LPS-induced RGS10 suppression in microglia.

1.10 Figures

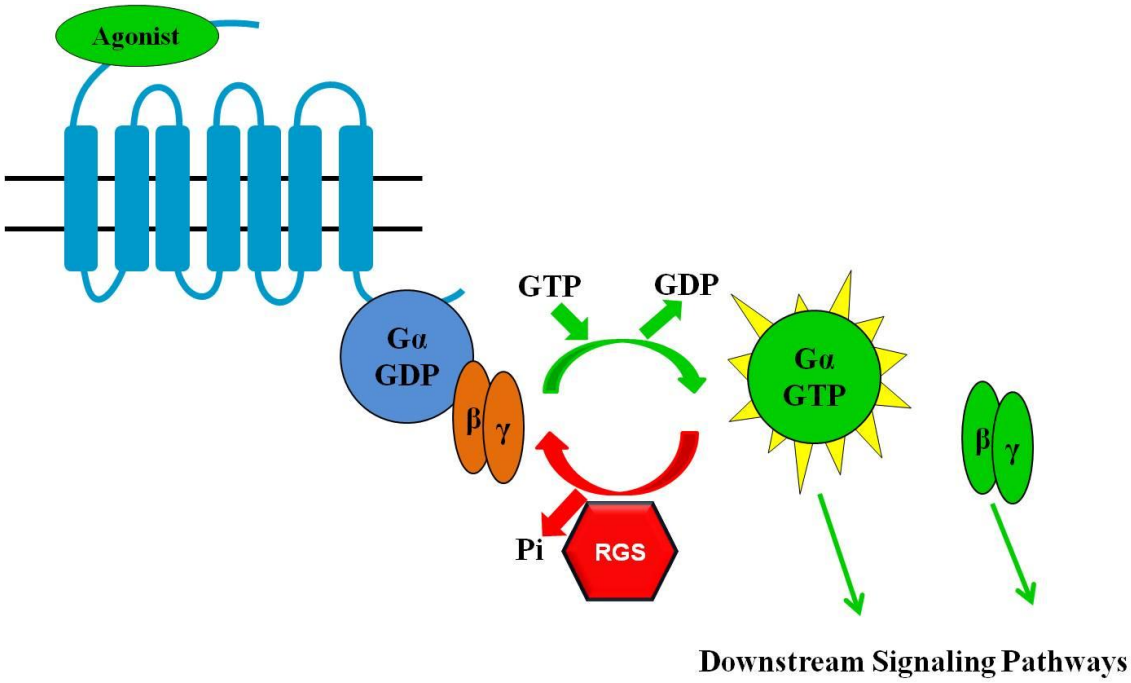

Figure 1.1: G-Protein Activation/Deactivation Cycle. Agonist binding to GPCRs results in the activation and release of GTP-bound Gα subunit and Gβγ dimer, which mediate downstream signaling pathways. RGS proteins accelerate the return to the inactive state.

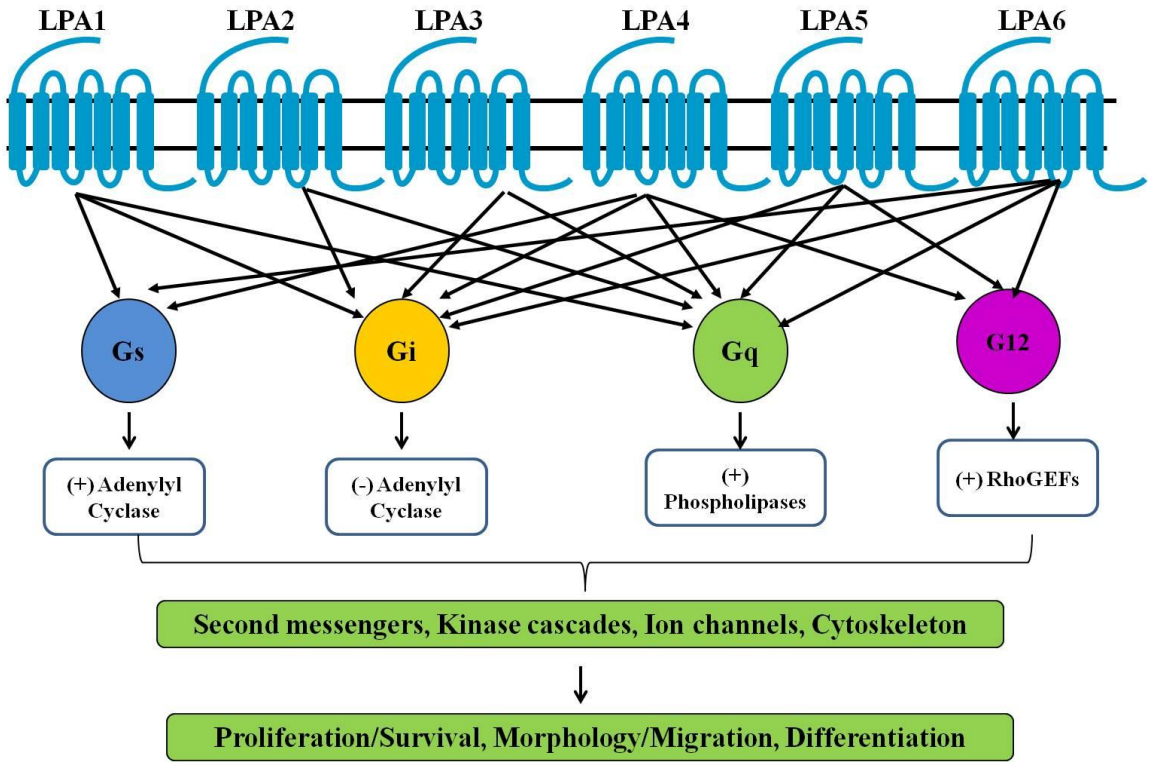

Figure 1.2: LPA Receptor Signaling Pathways. A single LPA receptor can couple to multiple G-proteins; hence agonist binding to one LPA receptor can trigger several signaling pathways.

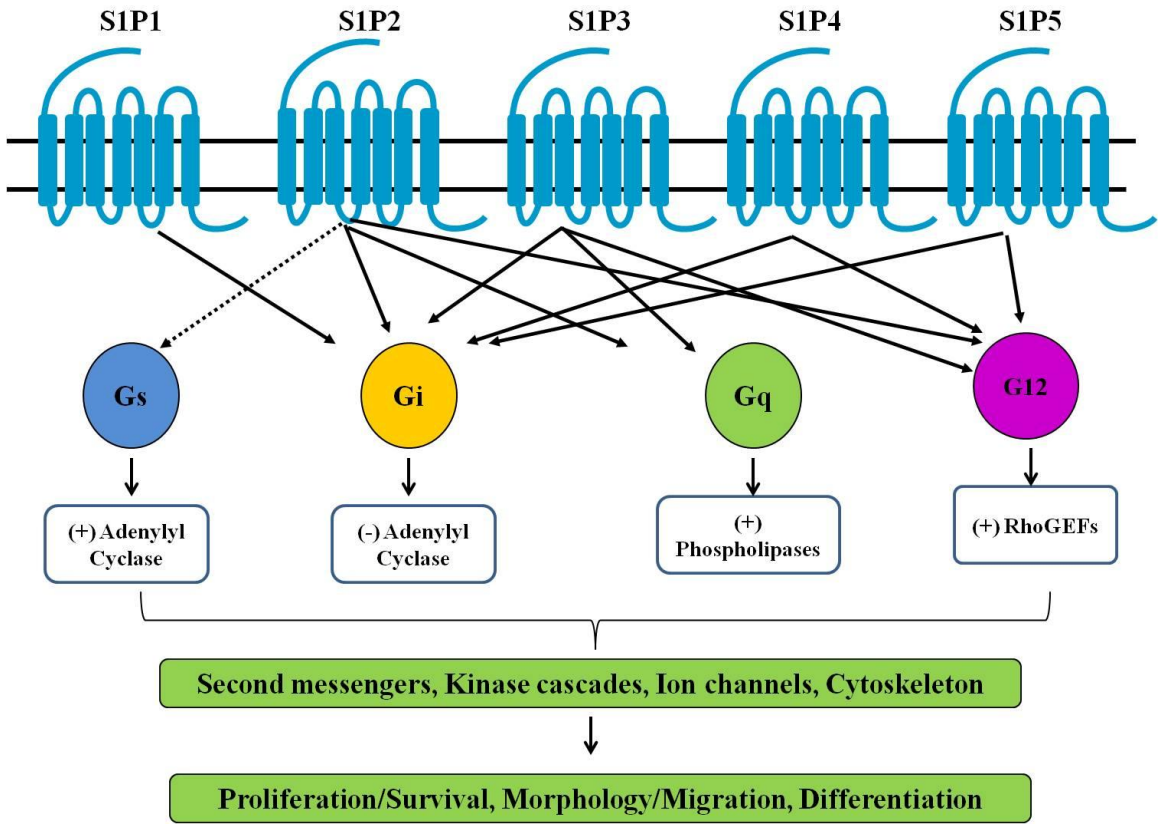

Figure 1.3: S1P Receptor Signaling Pathways. A single S1P receptor can couple to multiple G-proteins; hence agonist binding to one S1P receptor can trigger several signaling pathways.

CHAPTER 2

REGULATOR OF G-PROTEIN SIGNALING RGS10 AND RGS17 REGULATE AKT ACTIVATION IN OVARIAN CANCER CELLS

2.1. Background

Ovarian cancer is the leading cause of death from gynecological cancers and the fifth most common cause of cancer deaths in females. The estimated number of new cases in 2014 is 21,980 and the estimated number of deaths is 14,270 (Siegel et al., 2014). The disease is detected at an early stage in only 20% of patients where the disease is still confined to the ovaries, whereas in most of the patients the disease is detected at late stages. The high mortality rate is mainly due to the difficulty of detecting the disease at an early stage and the emergence of resistance to chemotherapy (Bast et al., 2009). Most of the attempts to identify biomarkers for the early detection of ovarian cancer have been unsuccessful due to patients' heterogeneity. Due to the lack of methods of an early detection for ovarian cancer, most patients are diagnosed after the disease has advanced to a later stage where the tumor has metastasized throughout the peritoneal cavity.

Current treatment approaches include cytoreductive surgery and adjuvant chemotherapy that consists of a platinum-based drug (cisplatin or carboplatin) and a taxane compound (paclitaxel or docetaxel). Approximately 70% of cancer patients will respond to the combination therapy; however, most of these patients will relapse within two years with treatment-refractory disease (Agarwal and Kaye, 2003). The recurrent tumors possess genetic and epigenetic mutations that allow them to survive

chemotherapeutic-induced cell death (Bast et al., 2009). Understanding the mechanisms by which ovarian cancer cells acquire chemoresistance is important for improving therapeutic outcomes for ovarian cancer patients.

Lysophosphatidic acid (LPA) is a major growth factor which enhances cell growth, migration and survival in ovarian cancer (Mills and Moolenaar, 2003, Yu et al., 2008). LPA is enriched in the ascites fluid that accumulates in the peritoneal cavity of ovarian cancer patients (Mills et al., 1988, Mills et al., 1990, Xu et al., 1995a, Xu et al., 1995b), suggesting that amplification of LPA-induced survival signaling could play a role in chemoresistance. LPA activates G-protein couple receptors (GPCRs) known as LPA receptors. Binding of extracellular LPA to these receptors activates heterotrimeric G-proteins, which results in the activation of signaling cascades such as MAP kinase growth pathways and AKT survival pathways (Hurst and Hooks, 2009a). LPA receptors initiate cellular responses by inducing the transition of Gα-proteins from the inactive GDP-bound form to the active GTP-bound form. Therefore, G-proteins mediate LPA signaling cascades, and proteins that regulate G-protein activity will in turn regulate the strength of LPA signaling in ovarian cancer.

An important step in the regulation of G-protein activity cycle is the hydrolysis of the active GTP-bound form, which results in the deactivation of G-proteins. This critical step is enhanced by GTPase activating proteins (GAPs). Regulator of G-protein signaling (RGS) proteins are a family of diverse proteins that terminate the G-protein cycle by acting as GAPs to accelerate the hydrolysis of the GTP-bound G-protein (Berman and Gilman, 1998, Hurst and Hooks, 2009b). Our lab has shown that RGS proteins blunt LPA-induced cell growth in ovarian cancer cells (Hurst et al., 2008, Hurst and Hooks,

2009a), and identified several RGS transcripts that are expressed in ovarian cancer cells, many of which are expressed at different levels in normal ovarian cells versus ovarian cancer cell lines (Hurst et al., 2009). Comparing RGS transcript expression in normal versus tumor tissue using existing databases shows significant changes in RGS expression during ovarian cancer progression, suggesting that RGS transcript levels change as ovarian epithelial cells undergo oncogenic transformation (Rhodes et al., 2004).

Given that LPA induces survival in ovarian cancer cells, RGS proteins blunt LPA-induced G-protein activity, and RGS expression changes in ovarian cancer cells compared to normal ovary suggest that changes in RGS expression during ovarian cancer progression may contribute to chemoresistance by controlling the strength of LPA-induced survival pathways. We hypothesized that LPA-induced survival pathways are enhanced by the suppression in RGS proteins, thus enabling ovarian cancer cells to overcome chemotherapeutic-induced cell death. In this chapter, we demonstrate that RGS10 and RGS17 suppress LPA-induced activation of the survival factor AKT, suggesting a mechanistic model for RGS10 and RGS17 control of chemoresistance in ovarian cancer cells (Hooks et al., 2010).

2.2. Methods

2.2.1. Cells and Reagents.

SKOV-3 cells were purchased from American Type Culture Collection (Manassas, VA). These cells were maintained in McCoy's 5A medium (Mediatech, Inc., Manassas, VA) supplemented with 10% FBS (PAA Laboratories, Inc., Etobicoke Ontario, Candada). Lysophosphatidic acid (18:1, 1-oleoyl-2-hydroxy-sn-glycero-3-

phosphate) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and reconstituted in 0.1% fatty acid-free bovine serum albumin (BSA) immediately prior to use. RGS plasmids were purchased from the UMR cDNA Resource Center (Rolla, MO).

2.2.2. RGS Gene Modulation.

Transient transfections were performed using Fugene 6 transfection reagent (Roche Diagnostics, Basel Switzerland), according to manufacturer's instructions, at a ratio of 2 μL Fugene 6 reagent to 1 μg plasmid DNA. SKOV-3 cells were plated in 24-well plates at 50,000 cells/well and transfected with 500 ng HA-tagged RGS17 plasmid DNA, 250 ng HA-tagged RGS10 plasmid DNA, or empty vector. Assays were performed 48 hours after transient transfections, and total protein was isolated and processed for immunoblotting to confirm expression with HA epitope antibodies.

2.2.3. Western Blot Analysis

SKOV-3 cells were plated in 24-well dishes at 50% confluency and transfected with Fugene 6 expression plasmid complexes as described above. Cells were serum-starved overnight, and treated with vehicle or 1 μM LPA for five minutes at 37ºC. Control wells were treated with LPA in the presence or absence of LY294002, an inhibitor of PI3-kinase. Media was then aspirated and cells were lysed in 100 μL SDS-PAGE sample buffer. The lysates were boiled for five minutes and analyzed using SDS-PAGE and immunoblotting. Membranes were incubated with phospho-AKT primary antibodies (Cell Signaling Technologies), and visualized using ECL reagents (Pierce). Membranes were subsequently blotted with GAPDH antibodies as a loading control.

2.2.4. Statistical Analysis.

Experimental data was analyzed for statistical differences using an analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison test or Tukey's test between groups. P values less than 0.05 were considered significant.

2.3. Results

2.3.1 Summary of Related Results

Microarray analysis of gene expression comparing chemoresistant ovarian cancer cells to their parental chemosensitive cells show that specific RGS transcripts (including RGS10 and RGS17) are significantly suppressed during acquired chemoresistance. The down-regulation of RGS10 and RGS17 transcript expression was confirmed in a third independent model of acquired chemoresistance using the ovarian cancer cell line, HeyA8 and its daughter chemoresistant cell line, MDR-HeyA8. This suggested a correlation between the down-regulation in RGS10 and RGS17 transcript expression and acquiring chemoresistance.

To confirm that the down-regulation in RGS10 and RGS17 transcripts expression was due to exposure to chemotherapeutics rather than selection for cells with reduced RGS10 and RGS17 expression, SKOV3 ovarian cancer cells were treated with an acute dose of cisplatin (IC_{80}:100 μM for 48 hours). Acute cisplatin treatment resulted in decreased RGS10 and RGS17 transcripts expression. To determine if the changes in transcript expression were transient, we further determined transcript expression after treatment with cisplatin for 48 hours as described above, followed by the removal of drug and growth in fresh media for additional 48 hours. RGS10 and RGS17 transcript

expression levels remained suppressed following drug removal, suggesting persistent effects of acute cisplatin treatment on RGS expression in ovarian cancer cells.

In order to investigate if direct loss of RGS proteins would recapitulate the observed chemoresistant effects, cell viability assays were conducted on SKOV3 cells following siRNA-mediated knockdown of RGS10 and/or RGS17. Loss of RGS10 and/or RGS17 caused a significant increase in ovarian cancer cell viability. Further, loss of RGS10 and/or RGS17 significantly enhanced cell viability following treatment of ovarian cancer cells with cisplatin, vincristine, or docetaxel. Similarly, RGS10 and/or RGS17 knockdown decreased cisplatin-mediated cell surface annexin V staining, a marker for cell death. These results suggest that endogenously expressed RGS10 and RGS17 blunt constitutive survival signals in ovarian cancer cells.

2.3.2. RGS10 and RGS17 Regulate LPA-Induced AKT Activation

The PI3K/AKT axis is an obvious candidate survival pathway since it is activated in ovarian cancer cells via Gαi-proteins, which are deactivated by RGS10 and RGS17 proteins (Hurst and Hooks, 2009a). Further, LPA, an established survival factor in ovarian cancer that is constitutively produced and released from ovarian cancer cells, activates AKT pathways (Long et al., 2005, Hurst and Hooks, 2009a). Given that RGS10 and RGS17 selectively deactivate Gαi-proteins (Hunt et al., 1996, Mao et al., 2004), we hypothesized that RGS10 and RGS17 may blunt LPA-stimulated AKT activation in ovarian cancer cells. To test this possibility, we serum-starved SKOV-3 cells transfected with control or RGS10 siRNA to remove serum-bound LPA, and then measured basal and acute LPA-stimulated AKT phosphorylation. We did not observe a difference in the ability of LPA to stimulate AKT phosphorylation between cells expressing endogenous

levels of RGS10 and cells with 75-80% knock-down of RGS10. However, basal AKT phosphorylation levels were slightly but consistently and significantly higher in cells with reduced RGS expression, suggesting that endogenous RGS10 expression levels may function to attenuate AKT-mediated survival signaling.

Next, we measured LPA-stimulated AKT activation in SKOV3 expressing basal levels of RGS proteins and cells over-expressing RGS10 or RGS17. The ability of LPA to stimulate AKT phosphorylation was reduced in cells over-expressing either RGS protein (Figure 2.2), suggesting that RGS10 and RGS17 proteins may deactivate Gαi-proteins necessary for LPA-induced AKT activation in SKOV3.

2.4. Discussion

Chemoresistance results in lower therapeutic outcomes in ovarian cancer patients. Understanding the mechanisms that control the emergence of chemoresistance in ovarian cancer patients is crucial for the development of new therapeutic approaches that could re-sensitize refractory tumors to chemotherapeutic agents. Results from this study suggest that RGS10 and RGS17 blunt the activation of LPA-induced AKT survival pathways. Based on our results, we propose that suppression of RGS10 and RGS17 is partly responsible for the molecular mechanism which allows ovarian cancer cells to resist chemotherapy. The working model of this mechanism suggests that autocrine or paracrine activation of Gαi-coupled receptors, such as LPA receptors activates survival pathways like those mediated by AKT that oppose chemotherapy-induced cell death. Thus, we hypothesize that RGS10 and RGS17 function as tumor suppressors by blunting endogenous survival pathways (Figure 2.2).

This study focused on the role of RGS10 and RGS17 in survival signaling in SKOV3 ovarian cancer cells. The RGS family of proteins consists of diverse proteins that range from simple proteins that are composed of only the RGS domain to large proteins with multiple targeting and regulatory domains (Hollinger and Hepler, 2002, Chidiac and Roy, 2003). RGS proteins function as GAPs that regulate the amplitude and the duration of G-protein signaling by accelerating the rate of hydrolysis/deactivation of the GTP-bound forms of Gα subunits up to 1000-fold (Posner et al., 1999).

RGS10 belongs to the R12/D subfamily of RGS10 proteins but it lacks the multiple regulatory domains that are found in the other members of that subfamily. RGS10 contains phosphorylation and palmitoylation sites that are important for its subcellular localization. RGS10 selectively deactivates members of the Gαi/o family of G-proteins (Hunt et al., 1996).

RGS17 belongs to the RZ/A RGS protein subfamily. Like RGS10, RGS17 also selectively deactivates Gαi/o family of G-proteins (Mao et al., 2004, Nunn et al., 2006). The N-terminus of RGS17 contains a palmitoylation site which regulates its subcellular localization, hence affecting its G-protein and receptor selectivity. Clinical expression data available on Oncomine database suggests that RGS17 is expressed at significantly lower level in ovarian tumor tissues compared to normal ovary, with RGS17 expression decreasing with the progression of the disease (Rhodes et al., 2004). This is consistent with our observation that RGS17 expression is suppressed during chemoresistance.

We focused on AKT pathway in this study because this pathway is known to be involved in ovarian cancer growth and survival (Long et al., 2005, Hurst and Hooks, 2009a). AKT phosphorylation occurs at two residues, Ser478 which is phosphorylated by

mTORC2 complex, and Thr308 which is phosphorylated by protein kinase PDK1 (Vanhaesebroeck and Alessi, 2000, Sarbassov et al., 2005). In this study, we observed that over-expression of either RGS10 or RGS17 blunted LPA-induced AKT activation suggesting that RGS10 and RGS17 proteins may deactivate Gαi proteins necessary for LPA-induced AKT activation in SKOV3.

2.5. Summary and Conclusions

Results from this study establish RGS10 and RGS17 as novel regulators of LPA-induced AKT survival pathway activation. Data presented in chapters 3 and 4 will further discuss the mechanisms regulating RGS10 expression in chemoresistant ovarian cancer cells. More studies are needed to define mechanisms of regulation of RGS17 in ovarian cancer, as well as in other cancers. The results presented in this chapter are in contrast with other reports showing that RGS17 promotes proliferation in cancer cells. Specifically, RGS17 has been reported to induce growth, migration and survival of lung and prostate cancers, with RGS17 transcript expression increasing up to 13-fold in prostate and lung tumor samples compared to patient-matched normal tissue samples (Mackie and Roman, 2011, Bodle et al., 2013). Future studies will define the role RGS17 plays in ovarian cancer.

2.6. Figures

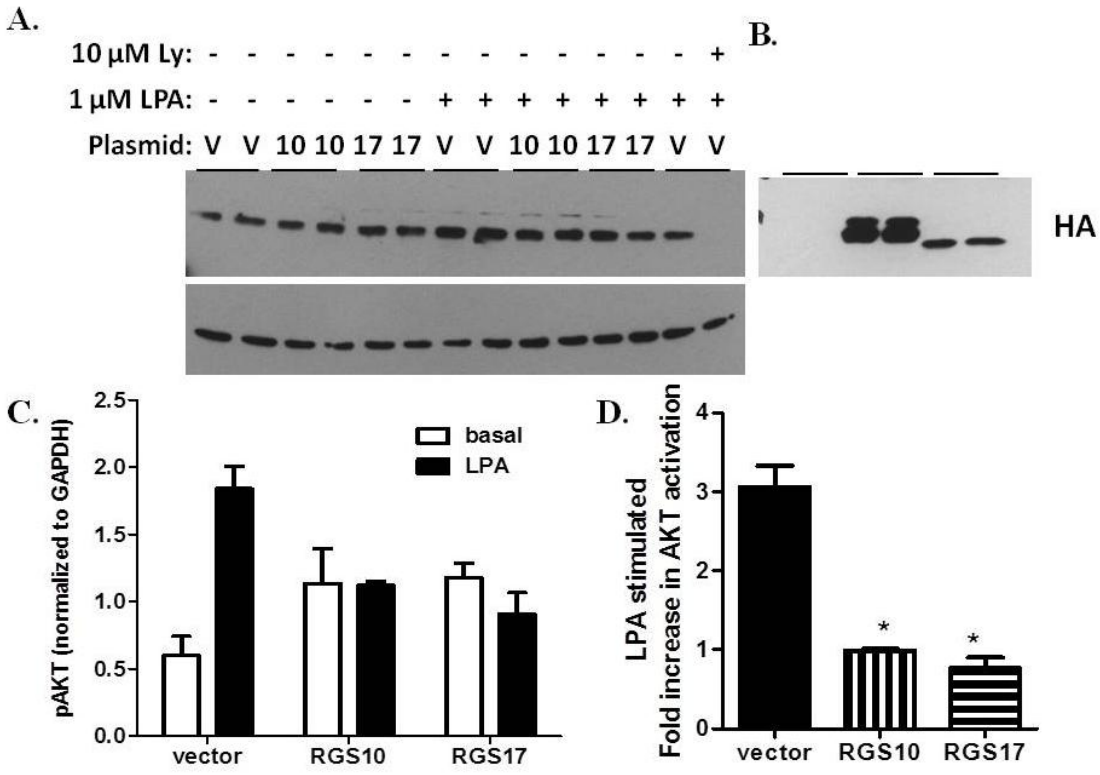

Figure 2.1: RGS10 and RGS17 Inhibit LPA-Stimulated AKT Activation. SKOV-3 cells were plated in 24-well dishes and transfected with either empty vector (V), plasmid encoding HA-tagged RGS10 (10), or plasmid encoding HA-tagged RGS17 (17). Cells were serum-starved overnight and treated with vehicle or LPA for 5 minutes at 37 ºC. Control cells were also treated with 10 µM LY294002 (LY), a PI3K inhibitor, to block AKT activation. Cell lysates were analyzed with SDS-PAGE and immunoblotting with anti-phospho-AKT antibodies (A) to measure AKT activation, or HA antibodies (B) to confirm transfection. Phospho-AKT blots were re-probed with GAPDH antibodies as loading controls. Activation of AKT was quantified by densitometry of bands and normalization to GAPDH controls (C). LPA-stimulated fold increase in AKT activation was quantified in the presence of empty vector, RGS10 plasmid, or RGS17 plasmid (D).

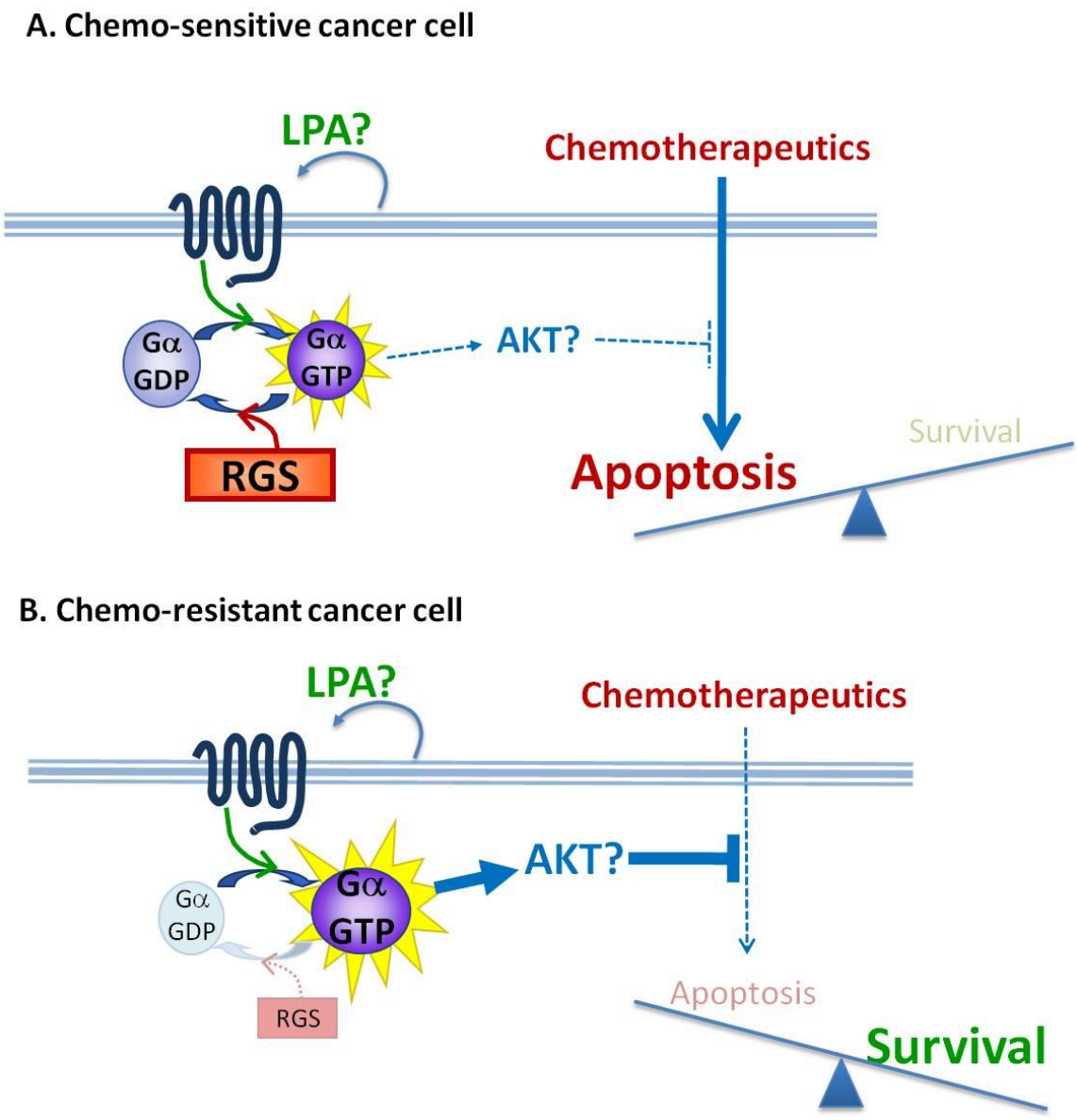

Figure 2.2: Model of Working Hypothesis for RGS Regulation of Acquired Chemoresistance. Autocrine activation of GPCRs in ovarian cancer cells activates AKT-dependent survival pathways. RGS10 and RGS17 inhibit LPA survival pathways by deactivating G-proteins. We hypothesize that suppression of RGS expression contributes to chemoresistance by indirectly amplifying LPA-mediated survival signals.

CHAPTER 3

TRANSCRIPTIONAL SUPPRESSION, DNA METHYLATION, AND HISTONE DEACETYLATION OF THE REGULATOR OF G-PROTEIN SIGNALING 10 (RGS10) GENE IN OVARIAN CANCER CELLS[1]

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[1]Ali, M.W., Cacan, E., Liu, Y., Pierce, J.Y., Creasman, W., Murph, M.M., Govindarajan, R., Eblen, S., Greer, S.F., and Hooks, S.B. 2013. PLoS One 2013:8(3)e60185.

Abstract

RGS10 regulates ovarian cancer cell growth and survival, and RGS10 expression is suppressed in cell models of ovarian cancer chemoresistance. However, the mechanisms governing RGS10 expression in ovarian cancer are poorly understood. Here we report RGS10 suppression in primary ovarian cancer and CAOV-3 ovarian cancer cells compared to immortalized ovarian surface epithelial (IOSE) cells, and in A2780-AD chemoresistant cells compared to parental A2780 cells. RGS10-1 and RGS10-2 transcripts are expressed in ovarian cancer cells, but only RGS10-1 is suppressed in A2780-AD and CAOV-3 cells, and the RGS10-1 promoter is uniquely enriched in CpG dinucleotides. Pharmacological inhibition of DNA methyltransferases (DNMTs) increased RGS10 expression, suggesting potential regulation by DNA methylation. Bisulfite sequencing analysis identified a region of the RGS10-1 promoter with significantly enhanced DNA methylation in chemoresistant A2780-AD cells relative to parental A2780 cells. DNA methylation in CAOV-3 and IOSE cells was similar to A2780 cells. More marked differences were observed in histone acetylation of the RGS10-1 promoter. Acetylated histone H3 associated with the RGS10-1 promoter was significantly lower in A2780-AD cells compared to parental cells, with a corresponding increase in histone deacetylase (HDAC) enzyme association. Similarly, acetylated histone levels at the RGS10-1 promoter were markedly lower in CAOV-3 cells compared to IOSE cells, and HDAC1 binding was doubled in CAOV- 3 cells. Finally, we show that pharmacological inhibition of DNMT or HDAC enzymes in chemoresistant A2780-AD cells increases RGS10 expression and enhances cisplatin toxicity. These data suggest that histone de-acetylation and DNA methylation correlate with RGS10 suppression and

chemoresistance in ovarian cancer. Markers for loss of RGS10 expression may identify cancer cells with unique response to therapeutics.

3.1. Background

Cancer cells exploit multiple receptor-mediated growth and survival signaling pathways to evade normal quiescence and cell death responses. Amplification of these pathways is a common mechanism in cancer progression. Activation of G-protein coupled receptors by the ligands lysophosphatidic acid (LPA), endothelin, stromal derived growth factor-1 (SDF1), prostaglandins, and thrombin contribute to the progression of multiple cancers, and drugs that block these receptors are currently in various stages of clinical trials as cancer therapeutics (Lappano and Maggiolini, 2011). These GPCRs initiate growth and survival signaling cascades by activating cellular G-proteins. G-protein activity is terminated by regulator of G-protein signaling (RGS) proteins that rapidly deactivate G-proteins and control the strength and duration of GPCR-initiated pathways (Hepler, 1999). RGS proteins that suppress oncogenic signals mediated by GPCR ligands are poised to inhibit cancer growth. Indeed, specific RGS proteins have been shown to suppress receptor-stimulated growth and survival signaling in breast, prostate, and ovarian cancer (Cao et al., 2006, Hurst et al., 2008, Liang et al., 2009).

Ovarian cancer is the leading cause of death from gynecological cancers and the fifth most common cause of cancer death in women. Less than 50% of ovarian cancer patients survive five years after their diagnosis (Jemal et al., 2009). Although ovarian cancer is characterized by a high response rate to chemotherapy, its high mortality rate is largely due to the development of resistance to the first-line chemotherapeutic agents (Pisano et al., 2009). The majority of patients who initially respond to chemotherapy will relapse with chemoresistant disease within two years (Agarwal and Kaye, 2003).

Understanding the molecular and genetic changes that drive ovarian cancer progression and the development of acquired chemoresistance may lead to strategies to predict and prevent the occurrence of refractory disease.

We have shown that endogenous RGS proteins suppress ovarian cancer cell growth, migration, and MAP kinase activation in response to LPA, a major autocrine growth factor in ovarian cancer (Hurst et al., 2008, Hurst and Hooks, 2009a). More recently, we have identified RGS10 as an important regulator of cell survival and chemoresistance. RGS10 transcript expression is down-regulated in multiple models of acquired chemoresistance in ovarian cancer, and RGS10 expression levels alter ovarian cancer cell sensitivity to cisplatin and taxane cytotoxicity (Hooks et al., 2010). These observations suggest that suppression of RGS10 expression may contribute to ovarian cancer progression and the development of chemoresistance by amplifying GPCR-mediated growth and survival signaling pathways. However, the mechanism of suppression of RGS10 expression in ovarian cancer has not been established.

RGS protein expression is dynamically regulated in neural and cardiovascular systems (Willars, 2006) and in cancer progression (Hurst and Hooks, 2009c), allowing for complex control over GPCR signaling pathways. Transcriptional and post-translational mechanisms for control of RGS expression are well defined (Fong et al., 2000, Anderson et al., 2007, Bodenstein et al., 2007, Cheng et al., 2008), while epigenetic control of RGS expression by covalent modifications to DNA or histones has been largely unexplored. Gene silencing by DNA methylation and histone deacetylation is an established mechanism in progression of many cancers (Esteller, 2007), including ovarian cancer (Huang et al., 2007, Nephew et al., 2009, Seligson et al., 2009). The addition of

methyl groups to CpG dinucleotides by DNA methyl transferase (DNMT) enzymes and the removal of acetyl groups on lysine residues in histone proteins by histone deacetylase (HDAC) enzymes coordinately suppress transcriptional activity (Steele et al., 2009a). DNA methylation and DNMT expression increase in ovarian cancer progression (Nephew and Huang, 2003), and histone deacetylases (HDACs) are also over-expressed in ovarian cancer tissues (Jin et al., 2008). This suggests that epigenetic regulation of RGS genes may also contribute to their dynamic expression in cancer progression.

In the current study, we investigated the epigenetic regulation of RGS10 expression in ovarian cancer cells. We focus on two models of RGS10 suppression– CAOV-3 ovarian cancer cells compared to benign ovarian epithelial cells, and chemoresistant A2780-AD cells and their chemosensitive parental cells. We identify significant increases in DNA methylation in chemoresistant cells, and marked decreases in histone acetylation and increases in HDAC1 association at the RGS10 promoter in both CAOV-3 and A2780-AD cells. Our results suggest that epigenetic histone modifications may contribute to the loss of RGS10 expression in ovarian cancer cells, and DNA methylation may contribute to further loss of expression during acquired chemoresistance.

3.2. Materials and Methods

3.2.1. Cells and Reagents

CAOV-3 and SKOV-3 cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (ATCC) and McCoy’s 5A medium (Mediatech, Inc.), respectively, supplemented with 10% FBS (PAA Laboratories, Inc.). The chemosensitive A2780 parental cell line and its multi-drug

resistant daughter counterpart A2780-AD cells were generously provided by Dr. Bob Brown, Imperial College London. These cells were maintained in RPMI 1640 medium (ATCC) supplemented with 10% FBS and 5mM L-glutamine. Chemoresistant cells were further maintained in 1.5 μM cisplatin. Immortalized ovarian surface epithelial cells (IOSE-80) were generously provided by Dr. Nelly Auersperg (University of British Columbia) and maintained in Media 199: MCDB 105 (1:1) supplemented with 15% FBS. All cells were grown in 5mM penicillin-streptomycin at 37°C with 5% carbon dioxide. 5-Aza-2′-deoxycytidine and cisplatin were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies recognizing histone H3 and acetylated histone H3 were from Millipore (Lake Placid, NY). Antibody recognizing histone H3 (acetyl K18) was from Abcam (Cambridge, MA). Antibodies recognizing RGS10 and HDAC1 were obtained from Santa Cruz (Santa Cruz, CA).

3.2.2. Cellular Viability Assays

10, 000 A2780 or A2780-AD cells were seeded in triplicate in 96-well plates and allowed to attach for 24 hours prior to treatment with the indicated concentrations of cisplatin for 48 hours. Cell viability assay was conducted in serum free media containing CellTiter-Blue® reagent (Promega Corporation) as previously described (Hooks et al., 2010).

3.2.3. Quantitative Real-Time PCR

mRNA was isolated using Trizol reagent (Invitrogen) and cDNA was synthesized from 2 μg of total RNA using the High Capacity Reverse Transcriptase cDNA kit (Applied Biosystems/ Life Technologies). Quantitative real-time polymerase chain reaction was performed using Superscript III kit for RT-PCR (Invitrogen) and Power

SYBR Green reagent (Applied Biosystems). Reactions were normalized using the housekeeping gene GAPDH and calculations were performed according to the $2^{-\Delta\Delta CT}$ method. Fold change in expression was determined in triplicate in three independent experiments, and experimental replicates were tested for significant differences between groups using paired T-tests. Primers used were based on algorithm-generated sequences from Primer Bank (http://pga.mgh.harvard.edu/primerbank/). RGS10 Forward: GAC CCA GAA GGC GTG AAA AGA, RGS10 Reverse: GCT GGA CAG AAA GGT CAT GTA GA, RGS10 variant-1 Forward: CCC GCG GCG ATG TTC AAC C, RGS10-variant-1 Reverse: CTC CAG GGA TGC CGC CCA TT, RGS10-variant-2 Forward: TGC GTG GAA CTT CTC AGG TGG ACA, RGS10 variant-2 Reverse: CCG CCC ATT TGG CTG TGC TCT, RGS2 Forward: AAG ATT GGA AGA CCC GTT TGA G , RGS2 Reverse: GCA AGA CCA TAT TTG CTG GCT, RGS5 Forward: CCC ACT CAT GCC TGG AAA GG, RGS5 Reverse: CTT GGC TGG TTT CTC TGG CT, GAPDH Forward: GCC AAG GTC ATC CAT GAC AAC T, GAPDH Reverse: GAG GGG CCA TCC ACA GTC TT.

To determine the effect of 5-Aza-2′-deoxycytidine exposure on RGS transcript expression, 7 x 105 SKOV-3 cells were plated in 100 mm tissue culture plates and allowed to attach overnight. The following day, media was aspirated and replaced with 20 μM 5-Aza-2′-deoxycytidine in DMSO or DMSO vehicle control. After 3, 5, 7 and 9 days of drug incubation, the media was aspirated and 7 mL Trizol reagent (Invitrogen) was added. RNA isolation, DNA synthesis, and qRT-PCR were performed as above.

3.2.4. Isolation of Ovarian Cancer Cells from Peritoneal Ascites

Peritoneal ascites from ovarian cancer patients at the Medical University of South Carolina (MUSC) were obtained under MUSC Institutional Review Board (IRB) protocol #18983, which included a review of the ethics of the study. Removal of peritoneal ascites is a standard of care for ovarian cancer patients. No patient identifying information was obtained by researchers in the laboratory. Peritoneal ascites were centrifuged at 1000 rpm for 10 minutes at room temperature and the cell pellets were washed with phosphate buffered saline (PBS). Red blood cells were lysed in RBC lysis buffer (eBioscience, San Diego, CA) for 5 min at room temperature. Lysis buffer was diluted with PBS, the cells centrifuged as above and resuspended in RPMI medium with 10% FBS. The cells were incubated for 1 hr at 37°C with 5% CO2 to allow attachment of fibroblasts and macrophages. Unattached epithelial cells were removed and incubated separately in complete RPMI medium containing 10% fetal bovine serum.

3.2.5. RGS10 Immunoblots

To evaluate RGS10 expression in primary ascites and IOSE cells, cell lysates were generated in RIPA buffer (50 mM Tris pH7.4, 10% glycerol, 150 mM NaCl, 1% Triton X100, 0.5% SDS, 0.5 mM EDTA, 0.5 mM EGTA, 5 mM Na4P2O7, 40 mM β-glycerophosphate, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride and aprotinin). After sonication and centrifugation, equal amounts of soluble protein were run on a 10-12% SDS PAGE gel, transferred to nitrocellulose, and immunoblotted with RGS10 antibody. To evaluate RGS10 expression in cell lines, 105 cells were lysed in SDS-PAGE sample buffer. The lysates were boiled for five minutes and analyzed using SDS-PAGE. Membranes were incubated with RGS10 primary antibodies (Santa Cruz

Biotechnology, Inc.) and HRP-conjugated rabbit secondary antibodies (Pierce) and visualized using ECL reagents (Pierce). Membranes were subsequently blotted with GAPDH antibodies (Life Technologies) as a loading control.

3.2.6. Bisulfite Sequencing

The Methprimer website (Li and Dahiya, 2002) http://www.urogene.org/methprimer/index1.html was used to analyze CpG content of RGS promoters and to design primers targeting different regions in the RGS10-1 promoter. Four different primer pairs were designed, RGS10-BS1, RGS10-BS2, RGS10-BS3 and RGS10-BS4: RGS10-BS1 forward: AAG AAA ATG GGG GTT AAT GAT ATT T, RGS10-BS1 reverse: TAC CTC TAA CAA AAC CTT CAA ACT C, RGS10-BS1 amplification region: -121,303,236 to -121,303,086. RGS10-BS2 forward: TGT TTT TAA AGT TAG AGA AGT GTT T, RGS10-BS2 reverse: CAC AAA CTA AAA AAC CTA AAC CTC, RGS10-BS2 amplification region: -121,303,076 to -121,302,726. RGS10-BS3forward: GAG GAG GTA AAG GTT ATA GGT TGG, RGS10-BS3 reverse: AAA TAC ACT AAC CCA AAA AAA ACC CC, RGS10-BS3 amplification region: -121,302,800 to -121,302,514. RGS10-BS4forward: GTT TGG TTA GGA GGA GG, RGS10-BS4reverse: CTC CAA TCT AAA AAA TAC CAC, RGS10-BS4 amplification region: -121,302,327 to -121,301,988.

Genomic DNA was harvested from cells and bisulfite-converted using EZ DNA Methylation-Direct Kit (Zymo Research Corp). ZymoTaq™ DNA polymerase (Zymo Research Corp) was used to amplify different regions in RGS10 promoter of bisulfite-treated genomic DNA and PCR products were analyzed with 2.5% DNA-agarose gels and purified using PureLink Quick Gel Extraction and PCR Purification Combo Kit

(Invitrogen). The purified products were ligated in plasmids using StrataClone PCR Cloning Kit (Agilent Technologies) which were then transformed into competent bacteria. 20 individual colonies were isolated from Carbenicillin LB-agar plates and expanded. QIAprep Spin Miniprep Kit (Qiagen Sample & Assay Technologies) was used to purify the plasmids from each colony, which were then sent for sequencing using T7 and/or T3 promoter sequencing primers at UGA Genomics Facility. Clone sequences were subjected to screens for quality and complete conversion, and aligned to genomic RGS10 promoter DNA using BiQ Analyzer software (Bock et al., 2005).

3.2.7. Chromatin Immunoprecipitation (ChIP) Assay

Cells were plated at a density of 2.5 x 106 in 15 cm-tissue culture plates and cross-linked with 1% formaldehyde for 8 minutes at room temperature. The cross-linking reaction was stopped by the addition of 0.125 M glycine for five minutes at room temperature. Cell nuclei were isolated and concentrated by lysing in fresh SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, dH2O) plus protease inhibitors for 25 minutes on ice followed by flash freezing in liquid nitrogen. Nuclei were sonicated using a Bioruptor water bath sonicator for 30 sec “On”, 30 sec “Off” 3X to generate an average of 500 bp of sheared DNA. DNA shearing was confirmed by subjecting lysates to 1% agarose gel electrophoresis and visualization by SYBR safe staining. 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 5µg of indicated antibody overnight at 4°C and the other half was immunoprecipitated with control antibody. Following an additional two hour immunoprecipitation with 60µl of 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, dH2O), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl, dH2O), LiCl (0.25M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris pH 8.0, dH2O), and 1xTE. DNA was eluted with SDS elution buffer (1% SDS, 0.1M NaHCO3, dH2O). Following elution, cross-links were reversed overnight with 5M NaCl at 65°C and 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 7900 (Applied Biosystems, Foster City, CA) using the following primers and probe for RGS10: forward, 5'-GGA ACC GCG AGT CCT CAC-3', reverse, 5'-CCC GGA GCT CTA GGT CCC-3' and probe, 5'-TGG CTA GGA GGA GGG CGG CG-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.

3.2.8. Statistical Analysis

Experimental data was analyzed for statistical differences using an analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison test or Tukey's test between groups, where indicated. *$p < 0.05$ **$p < 0.01$ and ***$p < 0.001$ indicate the levels of significance.

3.3. Results

3.3.1. Suppression of RGS10 Expression in Ovarian Cancer Cells.

Our previous data demonstrated down-regulation of RGS10 transcripts in ovarian cancer cell lines with acquired chemoresistance (Hooks et al., 2010). To determine if RGS10 is also down-regulated in ovarian cancer, we immunoblotted lysates from the benign, immortalized IOSE cell and from six primary epithelial ovarian cancer cell samples isolated from patient ascites (Figure 3.1A). RGS10 protein expression was markedly lower in cells from each patient, suggesting that RGS10 expression is suppressed in clinical ovarian cancer. Since patient samples are heterogeneous and non-renewable, their use in defining mechanisms of suppression is limited. To establish a renewable, homogeneous cell model of the loss of RGS10 expression in ovarian cancer, we compared RGS10 expression in IOSE cells and the serous epithelial ovarian cancer cell line CAOV-3 (Figure 3.1 B & C). RGS10 transcript and protein was significantly lower in CAOV-3 cells compared to IOSE control cells.

Our previous observation that RGS10 is suppressed in chemoresistant cells was made in published transcript expression datasets from chemosensitive and chemoresistant ovarian cancer cell pairs (Hooks et al., 2010). For the current study, we obtained A2780 ovarian cancer cells and their multi-drug resistant derivative A2780-AD. A2780-AD cells were derived from parental A2780 cells via chronic exposure to low-dose cytotoxic drug, and thus represent a model for acquired chemoresistance (Wang and Minko, 2004, de Jong et al., 2011). We confirmed the loss of sensitivity to cisplatin-induced cytotoxicity in A2780-AD cells, and demonstrated that RGS10 transcript and protein expression is reduced in A2780-AD cells compared to parental A2780 cells (Figure 3.1

D-F). Taken together, RGS10 transcript and protein expression is reduced in primary ovarian cancer cells and the CAOV-3 cancer cell line relative to immortalized ovarian epithelial cells, and in A2780 cells relative to parental cells. We focused the following studies on these two comparisons.

3.3.2. RGS10 Promoters

The human RGS10 gene resides on the negative strand of chromosome 10 and contains two transcriptional start sites, giving rise to two distinct transcripts and gene products (Figure 3.2A & B). The variants have unique first exons, and share four common exons. The longer transcript RGS10-1 gives rise to a 21kDa protein RGS10a containing 181 amino acids. The shorter transcript variant RGS10-2 gives rise to a 19.5 kDa protein RGS10b comprised of 167 amino acids. RGS10a is the only form of the protein detectable in ovarian cells (Figure 3.1). To determine if both transcripts are detectable and similarly suppressed in ovarian cancer, we performed qRT-PCR using variant-specific primers. Both the long and short transcripts were detected in all cell lines by qRT-PCR, but RGS10-2 was expressed at much lower levels than RGS10-1. RGS10-1 transcript expression in CAOV-3 ovarian cancer cells is approximately 20% of the expression level seen in IOSE cells, comparable to the fold reduction observed for total RGS10 transcript. However, the shorter transcript, RGS10-2, is not significantly different between the two cell lines (Figure 3.2C). Further, RGS10-1 transcript expression was down-regulated in the chemoresistant A2780-AD derivative cell line, while RGS10-2 levels were increased (Figure 3.2D). These results suggest that suppression of RGS10 transcript in CAOV-3 and A2780-AD ovarian cancer cells is unique to RGS10-1, and suggests that the mechanism may be targeted to the unique

promoter region. The following studies have focused on regulation of expression of RGS10-1 expression.

3.3.3. DNA Methylation of RGS10 Promoters in Ovarian Cancer Cells

Promoters containing G-C rich "CpG islands" typically have low levels of methylation in normal tissues, but become hypermethylated during cancer progression (Shi et al., 2002, Li et al., 2009b), suggesting that genes with CpG islands in their promoters are potential targets for transcriptional silencing by promoter DNA methylation in cancer cells. Analysis of a region 1 kilobase upstream of the transcriptional start sites and 0.5 kilobase downstream of the start sites of the RGS10-1 and RGS10-2 transcripts reveals a striking difference in the CG content and number of CpG dinucleotides between the two RGS10 promoter regions (Figure 3.3A). The promoter region of RGS10-1 contains 60-80% CG content and includes approximately 120 CpG dinucleotides, while the RGS10-2 promoter contains less than 30. In comparison, analysis of the RGS2 promoter has CpG content similar to RGS10-1, while the promoter of RGS5 contains few CpG dinucleotides.

The high concentration of CpG dinucleotides in the RGS10-1 promoter suggests that the RGS10 gene is a potential target for regulation by DNMT enzymes and may be suppressed in ovarian cancer progression by enhanced DNA methylation. To test this prediction, we determined the effect of inhibiting DNA methylation on RGS10 expression. The DNMT inhibitor 5-Aza 2'deoxycytidine (5-Aza) blocks the addition of methyl groups to CpG dinucleotides in newly synthesized DNA of proliferating cells (Koh et al., 2007). Thus, the effects of 5-Aza on DNA methylation and gene expression are manifest after multiple rounds of cell division. Cells were treated with vehicle or 5-

Aza for a total of nine days, and the effect on the transcript levels of RGS10-1, RGS2 and RGS5 was determined every two days. Consistent with CpG island predictions, RGS5 expression does not change with 5-Aza treatment, while RGS10-1 and RGS2 transcript levels are approximately 8-fold higher in 5-Aza treated cells compared to vehicle treated cells (Figure 3.3B). This result suggests that DNMT enzymes likely contribute to suppression of RGS10-1 transcript levels.

3.3.4. Bisulfite Sequencing of RGS10-1 Promoter

We further predicted that the frequency of methylation in RGS10-1 promoters would be higher in ovarian cancer cells with lower RGS10-1 expression levels. Methylated and un-methylated cytosine residues are distinguishable by treatment with bisulfite, which converts unmethylated but not methylated cytosine bases to uracil. We first performed bisulfite sequencing to compare the frequency of DNA methylation at RGS10-1 promoters between parental A2780 and A2780-AD chemoresistant cells. Bisulfite-treated genomic DNA was amplified using four overlapping primer pairs designed to fully cover a region from 1000 base pairs upstream to 200 base pairs downstream of the RGS10-1 transcriptional start site. Isolated clones of bisulfite treated genomic DNA were sequenced and aligned to genomic DNA using BiQ Analyzer software to determine the methylation status of each CpG site in the RGS10-1 promoter in at least 10 clones. The results obtained with primer pairs BS10-1, BS10-2, BS10-3, and BS10-4 are shown in conventional lollipop format in figures 3.4, 3.5, 3.6, and 3.7, respectively.

Using the bisulfite sequencing data, we determined the rates of methylation of CpG sites across the RGS10-1 promoter in A2780 and A2780-AD cells (Figure 3.8). The

frequency of methylation at CpG sites across the RGS10-1 promoter was low; the majority of CpG sites was completely unmethylated or were methylated in 10-20% of clones. An exception was the dinucleotide at position -121,030,162, which was highly methylated in both cell lines. Over the entire promoter, the rate of methylation was slightly higher in A2780-AD cells than in parental A2780 cells (Figure 3.8, inset). This difference was more pronounced in multiple adjacent CpG sites in region -121,303,155 →-121,303,007 (indicated by dotted horizontal bar, Figure 3.8A). The overall rate of methylation across this region was doubled in the chemoresistant cells compared to parental cells (Figure 3.8, inset). These data suggest that local enhanced DNA methylation in a region approximately 800 basepairs upstream of the transcriptional start site correlates with loss of RGS10 expression in acquired chemoresistance. We next performed the same analysis on RGS10-1 promoters in IOSE and CAOV-3 cells. Methylation rates across the entire RGS10-1 promoter and in the region identified above were similar in IOSE and CAOV-3 cells (Figure 3.8B). Thus, enhanced DNA methylation of the RGS10-1 promoter does not account for transcriptional suppression in CAOV-3 cells.

3.3.5. Histone Modifications at RGS10 Promoters in Ovarian Cancer Cells

To explore additional mechanisms of regulation of RGS expression, we assessed histone modifications at RGS10-1 promoters using chromatin immunoprecipitation (ChIP) experiments. We compared acetylation at histones associated with the RGS10-1 promoter in A2780 and A2780-AD cells, using the GAPDH promoter as a control. Total H3 histone binding was similar at RGS10-1 and GAPDH promoters (data not shown). In contrast, acetylated H3 histone levels were significantly lower at RGS10-1 promoters in

the chemoresistant A2780-AD cells, while similar levels of acetylated histone H3 were associated with the GAPDH promoter in both cell types (Figure 3.9A). 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 et al., 2005, Seligson 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 RGS10-1 promoter in chemoresistant cells as compared to A2780 parental cells, with no change at the GAPDH control promoter (Figure 3.9B).

Histone acetylation is dynamically regulated in cells by the opposing actions of histone acetyltransferases (HATs) that add the acetyl functional group to histones, and histone deacetylases (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 et al., 2008). We observed a striking increase in HDAC1 association with RGS10-1 promoters in A2780-AD cells as compared to parental A2780 cells. This increase reflects a specific recruitment to the RGS10-1 promoter, as HDAC1 association with GAPDH promoters was unchanged between cell lines (Figure 3.9C), and total HDAC1 expression levels were not higher in A2780-AD cells (Figure 3.9D).

To determine if histone modifications at RGS10-1 promoters may account for the difference in expression in IOSE and CAOV-3 cells, we performed ChIP assays to compare histone acetylation. Again, total histone H3 levels at the RGS10-1 promoter were unchanged between the cell lines, while the level of acetylated histone H3

associated with the RGS10-1 promoter in CAOV-3 cancer cells was half that observed in IOSE normal ovarian epithelial cells (Figure 3.10A). Finally, we compared the association of HDAC1 with RGS10-1 promoters in IOSE and CAOV-3 cells. The level of HDAC1 associated with the control promoter GAPDH was unchanged between cell lines, but was more than doubled at RGS10-1 promoters in the cancer cell line, compared to IOSE cells (Figure 3.10B). These data show that decreased RGS10-1 expression in CAOV-3 ovarian cancer cells correlates with enhanced HADC1 binding and loss of histone acetylation at the at the RGS10-1 promoter as compared to IOSE cells.

3.4. Discussion

G-proteins are an important class of signal mediators, critical in the regulation of basic function of the nervous system, cardiovascular system, immune system, and malignancies (Tyndall and Sandilya, 2005). The essential mechanism by which G-proteins are activated to initiate these events is by ligand binding to G-protein coupled receptors (GPCRs). G-proteins are negatively regulated by cellular RGS proteins, which deactivate G-proteins through their GTPase activating protein (GAP) activity (Srinivasa et al., 1998). Therefore, the strength of G-protein signaling cascades is determined by the balance of activity of GPCRs and RGS proteins, requiring that both GPCRs and RGS proteins be tightly regulated. In the case of GPCRs, activity is controlled by binding of endogenous ligands to the extracellular surface of the receptors. Growing evidence suggests that RGS activity is regulated by multiple mechanisms controlling the expression and localization of RGS proteins. The current study marks the first description of the regulation of expression of an RGS gene by histone deacetylation and

establishes histone deacetylation as an additional mechanism by which RGS expression–and indirectly G-protein activity–is regulated.

We have previously reported that expression of RGS10, which normally suppresses growth and survival signaling pathways triggered by G-protein coupled receptors, is suppressed as ovarian cancer cells develop chemoresistance (Hooks et al., 2010). This suppression indirectly amplifies G-protein mediated cell growth and survival signaling and contributes to chemoresistance. In the current study we have performed a detailed analysis of the expression of RGS10 isoforms in normal and cancer-derived ovarian cells and determined the changes in epigenetic marks on RGS10 promoter DNA and histones in cells with different RGS10 expression levels. To probe the mechanisms responsible for suppressing RGS10 expression, we focused on two comparisons. First, we compared IOSE immortalized ovarian surface epithelial cells versus CAOV-3 ovarian cancer cells, as these cells displayed the greatest fold difference in RGS10 expression. However, because these cells are derived from two different patients, the difference in RGS10 expression may represent multiple differences in the epigenetic and transcriptional machinery. Thus, we also compared A2780 and A2780-AD cells. While the change in RGS10 expression is relatively modest between these two cell lines, the fact that they are a parent-daughter cell line pair with common genetic background suggests this model may reveal more subtle and acute modifications to the RGS10 gene and its regulation.

We predicted that the RGS10-1 promoter may be epigenetically regulated by DNA methylation for multiple reasons. First, silencing of tumor suppressors via DNA hypermethylation of their promoter regions is a major mechanism for cancer progression

in general (Costello et al., 2000, Tsou et al., 2002). Second, DNA methylation is implicated in ovarian cancer chemoresistance, as global DNA methylation and DNA methyl transferase expression are both increased in cisplatin resistant A2780 ovarian cancer cells (Li et al., 2009b). Further, inhibitors of DNA methylation re-sensitize previously resistant ovarian cancer cells to cisplatin (Steele et al., 2009b). Third, a recent report by Tu et al. reported epigenetic silencing of RGS2 in prostate cancer cells by promoter DNA methylation (Wolff et al., 2011). Our findings that the promoter of RGS10-1 was distinctly enriched in CpG dinucleotides and that inhibition of DNMT activity dramatically increased RGS10-1 expression supports the hypothesis that RGS10-1 transcription may be negatively regulated by DNA methylation. Further, we observed an increase in the methylation frequency of the RGS10-1 promoter in A2780-AD cells compared to parental cells, which was most prominent in a region approximately 800 basepairs upstream of the transcriptional start site. Recently released ENCODE transcription factor ChIP-Seq datasets suggest HEY-1 and c-myc transcription factors may interact with this region, as well as possible Pol-2 and Pol3 interactions (Consortium et al., 2011). Additional studies are needed to define the specific contribution of this region to the regulation of RGS10-1 expression in ovarian cancer cells and in clinical chemoresistance. In contrast, no change in RGS10-1 promoter methylation was observed between IOSE and CAOV-3 cells, suggesting that this mechanism may specifically correlate to loss of RGS10-1 expression in acquired chemoresistance.

Our results clearly demonstrate loss of histone acetylation and gain of HDAC-1 binding at RGS10-1 promoters in ovarian cancer cells with low RGS10-1 expression. This result is consistent with abundant evidence that acetylation of lysine residues in the

N-terminus tails of histones H3 and H4 is frequently reduced in cancers (Huang et al., 2007, Seligson et al., 2009). Further, Class I HDACs 1-3 are over-expressed in ovarian cancer tissues (Jin et al., 2008), and aberrant HDAC expression is associated with poor responses to chemotherapy (Witt et al., 2009a). HDAC inhibitors can inhibit cancer cell growth in vitro and in vivo, revert oncogene-transformed cell morphology, induce apoptosis, and enhance cell differentiation (Bolden et al., 2006, Frew et al., 2009). The class I selective HDAC inhibitor romidepsin (FK228) is effective in reducing ovarian cancer cell proliferation at nanomolar concentrations (Lech-Maranda et al., 2007), and multiple HDAC inhibitors are in ongoing cancer clinical trials (Mackay et al., 2010, Takai and Narahara, 2010).

Given that RGS10 down-regulation correlates with acquired chemoresistance and RGS10 knock-down directly enhances cell growth and survival, it is possible that enhancing RGS10 expression will have therapeutic benefit. Our results suggest that DNMT and HDAC enzymes may suppress RGS10 expression in ovarian cancer cells, and therefore inhibition of DNMT and HDAC enzymes should enhance RGS10 expression. HDAC inhibitors induce apoptosis in chemoresistant ovarian cancer cells (Muscolini et al., 2008), and DNMT inhibitors can re-sensitize chemoresistant ovarian cancer cells to cisplatin (Balch et al., 2005). Future studies will determine if HDAC inhibition and DNMT inhibition can synergistically increase RGS10-1 expression, and define the role that RGS10 expression may play in the therapeutic effects of HDAC and DNMT inhibitors in ovarian cancer.

Ovarian cancer is a notoriously heterogeneous disease, representing multiple cellular strategies for evading normal quiescence and apoptotic signals. It is noteworthy

that while CAOV-3 cells show dramatically reduced RGS10-1 expression compared to IOSE cells, RGS10 is not significantly suppressed in SKOV-3 serous epithelial ovarian cancer cells. Therefore, loss of RGS10 is not a universal feature of ovarian cancer cells. Understanding the molecular events that account for loss of RGS expression in certain ovarian cancer cells may lead to important diagnostic tools to identify populations of cells with greater chemoresistance. We predict that loss of RGS expression may define a subclass of ovarian cancer cells that have enhanced sensitivity to G-protein coupled growth and survival signals. Determining the epigenetic status of these RGS genes in individual patient tumors may lead to an (epi)genetic biomarker for tumors with resistance to traditional chemotherapy, but with enhanced sensitivity to newer GPCR-blocking drugs, such as LPA receptor antagonists. Finally, while our studies have focused on RGS10-1 suppression in ovarian cancer, our results have broader implications. RGS10 GAP activity selectively targets Gαi-family G-proteins, and the receptors for LPA, endothelin, and SDF-1 all strongly couple to Gαi proteins to mediate growth and survival responses in multiple cancers (Wu et al., 2008, Growcott, 2009, Li et al., 2009a, Liu et al., 2009). This suggests that RGS10 expression may suppress cancer cell growth and survival in a variety of tumors. Additional work is needed to determine if the epigenetic marks described here contribute to regulation of RGS10 expression in other cancers.

3.5. Summary and Conclusions

Loss of RGS10 expression enhances cell growth and resistance to chemotherapeutic drugs in ovarian cancer cells, and RGS10 expression is suppressed in multiple models of acquired chemoresistance in ovarian cancer cells. However, the mechanism for suppression of RGS10 expression is unknown. In the current study, we

demonstrate that expression of RGS10 is suppressed in primary ovarian cancer and in CAOV-3 ovarian cancer cells compared to benign immortalized ovarian surface epithelial (IOSE) cells, and is suppressed in A2780-AD chemoresistant cells compared to parental A2780 cells. The RGS10-1 promoter is enriched in CpG dinucleotides, and inhibition of DNA methyl transferases increases RGS10 expression, suggesting DNA methylation suppresses RGS10 expression. We performed bisulfite sequencing across the RGS10 promoter region, and identified a region with significantly enhanced DNA methylation in A2780-AD chemoresistant cells compared to parental cells. No differences in methylation were observed between IOSE and CAOV-3 cells. We further found significantly less acetylated histone H3 associated with RGS10-1 promoters in A2780-AD cells compared to parental cells, and a corresponding increase in histone deacetylase 1 (HDAC1) enzyme association with the RGS10-1 promoter in the resistant cells. Similarly, acetylated histone H3 levels were markedly lower in CAOV-3 ovarian cancer cells compared to IOSE cells, and HDAC1 binding was doubled in CAOV-3 cells. Our results suggest that loss of histone acetylation correlates with suppressed expression of RGS10 in ovarian cancer cells, while DNA methylation may contribute to further loss of RGS10 expression in chemoresistance. This study marks the first report of the regulation of an RGS gene by histone deacetylation.

3.6. Figures

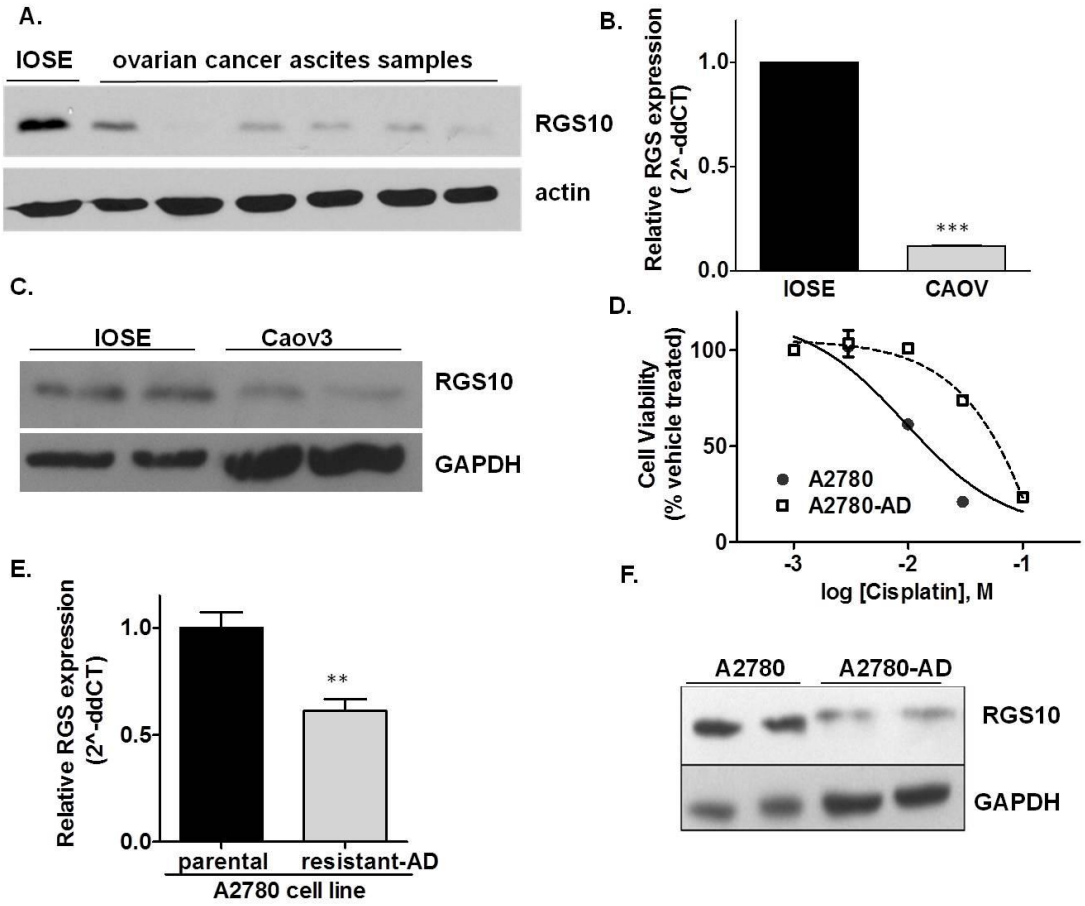

Figure 3.1: Loss of RGS10 Expression in Ovarian Cancer Cells. (A): Ovarian cancer cells were isolated from patient malignant ascites and RGS10 expression levels were compared to IOSE cells via western blotting. B.-C. RGS10 transcript (B) and protein (C) expression levels were compared in CAOV-3 ovarian cancer cell lines and IOSE benign ovarian epithelial cells using qRT-PCR and western blotting. (D): Cisplatin dose response curves were determined using CellTiter-Blue viability assays in A2780 and A2780-AD cells. E.-F.RGS10 transcript (E) and protein (F) levels were compared in chemoresistant A2780-AD cells relative to their parental chemosensitive cell line A2780. **: $p<0.01$, ***: $p<0.0001$.

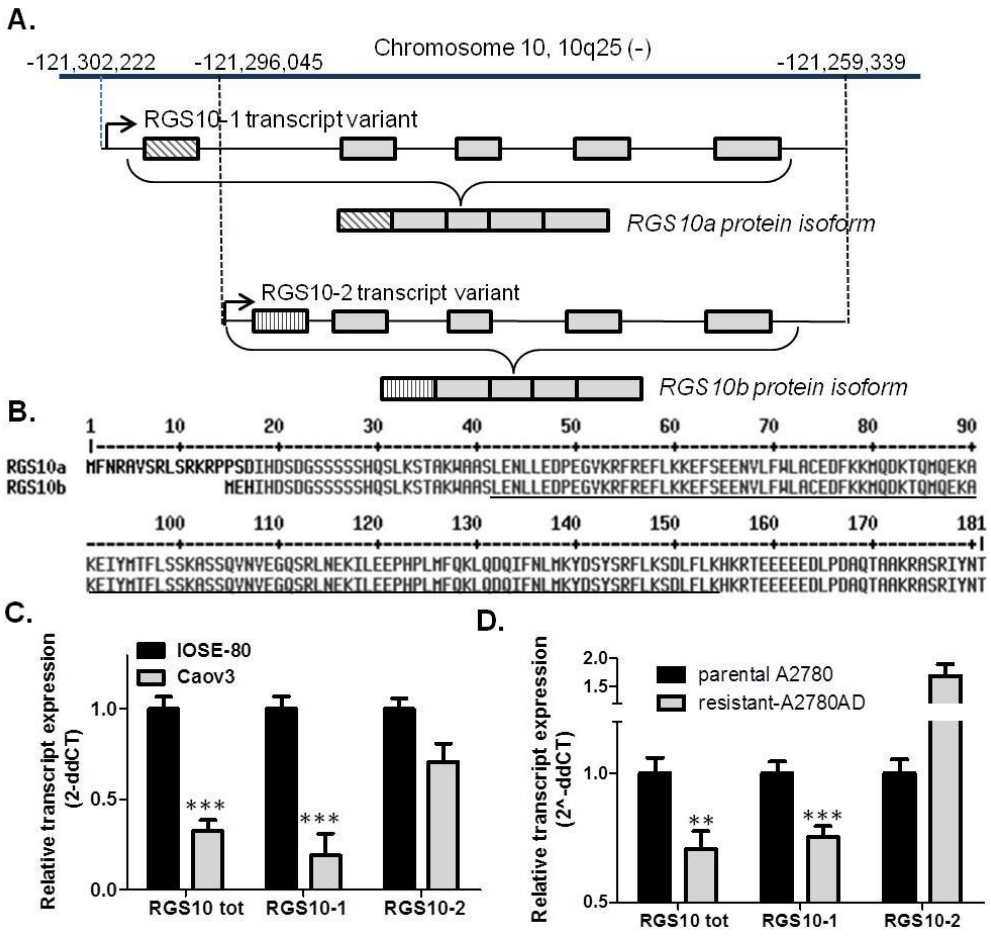

Figure 3.2: RGS10 Gene Structure and RGS10 Transcript Expression in IOSE, CAOV-3, A2780, and A2780-AD Cells. (A): The RGS10 gene (geneID:6001) is located on the negative strand of chromosome 10 (NCBI accession: NC_000010.10) at position -121,302,222 to -121,259,339. Two transcription variants RGS10-1 (accession:NM_001005339) and RGS10-2 (accession:NM_002925) have been reported for RGS10 based on alternate start sites that result in distinct first exons. (B): The resulting protein isoforms RGS10a (accession:NP_001005339) and RGS10b (accession:NP_002916) vary by only the first 18 or three amino acids. The conserved RGS domain is underlined. The expression of total RGS10 transcript (RGStot), RGS10-1,

and RGS10-2 were determined in IOSE and CAOV-3 cells (C) and in parental A2780 cells and chemoresistant A2780-AD cells (D). **: $p<0.01$, ***: $p<0.0001$.

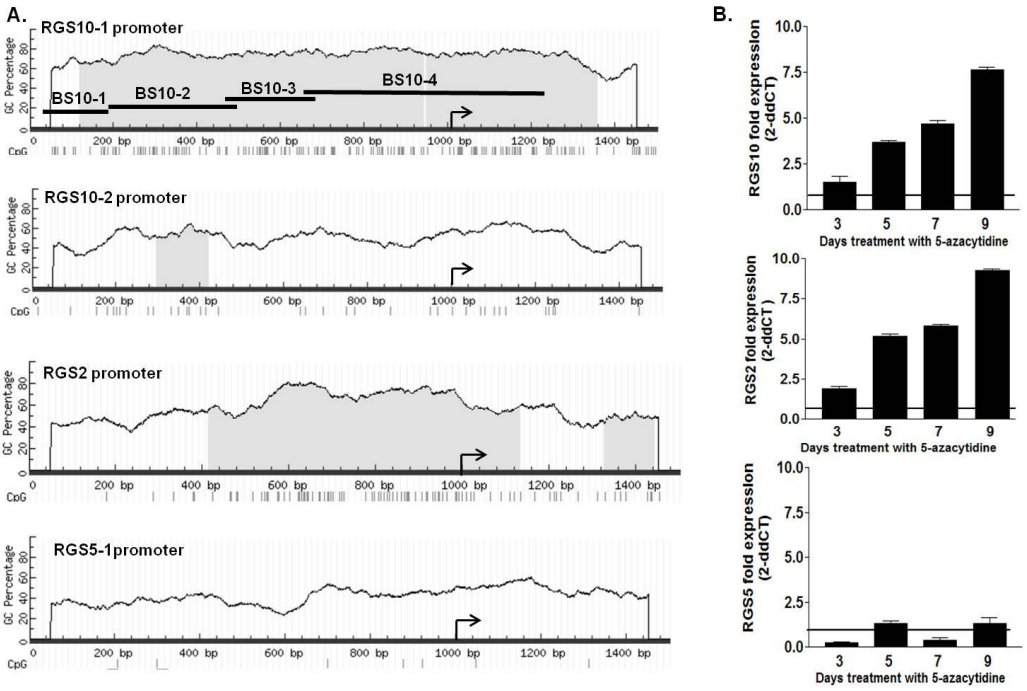

Figure 3.3: Regulation of RGS Genes by DNA Methylation. (A) The promoter regions of RGS10-1, RGS10-2, RGS2, and RGS5 were analyzed for CpG content using the website Methprimer. For each promoter, a region of genomic DNA 1000 basepairs 5' of the transcriptional start site and 500 basepairs 3' of the start site were evaluated for percent GC content and individual CpG dinucleotides. Nucleotide position is indicated along the x-axis and GC content is graphed on the y-axis; CpG islands are indicated with shading. Each CpG dinucleotide is indicated by a hash mark below the nucleotide numbering, and the transcriptional start site is indicated with an arrow. Amplification regions for four bisulfite sequencing primer pairs are indicated by horizontal bars (BS10-1, BS10-2, BS10-3, BS10-4). (B): SKOV-3 cells were treated with vehicle or the DNMT inhibitor 5-Aza for nine days, and the transcript levels of the indicated RGS and GAPDH controls were measured at 3, 5, 7, and 9 days of treatment. The RGS transcript was normalized to GAPDH, and is graphed relative to expression in vehicle treated controls at each time point.

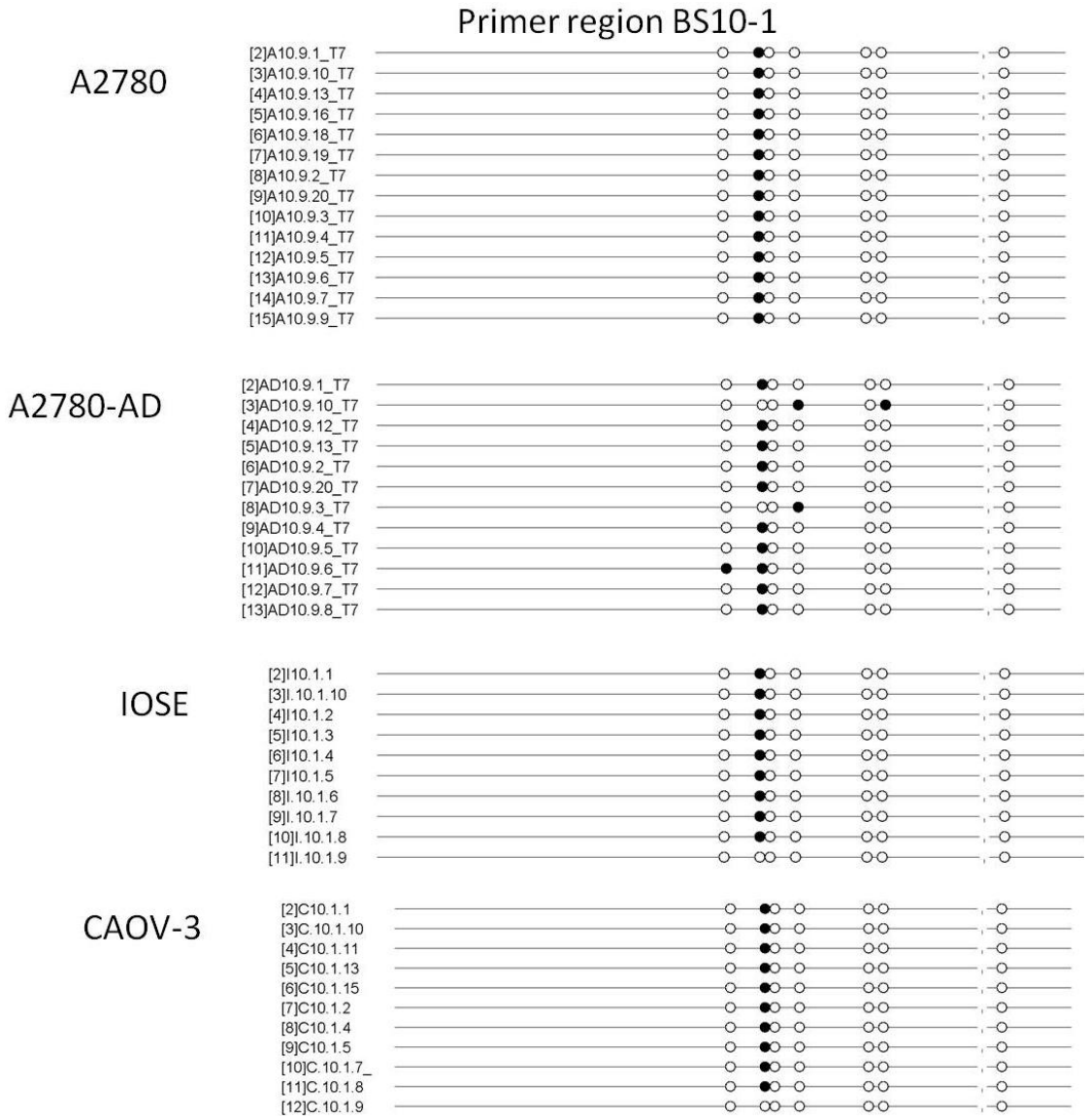

Figure 3.4: Bisulfite Sequencing of RGS10-1 Promoter-RegionBS10-1. RGS10 promoter genomic DNA was aligned with individual sequences of cloned PCR products from primer pair BS10-1 amplification of bisulfite treated genomic DNA from the indicated cell lines. Sequences were subjected to quality control analysis and aligned using BiQ Analyzer software. In this conventional 'lollipop' representation, each CpG site in the region (-121,303,236 to -121,303,086) is indicated with a circle; filled circles are methylated, unfilled circles are unmethylated.

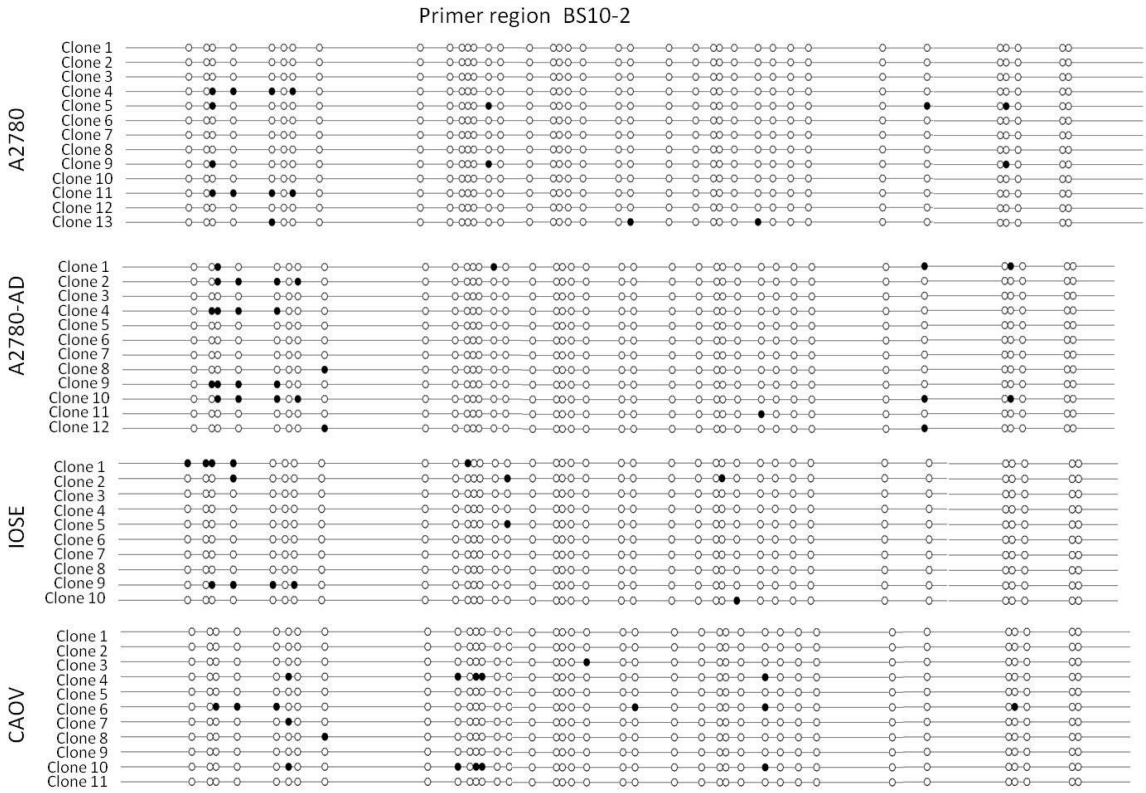

Figure 3.5: Bisulfite Sequencing of RGS10-1 Promoter-RegionBS10-2. RGS10 promoter genomic DNA was aligned with individual sequences of cloned PCR products from primer pair BS10-2 amplification of bisulfite treated genomic DNA from the indicated cell lines. Sequences were subjected to quality control analysis and aligned using BiQ Analyzer software. In this conventional 'lollipop' representation, each CpG site in the region (-121,303,076 → -121,302,726) is indicated with a circle; filled circles are methylated, unfilled circles are unmethylated.

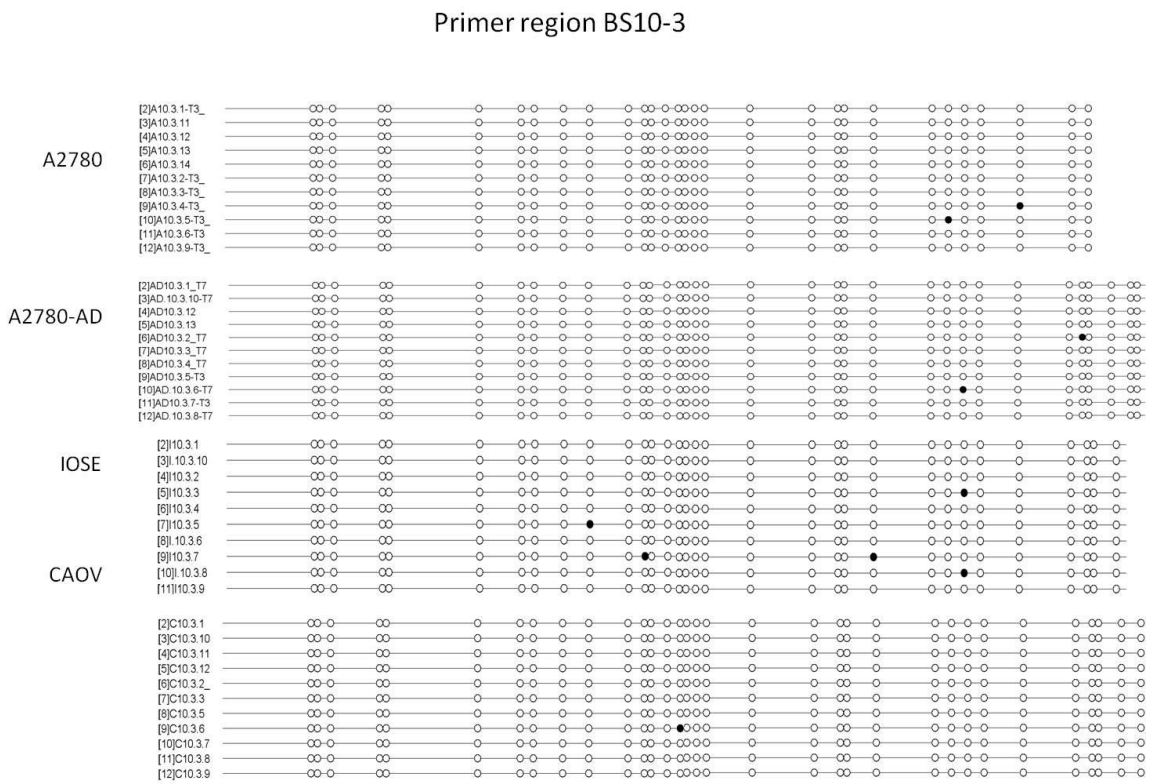

Figure 3.6: Bisulfite Sequencing of RGS10-1 Promoter-RegionBS10-3. RGS10 promoter genomic DNA was aligned with individual sequences of cloned PCR products from primer pair BS10-3 amplification of bisulfite treated genomic DNA from the indicated cell lines. Sequences were subjected to quality control analysis and aligned using BiQ Analyzer software. In this conventional 'lollipop' representation, each CpG site in the region (-121,302,800 to -121,302,514) is indicated with a circle; filled circles are methylated, unfilled circles are unmethylated.

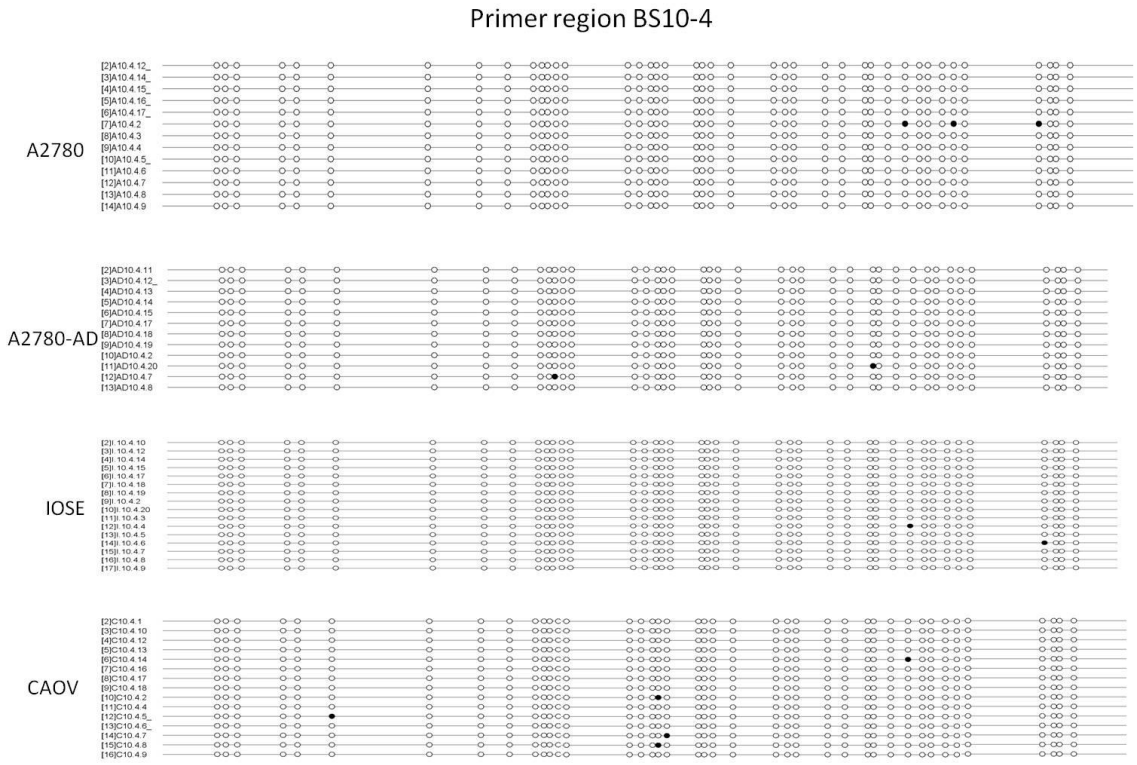

Figure 3.7: Bisulfite Sequencing of RGS10-1 Promoter-RegionBS10-4. RGS10 promoter genomic DNA was aligned with individual sequences of cloned PCR products from primer pair BS10-4 amplification of bisulfite treated genomic DNA from the indicated cell lines. Sequences were subjected to quality control analysis and aligned using BiQ Analyzer software. In this conventional 'lollipop' representation, each CpG site in the region (-121,302,327 to -121,301,988) is indicated with a circle; filled circles are methylated, unfilled circles are unmethylated.

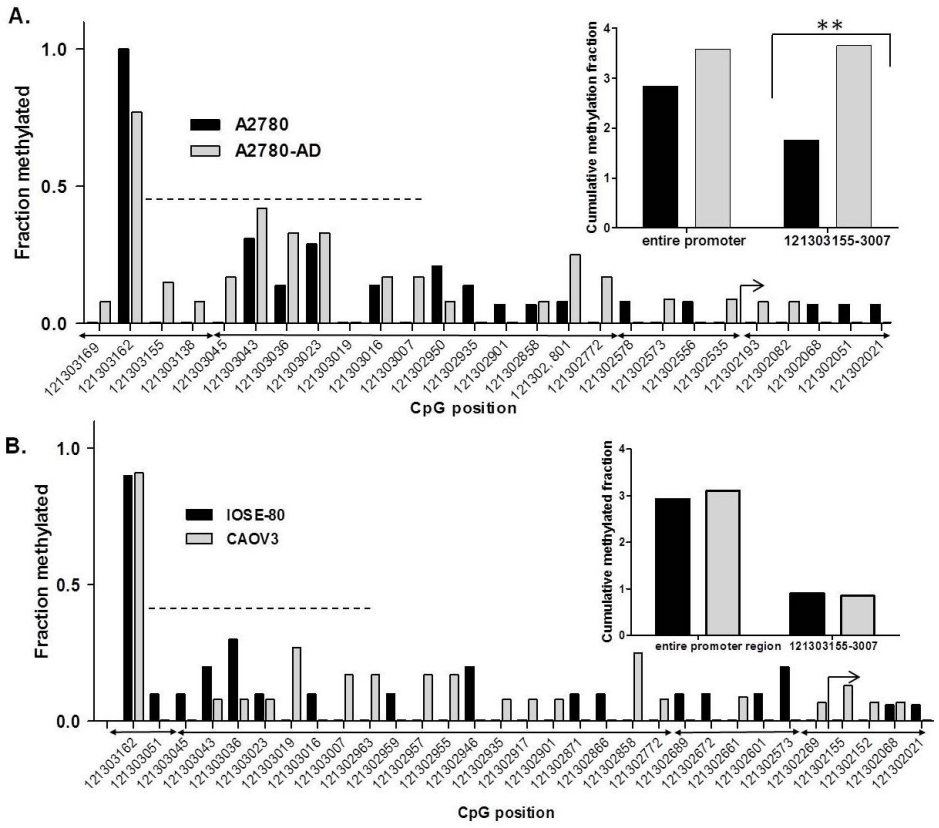

Figure 3.8: Methylated Fraction of CpG Dinucleotides Across the RGS10-1 Promoter in Ovarian Cell Lines. The fraction of clones that were methylated at individual CpG dinucleotides across the RGS10 promoter is shown. CpG dinucleotides are labeled by their position on chromosome 10(-). Nucleotides that were not methylated in either cell line are not shown. (A): Methylation rates are compared between A2780 and A2780-AD cells. (B): Methylation rates are compared between IOSE and CAOV03 cells. Dotted horizontal bar: region -121,303,155 → -121,303,007. Insets: The cumulative fraction of DNA methylation is shown for the entire RGS10-1 promoter and for the indicated region. **: $p<0.01$. Bent arrow: transcriptional start site. Arrows below x-axis: sites contained within each primer pair (left to right: BS10-1, BS10-2, BS10-3, BS10-4).

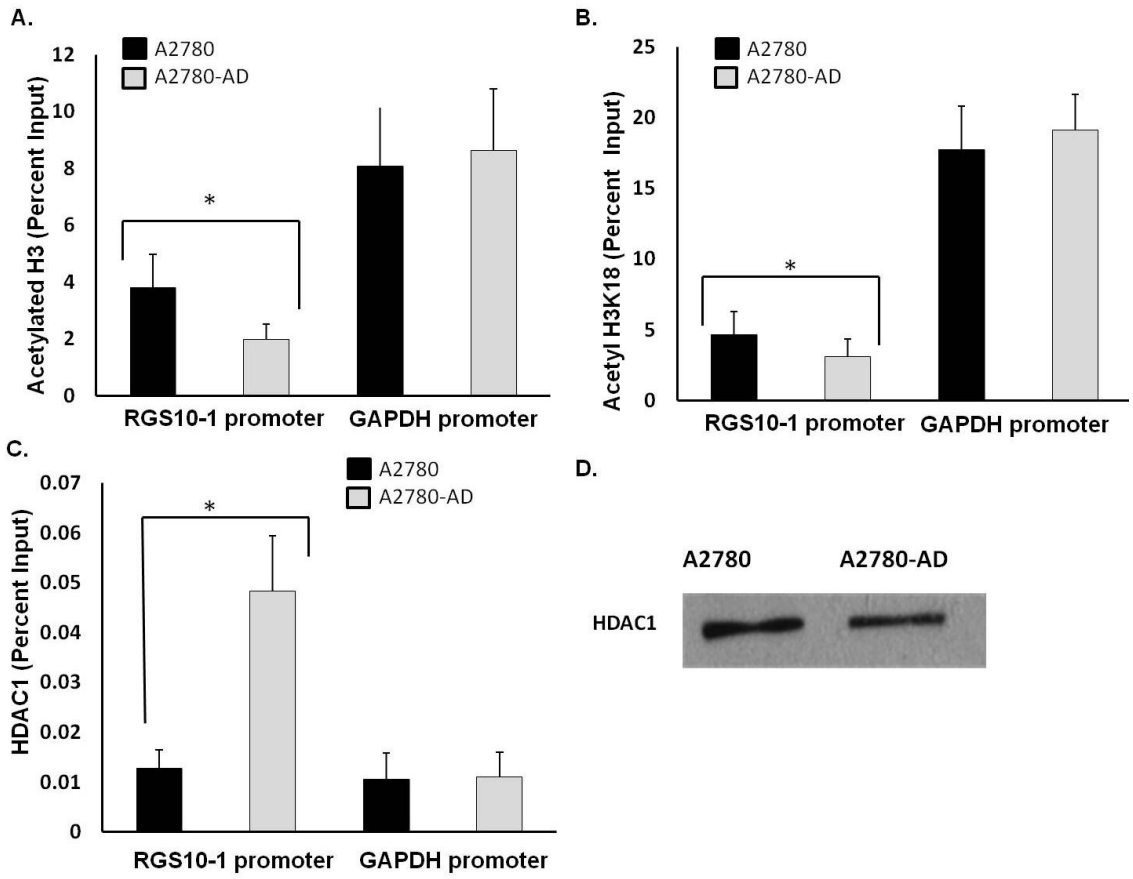

Figure 3.9: Histone Acetylation and HDAC Binding at the RGS10-1 Promoters in Chemoresistant A2780-AD Cells and Parental A2780 Cells. Lysates were immunoprecipitated with control, anti-acetyl histone H3, anti-acetyl H3K18, or anti-HDAC1 antibody. Associated DNA was isolated and analyzed via real time PCR using primers spanning the RGS10-1 and GAPDH promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). * $P<0.05$. (A): Global levels of Histone H3 acetylation associated with RGS10 and GAPDH promoters in A2780 and A2780-AD ovarian cancer cells. Values represent mean ± SEM of four independent experiments. (B): Levels of histone H3 acetylated at lysine 18 associated with RGS10-1 and GAPDH promoters in A2780 and A2780-AD ovarian cancer cells. Values represent mean ± SEM of four independent experiments. (C): Levels of HDAC1

associated with RGS10 and GAPDH promoters in A2780 and A2780-AD ovarian cancer cells. Values represent mean ± SEM of three independent experiments. (D): Western blot analysis of global HDAC1 levels in A2780 and A2780-AD cells.

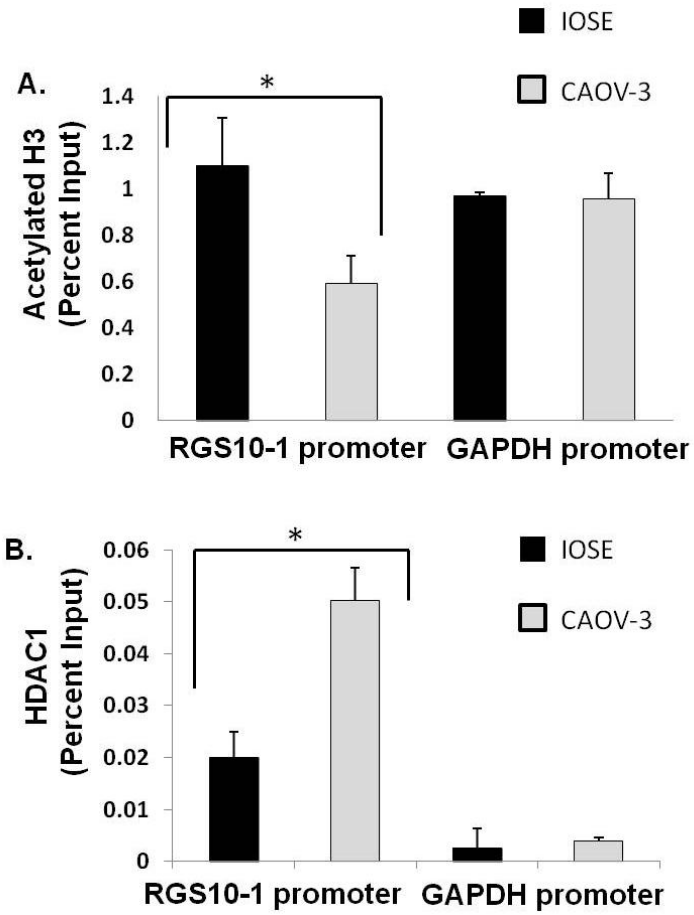

Figure 3.10: Histone Acetylation and HDAC Binding at the RGS10-1 Promoters in IOSE and CAOV-3 Ovarian Cells. Lysates were immunoprecipitated with control antibody, anti-acetyl histone H3 antibody, or with anti-HDAC1 antibody. Associated DNA was isolated and quantified via real time PCR using primers spanning the RGS10 and GAPDH promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). * $P<0.05$. (A): Global levels of Histone H3 acetylation associated with RGS10 and GAPDH promoters in normal and chemosensitive ovarian cancer cells. Values for histone H3 acetylation represent mean ± SEM of two independent experiments. (B): HDAC1 levels associated with RGS10 and GAPDH

promoters in normal and chemosensitive ovarian cancer cells. Values for HDAC1 binding are representative data. Error bars show deviation between technical errors.

CHAPTER 4

HISTONE DEACETYLATASE HDAC1 AND DNA METHYLTRANSFERASE DNMT1 REGULATE EXPRESSION LEVEL OF REGULATOR OF G-PROTEIN SIGNALING RGS10 AND CELL VIABILITY IN CHEMORESISTANT OVARIAN CANCER CELLS A2780-AD

4.1. Background

Ovarian cancer is the deadliest type of gynecological cancers, and represents the fifth leading cause of cancer-related deaths in females. The disease is often detected at late stages, with almost 70% of patients diagnosed in stages III or IV (Bast et al., 2009). The high mortality rate of ovarian cancer is due largely to the high rate of chemoresistance (Bast et al., 2009). The majority of cancer patients respond to the initial chemotherapeutic therapy; however, most of these patients will relapse with refractory disease within two years (Agarwal and Kaye, 2003).

In chapter 3, our results showed that RGS10 is suppressed in chemoresistant ovarian cancer cells through DNA hypermethylation and histone deacetylation, two important mechanisms that contribute to silencing of tumor suppressor genes during cancer progression. DNA methyl transferases (DNMTs) maintain DNA methylation (Rhee et al., 2000) and histone deacetylation is maintained by histone deacetylases (HDACs) (Ito et al., 2000). These two enzymes usually work in coordination to suppress gene transcription (Ghoshal et al., 2002, Cai et al., 2013), with DNMT1 been shown to bind HDAC1 (Fuks et al., 2000). The goal of studies in this chapter was to investigate the

molecular mechanisms by which RGS10 is epigenetically silenced in chemoresistant ovarian cancer cells, A2780-AD cells. Our results demonstrated that over-expressing HDAC1 in A2780-AD cells significantly reduced RGS10 transcript expression. Further, the pharmacological inhibition of HDAC1 using trichostatin A (TSA) and/or DNMT1 inhibition using 5-aza-2'-deoxycytidine (5-Aza-dC) in A2780-AD cells resulted in significant increase in RGS10 transcript expression. This correlates with enhanced cisplatin-induced cell death under the same conditions. These results suggest that HDAC1 and DNMT1 cooperatively work to suppress RGS10 during acquired chemoresistance and support the growing evidence that HDAC1/DNMT1 inhibition could be a novel therapeutic approach to re-sensitizing ovarian cancer patients to chemotherapeutic drugs.

4.2. Methods

4.2.1. Cells and Reagents.

The chemosensitive A2780 parental cell line and their derivative chemoresistant A2780-AD cells (derived as described (Louie 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. 5-Aza-2′-deoxycytidine (5-Aza-dC), trichostatin A (TSA), and cisplatin were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2. RGS Gene Modulation.

Transient transfections were performed using Fugene 6 transfection reagent (Roche Diagnostics, Basel Switzerland), according to manufacturer's instructions, at a

ratio of 2 μL Fugene 6 reagent to 1 μg plasmid DNA. A2780-AD cells were plated in 24-well plates at 50,000 cells/well and transfected with 500 ng FLAG-tagged HDAC1 or empty vector. Assays were performed 48 hours after transient transfections. Success of transfection was confirmed via qRT-PCR using HDAC1 and actin primers

4.2.3. 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'

To determine the effect of 5-Aza-dC and TSA exposure on RGS10 transcript expression, 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 vehicle, with or without 30 μM cisplatin for the final 12 hours. 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 vehicle, with or without 30 μM cisplatin for the final 12 hours. Cells were RNA isolation and DNA synthesis were performed as described above. Gene expression was normalized to GAPDH gene expression.

4.2.4. Cell Viability Assay

To determine the effect of 5-Aza-dC and TSA exposure on cell viability following cisplatin exposure, A2780-AD cells were plated in 96-well plates and treated with 5 μM 5-aza-2'-deoxycytidine (5-Aza) for 5 days, 500 nM Trichostatin A (TSA) for 36 hours, a combination of 5 μM 5-Aza for 5 days and 500 nM TSA for the final 36 hours or vehicle, with or without 30 μM cisplatin for the final 12 hours. Cell survival was assessed using CellTiter-Blue fluorimetric viability assays.

4.2.5. Statistical Analysis

Experimental data was analyzed for statistical differences using an analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison test or Tukey's test between groups, where indicated. *$p < 0.05$ **$p < 0.01$ and ***$p < 0.001$ indicate the levels of significance.

4.3. Results

4.3.1. HDAC1 and DNMT1 Suppresses RGS10 Expression in Chemoresistant Ovarian Cancer Cells

Results from the previous chapter demonstrated that HDAC1 enzyme binding to the RGS10 promoter was significantly increased in chemoresistant A2780-AD cells compared to parental chemosensitive A2780 cells (Ali et al., 2013). In order to investigate molecular roles for HDAC1 in regulating RGS10 transcript expression in chemoresistant ovarian cancer cells, A2780-AD cells were transfected with HDAC1. HDAC1 over-expression significantly reduced RGS10 transcript expression (Figure 4.1). This suggests that HDAC1 accumulation at the RGS10 promoter contributes to RGS10 suppression in chemoresistant ovarian cancer cells. In a reciprocal experiment performed by our collaborators, Dr. Suzanna Greer and Ercan Cacan, siRNA-mediated HDAC1 knockdown significantly increased RGS10 transcript and protein levels in A2780-AD cells. Together, these data suggest that HDAC1 may be a key regulator of RGS10 gene expression in chemoresistant ovarian cancer cells.

The RGS10 promoter contains high concentration of CpG dinucleotides, making it a potential target for regulation by DNMT1 maintenance methylation during ovarian cancer progression. To explore the involvement of DNMT1 in suppression of RGS10 expression, ChIP assays were carried out in A2780 and A2780-AD cells in order to quantify the level of DNMT1 at the RGS10 promoter in both cell lines. ChIP assays revealed a significantly higher amount of DNMT1 at the RGS10 promoter in A2780-AD cells compared to A2780 cells. Additionally, western blot analysis revealed that global protein levels of DNMT1 in both cell lines were similar, suggesting that the increase in

DNMT1 binding at the RGS10 promoter in A2780-AD compared to A2780 cells is due to increased recruitment, rather than an increase in global DNMT1 level. Together, these results suggest that binding of DNMT1 at the RGS10 promoter may contribute to RGS10 suppression in chemoresistant ovarian cancer cells.

To determine if DNMT1 binding at the RGS10 promoter affects RGS10 expression in chemoresistant ovarian cancer cells, A2780-AD cells were transfected with DNMT1 siRNA. DNMT1 knockdown significantly enhanced RGS10 transcript and protein levels, which suggests that DNMT1 is implicated in the suppression of RGS10 in chemoresistant ovarian cancer cells. Taken together, these data suggest that HDAC1 and DNMT1 are important regulators of RGS10 expression in chemoresistant ovarian cancer cells.

To determine if crosstalk exists between DNMT1 and HDAC1, ChIP assays quantifying HDAC1 binding at the RGS10 promoter were carried out in A2780-AD cells in the presence or absence of DNMT1 siRNA. DNMT1 knockdown significantly reduced HDAC1 binding at the RGS10 promoter in A2780-AD cells, whereas DNMT1 knockdown did not change the global levels of HDAC1 protein. These data suggest that DNMT1 is required for the binding of HDAC1 at the RGS10 promoter, and point out the potential cooperative crosstalk between HDAC1 and DNMT1 in the chemoresistant ovarian cancer cells, A2780-AD.

4.3.2. Inhibition of HDAC1 and DNMT1 Enhances RGS10 Expression and Decreases Ovarian Cancer Cell Viability

The aim of this experiment was twofold: first, to determine if pharmacological inhibition of histone acetylation or DNA methylation affect RGS10 expression in

chemoresistant ovarian cancer cells, and second, to investigate potential synergistic roles for HDAC1 and DNMT1 in regulating RGS10 expression. HDAC inhibitor trichostatin A (TSA) and DNMT inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) were used to inhibit HDACs and DNMTs, respectively. A2780-AD cells were treated with 5-aza-dC, TSA, a combination of 5-aza-dC and TSA or vehicle, in the presence or absence of cisplatin. The relative expression of RGS10 transcript was quantified by qRT-PCR and normalized to GAPDH transcript expression. TSA or 5-aza-2dC alone increased RGS10 expression in A2780-AD cells, and the combination of these two drugs causes a fold increase in RGS10 expression greater than the sum of the individual effects, suggesting a potential cooperative effect (Figure 4.2). Next, the cooperative roles of HDAC1 and DNMT1 in cell growth and chemoresistance were investigated. A2780-AD cells were treated with TSA and/or 5-aza-dC in the presence of cisplatin and cell viability assays were performed (Figure 4.3). 5-aza-dC alone decreased cell growth by approximately 40%, while TSA alone had a modest but significant effect on cell viability. Interestingly, the combination of TSA and 5-aza-dC inhibited cell viability by 90%. As expected, A2780-AD cells were resistant to cisplatin toxicity, but pretreatment with either TSA or 5-azadC partially re-sensitized the cells to cisplatin-mediated cytotoxicity. Taken together, these data suggest that DNMT1 acts coordinately with HDAC1 to increase RGS10 expression in chemoresistant A2780-AD ovarian cancer cells. This correlates with a lower cell viability and increased sensitivity of these cells to the cytotoxic effects of cisplatin.

To determine changes mediated by HDAC1 in cell survival of chemoresistant ovarian cancer cells, cisplatin-resistant A2780-AD cells were transfected with HDAC1 siRNA. Following HDAC1 siRNA transfection, cells were treated with cisplatin and

apoptosis was assessed using annexin V:PE detection. 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. 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 cytometry was used to distinguish between populations of unlabeled and singly- or doubly-labeled cells. HDAC1 knock down significantly increased the population of cisplatin-treated cells that are positive for both annexin V-PE and 7-AAD (late apoptotic or dead cells). These data suggest that HDAC1-mediated reduction of RGS10 expression correlates with the decreased ability of cisplatin to induce cell death in A2780/AD ovarian cancer cells.

4.3.3. Suppression of RGS10 Expression Did Not Reverse the Effect of HDAC/DNMT Inhibitors on Ovarian Cancer Cell Viability.

In order to determine the contribution of enhanced RGS10 expression on reduced cell viability in ovarian cancer cells following treatment with HDAC and DNMT enzymes inhibitors, A2780/AD cells were transfected with negative control siRNA or RGS10 siRNA duplexes, then dosed with a combination of 5-aza for and TSA. Cisplatin or vehicle was added for the last 12 h, and cell survival was assessed using CellTiter-Blue fluorimetric viability assays.

If HDAC/DNMT effects on cell viability were due to enhanced RGS10 expression, RGS10 siRNA should rescue cell survival in the presence of HDAC/DNMT inhibitors compared to cells transfected with negative control siRNA; however, our results showed that RGS10 knockdown did not alter ovarian cancer cell viability under

the aforementioned conditions (Figure 4.4A). Loss of RGS10 protein was confirmed using western blot analysis (Figure 4.4B). These results suggest that RGS10 is not the only driver of chemoresistance in A2780-AD cells.

4.4. Discussion

GPCRs have been identified as drug targets for the treatment of multiple cancers (Hurst and Hooks, 2009b, Carrieri et al., 2013, Xie et al., 2013). RGS proteins, including RGS10-the focus of this study, negatively regulate GPCR-mediated signaling pathways (Hunt et al., 1996, Popov et al., 1997, Gold et al., 2002, Shi et al., 2004). We previously linked the suppression of RGS10 expression to cell survival and chemoresistance in ovarian cancer cells, and further showed that RGS10 knockdown increases cell growth and survival (Hooks et al., 2010), and that RGS10 gene is epigenetically silenced in chemoresistant ovarian cancer cells via increased DNA methylation and decreased histone acetylation (Ali et al., 2013). DNA methylation and histone deacetylation are often associated with transcriptional repression (Fuks, 2005, Robertson, 2005) and with lower responsiveness to chemotherapy (Esteller et al., 2000, Giacinti et al., 2008). Our lab and others have shown that DNA hypermethylation and histone deacetylation contribute to chemoresistance in ovarian cancer via amplification of cell survival proteins (Terasawa et al., 2004, Stronach et al., 2011, Zeller et al., 2012, Ali et al., 2013). Recent studies suggest that resistance to cisplatin in ovarian cancer is driven by epigenetic mechanisms (Terasawa et al., 2004). Studies in this chapter demonstrate that DNMT1 and HDAC1, two important epigenetic factors, coordinate the suppression of RGS10 expression in chemoresistant ovarian cancer cells (Cacan et al., 2014).

HDACs remove acetyl groups from substrates, including histones of chromatin. HDAC inhibitors on the other hand preserve the acetylation status of proteins and induce apoptosis of cancer cells (Hassig and Schreiber, 1997, Kruhlak et al., 2001, Barneda-Zahonero and Parra, 2012). HDAC inhibitors have been shown to be effective anti-tumor drugs in clinical trials (Marks et al., 2003) and growing evidence (Arts et al., 2007, Witt et al., 2009b, Wang et al., 2011) demonstrates that HDAC inhibitors show great promise against ovarian cancer.

Multiple epigenetic modifications are commonly disrupted during oncogenesis. Oncogenes transcriptional expression is increased through DNA hypomethylation and histone acetylation (Jin et al., 2009, Muller et al., 2013). On the other hand, tumor suppressor genes are transcriptionally suppressed by enhanced DNA methylation and decreased histone acetylation (Nguyen et al., 2001, Herman and Baylin, 2003, Esteller, 2007). DNA methylation and histone deacetylation act synergistically to silence cancer-associated genes in ovarian cancer (Fuks et al., 2000, Ghoshal et al., 2002, Meng et al., 2011, Cai et al., 2013) and ovarian cancer is usually associated with increased expression levels of DNMTs and HDACs (Gu et al., 2013).

Studies presented here show that HDAC inhibition by TSA and DNMTs inhibition by 5-aza-dC induced significant increase in RGS10 transcript expression in chemoresistant ovarian cancer cells (Cacan et al., 2014), which correlated with HDAC/DNMT loss of cell viability. To determine if RGS10 increased expression was causative of decreased cell viability, RGS10 expression was blocked in the presence of HDAC/DNMT inhibitors. siRNA-mediated RGS10 knockdown did not increase cell viability under tested conditions, suggesting that RGS10 up-regulation does not account

for the observed HDAC/DNMT effects. Indeed, it is not expected that RGS10 is the sole driver of chemoresistance in ovarian cancer cells. Multiple RGS transcripts are epigenetically regulated, therefore other RGS proteins, which also negatively regulate survival pathways in ovarian cancer cells could be involved as well in the development of chemoresistance. Further, many proteins from outside the RGS family are expected to contribute to the development of chemoresistance in ovarian cancer cells.

Recent studies show that tumorigenecity and metastasis of ovarian cancer is suppressed by a combination of TSA and 5-aza-dC in xenograft mouse models (Meng et al., 2013). Thus, in this study the effects of TSA and 5-aza-dC combination treatment on RGS10 expression and cell viability in chemoresistant ovarian cancer cells were determined. The combination of these two drugs cooperatively enhanced RGS10 transcript expression and decreased chemoresistant cell viability.

In line with data presented in this chapter, a phase I clinical trial showed that a combination of methylation and histone deacetylation inhibitors enhanced clinical outcomes (Carafa et al., 2011, Falchook et al., 2013), and preselecting patients based on their methylation status could optimize their treatment responses (Falchook et al., 2013).

4.5. Summary and Conclusions

Results from this study support that the RGS10 gene is a target for DNA demethylating and histone acetylating therapeutic approaches and links RGS10 expression levels as a contributing mechanism to the clinical efficacy of DNMT and HDAC inhibitors in chemoresistant ovarian cancer cells (Figure 4.5).

4.6. Figures

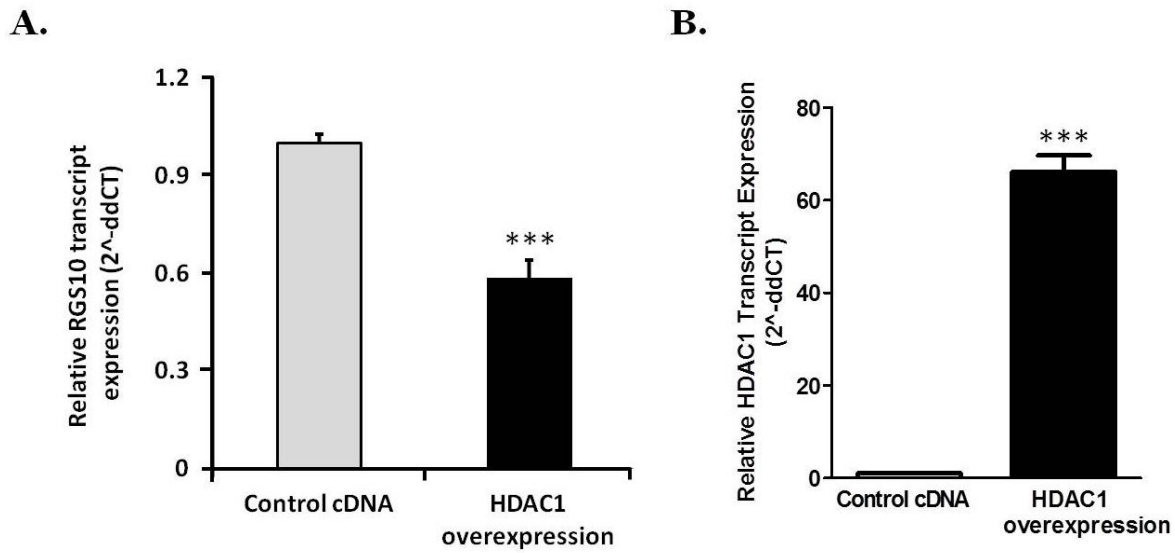

Figure 4.1: HDAC1 Over-expression Decreases RGS10 Expression in Chemoresistant Cells. A2780/AD cells were plated in 24-well plate and allowed to attach overnight. Cells were transfected with 500 ng HDAC1 or empty vector using FuGene 6 reagent (Promega) according to the manufacturer's protocol. Following 48 hours incubation, cells were harvested in TRIzol (Invitrogen) and the expression of HDAC1 (A) and RGS10 (B) genes was assessed using RT-PCR as described, and normalized to actin gene expression. Values represent mean ± SEM of four independent experiments ***$p<0.0001$.

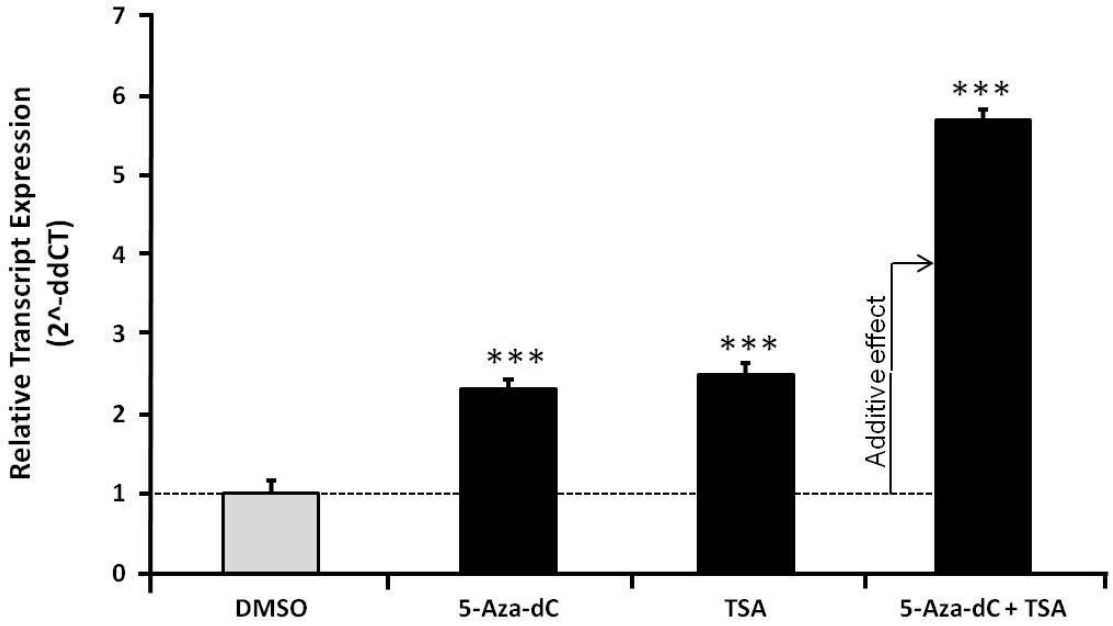

Figure 4.2: Effects of HDAC Inhibitor Trichostatin A (TSA) and DNMT Inhibitor 5-Aza-2′-deoxycytidine (5-Aza-dC) on RGS10 Transcript Expression. Total RNA was isolated from untreated control cells and TSA or 5-Aza-dC treated cells. The relative expression of RGS10 mRNA was quantified by qRT-PCR and normalized to GAPDH transcript expression. (***: $p<0.001$). The dotted box indicates the cell viability predicted by an additive effect of TSA and 5-Aza-dC. 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.

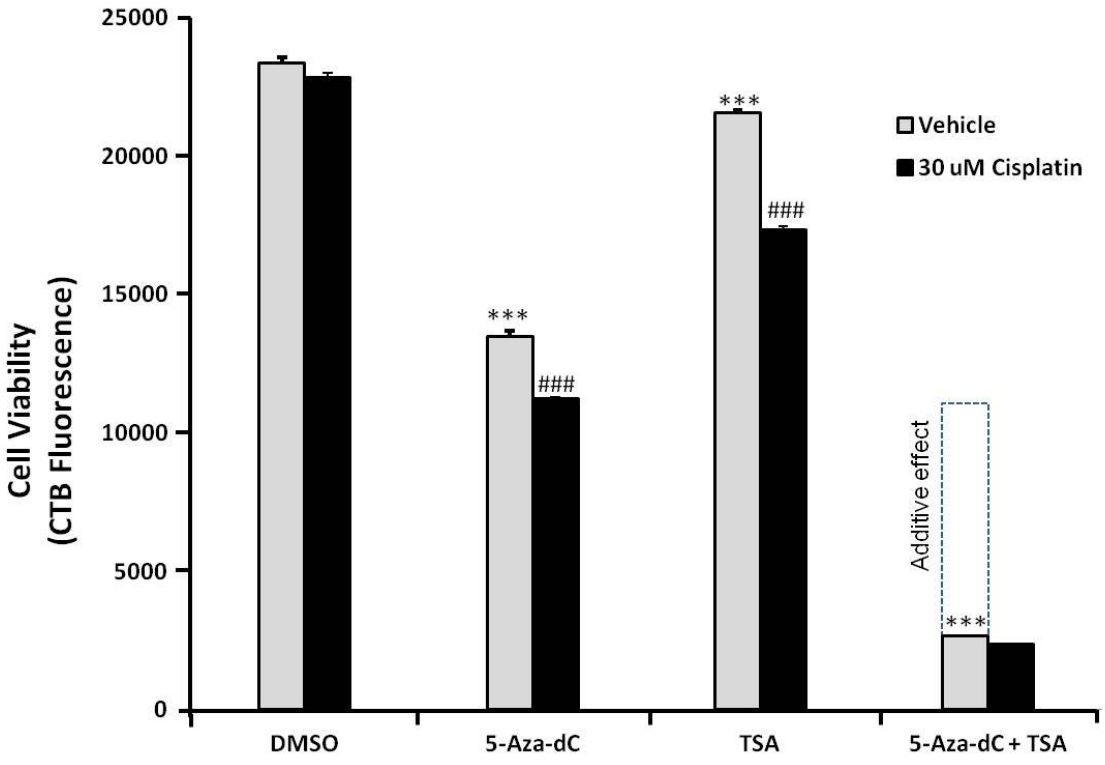

Figure 4.3: Effects of HDAC Inhibitor Trichostatin A (TSA) and DNMT Inhibitor 5-Aza-2′-deoxycytidine (5-Aza-dC) on Cell Viability in Chemoresistant Ovarian Cancer Cells. A2780-AD cells were treated under the same conditions as figure 4.2 with or without 30 μM cisplatin for the final 12 hours. Cell survival 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.

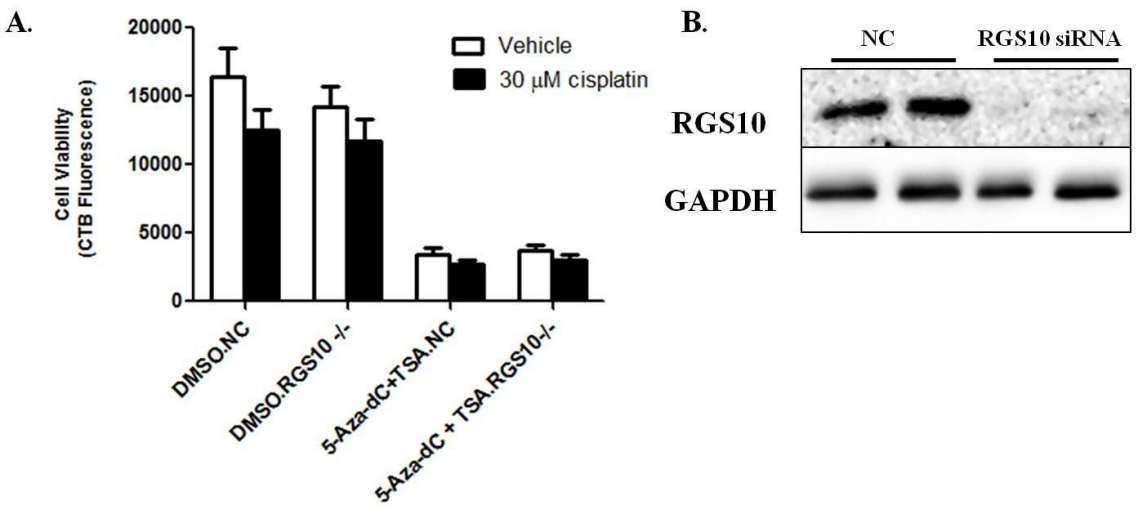

Figure 4.4: Suppression of RGS10 Expression Did Not Reverse the Effect of HDAC/DNMT Inhibitors On Ovarian Cancer Cell Viability. (A): A2780/AD cells (5000 cells/well) were plated in 96-well plate and allowed to attach overnight. Cells were dosed with a combination of 5 µM 5-aza-2'deoxycytine (5-aza) for 3 days and 500 nM trichostatin-A (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. (B): Western blot analysis showing efficiency of siRNA-mediated knockdown of RGS10 gene in A2780/AD cells. 5000 cells/well were plated in 96-well plate and allowed to attach overnight. Cells were transfected with negative control or RGS10 siRNA duplexes (Ambion Grand Island, NY) as per the manufacturer's protocol using Dharmafect1 transfection reagent (Dharmacon). 48 hours post-transfection cells were harvested in sample buffer and run on SDS-PAGE gels and blotted RGS10 protein using goat-antiRGS10 antibody (Santa Cruz) with GAPDH as loading control (Ambion).

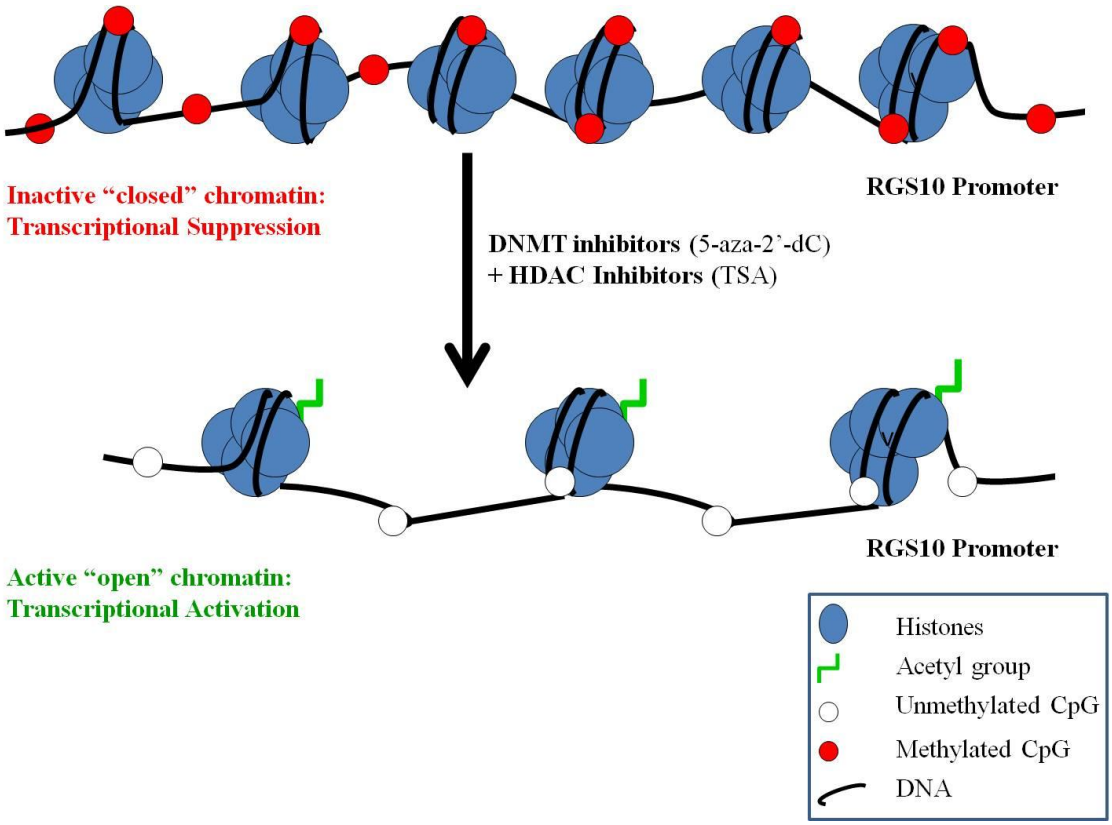

Figure 4.5: Effects of Epigenetic Drugs on RGS10 Expression. Increased DNA methylation and decreased histone acetylation induce a "closed" chromatin structure and cause gene suppression. Conversely, DNMT and HDAC inhibitors cause an "open" chromatin structure and drive gene expression.

CHAPTER 5

EXPRESSION AND FUNCTION OF REGULATOR OF G-PROTEIN SIGNALING 10 (RGS10) IN MICROGLIA

5.1. Background

Microglia are brain-resident macrophages that are responsible for homeostatic functions, such as combating brain infections (Gehrmann et al., 1995) and clearing cellular debris (Trang et al., 2011), as well as developmental functions such as synaptic pruning in developing brain (Stevens et al., 2007). However, uncontrolled microglial activation has been implicated in the initiation and progression of several neurodegenerative disorders such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease (see review: (Fu et al., 2014)). Finding novel ways to control excessive microglial activation could be very beneficial in the prevention and/or treatment of such neurodegenerative disorders.

Recently, regulator of G-protein signaling 10 (RGS10) protein has emerged as an important neuroprotective factor. RGS proteins are a highly diverse group of proteins that regulate signaling pathways downstream of GPCRs. The main role of RGS proteins is to regulate the duration and amplitude of G-protein signaling through their ability to function as GTPase activating proteins (GAPs), where RGS proteins accelerate the deactivation of G-proteins through stabilizing Gα-GTP transition state, hence increasing GTP hydrolysis by up to 1000-fold (Posner et al., 1999). All RGS proteins contain an

RGS domain that consists of approximately 120 amino acids, which is sufficient for interaction with Gα proteins and GAP activity (De Vries et al., 1995).

RGS10 belongs to the R12/D subfamily of RGS proteins, and contains phosphorylation and palmitoylation sites that are important for regulating its subcellular localization. RGS10 selectively deactivates Gαi family of G-proteins (Hunt et al., 1996), and it is expressed at high levels in the brain regions of hippocampus, striatum, and dorsal raphe (Gold et al., 1997), which are involved in higher brain functions. RGS10 is also abundantly expressed in immune tissues such as thymus and spleen, and RGS10 transcript expression level is increased upon T-cell activation (Haller et al., 2002). RGS10 is expressed in microglia and is localized in different subcellular compartments including the nucleus (Waugh et al., 2005).

Recent reports have shown that RGS10 normally suppresses cytokine production and that lipopolysaccharide (LPS)-induced microglial activation suppressed RGS10 expression. Brains of RGS10 knockout mice exhibited increased microglial activation, increased cytokine production, and higher vulnerability toward neuroinflammation-induced neuronal cell death (Lee et al., 2008). These results suggest a mechanism by which RGS10 suppression in microglia enhances inflammatory neuronal cell death. However, the mechanisms of RGS10 suppression in activated microglia, as well as the mechanisms by which RGS10 exerts its anti-inflammatory effects are not fully understood. Understanding such mechanisms could help in the development of new strategies for the prevention and/or treatment of neurodegenerative diseases that are characterized by excessive microglial activation.

Sphingolipid signaling, especially that of sphingosine-1-phosphate (S1P), is an emerging subject in the field of regulation of microglial function. There is increasing evidence that S1P is involved in microglial activation, and that targeting of S1P receptors could be beneficial in the treatment of neuroinflammatory and neurodegenerative disorders that are characterized by excessive microglial activation. The aim of the studies in this chapter is twofold: first, to investigate the mechanisms that drive RGS10 suppression in LPS-activated microglia, and second, to explore the mechanism by which RGS10 regulates inflammatory signaling in microglia. Our results suggest that the RGS10 gene is epigenetically silenced in activated microglia via decreased histone acetylation. Our data also suggest that RGS10, through its ability to negatively regulate Gαi-proteins, increases PKA activation. This in turn decreases glycogen synthase kinase 3-beta (GSK-3β) activation, which presumably leads to decreased nuclear factor-kappa B (NF-κB) activation and lower pro-inflammatory cytokine production (Wang et al., 2010).

5.2. Materials and Methods

5.2.1. Cells and Reagents

Murine microglial BV2 cell line was a generous gift from Dr. George Hasko at University of Medicine and Dentistry of New Jersey. BV2 cells were maintained in Dulbecco's Modified Eagle's Medium (ATCC) supplemented with 10% FBS (PAA Laboratories, Inc.). Lipopolysaccharide (LPS), H89, and trichostatin A (TSA) were obtained from Sigma-Aldrich (St. Louis, MO). Pertussis toxin (Ptx) was purchased from List Biological Laboratories, Inc. (Campbell, CA). For western blot analysis, the following antibodies were used: anti-RGS10 antibody, anti-phospho AKT (S 473), anti-phospho ERK1/2 p44/42 (T202/Y204), anti-phospho P38 (T180/Y182), anti-phospho

glycogen synthase kinase-3 beta (pGSK-3β), and anti-phospho ser/thr PKA substrate (Cell Signaling Technologies, Beverly, MA). Anti-GAPDH antibody (Ambion/Life Technologies, Carlsbad, CA). Anti-histone H3 and anti-acetylated histone H3 (Millipore, Lake Placid, NY). Anti-HDAC1 (Santa Cruz Biotechnology, Santa Cruz, CA).

5.2.2. Quantitative real-time PCR

mRNA was isolated using Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA) and cDNA was synthesized from 2 μg of total RNA using the High Capacity Reverse Transcriptase cDNA kit (Applied Biosystems/ Life Technologies). Quantitative real-time polymerase chain reaction was performed using Superscript III kit for RT-PCR (Invitrogen/Life Technologies, Carlsbad, CA) and Power SYBR Green reagent (Applied Biosystems). Reactions were normalized using the housekeeping gene actin and calculations were performed according to the $2^{-\Delta\Delta CT}$ method. Fold change in expression was determined in triplicate in three independent experiments. Primers used were based on algorithm-generated sequences from Primer Bank (http://pga.mgh.harvard.edu/primerbank/). RGS10 Forward: CCT GGA GAA TCT TCT GGA AGA CC, RGS10 Reverse: CTG CTT CCT GTC CTC CGT TTT C, TNFα Forward: CCT GTA GCC CAC GTC GTA G, TNFα Reverse: GGG AGT AGA CAA GGT ACA ACC C, IL1β Forward: GAA ATG CCA CCT TTT GAC AGT G, IL1β Reverse: TGG ATG CTC TCA TCA GGA CAG, Actin Forward: GGC TGT ATT CCC CTC CAT CG, Actin Reverse: CCA GTT GGT AAC AAT GCC ATG T. All primers were purchased from Integrated DNA Technologies, IDT (Coralville, IA).

5.2.3. Western Blot Analysis

To evaluate protein expression in cell lines, 100, 000 cells were lysed in SDS-PAGE sample buffer. The lysates were boiled for five minutes and analyzed using SDS-PAGE. Membranes were incubated with primary antibodies and appropriate HRP-conjugated rabbit secondary antibodies (Pierce) and visualized using ECL reagents (Pierce). Membranes were subsequently blotted with GAPDH antibodies (Millipore Technologies) as a loading control.

5.2.4. siRNA Knockdown Assay.

siRNA knockdown experiments were performed in 24-well plates. BV2 cells were plated (100,000 cells/well) and reverse transfected with 60 nM of mouse RGS10 siRNA or control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using Lipofectamine LTX transfection reagent (Life Technologies, Carlsbad, CA). Protein analysis was carried out 48 hours after transfection.

5.2.5. Statistical Analysis

Experimental data was analyzed for statistical differences using an analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison test or Tukey's test between groups, where indicated. *$p < 0.05$ **$p < 0.01$ and ***$p < 0.001$ indicate the levels of significance.

5.3. Results

5.3.1. LPS Effects on RGS10 Expression in Microglia

Previous reports show that RGS10 is down-regulated in microglia following low dose LPS activation (Lee et al., 2008). To determine the time and dose dependency of RGS10 suppression in our model, the murine microglial BV2 cells, we analyzed RGS10

transcript and protein levels after treating the cells with increasing doses of LPS using qRT-PCR and western blotting techniques, respectively. LPS resulted in RGS10 transcript (measured at 4 hours) and protein (measured at 24 hours) suppression in BV2 cells in a dose-dependent fashion (Figure 5.1A & B). We simultaneously measured inflammatory cytokine production, and saw that LPS, with a similar dose-response, enhanced pro-inflammatory cytokines TNFα and IL-1β in BV2 cells (Figure 5.1D & E). These results show correlation between RGS10 expression levels and pro-inflammatory cytokines production. Time course analysis revealed that RGS10 transcript was optimally inhibited by 100 ng/ml LPS at 6 hours and remained suppressed up to 72 hours (Figure 5.1C).

5.3.2. Effects of HDAC Inhibition on RGS10 Expression in Microglia

We previously reported that the RGS10 gene is epigenetically suppressed in chemoresistant ovarian cancer cells via increased recruitment of histone deacetylase1 (HDAC1) and decreased histone acetylation at the RGS10 promoter (Ali et al., 2013). To test whether RGS10 expression is regulated via a similar mechanism in BV2 cells, we analyzed RGS10 transcript expression following pharmacological inhibition of HDAC enzymes using the HDAC inhibitor, trichostatin A (TSA). RGS10 transcript expression was increased dose-dependently following TSA treatment (Figure 5.2A), suggesting that RGS10 gene expression could be epigenetically regulated by histone acetylation.

Next, we tested if TSA could blunt LPS-induced RGS10 suppression in BV2 cells. To that end, we treated BV2 cells with vehicle, LPS (1 μg/ml for 4h), TSA (250 nM for 24h), or TSA (250 nM pretreatment for 20 hrs) followed by LPS (1μg/ml for 4 hrs). HDAC inhibition blocked LPS-induced RGS10 transcript suppression (Figure 5.2B).

This result suggests that LPS-induced RGS10 suppression in BV2 cells requires HDAC activity. In parallel, we quantified TNFα and IL-1β transcript expression, and found that HDAC inhibition significantly blunted LPS-induced cytokine production in BV2 cells (Figure 5.2C & D). Together, these results show correlation between HDAC-mediated effects on RGS10 expression and pro-inflammatory cytokine expression, and suggest an increase in RGS10 expression caused by HDAC inhibition.

5.3.3. LPS Effects on Histone Acetylation and HDAC1 Recruitment at the RGS10 Promoter in Microglia

To further investigate whether the RGS10 gene is epigenetically silenced in LPS-activated microglial cells via histone deacetylation, our collaborators, Dr. Suzanna Greer and Ercan Cacan performed chromatin immunoprecipitation (ChIP) assays to quantify the amount of acetylated histones and HDAC1 enzyme bound to the RGS10 promoter before and after LPS treatment (100 ng/ml, 24 hours) in BV2 cells. Results reveal that LPS treatment significantly decreased total histone H3 acetylation at the RGS10 promoter following LPS treatment, whereas total histone H3 levels remained unchanged, showing that the decrease in H3 acetylation is due to a decreased acetylation reaction rather than mere decrease in total histone H3 proteins. HDAC1 ChIP assay demonstrated that the decreased acetylation was accompanied by an increase in the level of HDAC1 bound at the RGS10 promoter in LPS-treated cells. These results suggest that the RGS10 gene is epigenetically silenced in microglia following LPS stimulation via HDAC1 binding and decreased histone H3 acetylation at the RGS10 promoter.

5.3.4. Role of Gαi-Protein Signaling in LPS-Mediated Effects in BV2 Cells

Given that RGS10 has been shown to blunt LPS-induced cytokine production in microglia (Lee et al., 2008), and that RGS10 is known to selectively deactivate Gαi-family G-proteins (Hunt et al., 1996), we investigated whether LPS-induced cytokine production in BV2 cells is regulated by Gαi-proteins. If LPS effects in microglia are regulated by Gαi-proteins, this would suggest that the anti-inflammatory effects of RGS10 could be due to its negative regulation of Gαi-proteins. BV2 cells with pertussis toxin, a Gαi-protein inhibitor, and then cells were treated in media containing full serum (10% FBS) with vehicle, LPS, LPS plus pertussis toxin, or pertussis toxin alone. Expression of the pro-inflammatory cytokines TNFα and IL-1β transcript was analyzed using qRT-PCR. Inhibition of Gαi-protein signaling in BV2 cells significantly blunted LPS-induced pro-inflammatory cytokine production (Figure 5.3). These data suggest that LPS-mediated effects in BV2 cells are partially Gαi-protein-dependent, and that a ligand present in serum-containing media or generated by BV2 cells for autocrine signaling, which activates Gαi-protein, may facilitate LPS-induced cytokine production. Thus, an endogenous Gαi-coupled GPCR pathway could be a target for regulation by RGS10 protein.

5.3.5. RGS10 Knockdown Regulates GSK-3β Downstream of Serum and LPS

Next, we sought to define RGS10 effects on specific inflammatory signaling pathways. Glycogen synthase kinase-3 beta (GSK-3β) is a known regulator of inflammatory cytokine expression in microglia and is regulated by GPCRs and LPS; therefore, we predicted that GSK-3β may be regulated by RGS10. To address regulation of GSK-3β by RGS10 following treatment with serum or LPS, BV2 cells were starved in

serum-free media (SFM), and then dosed with 1% serum, 10% serum, or LPS in the presence of siRNA RGS10 (R10) or negative control siRNA (NC). LPS has been shown to enhance or suppress GSK-3β phosphorylation in various systems. In serum-starved BV2 cells, both serum and LPS strongly enhanced GSK-3β phosphorylation (see discussion). Knocking down RGS10 protein expression resulted in a slight decrease in basal GSK-3β phosphorylation, and a significant decrease in serum-, and LPS-activated GSK-3β phosphorylation (Figure 5.4). This suggests that endogenous RGS10 enhances phosphorylation and deactivation of GSK-3β downstream of multiple stimuli.

5.3.6. Effects of RGS10 Knockdown on AKT, ERK and P38 Activation

AKT has been shown to directly phosphorylate GSK-3β in many systems (Cross et al., 1995). Thus, we predicted AKT may be activated by upstream of GSK-3β phosphorylation by serum and LPS, and inhibited by RGS10 siRNA to account for the observed regulation of GSK-3β. To test this prediction, we blotted for phospho-AKT (Figure 5.5) following treatment with serum or LPS in the presence or absence of RGS10 knockdown. LPS strongly enhanced AKT phosphorylation; however, the effect of serum and RGS10 knockdown on AKT phosphorylation did not correlate with the effects seen with GSK-3β phosphorylation. Specifically, serum caused significant inhibition of AKT phosphorylation, and RGS10 knockdown lowered basal AKT phosphorylation but did not have an effect on serum- or LPS-stimulated AKT activity (Figure 5.5). Thus, regulation of AKT does not account for observed changes in GSK-3β phosphorylation downstream of serum and RGS10 siRNA. Similarly, we measured ERK1/2 MAPK and P38 MAPK phosphorylation under these conditions. RGS10 knockdown did not regulate phosphorylation of either kinase.

5.3.7. GSK-3β Phosphorylation Is Regulated by PKA and Gαi-Proteins

In addition to AKT, the kinase PKA has also been shown to directly phosphorylate GSK-3β (Fang et al., 2000). In order to determine if PKA is similarly regulated by serum, LPS, and RGS10 siRNA, we blotted for PKA substrate phosphorylation following serum or LPS treatment in the presence or absence of RGS10 siRNA. Serum and LPS stimulated PKA activation, and RGS10 knockdown significantly decreased PKA activation under all tested conditions (Figure 5.6A). The pattern of PKA activation correlates with the effects seen with GSK-3β phosphorylation, with more robust effects observed following RGS10 siRNA. These results are consistent with PKA-mediated phosphorylation of GSK-3β and suggest that endogenous RGS10 enhances PKA activity and upstream of GSK-3β phosphorylation, to confirm that PKA is involved in GSK-3β phosphorylation. We tested if H89, a pharmacological inhibitor of PKA would reduce LPS-induced GSK-3β phosphorylation. Indeed, pretreating BV2 cells with H89 significantly decreased GSK-3β phosphorylation following LPS treatment (Figure 5.6B).

We further predicted that inhibition of Gαi-proteins, which block adenylyl cyclase activity and downstream cAMP-PKA activity, would enhance GSK-3β phosphorylation. Consistent with our prediction, Gαi-protein inhibition via pertussis toxin enhanced LPS-mediated GSK-3β phosphorylation (Figure 5.6C). These data suggest that LPS regulates GSK-3β phosphorylation in BV2 cells through a PKA-dependent signaling pathway.

5.3.8. Effect of RGS10 on GSK-3β Phosphorylation Is Regulated by PKA and Gαi-Proteins

The direct target for RGS10 activity is Gαi, which inactivates adenylyl cyclase and cAMP production and subsequent PKA activity. This suggests that reduction of RGS10 expression indirectly blocks PKA by enhancing Gαi activity. Given that PKA is upstream of GSK-3β phosphorylation, we hypothesized that the ability of RGS10 to regulate GSK-3β may be mediated by its effect on Gαi-proteins. Specifically, we predicted that knockdown of RGS10 may result in enhanced Gαi activity, resulting in reduced PKA activity and reduced GSK-3β phosphorylation. To test this prediction, we measured GSK-3β phosphorylation following RGS10 knockdown in the presence of PKA and Gαi-protein inhibitors. Our results show that the PKA inhibitor H89 enhanced the RGS10 knockdown-mediated decrease in GSK-3β phosphorylation following LPS treatment (Figure 5.7A). Similarly, we predicted that inhibition of Gαi-proteins would reverse the effect of RGS10 knockdown on GSK-3β phosphorylation after LPS treatment. Indeed, we show that pertussis toxin partially blocked RGS10 siRNA-mediated inhibition of GSK-3β phosphorylation following LPS treatment (Figure 5.7B). This suggests that the anti-inflammatory effect exerted by RGS10 in BV2 cells may be mediated in part through the negative regulation of Gαi-proteins, which results in PKA activation and the subsequent deactivation of GSK-3β by phosphorylation.

5.3.9 S1P Signaling Pathways as Potential Targets of RGS10 Anti-Inflammatory Effects in BV2 Cells

Next, the effect of RGS10 on specific GPCR coupled pathways in microglia was investigated. S1P is an established regulator of inflammatory signaling in microglia, and

couples to five distinct receptors with broad G-protein coupling. First, we tested whether S1P induced pro-inflammatory cytokine expression in our model. We show that S1P enhanced pro-inflammatory TNFα and IL-1β transcript expression in BV2 cells, but with a significantly lower level and slower time course from LPS, suggesting S1P inflammatory signaling serves a modulatory role (Figure 5.8 A&B).

Next, the effects of S1P on specific signaling cascades were explored. Given that S1P increased pro-inflammatory cytokine production in microglia, we predicted that S1P would oppose PKA-mediated GSK-3β phosphorylation. However, we observed that S1P significantly increased GSK-3β phosphorylation, as observed with total serum, and RGS10 knockdown blunted the effect of S1P on GSK-3β phosphorylation, suggesting that endogenous RGS10 facilitates, rather than opposes S1P effects on GSK-3β. This suggests that S1P effects are not mediated by Gαi but by a Gαs-like PKA activating pathway (Figure 5.8C). To test this, S1P effects on PKA substrate phosphorylation were measured, and found that S1P enhanced PKA activation, and RGS10 knockdown decreased S1P-mediated PKA activation (Figure 5.8D). In contrast, S1P significantly decreased AKT phosphorylation, and RGS10 knockdown further suppressed AKT phosphorylation (Figure 5.8E). Taken together, these results suggest that S1P activates PKA and GSK-3β phosphorylation, and that this effect is enhanced by the activity of endogenous RGS10. Further, S1P, like serum, has opposing effects on AKT and GSK-3β, while the activity of PKA correlates with GSK-3β phosphorylation. This suggests that PKA, but not AKT, is upstream of GSK-3β phosphorylation following activation by S1P in microglia. To test this hypothesis, we determined if the PKA inhibitor, H89 could reduce GSK-3β phosphorylation following S1P treatment in BV2 cells. Indeed,

pretreating BV2 cells with H89 significantly reduced S1P-mediated GSK-3β phosphorylation (Figure 5.9). These results suggest that S1P regulates GSK-3β phosphorylation in BV2 cells through a PKA signaling pathway. This also suggests that the anti-inflammatory effects of RGS10 in BV2 cells could be due to negative regulation of Gαi-proteins, and the subsequent activation of PKA and GSK-3β phosphorylation.

5.4. Discussion

Microglia, the brain's resident macrophages, have been implicated in the initiation and/or progression of multiple neuroinflammatory and neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease (Schwartz et al., 2013b), and regulation of microglia-mediated neuroinflammation could be beneficial in the prophylaxis and/or treatment of such diseases. RGS10 protein has emerged in recent years as an important regulator of pro-inflammatory cytokine production in microglia, hence functioning as an important neuroprotective factor. LPS-activated microglia show suppressed levels of RGS10 proteins, and restoring RGS10 levels has been shown to reduce pro-inflammatory cytokine production in microglia (Lee et al., 2008, Lee et al., 2011). In this chapter, the mechanisms by which RGS10 is suppressed in activated microglia, as well as the mechanisms by which RGS10 regulates signaling pathways in microglia were investigated.

Lee et. al reported that a low dose of LPS (10 ng/ml, 24h) decreased RGS10 protein in BV2 cells. On the other hand, a higher dose of LPS (1 μg/ml, 24h) did not change RGS10 protein level (Lee et al., 2008). Here we report that RGS10 transcript and protein levels were suppressed in BV2 cells in a dose-dependent fashion (Figure 5.1A&B), with maximal suppression observed at 1 μg/ml. We also report that RGS10

transcript expression was suppressed as early as 4 hours and remained suppressed up to 72 hours following LPS treatment (Figure 5.1C). The experiments described in this chapter were performed using the murine cell line, BV2 cells, a well-established model that is used to study microglial functions (Henn et al., 2009). The BV2 cell line was generated by infecting primary microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus (J2) (Blasi et al., 1990). Future experiments will test the findings from this study in another microglial cell line, the mouse microglial N9 cell line, and in primary microglia.

We have previously reported that the RGS10 gene is epigenetically silenced through increased DNA methylation and decreased histone acetylation of its promoter in chemoresistant ovarian cancer cells (Ali et al., 2013). We predicted that the suppression of RGS10 expression in BV2 cells could be due to a similar epigenetic silencing mechanism. To test this possibility, we determined the effects of the pharmacological inhibitor of histone deacetylases (HDACs), trichostatin A (TSA) on RGS10 transcript expression. Here we show that TSA significantly enhances RGS10 transcript expression and suggests that RGS10 expression in BV2 cells could be regulated via histone acetylation. We predicted that LPS-mediated RGS10 suppression in BV2 cells could be mediated through decreased histone acetylation. Supporting this prediction, we observed that TSA pretreatment blocked LPS-induced RGS10 suppression in microglia (Figure 5.2B). This result suggests that LPS-induced RGS10 gene silencing is mediated by histone deacetylation in microglia. Consistent with this possibility, LPS has been reported to stimulate an increase in HDAC expression (Kannan et al., 2013). Further, HDAC inhibitors have been reported to possess anti-inflammatory effects by lowering pro-

inflammatory cytokines production in microglia (Suh et al., 2010, Kannan et al., 2013). For instance, HDAC inhibitors, SAHA and ITF2357, reduced the production of pro-inflammatory cytokines production in activated microglia (Faraco et al., 2009). Another HDAC inhibitor, trichostatin A (TSA), reduced pro-inflammatory cytokine production in microglia, and reduced brain injury in a model of LPS-sensitized neonatal hypoxic-ischemia (Fleiss et al., 2012). Here, we report that HDAC inhibition caused a decrease in LPS-mediated pro-inflammatory cytokine (TNFα and IL-1β) production in microglia. In order to further investigate the possibility that RGS10 could be epigenetically silenced by decreased histone acetylation and HDAC1 binding, ChIP assays quantifying acetylated H3 histones and HDAC1 at the RGS10 promoter were carried out. These results reveal that LPS increases HDAC1 recruitment and decreases histone H3 acetylation at the RGS10 promoter, which further strengthen the notion that RGS10 gene is epigenetically silenced via histone deacetylation in LPS-activated microglia. Different epigenetic mechanisms have been reported to co-operatively regulate gene expression in many cells (Yang et al., 2001, Ghoshal et al., 2002), including microglia (Garden, 2013). Future studies will explore whether other epigenetic mechanisms, such as DNA methylation, are involved in the regulation of RGS10 gene expression in microglia.

In order to respond to a wide array of external stimuli, microglia express multiple receptor types, including GPCRs, and activation of these receptors by different ligands in the microglial microenvironment results in tight regulation of inflammatory cytokines such TNFα and IL-1β. Activation of microglial Gαi-coupled GPCRs, such as the purinergic receptor P2Y12 has been implicated in microglia-mediated inflammatory responses (Webster et al., 2013). Additionally, activation of toll-like receptors (TLRs) by

LPS has been shown to initiate cross talk with the Gαi-coupled P2Y1 purinergic receptors in rat microglial cells (Seo et al., 2008). Gαi-protein signaling has been shown to be implicated in inflammatory cytokine release in microglia (Yin et al., 2010). To investigate if LPS-induced pro-inflammatory cytokine production in our cell model is regulated through a Gαi-protein-dependent signaling pathway, we determined the effect of Gαi-protein inhibition in BV2 cells using pertussis toxin on pro-inflammatory cytokine production following LPS stimulation. We show that inhibition of Gαi-proteins significantly reduced TNFα and IL-1β transcript expression (Figure 5.3). These data suggest that LPS-mediated effects in BV2 cells are enhanced by a Gαi-protein-dependent pathway, that a ligand present in serum-containing media or generated by BV2 cells for autocrine signaling, which activates Gαi-protein, may facilitate LPS-induced cytokine production.

Glycogen synthase kinase-3 beta (GSK-3β) is a known regulator of inflammatory cytokine expression in microglia and is regulated by GPCRs and LPS (Martin et al., 2005). LPS has been shown to increase GSK-3β expression and activity, and pro-inflammatory cytokine levels in rat glial-enriched cortical cultures. Inhibition of GSK-3β reduces LPS-induced pro-inflammatory cytokine production in multiple systems (Green and Nolan, 2012). However, LPS has also been shown to enhance GSK-3β phosphorylation under certain conditions (Martin et al., 2005, Lee et al., 2012, Ke et al., 2013, Koide et al., 2013, Wang et al., 2013, Wakayama et al., 2014). Indeed, we observed LPS induced GSK-3β phosphorylation in serum-starved BV2 cells. We predict that depletion of serum alters the signaling networks downstream of LPS by removing multiple GPCR inputs, revealing regulation of LPS signaling by G-protein pathways.

Current studies to evaluate the effect of serum depletion on signaling pathways in BV2 cells are underway. The ability of LPS to deactivate GSK-3β via phosphorylation may provide a negative feedback mechanism to control the duration of inflammation, and the strength of this feedback mechanism appears to be regulated by RGS10.

AKT has been shown to directly phosphorylate GSK-3β in many systems (Cross et al., 1995), and LPS treatment in microglial cells increases AKT activation (Moon et al., 2007, Jeong et al., 2010, Park et al., 2011, Jang et al., 2013, Lee et al., 2013, Jayasooriya et al., 2014). Therefore, we predicted that AKT was upstream of GSK-3β phosphorylation; however, we observed inhibition of AKT following serum or S1P treatment conditions that enhanced GSK-3β phosphorylation. While these results show that AKT and GSK-3β are not regulated by a common pathway in microglia, the observed inhibition of AKT is consistent with previous reports that S1P2 receptors has been shown to activate phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which would mediate AKT dephosphorylation (Sanchez et al., 2005). Taken together, these results suggest that AKT activation does not account for GSK-3β phosphorylation in BV2 cells following serum or LPS activation, or RGS10 knockdown.

Similarly, LPS is known to stimulate ERK and P38 MAPK activation in microglia (Moon et al., 2007, Jeong et al., 2010, Jeong et al., 2014, Miyake et al., 2014). To test if LPS-induced ERK or P38 MAPK activation accounts for effects seen on GSK-3β phosphorylation, we blotted for phospho-ERK or phospho-P38 following serum and LPS treatments in the presence or absence of RGS10 siRNA. We did not see any significant effect of RGS knockdown on these kinases. This suggests that ERK and P38 MAPKs are

not targets for RGS10 effects in microglia and do not account for the effect on GSK-3β phosphorylation.

Further, Protein kinase A (PKA) also has been reported to directly phosphorylate GSK-3β (Fang et al., 2000). We found that the regulation of PKA activity in BV2 cells closely mirrored that of GSK-3β phosphorylation: serum- and LPS-induced PKA activation and RGS10 knockdown significantly decreased serum- and LPS-induced PKA activation (Figure 5.6A). These results suggest that regulation of PKA activity could account for GSK-3β regulation by RGS10 in our cell model. This finding is in line with reports that PKA mediates anti-inflammatory effects in microglia (Liu et al., 2011b, Park et al., 2014). Further, the PKA inhibitor, H89 significantly blunted GSK-3β phosphorylation stimulated by LPS. This suggests that GSK-3β phosphorylation is mediated by PKA under these conditions.

PKA activity is negatively regulated by Gαi-proteins, which inhibit adenylyl cyclase, hence reducing intracellular levels of cyclic adenosine mono-phosphate (cAMP), the second messenger that activates PKA. To test if GSK-3β phosphorylation is regulated by a Gαi-protein-dependent signaling pathway, we pretreated BV2 cells with pertussis toxin, an established inhibitor of Gαi-proteins, then blotted for phospho-GSK-3β following LPS or serum treatments. Gαi-protein inhibition significantly enhanced GSK-3β phosphorylation following stimulation (Figure 5.6C), suggesting that endogenous/autocrine Gαi-coupled signaling opposes the phosphorylation of GSK-3β downstream of LPS.

Further, we sought to determine if Gαi-proteins and PKA were involved in regulation of GSK-3β following RGS10 knockdown. Specifically, we predicted that

RGS10 knockdown relieves inhibition of Gαi, thus decreasing PKA activity. Thus, we hypothesized that PKA inhibition would enhance the effect of RGS10 knockdown on GSK-3β phosphorylation, whereas Gαi-protein inhibition would reverse that effect. We observed that Gαi-protein inhibition blunted the ability of RGS10 knockdown to block LPS-stimulated GSK-3β phosphorylation. Taken together, our results suggest that RGS10 effects in BV2 cells are partially due to its ability to negatively regulate Gαi-proteins, which results in increased PKA activation. This in turn deactivates GSK-3β by increased phosphorylation, which leads to reduced pro-inflammatory cytokine production, presumably through decreased NF-κB activity (Wang et al., 2010). Ongoing studies will determine whether NF-κB is involved in the signaling cascade downstream of GSK-3β and RGS10. However; two critical questions remain: first, what is the endogenous driver of Gαi activity in microglia. Currently in our laboratory, multiple Gαi-coupled agonists are being tested for potential counter-regulation of GSK-3β and inflammatory signaling. Second, why is the effect of RGS10 knockdown only partially rescued by Gαi-protein inhibition? If RGS10 effects were entirely mediated by inhibiting Gαi, then the effect of RGS10 knockdown should be fully rescued by RGS10 a Gαi-inhibitor. We are currently exploring Gαi-independent mechanisms of RGS10 activity that may complement Gαi-inactivation to regulate signaling.

S1P is known to activate Gαi-coupled receptors (Rosen, 2005), and S1P has become an emerging key regulator of microglial activity (Tham et al., 2003, Nayak et al., 2010). Targeting S1P receptors with drugs such as the S1P1 analogue fingolimod (FTY720-Gilenya®) has proven beneficial in the neurodegenerative disorder, multiple sclerosis, which is characterized by excessive microglial activation (Cohen et al., 2010,

Kappos et al., 2010, Groves et al., 2013). We have shown that S1P mimics the effects of serum on GSK-3β and PKA, but surprisingly this effect is not Gαi-mediated, but rather is consistent with a Gαs effect. S1P has been shown to have Gαs-like increase in cAMP activity in other systems, but the receptor responsible has not been defined (Lagadari et al., 2009, Shen et al., 2013). We will address this in future experiments. Further, while S1P does indeed cause enhanced cytokine transcription, it does so at lower levels and much more slowly than LPS. Indeed, we predict that S1P effects shown here are likely part of a regulatory mechanism that limits cytokine production.

5.5. Summary and Conclusions

Results discussed in this chapter describe the cause and consequences of suppressed RGS10 expression in microglia. We show that the RGS10 gene is epigenetically silenced via decreased histone H3 acetylation and increased HDAC1 recruitment in LPS-activated microglia. Identifying novels ways of restoring RGS10 protein in activated microglia could help protect and/or treat neuroinflammation, a characteristic feature of several neurodegenerative diseases such as multiple sclerosis. Our results suggest that indirect activation of PKA and the subsequent deactivation of GSK-3β may be the target for RGS10 anti-inflammatory effects in microglia (Figure 5.10). Understanding the mechanism by which RGS10 reduces pro-inflammatory cytokine production in microglia could help design novel therapeutics for neuroinflammatory and neurodegenerative diseases that are associated with excessive microglial activation.

5.6. Figures

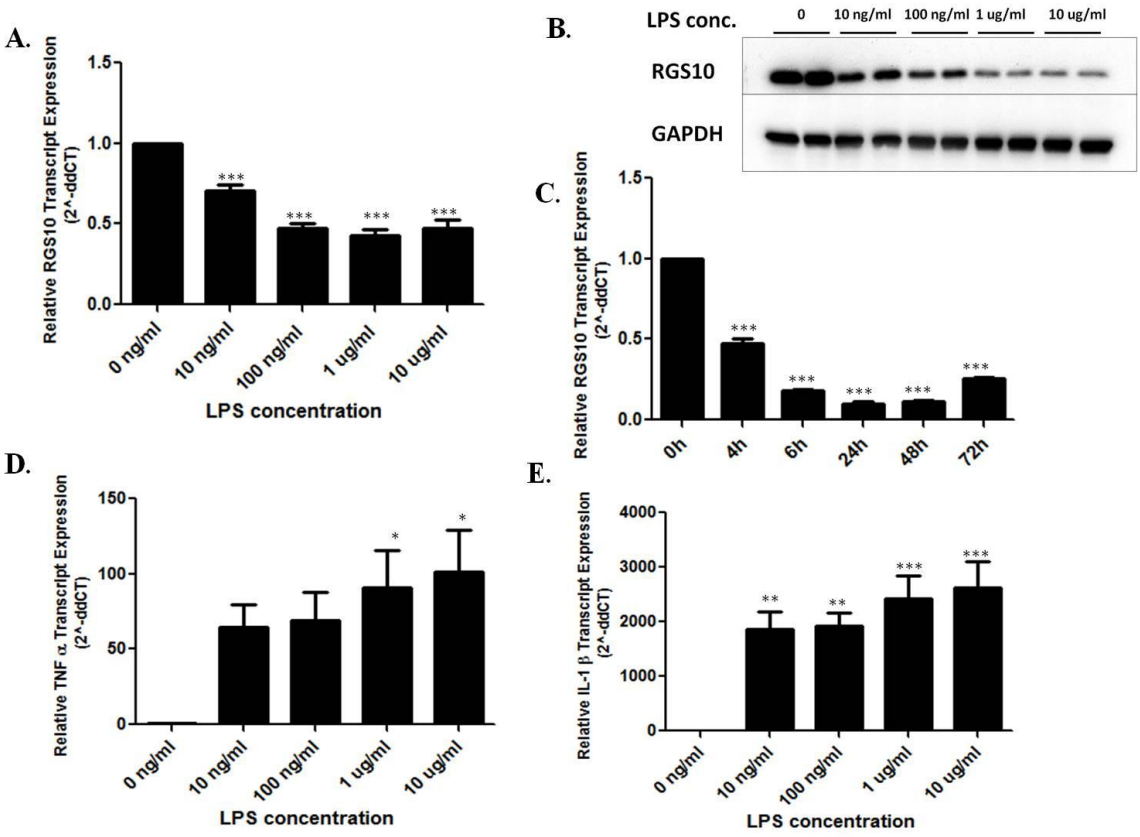

Figure 5.1: Effects of LPS Treatment in Microglial BV2 Cells. RGS10 transcript (measured at 4h) (A) and protein (measured at 24h) (B) were down-regulated following LPS treatment in a dose-dependent fashion. (C): Time course of RGS10 transcript expression in BV2 cells following LPS treatment (100 ng/ml). (D) & (E): LPS dose-dependently enhanced pro-inflammatory cytokine (TNFα and IL-1β) production in BV2 cells. BV2 cells were treated with vehicle, 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml LPS for 4 hours. Cells were harvested in Trizol and RNA was isolated. RGS10, TNFα, and IL-1β transcripts were quantified and normalized to the housekeeping gene actin. *p<0.05, **p<0.01, ***p< 0.001

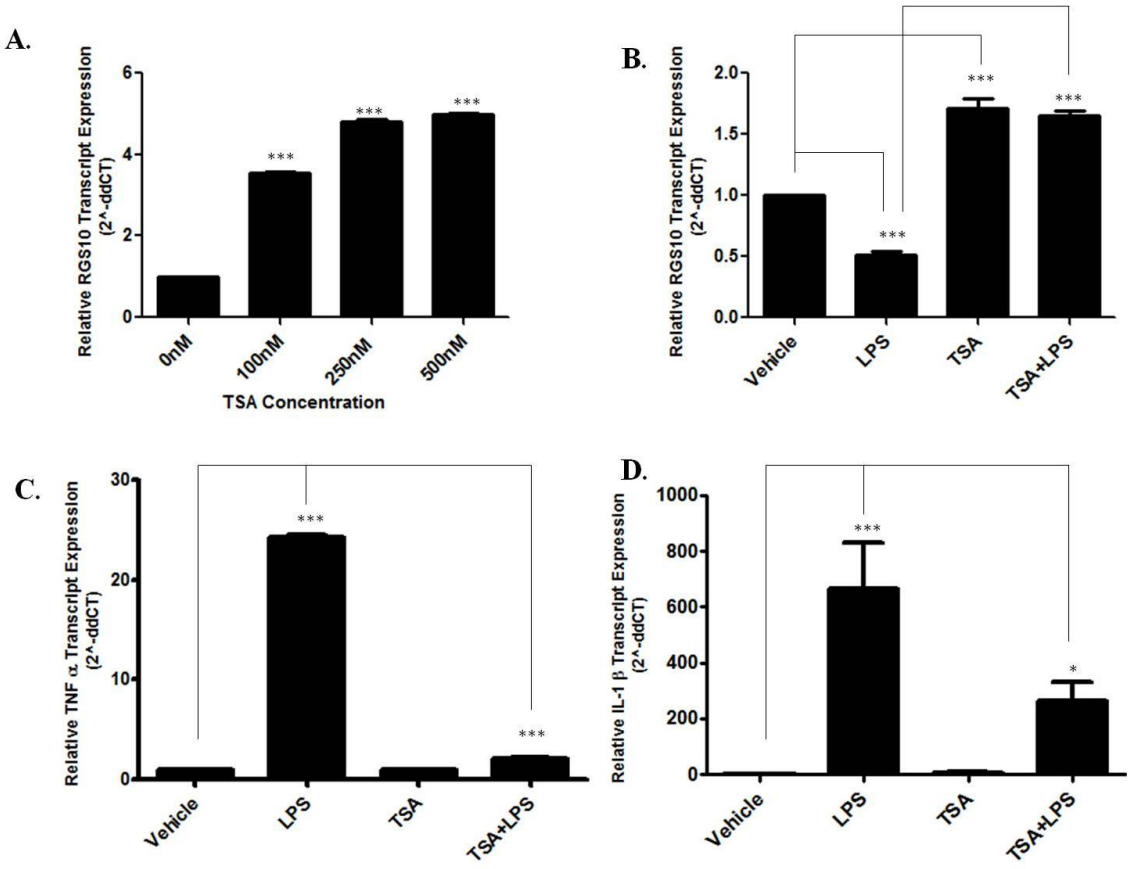

Figure 5.2: Effects of HDAC Inhibition on RGS10 Expression. (A): HDAC pharmacological inhibition enhanced RGS10 transcript expression. BV2 Cells were treated with vehicle, 100, 250, and 500nM TSA for 24 hours. RGS10 transcript was normalized to actin. (B): TSA blocked LPS-induced RGS10 transcript suppression in BV2 cells. BV2 cells were treated with vehicle (24 hrs), vehicle pretreatment (20h) followed by LPS (1μg/ml, 4h), TSA (250 nM, 24h), and TSA pretreatment (250 nM, 20h) followed by TSA plus LPS (1μg/ml, last 4h). (C) & (D): Production of TNFα and IL-1β transcripts correlates with HDAC inhibition. *$p<0.05$, ***$p< 0.001$

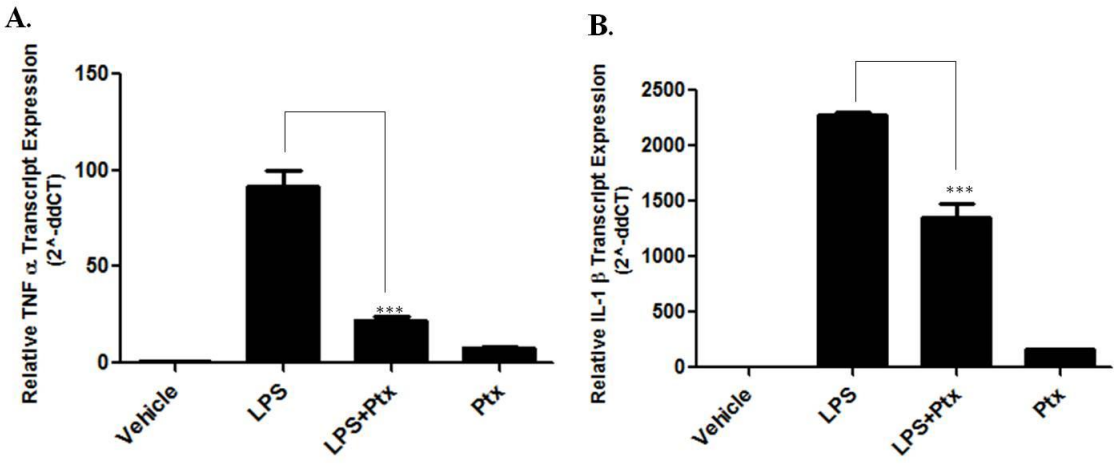

Figure 5.3: Role of Gα*i*-Proteins in LPS-Induced Cytokine Production in BV2 Cells. BV2 cells were treated with vehicle (media containing 10% serum), vehicle pretreatment (12h) + LPS (1 μg/ml, 6h), pertussis toxin pretreatment (Ptx 100 ng/ml, 12h) + LPS (1 μg/ml, last 6h), or Ptx (100 ng/ml 18h). Cells were harvested in Trizol and RNA was isolated. TNFα and IL-1β transcripts were quantified and normalized to the housekeeping gene actin (***$p < 0.001$). Pharmacological inhibition of Gαi-protein in BV2 cells significantly blunted LPS-induced pro-inflammatory cytokine TNFα (A) and IL-1β (B) transcript expression.

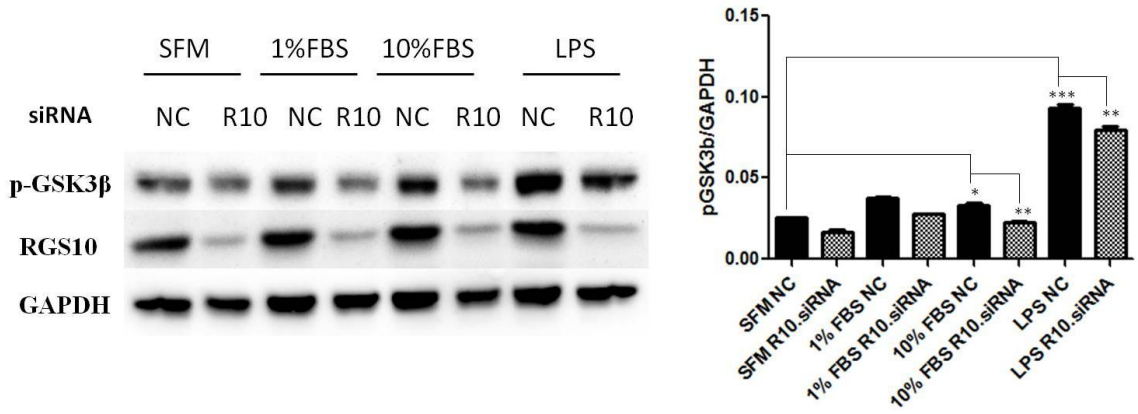

Figure 5.4: Effects of RGS10 Knockdown on GSK-3β in BV2 Cells. BV2 cells were transfected with RGS10 siRNA (R10) or negative control siRNA (NC), then starved for 12 hours in serum-free media, then dosed with 1% FBS, 10% FBS, or 1 μg/ml LPS for 50 minutes. Densitometry graphs represent data from two independent experiments: *p<0.05, **p<0.01, ***p< 0.001. 10% serum and LPS induces GSK-3β phosphorylation, and RGS10 knockdown significantly blunts GSK-3β phosphorylation.

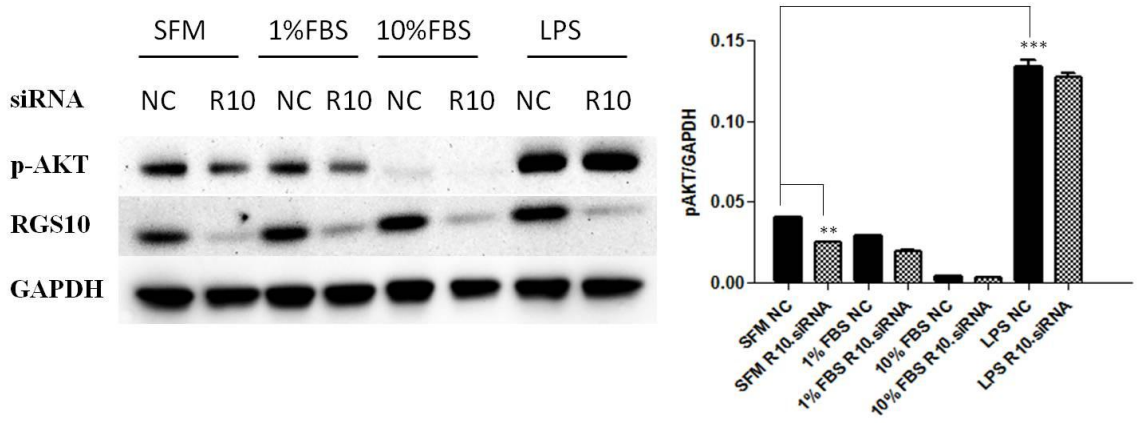

Figure 5.5: Effects of RGS10 Knockdown on AKT Phosphorylation in BV2 Cells. BV2 cells were transfected with RGS10 siRNA (R10) or negative control siRNA (NC), then starved for 12 hours in serum-free media, then dosed with 1% FBS, 10% FBS, 1 μg/ml LPS. Densitometry graphs represent data from two independent experiments: **p<0.01, ***p< 0.001. Serum significantly decreased AKT phosphorylation, and RGS10 knockdown lowered basal AKT phosphorylation. RGS10 knockdown did not have an effect on serum-mediated AKT inhibition. LPS stimulated AKT phosphorylation, and RGS10 knockdown did not have an effect on LPS-stimulated AKT activity.

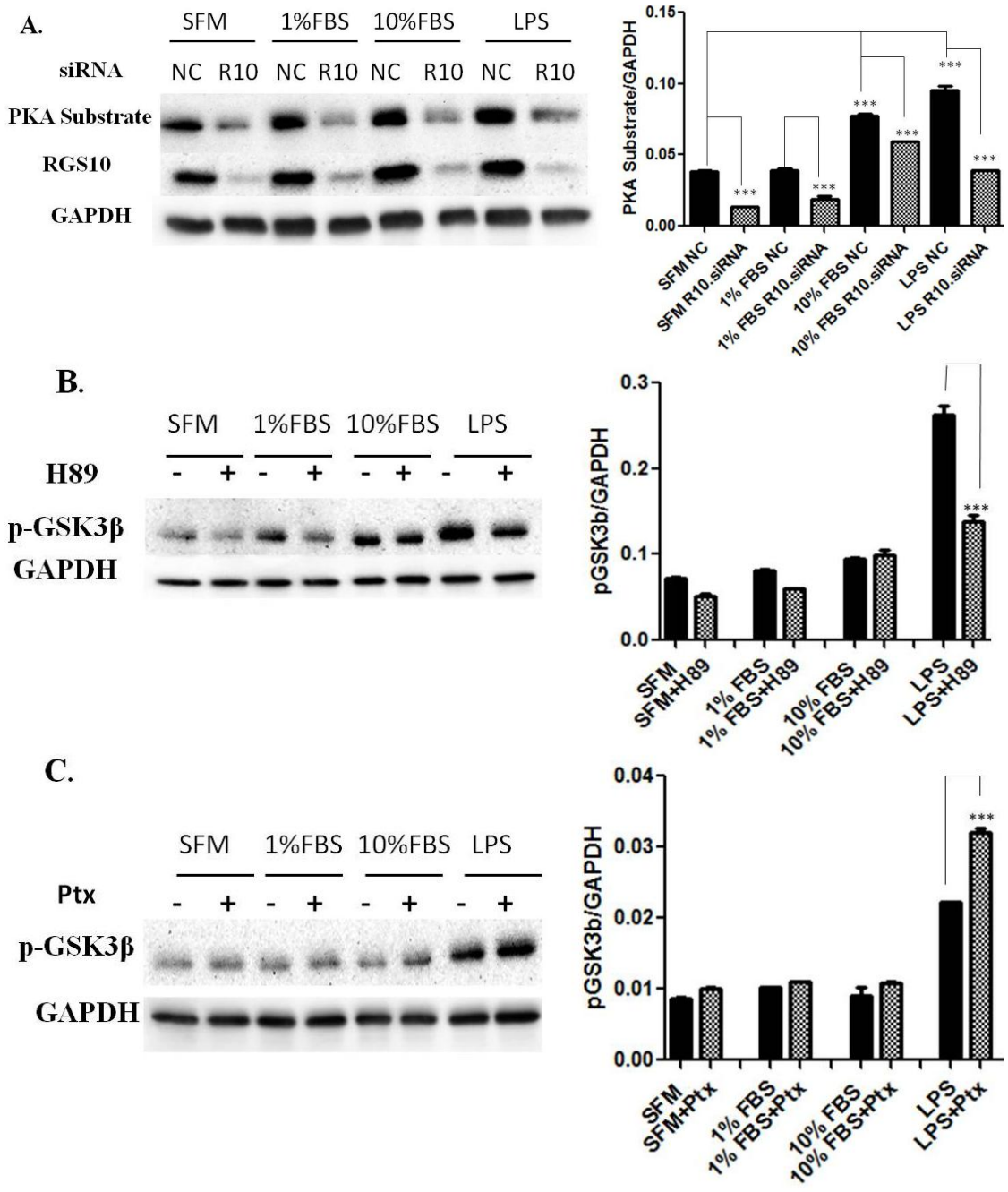

Figure 5.6: GSK-3β Phosphorylation Is Regulated by Gαi-Proteins and PKA. BV2 cells were starved for 12 hours in serum-free media, then dosed with 1% FBS, 10% FBS, 1 μg/ml LPS, or 10 μM S1P for 30 minutes. Densitometry graphs represent data from two independent experiments: ***p< 0.001. (A): Serum and LPS induced PKA activation,

whereas RGS10 siRNA lowered basal, serum-, and LPS-mediated PKA activation. BV2 cells were starved 12 hours in serum-free media (SFM), then cells were dosed with SFM, 1%, 10% fetal bovine serum (FBS), or LPS (1 ug/ml) for 50 minutes. H89 (10 uM) or Ptx (100 ng/ml) were added 1 hour or 12 hours before drug treatments, respectively. Densitometry graphs represent data from two independent experiments: ***$p < 0.001$ (B): The Pharmacological inhibitor of PKA, H89 significantly decreased LPA-induced GSK-3β phosphorylation. (C): Gαi-protein inhibition, via pertussis toxin (Ptx) significantly enhanced LPA-induced GSK-3β phosphorylation.

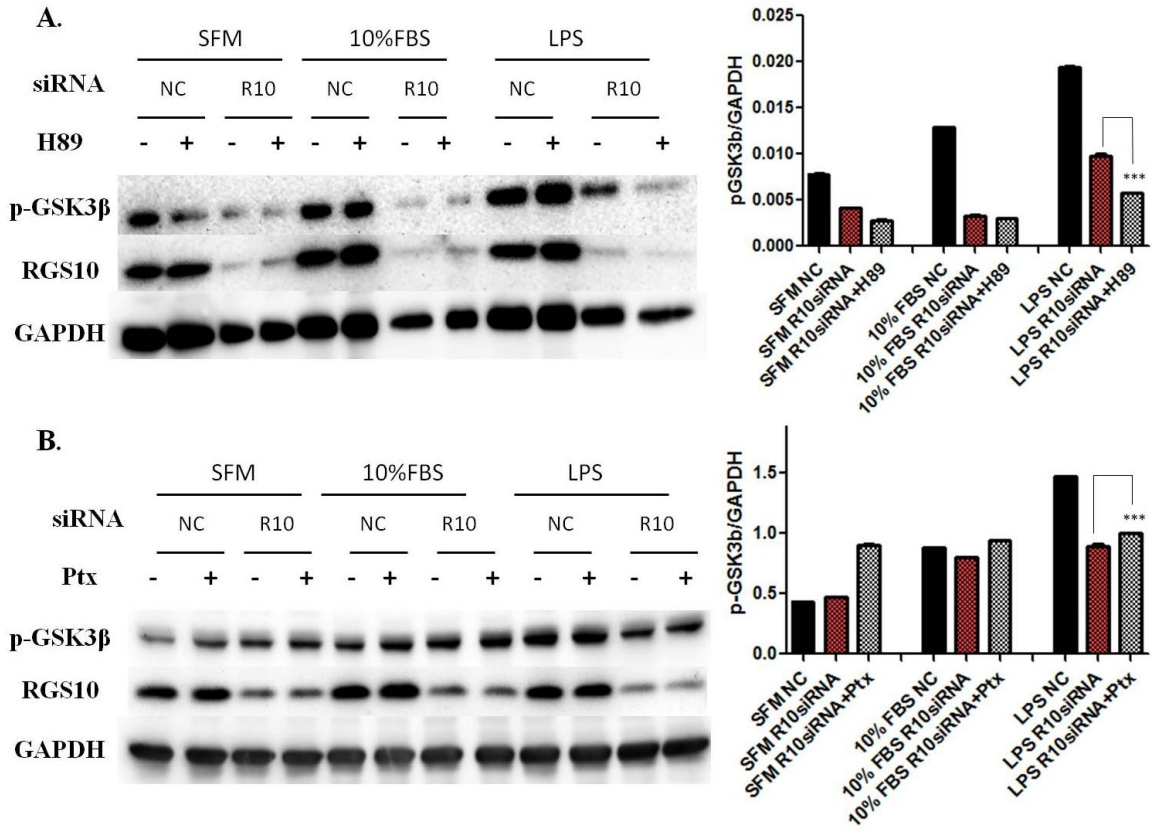

Figure 5.7: Effect of RGS10 on GSK-3β Phosphorylation Is Regulated by PKA and Gα*i*-Proteins. BV2 cells were transfected with RGS10 siRNA or negative control (NC), then cells were then dosed with SFM, 10% FBS, or LPS (1 μg/ml) for 50 minutes. H89 (10 μM) or Ptx (100 ng/ml) were added 1 hour or 12 hours before drug treatments, respectively. Densitometry graphs represent data from two independent experiments: ***p< 0.001. (A): H89 augmented the effect of RGS10 knockdown on lowering GSK-3β phosphorylation following LPS treatment. (B): Ptx reversed RGS10 siRNA-mediated decrease in GSK-3β phosphorylation following LPS treatment.

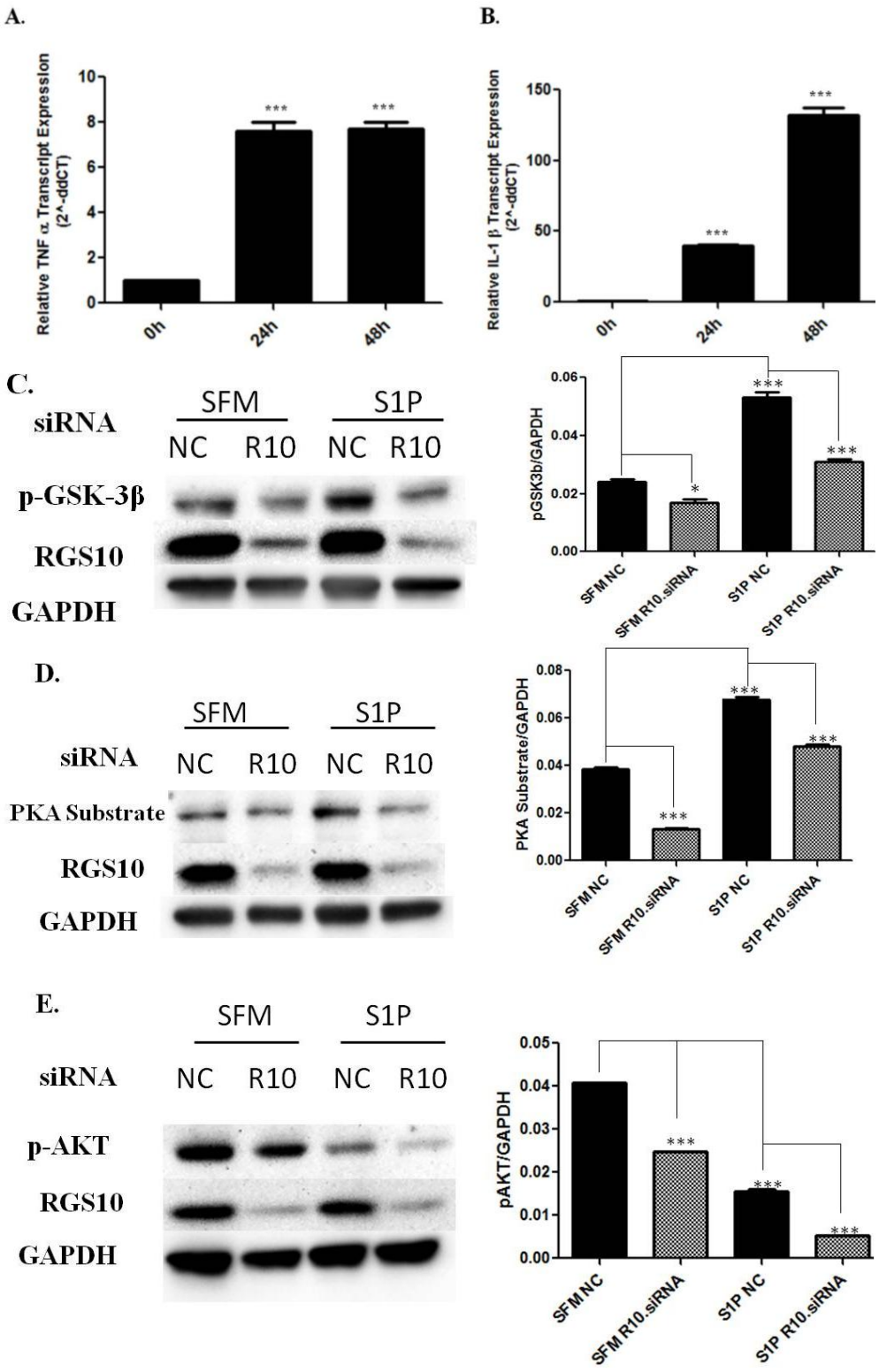

Figure 5.8: S1P as a Potential Target of RGS10 Anti-Inflammatory Effects in BV2 Cells. S1P increases TNFα (A) and IL-1β (B) cytokines production. BV2 cells were

treated with 10 μM S1P for 24 or 48 h. RNA was isolated in Trizol and TNFα and IL1β transcript expression was normalized to actin (***p< 0.001). (C): S1P significantly increased GSK-3β phosphorylation, and RGS10 knockdown blunted S1P-induced GSK-3β phosphorylation. (D): S1P induces PKA activation, whereas RGS10 siRNA S1P-mediated PKA activation. (E): S1P decreases AKT phoshorylation, and RGS10 knockdown further reduces AKT phoshorylation. BV2 cells were starved for 12 hours in serum-free media (SFM), and then dosed with SFM or 10 μM S1P for 15 minutes (p-AKT blots), 30 minutes (PKA substrate blots), or 50 minutes (p-GSK-3β blots). Densitometry graphs represent data from two independent experiments: *p<0.05, **p<0.01, ***p< 0.001

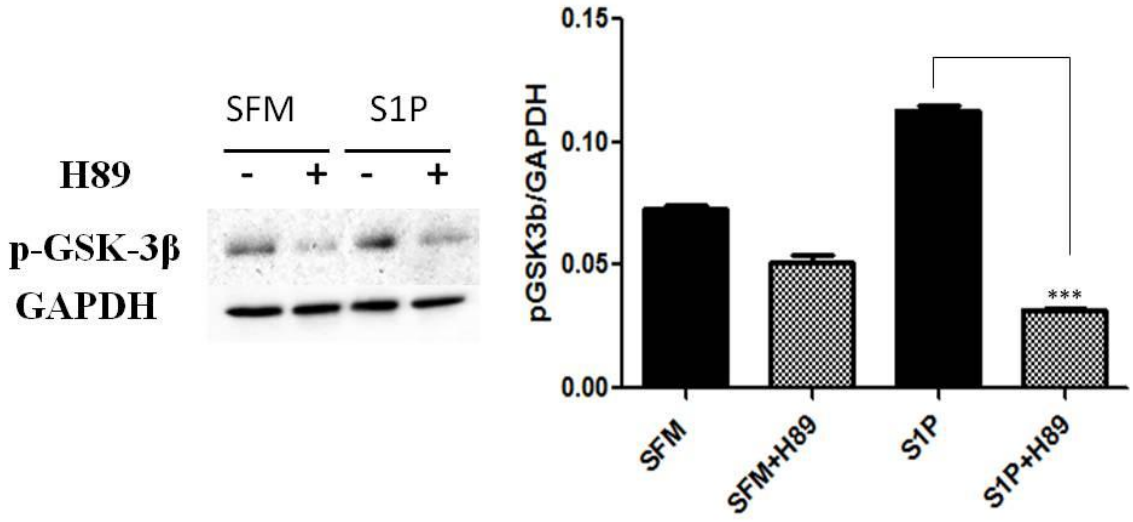

Figure 5.9: PKA Regulates S1P-Induced GSK-3β Phosphorylation. BV2 cells were starved 12 hours in serum-free media (SFM), then cells were dosed with SFM or S1P (10 uM) for 50 minutes. H89 (10 uM) was added 1 hour before drug treatments. Densitometry graphs represent data from two independent experiments: ***p< 0.001. Pharmacological inhibition of PKA via H89 significantly decreased S1P-induced GSK-3β phosphorylation.

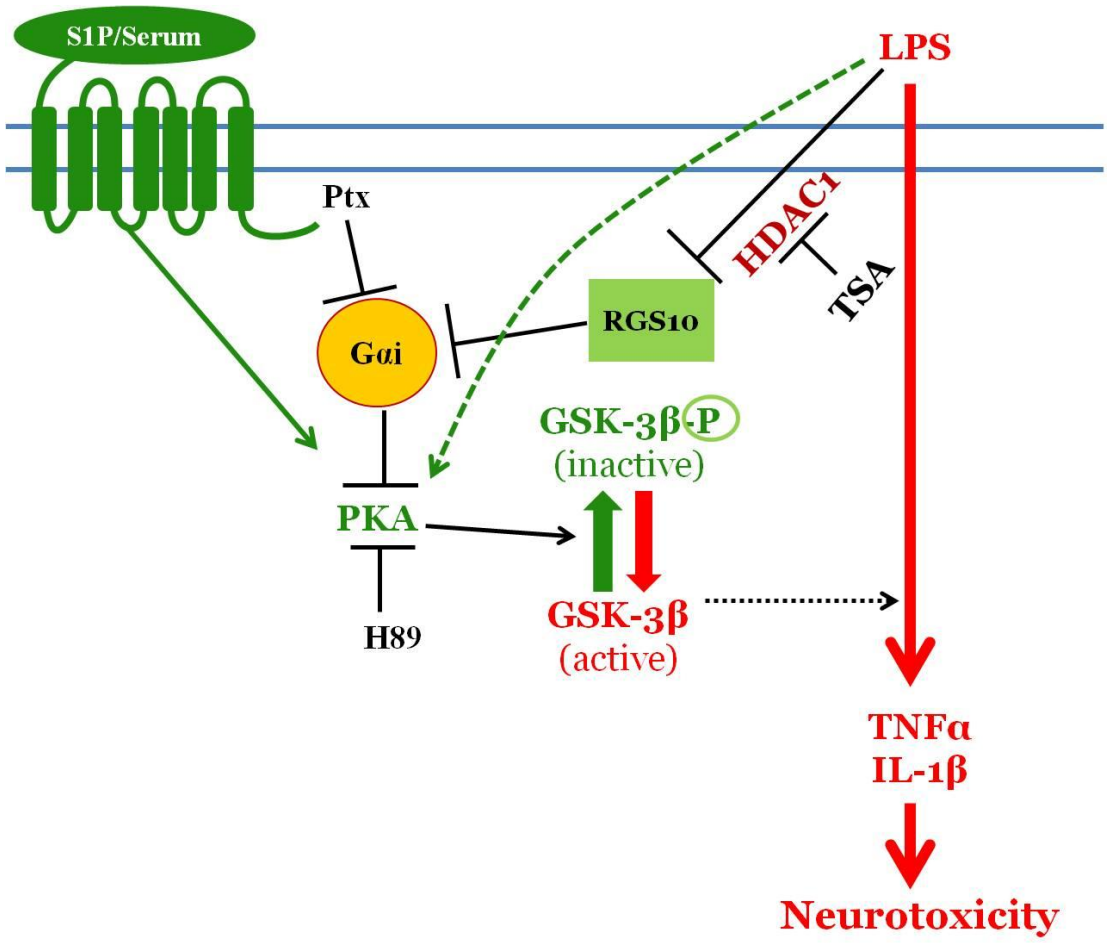

Figure 5.10: Model of Working Hypothesis for RGS10 Expression and Function in Activated Microglia. LPS induces RGS10 suppression via decreased histone acetylation and increased HDAC1 recruitment at RGS10 promoter. RGS10 anti-inflammatory effect is due to inhibition of Gαi-proteins, which indirectly results in PKA activation and GSK-3β inhibition. We predict that RGS10 activity enhances PKA activity to inactivate GSK-3β by inhibiting Gαi activity. This activity enhances PKA-driven signaling by GPCRs downstream of multiple receptors including S1P and LPS.

CHAPTER 6

SUMMARY

6.1. Summary and Conclusions

The goal of this research was to characterize the function and expression of regulator of G-protein signaling 10 (RGS10) in ovarian cancer cells and microglia. We have established RGS10 as a novel regulator of survival pathways in ovarian cancer cells. Lysophosphatidic acid (LPA) is the predominant growth factor in ovarian cancer (Mills et al., 1988, Mills et al., 1990, Moolenaar and van Corven, 1990), mediating cell proliferation (van Corven et al., 1989), migration (Huang et al., 2002), invasion, and survival (Frankel and Mills, 1996, Goetzl et al., 1999, Yu et al., 2008) via activation of LPA receptors. Our laboratory has previously demonstrated that RGS proteins negatively regulate LPA-mediated proliferation and migration in ovarian cancer cells (Hurst et al., 2008, Hurst and Hooks, 2009a). Here we show that RGS10, as well as RGS17, regulate LPA-mediated AKT survival pathways in SKOV3 ovarian cancer cells. Further, we demonstrate that RGS10 transcript expression is suppressed in models of acquired chemoresistance in ovarian cancer cells, and that RGS10 expression level regulates chemotherapeutic-mediated cell death in ovarian cancer cells (Hooks et al., 2010).

We have further identified that RGS10 is epigenetically silenced via increased DNA methylation and decreased histone acetylation of its promoter in chemoresistant ovarian cancer cells (Ali et al., 2013). This is the first report to the regulation of RGS expression by histone deacetylation. We demonstrate that expression of RGS10 is

suppressed in primary ovarian cancer and in CAOV-3 ovarian cancer cells compared to benign immortalized ovarian surface epithelial (IOSE) cells, and is suppressed in A2780-AD chemoresistant cells compared to parental A2780 cells. The RGS10-1 promoter is enriched in CpG dinucleotides, and inhibition of DNA methyl transferases increases RGS10 expression, suggesting that DNA methylation suppresses RGS10 expression. We performed bisulfite sequencing across the RGS10 promoter region, and identified a region with significantly enhanced DNA methylation in A2780-AD chemoresistant cells compared to parental cells. We further found significantly less acetylated histone H3 associated with RGS10-1 promoters in A2780-AD cells compared to parental cells, and a corresponding increase in histone deacetylase 1 (HDAC1) enzyme association with the RGS10-1 promoter in the resistant cells. Similarly, acetylated histone H3 levels were markedly lower in CAOV-3 ovarian cancer cells compared to IOSE cells, and HDAC1 binding was doubled in CAOV-3 cells.

We further investigated the molecular mechanisms that drive the suppression of RGS10 expression in the chemoresistant ovarian cancer cell line, A2780-AD cells, and determined the effect of altering HDAC1 and DNMT1 levels on RGS10 expression and on cisplatin-induced cell death in these cells. Over-expression of HDAC1 significantly reduced RGS10 expression. Pharmacological inhibition of HDAC1 and/or DNMT1 significantly increases RGS10 expression and increased cisplatin-induced cell death in chemoresistant A2780-AD ovarian cancer cells (Cacan et al., 2014). Our results suggest that loss of histone acetylation correlates with suppressed expression of RGS10 in ovarian cancer cells, while DNA methylation may contribute to further loss of RGS10 expression in chemoresistance.

Overall, these studies have characterized the function of RGS10 protein in ovarian cancer cells, identifying RGS10 as a novel regulator of AKT survival pathways in ovarian cancer cells. Results from this study show, for the first time, that RGS10 is epigenetically suppressed in chemoresistant ovarian cancer cells, and may define a population of ovarian cancer cells with unique response to therapeutics. Further, our results suggest that HDAC1 and DNMT1 contribute to RGS10 suppression during acquired chemoresistance and support the use of inhibitors of HDAC1 and DNMT1 as an adjuvant therapeutic approach to overcome ovarian cancer chemoresistance.

Recently, RGS10 protein has emerged as an important neuroprotective factor. Reports have shown that RGS10 normally suppresses cytokine production following microglial activation and that lipopolysaccharide (LPS)-induced microglial activation suppressed RGS10 expression. However, the mechanisms of RGS10 suppression in activated microglia have not been defined. In chapter 5, such mechanisms are explored. Our results suggest that RGS10 is epigenetically silenced through decreased histone acetylation of its promoter, and that RGS10 negatively regulates Gαi-proteins, hence regulating protein kinase A (PKA) activation, which could be involved in the production of pro-inflammatory cytokines in activated microglia. Ongoing studies will define how RGS10 regulation of glycogen synthase kinase-3 beta (GSK-3β) leads to regulation of nuclear factor-kappa B (NF-κB) signaling and regulation of cytokine expression. Further, we will define specific GPCRs upstream of RGS10 activity and define if Gαi regulation fully accounts for RGS10 effects

Finally, our studies characterize the mechanism by which the RGS10 is suppressed in microglia, and identify the mechanism by which RGS10 reduces pro-

inflammatory cytokines production in microglia. Understanding such mechanisms could help develop new strategies for the prevention and/or treatment of neurodegenerative diseases that are characterized by excessive microglial activation. The models of working hypotheses for epigenetic silencing of RGS10 in chemoresistant ovarian cancer and activated microglia are summarized in figure 6.1. Epigenetic silencing of RGS10 in ovarian cancer cells drives ovarian cancer progression and chemoresistance, whereas epigenetic suppression of RGS10 in activated microglia enhances pro-inflammatory cytokine signaling.

6.2. Figures

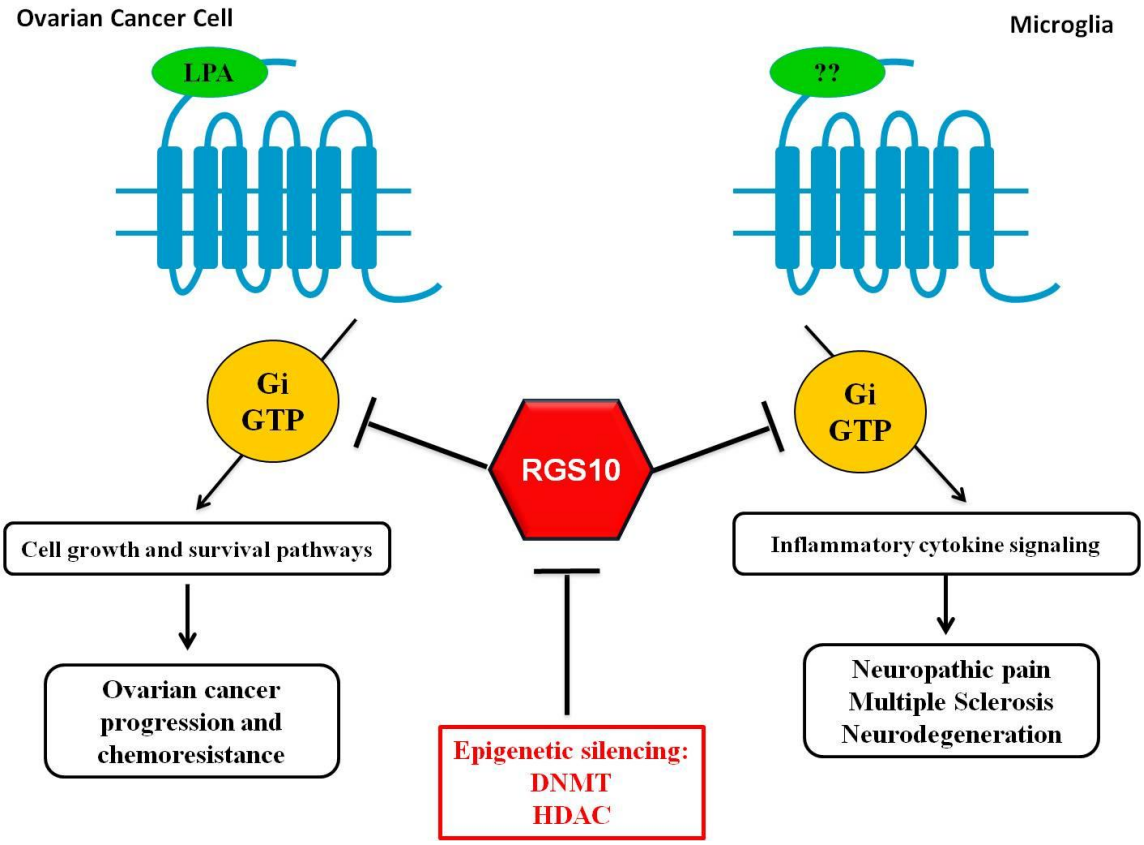

Figure 6.1: Epigenetic Silencing of RGS10 in Ovarian Cancer and Activated Microglia. Epigenetic silencing of RGS10 in ovarian cancer cells drives ovarian cancer progression and chemoresistance, whereas RGS10 epigenetic suppression in activated microglia enhances pro-inflammatory cytokine signaling.

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