REGULATION OF LYSOPHOSPHOLIPID SIGNALING IN DEVELOPMENT AND CANCER

by

JILLIAN H. HURST

(Under the Direction of Shelley B. Hooks)

ABSTRACT

Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are bioactive phospholipids that mediate their effects through the activation of G-protein coupled receptors (GPCRs). LPA and S1P mediate a broad range of cellular activities including proliferation, survival, motility, adhesion, cytoskeletal changes, and differentiation. Further, LPA and S1P signaling play a role in multiple physiological and pathophysiological processes. The goal of this research was to define the regulation of LPA and S1P signaling pathways in neural development and ovarian cancer.

LPA and S1P are required for proper development of the central nervous system. A stable, adherent human embryonic stem cell-derived neuroepithelial (hES-NEP) cell line has recently been established. We therefore characterized the responses to LPA and S1P in these cells to determine 1) if hES-NEP cells express lysophospholipid receptors and 2) if LPA and S1P mediate cellular responses critical for neural development. Our data demonstrate that hES-NEP cells express functional LPA and S1P receptors, which modulate hES-NEP cell growth and morphology through distinct mechanisms. Further, these data establish hES-NEP cells as a model system for studying the role of lysophospholipids in human neural progenitors.

LPA is the predominant growth factor in ovarian cancer, promoting growth, survival, migration, and invasion. The goal of this study was to characterize the signaling pathways regulating LPA signaling in ovarian cancer. Using pharmacological inhibitors we demonstrated that LPA-stimulated cell growth is mediated by distinct sets of receptors and signaling intermediates in two different model ovarian cancer cell lines. We also explored the role of Regulator of G-protein Signaling (RGS) proteins in the regulation of LPA signaling in ovarian cancer cells. RGS proteins are a diverse group of multifunctional proteins which regulate signaling downstream of GPCRs. Our data establish RGS proteins as novel regulators of LPA signaling in ovarian cancer cells and demonstrate that RGS transcripts are differentially expressed in benign and cancerous ovarian tissue.

INDEX WORDS: lysophospholipids, lysophosphatidic acid, sphingosine-1-phosphate, G-protein coupled receptors, Regulator of G-protein Signaling proteins, ovarian cancer, neural progenitors, human embryonic stem cells

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DEDICATION

I would like to dedicate this work to my family, friends, and teachers. This degree has been a team effort.

To my parents, Jules and Susan Hurst, who have supported me throughout any project I have decided to undertake.

To my brother, Jay, who has always been my best competition. Thank you for pushing me to work harder and for encouraging me when I doubted myself.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW:
BIOLOGY OF LYSOPHOSPHOLIPID SIGNALING

Lysophosphatidic acid (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate, LPA) and sphingosine-1-phosphate (1-phosphate-2-amino-4-cis-octadecene-l,3-diol, S1P) are two distinct biologically-active phospholipids that produce a wide range of cellular effects by acting as ligands for G-protein coupled receptors (GPCRs). While both molecules have long been known as metabolic intermediates in membrane phospholipid synthesis pathways, the discovery that LPA and S1P act as ligands for cell surface G-protein coupled receptors has established these phospholipids as signaling molecules. The following chapter will provide a brief introduction to the structure, production, and biological functions of LPA and S1P.

Lysophosphatidic Acid Structure and Metabolism

LPA is a simple glycerophospholipid, consisting of a glycerol backbone with a phosphate head group at the *sn*-3 position, a fatty acyl chain at either *sn*-1 or *sn*-2, and a hydroxyl group at the remaining position (**Figure 1.1A**). Structural diversity of LPA is achieved by variability in the length and saturation of the hydrocarbon chain as well as linkage of the hydrocarbon chain to the glycerophosphate backbone. LPA species commonly found in human serum are acyl LPA with palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and arachidonic acids (20:4) esterified to the *sn*-1 position (Baker et al., 2001).

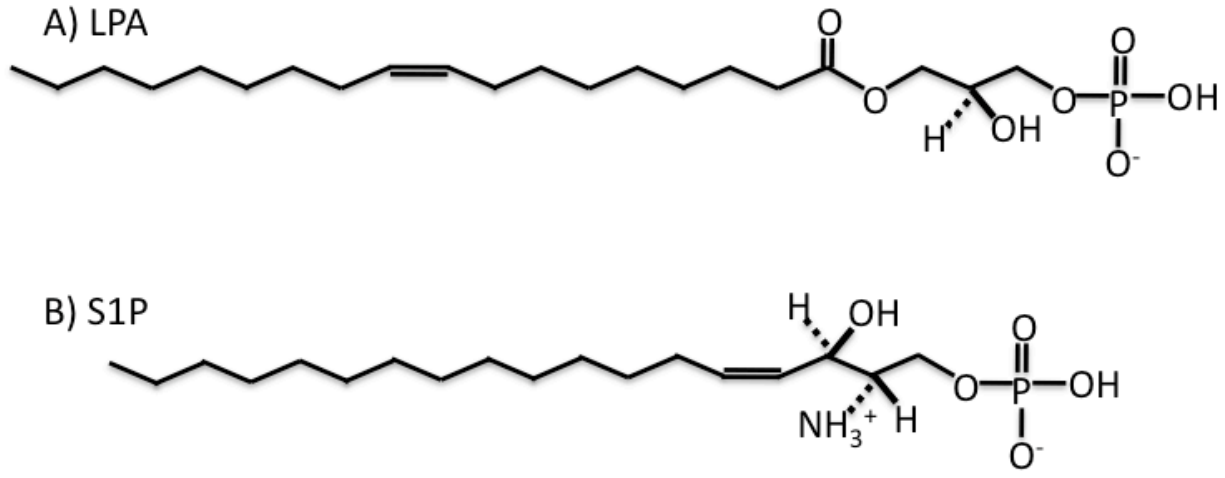

Figure 1.1: Structure of lysophospholipids.
A) Lysophosphatidic acid (LPA) and B) sphingosine-1-phosphate (S1P).

LPA is produced in multiple tissues and biological fluids, particularly serum, saliva, and follicular fluid (Rivera and Chun, 2008). LPA-secreting cells include activated platelets (Mauco et al., 1978), Schwann cells (Weiner et al., 2001), mature neurons (Fukushima et al., 2000), adipocytes (Pages et al., 2001), fibroblasts (Jalink et al., 1990), and ovarian cancer cells (Xu et al., 1995c). Synthesis of LPA primarily involves phospholipases A_1 and A_2, and lysophospholipase D, also known as autotaxin (ATX/phosphodiesterase Iα/(ecto)nucleotide pyrosphosphatase/phosphodiesterase 2), a multifunctional ectoenzyme that was originally discovered as a tumor cell motility factor (Stracke et al., 1992). ATX mediates the production of LPA by cleavage of the choline head group from lysophosphatidylcholine (LPC). LPA can inhibit autotaxin, thus regulating its own biosynthesis (van Meeteren et al., 2005). PLA_1 and PLA_2 are believed to be primarily responsible for the generation of LPC, which then serves as a substrate for autotaxin (Aoki et al., 2002; Sano et al., 2002). LPA is degraded by lipid phosphate phosphatases (LPPs), LPP-1, LPP-2, and LPP-3, a group of ectoenzymes responsible for the dephosphorylation of LPA, phosphatidic acid, S1P, and ceramide-1-phosphate (Kai et al.,

1996; Kai et al., 1997; Hooks et al., 1998; Roberts and Morris, 2000; Brauer et al., 2003). See **Figure 1.2** for a schematic of LPA metabolism.

S1P Structure and Metabolism

S1P, like LPA, consists of a mono-phosphorylated backbone, in this case an amide, linked to an unsaturated hydrocarbon chain of 18 carbons (**Figure 1.1B**). S1P is produced by activated platelets (Igarashi and Yatomi, 1998; Yatomi et al., 2000) and several types of hematopoietic cells including erythrocytes, neutrophils, and peripheral mononuclear cells (Yang et al., 1999). Thus, S1P is found in blood and, like LPA, in serum, plasma (Yatomi et al., 1997a), follicular fluid (von Otte et al., 2006), and ovarian cancer ascites (Westermann et al., 1998). S1P is generated by sphingosine kinase-mediated phosphorylation of sphingosine, a lipid synthesized via deacylation of ceramide (Park and Schuchman, 2006). There are two sphingosine kinases, SPHK1 and SPHK2 (Kohama et al., 1998; Liu et al., 2000a) and are responsible for the majority of S1P synthesis. Interestingly, autotaxin can hydrolyze sphingosylphosphorylcholine to S1P; however, the efficiency is about 1000-fold lower than the generation of LPA from LPC and the biological significance of this activity is unclear (Clair et al., 2003). Like LPA, S1P also inhibits autotaxin (van Meeteren et al., 2005). S1P degradation is catalyzed by LPPs and S1P lyase (Mandala et al., 2000; Le Stunff et al., 2002; Ogawa et al., 2003). See **Figure 1.3** for a schematic of S1P metabolism.

Discovery of LPA and S1P Signaling Pathways

As early as the 1960s, LPA and S1P were recognized as having significant physiological effects (Kirschner and Vogt, 1961). LPA was demonstrated to modulate blood pressure in cats, guinea pigs, rats, and rabbits and was later shown to induce smooth muscle contraction (Tokumura et al., 1978; Tokumura et al., 1980). Further, both LPA and S1P induce platelet aggregation

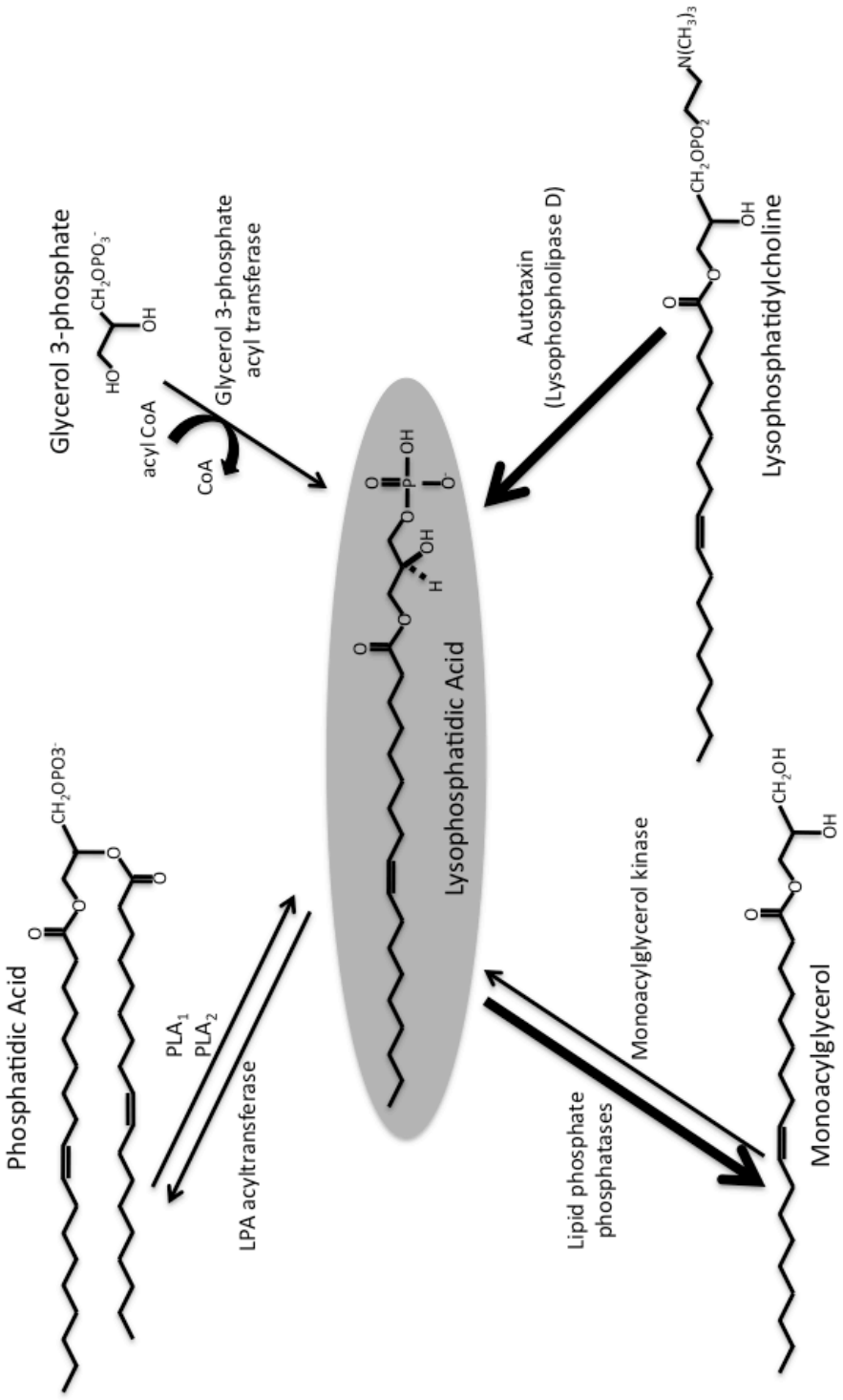

Figure 1.2: Pathways of LPA metabolism.
Key enzymes for the formation and degradation of LPA are shown.

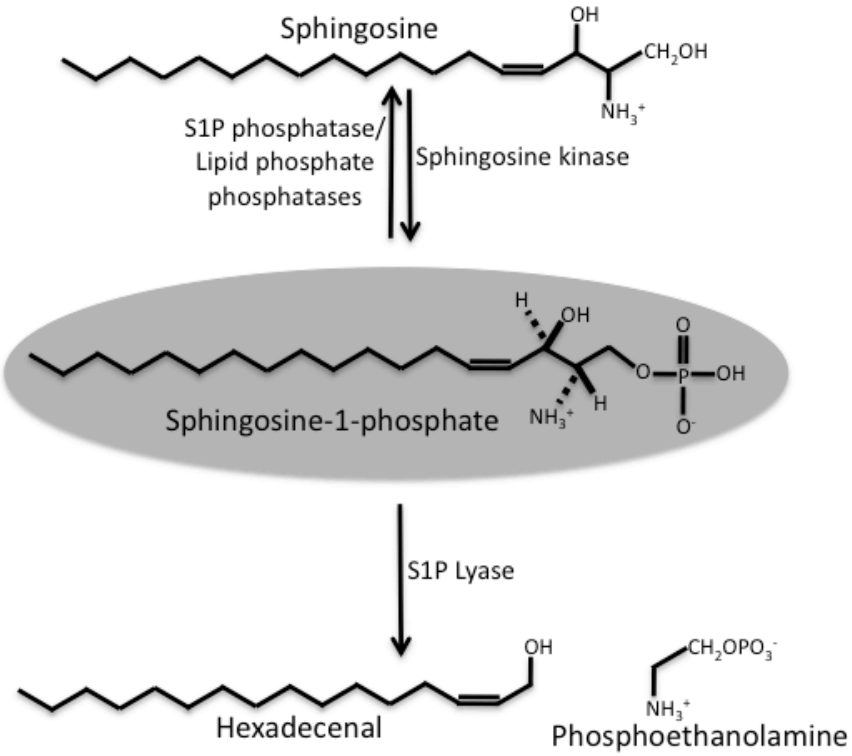

Figure 1.3: Pathways of S1P metabolism.
Key enzymes for the formation and degradation of S1P are shown.

(Gerrard et al., 1979; Yatomi et al., 1997b), and increased migration of neutrophils (Gerrard et al., 1980). Due to the detergent like structures of LPA and S1P, there were concerns that the effects of these lysophospholipids could be non-specific; however, evidence for lysophospholipidreceptors began to appear in the late 1980s, when Wouter Moolenaar's group demonstrated that LPA's effects were GTP-dependent and that LPA treatment either directly or indirectly activated Gi and Gq family heterotrimeric G-proteins (van Corven et al., 1989).

Additionally, LPA stimulated calcium mobilization, inositol phosphate formation, gene transcription, and proliferation in fibroblasts. These effects were evident at nanomolar to micromolar concentrations of LPA (below the critical micelle concentration), displayed a saturable dose-response which mimicked first-order kinetics, was subject to homologous

desensitization, and was cell-type specific (Moolenaar et al., 1986; Jalink et al., 1990; Moolenaar and van Corven, 1990). In addition to its effects in mammalian cells, LPA also serves as a chemoattractant for the slime mold *Dictyostelium discoideum* (Jalink et al., 1993b) and is capable of mobilizing intracellular calcium in Xenopus oocytes (Fernhout et al., 1992). In 1992, van der Bend and colleagues performed radioligand binding assays that identified a putative LPA receptor in neuronal cells, brain homogenates, cancer cells, and fibroblasts (van der Bend et al., 1992). Based on these data, it became apparent that LPA was a bioactive molecule, the effects of which were likely mediated by some form of guanine nucleotide-dependent receptor.

In 1996, Jerold Chun's lab identified the first LPA receptor in cortical neuroblasts from the mouse ventricular zone in a screen for GPCRs that were related to cortical neurogenesis (Hecht et al., 1996). *Vzg-1*, a gene previously cloned and characterized as *Edg-2* (Masana et al., 1995), induced "cell rounding" when over-expressed in cortical neuroblasts in a serum-dependent manner. Hecht and colleagues next identified the serum component that was capable of reproducing the serum effect. Out of all the lipids and growth factors found in serum, LPA alone caused cell rounding. Further, over-expression of *vzg-1* lowered the EC_{50} for LPA-induced morphological changes. Finally, the effect was demonstrated to be sensitive to pertussis toxin (Ptx), a known inhibitor of Gi-family G-proteins (Hecht et al., 1996). In 1998, Chun and colleagues used a heterologous expression system in neuronal and non-neuronal cell lines to demonstrate the ability of *vgz-1* to serve as an LPA membrane binding site and to mediate LPA-dependent G-protein activation, stress fiber formation, neurite retraction, transcriptional serum response element (SRE) activation, and increased DNA synthesis (Fukushima et al., 1998). Shortly thereafter, two more LPA receptors (Edg4/LPA2 (An et al., 1998) and Edg7/LPA3 (Bandoh et al., 1999)), a *Xenopus laevis* LPA receptor (Guo et al., 1996).

S1P was identified as a signaling molecule shortly after the first LPA receptor was cloned in 1996 (Hecht et al., 1996). In 1990, Ghosh and colleagues demonstrated that sphingosine metabolites, and S1P in particular, induce calcium mobilization and activation of protein kinase C (PKC) (Ghosh et al., 1990). Shortly afterwards, sphingosine and S1P were shown to promote DNA synthesis, intracellular calcium mobilization, and morphological changes in Swiss 3T3 fibroblasts, leading the authors to conclude that S1P was part of the intracellular second messenger system that was mediating the effects of sphingosine (Zhang et al., 1991; Desai et al., 1992; Wu et al., 1995). Additionally, S1P was shown to reduce tumor cell motility and invasiveness, suggesting that S1P may mediate cytoskeletal changes (Sadahira et al., 1992; Spiegel et al., 1994). Like LPA, S1P was also demonstrated to provoke calcium mobilization in Xenopus oocytes (Durieux et al., 1993). Evidence of GPCR involvement became apparent when S1P-stimulated, but not bradykinin-stimulated, DNA synthesis, inhibition of cAMP formation, inositol phosphate production, and activation of mitogen-activated protein kinase (MAPK) cascades was shown to be pertussis toxin (Ptx) sensitive, indicating that these effects are mediated by a Gi-family G-protein (Goodemote et al., 1995; Wu et al., 1995; Okajima et al., 1996; van Koppen et al., 1996). The signaling activity of S1P is highly similar to that of LPA, indicating that the effects of LPA and S1P could be mediated by the same receptors or signaling intermediates; however, Moolenaar and colleagues demonstrated that while both LPA and S1P induce neurite retraction and cell rounding in NIE-115 neuronal cells, S1P is ~100-fold more potent than LPA, and that LPA and S1P do not cross-desensitize, suggesting that LPA and S1P bind to different receptors (Postma et al., 1996). The first S1P receptor was finally identified as a ligand for the orphan receptor Edg-1 (Lee et al., 1998; Zondag et al., 1998), a receptor that is highly homologous to LPA1/Edg-2/Vgz-1 (Hla and Maciag, 1990; Lee et al., 1996). Four more

S1P receptors were subsequently cloned based on their homology to Edg-1/S1P1 (An et al., 1997a; Hla et al., 1997; Sato et al., 1998; Sato et al., 1999b; Windh et al., 1999; Kimura et al., 2000; Yamazaki et al., 2000; Im et al., 2001).

LPA receptors and signaling pathways

It is now known that LPA signaling is mediated by at least five different GPCRs: LPA1-5. These receptors belong to two different gene families based on their primary structures. LPA1-3 (formerly Edg2, Edg4, and Edg7, respectively) belong to the endothelial differentiation gene (*edg*) family, while LPA4 (GPR23/P2Y9) (Noguchi et al., 2003) and LPA5 (GPR92) (Kotarsky et al., 2006; Lee et al., 2006a) belong to the P2Y family of receptors. Recently, three more receptors have been identified but have not yet been officially designated LPA receptors: GPR87 (Tabata et al., 2007), P2Y5 (Pasternack et al., 2008), and P2Y10, which has been identified as both an LPA and an S1P receptor (Murakami et al., 2008). Notably, LPA receptors belonging to the P2Y family have only 20-24% peptide sequence homology with LPA1-3 (Noguchi et al., 2003). Further, LPA-induced responses in the absence of Edg-family LPA receptors such as adenylyl cyclase inhibition, inositol phosphate production, calcium mobilization, and stress fiber formation required much higher (micromolar) concentrations of LPA when mediated by LPA4 or LPA5 (Noguchi et al., 2003; Lee et al., 2006a). Further studies will be required to determine if LPA is the primary ligand for LPA-responsive receptors in the P2Y family.

In addition to the cell surface GPCRs, LPA can also activate the intracellular receptor peroxisome proliferator-activated receptor γ (PPARγ) (McIntyre et al., 2003). PPARγ is part of a family of nuclear hormone receptors and serves as a transcription factor that generally controls energy metabolism (Auwerx, 1999). PPARγ signaling has been predominately linked to insulin

and lipid storage and adipocyte differentiation, but it also appears to play a role in carcinogenesis, inflammation, cell cycle control, and artherosclerosis (Fajas et al., 2001). The focus of the studies discussed here, however, will be on cell surface GPCRs.

LPA receptors are widely expressed in multiple tissues and at multiple stages of development. LPA1, which was originally identified as a highly expressed receptor in the ventricular zone of the embryonic brain (Hecht et al., 1996), is also expressed in white matter tracts and is associated with myelination. It is highly expressed in oligodendrocytes in adult brain (Weiner et al., 1998). Additionally, LPA1 mRNA is abundant in a wide range of adult tissues including brain, heart, colon, small intestine, placenta, prostate, ovary, pancreas, testis, spleen, skeletal muscle, and kidney (Anliker and Chun, 2004a). In adult mice, LPA2 mRNA is most abundant in kidney, testis, and leukocytes, and is also found in pancreas, thymus, spleen, and prostate. In contrast with LPA1, it is only weakly expressed in heart, lung, liver, kidney, intestines, colon, muscle, placenta, and ovary (Anliker and Chun, 2004a). Expression of LPA2 is very high in embryonic brain, but is very weakly expressed in adult brain (Contos and Chun, 2000). LPA3 is detectable at the highest levels in heart, pancreas, prostate, testes, and is also found at moderate levels in ovary and lung (Bandoh et al., 1999; Im et al., 2000b).

LPA4/P2Y9/GPR23 is most highly expressed in ovary with only weak expression in other tissues (Noguchi et al., 2003), while LPA5/GPR92 has a low level of expression in multiple tissues, but is enriched in embryonic brain and embryonic stem cells, intestine, and dorsal root ganglia (Lee et al., 2006a). GPR87 is most highly expressed in brain and testis, with lower levels of transcript found in placenta, ovary, skeletal muscle, and prostate. Transcript was not found in heart, kidney, lung, intestine, or liver (Tabata et al., 2007). Additionally, GPR87 was shown to be over-expressed in human squamous cell carcinoma of the lung and to contribute

to tumor cell motility (Glatt et al., 2008; Gugger et al., 2008). Expression patterns have not been reported for P2Y5. P2Y10 expression is reported in brain, lung, skeletal muscle, placenta, uterus, testes, and prostate (Murakami et al., 2008)

All LPA receptors are capable of activating multiple families of heterotrimeric G-proteins. LPA1 and LPA2 couple to Gi, Gq, and G12 family G-proteins, while LPA3 only couples to Gi and Gq (Anliker and Chun, 2004a). LPA4 couples to Gi and Gs-family G-proteins (Noguchi et al., 2003); LPA5 couples to G12 and Gq-family G-proteins (Lee et al., 2006a); G-protein coupling has not yet been reported for GPR87 (Tabata et al., 2007); P2Y5 couples to G12-family G-proteins (Yanagida et al., 2009); G-protein coupling has not yet been reported for P2Y10 (**Figure 1.4**). Further studies on these newest LPA receptors will be required to confirm that they cannot interact with members of other heterotrimeric G-protein families. Gs family G-proteins stimulate adenylyl cyclase, increasing cellular levels of cAMP, while Gq family G-proteins stimulate phospholipases to generate inositol phosphates (IPs) and diacylglycerol (DAG), inducing intracellular calcium mobilization. Activation of Gi family G-proteins can inhibit adenylyl cyclase and activate phosphoinositide-3-kinase (PI3K), phospholipase $C\beta_2$, or the small G-protein Ras, which can activate MAP kinase pathways. G12 family G-proteins modulate activity of the small G-protein Rho. Common effects observed after activation of these pathways results in cell proliferation, cell survival, and changes in cell morphology (Goetzl et al., 2000; Swarthout and Walling, 2000).

Studies of mouse embryonic fibroblasts (MEFs) isolated from wild-type and LPA receptor knockout animals and over-expression of individual LPA receptors in cell lines have been used to determine which pathways are activated by different LPA receptors. LPA treatment of cell lines over-expressing LPA1 causes activation of phospholipase C (PLC), inhibition of

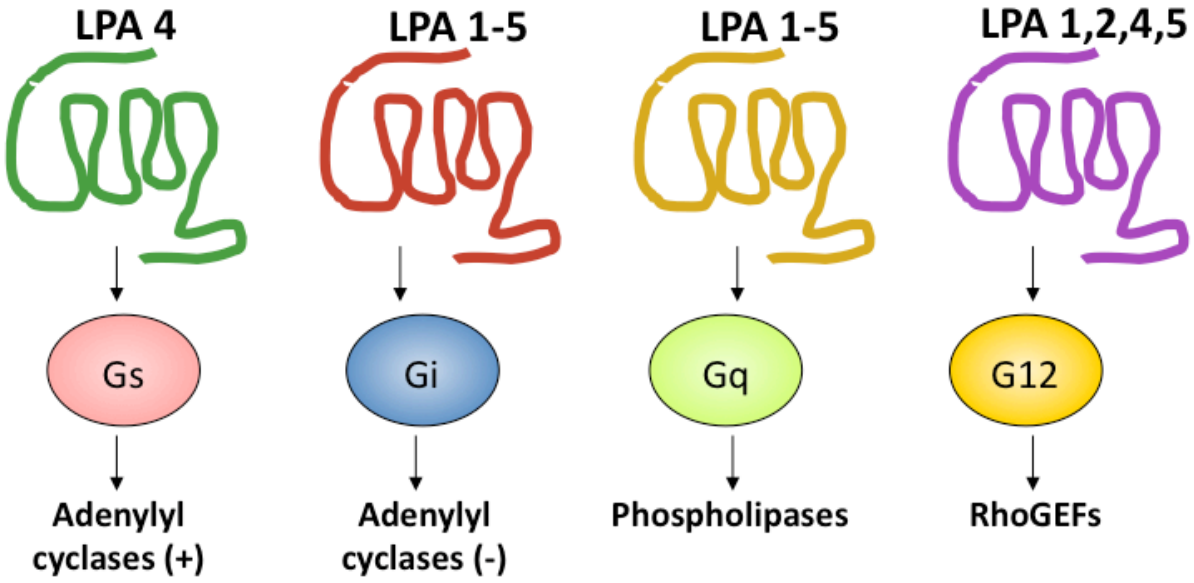

Figure 1.4: Heterotrimeric G-protein coupling of LPA receptors.
Common effectors are shown.

adenylyl cyclase (AC), and cell rounding (Hecht et al., 1996; Fukushima et al., 1998; Ishii et al., 2000); conversely, knockdown of LPA1 partially inhibits PLC activation and completely blocks inhibition of AC while morphology changes are reduced (Contos et al., 2002). Further, LPA1-mediated LPA signaling induces activation of serum response element (SRE), which is sensitive to pertussis toxin (Ptx) and C3 exoenzyme treatment, indicating the involvement of both Gi G-proteins and Rho.

LPA1-null mice demonstrate a critical role for LPA1 in development. The loss of LPA1 in mice results in a 50% reduction in litter size and the majority of LPA1-null mice die between birth and 3 weeks of age, likely due to abnormal suckling behavior, which is believed to be caused by an olfactory defect. Some LPA1-null neonatal mice have frontal hematomas or exencephaly. LPA1-null mice that survive into adulthood have craniofacial defects including a shortened snout and widely spaced eyes as well as reduced body mass (Contos et al., 2002)

LPA2 over-expression has similar effects to what are seen with the over-expression of LPA1 (An et al., 1998; Ishii et al., 2000). Knockdown of LPA2 in MEFs results in a greater reduction of LPA-stimulated PLC activity compared with MEFs lacking LPA1, and LPA-stimulated PLC activity is completely abolished when both LPA1 and LPA2 are knocked out, indicating that both LPA1 and LPA2 are involved in PLC activation, but that LPA2 plays a greater role than LPA1 (Contos et al., 2002). Knockdown of LPA2 in MEFs did not have an effect on inhibition of AC, suggesting that LPA2 is not coupled to this pathway in these cells (Contos et al., 2002). Further, LPA2 knockout mice do not have an obvious phenotype, and LPA1/2 double knockouts do not have a more severe phenotype than LPA1 knockout mice (Contos et al., 2002). Taken together, these data suggest that LPA1 and LPA2 signaling are somewhat redundant.

LPA3 signaling is distinct from LPA1 and LPA2 signaling pathways. While LPA1 and LPA2 cause cell rounding and process retraction, LPA3 over-expression in the B103 neuroblastoma cell line inhibits cell rounding and causes neurite elongation. Similar to LPA1 and LPA2, LPA3 over-expression also stimulates activation of PLC, mediated by Gq-like G-proteins, and inhibition of AC and activation of MAP kinase cascades, both of which are inhibited by Ptx-treatment, indicating that these pathways are mediated by Gi family G-proteins (Ishii et al., 2000). LPA3 null mice do not have neurological defects; however, they display abnormalities in embryo implantation and deficiencies in prostaglandin synthesis (Ye et al., 2005).

There is limited data available on the pathways activated by LPA receptors belonging to the P2Y family. Unlike the first three LPA receptors, LPA4 mediates activation of AC and increases in intracellular calcium (Noguchi et al., 2003); however, over-expression of LPA4 in

rat neuroblastoma B103 cells did not affect adenylyl cyclase activity. Additionally, LPA treatment of B103-LPA4 cells caused Gq-dependent calcium mobilization and G12/Rho/Rho-associated kinase (ROCK)-dependent morphology changes including cell rounding, aggregation, and cadherin-dependent cell adhesion (Yanagida et al., 2007). MEFs from LPA4 knockout mice showed increased migration in response to LPA compared to MEFs from wild-type animals, suggesting that LPA4 negatively regulates LPA-stimulated cellular migration (Lee et al., 2008). In studies of cells over-expressing the receptor, LPA5 mediated LPA-stimulated neurite retraction in B103 cells and stress fiber formation in RH7777 cells through RhoA and p160ROCK (Lee et al., 2006a). Additionally, LPA increased cAMP levels and intracellular calcium in LPA5 expressing cells through a pathway that was sensitive to Gq but not Gi blockade, suggesting that LPA5 couples to Gq and G12 family G-proteins Further studies will be required to define the signaling pathways linked to P2Y5, P2Y10, and GPR87 (Tabata et al., 2007; Murakami et al., 2008; Yanagida et al., 2009).

Physiological and Pathophysiological Functions of LPA

LPA has been linked to multiple physiological and pathophysiological processes. In the cardiovascular system, LPA is a critical regulator of vascular morphogenesis and angiogenesis, directing the migration and invasion of endothelial cells. While lack of individual LPA receptors (LPA1-3) does not appear to severely disrupt vascular development, mice lacking autotaxin die early on in embryonic development due to impaired blood vessel formation (Argraves et al., 2004; van Meeteren et al., 2006). LPA has also been reported to play a role in artherosclerosis. Artherosclerotic plaques contain elevated levels of LPA, which is known to promote the activation of platelets, vascular smooth muscle cells, endothelial cells, and macrophages

involved in the initiation and progression of the disease (Siess et al., 1999; Rother et al., 2003; Siess and Tigyi, 2004; Smyth et al., 2008).

LPA's ability to modulate the activity of endothelial cells, smooth muscle cells, fibroblasts, and platelets also allows it to play a role in wound healing (Lee et al., 2000; Siess, 2002; Pilquil et al., 2006). LPA has been shown to promote wound healing in the skin, cornea, intestinal epithelia, and in human periodontal ligaments and gingiva (Balazs et al., 2000; Sturm and Dignass, 2002; Cerutis et al., 2007; Xu et al., 2007). LPA is implicated as a pro-inflammatory factor. It has been shown to stimulate an inflammatory cascade in airway epithelial cells, which is involved in asthma and allergen responses (Barekzi et al., 2006; Kassel et al., 2009). Additionally, LPA mediates migration and adhesion of human monocytes and neutrophils and the association of monocytes with endothelial cells, thus promoting inflammation processes (Zhang et al., 2006b; Lin et al., 2007). LPA has also been shown to play a role in the development of neuropathic pain. Unlike their wild-type counterparts, mice lacking LPA1 do not develop allodynia and hyperalgesia after peripheral nerve injury (Inoue et al., 2004)

LPA also regulates the mammalian reproductive system. It is present in follicular fluid and increases during pregnancy (Ye, 2008). Further, LPA3-null mice exhibit delayed uterine implantation, uneven embryo spacing, prolonged pregnancy, delayed embryonic development, and increased embryonic lethality (Ye et al., 2005). LPA also influences the fertility of male mice. LPA1, LPA2, and LPA3 are enriched in the testes and loss of any of the three receptors results in decreased mating activity and spermatogenesis (Ye et al., 2008).

The putative LPA receptors, P2Y5 and GPR87, have been linked to physiological and pathophysiological conditions. P2Y5 has been linked to the maintenance of human hair growth. A mutation in the receptor is associated with a condition known as "wooly hair syndrome" and

hypotrichosis (Pasternack et al., 2008; Shimomura et al., 2009; Yanagida et al., 2009). GPR87 is associated with tumor cell viability and is over-expressed in squamous cell lung carcinoma (Glatt et al., 2008; Gugger et al., 2008). Further studies will be required to determine functions for P2Y10.

In addition to the functions decribed above, LPA plays significant roles in brain development and oncogenesis, which will be discussed in detail in later sections.

S1P Receptors and Signaling Pathways

S1P signaling is mediated by five different GPCRs all belonging to the endothelial differentiation gene (*edg*) family. They are designated S1P1/Edg1, S1P2/Edg5, S1P3/Edg3, S1P4/Edg6, and S1P5/Edg8. Like LPA receptors, S1P receptors are widely expressed in multiple tissues and at different stages of development. The S1P1/Edg1 receptor was initially identified as a gene that was up-regulated during phorbol 12-myristate 13-acetate (PMA) induced differentiation of human endothelial cells (Hla and Maciag, 1990; Lee et al., 1996). It is expressed most highly in adult brain, liver, heart, spleen, and lung, and in immune system cells. In the embryo, S1P1 is highly expressed in the brain, particularly neocortical areas, the skeletal system, the aorta, limb buds, and capillaries and blood vessels (McGiffert et al., 2002; Ishii et al., 2004; Ohuchi et al., 2008). S1P2/Edg5 was first found in rat cDNA libraries from hippocampus and aortic smooth muscle (Okazaki et al., 1993; MacLennan et al., 1994). It is found at high levels in embryonic brain and also in adult thymus, lung, heart, kidney, and spleen (Okazaki et al., 1993; MacLennan et al., 1994; Yamaguchi et al., 1996; Ishii et al., 2001; Ishii et al., 2002; McGiffert et al., 2002). S1P3/Edg3 was identified in a human genomic DNA library using degenerate primers to cannabinoid receptors (Yamaguchi et al., 1996) and is most prominently expressed in embryonic brain and adult pancreas, lung, heart, and kidney (Zhang et al., 1999;

McGiffert et al., 2002). S1P4/Edg6 was found using degenerate chemokine receptor primers in human dendritic cells (Graler et al., 1998) and is only expressed in lymphoid cells and tissues (Graler et al., 1998; Graler et al., 1999). S1P5/Edg8/Nrg1 was cloned from rat cDNA and identified in expressed sequence tag databases (Glickman et al., 1999; Im et al., 2000b). Its expression is also relatively restricted to brain, lung, spleen, and skin (Niedernberg et al., 2002; Toman and Spiegel, 2002; Yu et al., 2004; Jaillard et al., 2005; Ohuchi et al., 2008).

Except for S1P1, S1P receptors couple to multiple heterotrimeric G-proteins. S1P1 only couples to Gi family G-proteins (Okamoto et al., 1998); S1P2 and S1P3 couple to Gi, Gq, and G12 family G-proteins (Okamoto et al., 1999; An et al., 2000; Okamoto et al., 2000; Meacci et al., 2002); S1P4 and S1P5 couple to Gi and G12/13 family G-proteins (Im et al., 2000a; Malek et al., 2001; Niedernberg et al., 2002; Siehler and Manning, 2002; Graler et al., 2003) (**Figure 1.5**). Additonally, S1P can act intracellularly to regulate calcium mobilization, proliferation and survival (Payne et al., 2002).

MEFs and other cells from S1P receptor knockout animals and cell lines over-expressing individual S1P receptors have been used to determine the signaling functions of the S1P receptor family members. Stimulation of the Gi-coupled S1P1 receptor results in PLC activation, inhibition of AC, and activation of MAP kinases (Lee et al., 1996; Okamoto et al., 1998; Zondag et al., 1998). S1P1-mediated calcium mobilization did not occur in all cell lines (Okamoto et al., 1998; Zondag et al., 1998; Ancellin and Hla, 1999). S1P1 consistently induced activation of Rho; this signaling pathway appears to play a role in cellular migration, cell-cell contacts, and adhesion (Lee et al., 1998; Paik et al., 2001). S1P-stimulated Rac activation and migration are

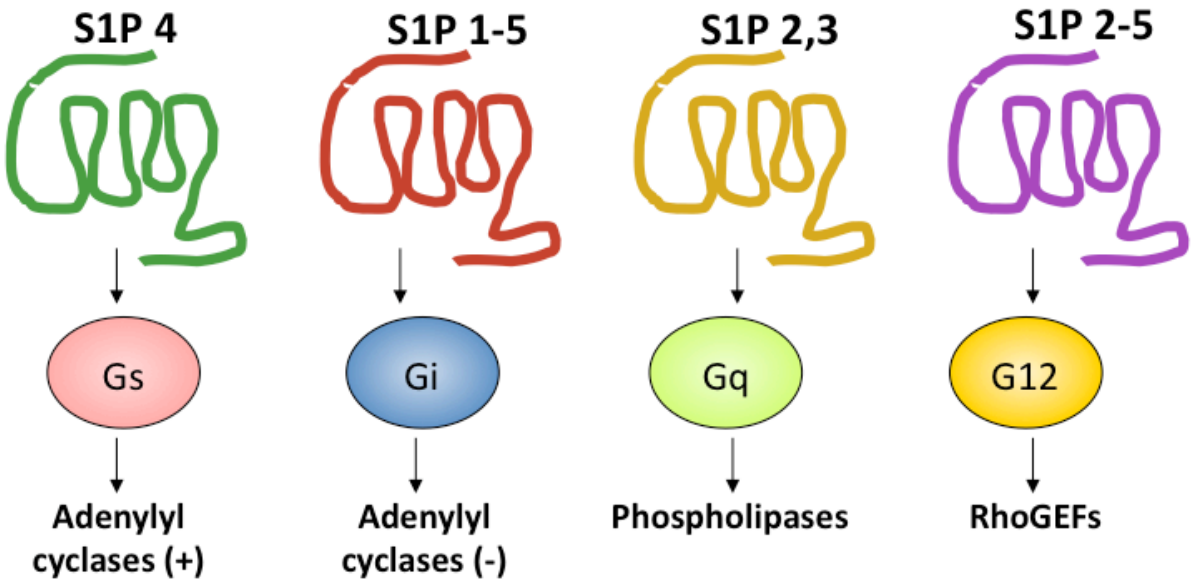

Figure 1.5: Heterotrimeric G-protein coupling of S1P receptors.
Common effectors are shown.

ablated in fibroblasts from S1P1 knockout mice (Liu et al., 2000b), explaining the migration and cell-cell interaction defects seen in endothelial cells, vascular smooth muscle cells, and immune system cells seen in these animals (Liu et al., 2000b; Graler and Goetzl, 2002; Goetzl et al., 2004; Matloubian et al., 2004; Wang et al., 2004a; Kono et al., 2008).

Cell lines over-expressing S1P2 exhibit S1P-stimulated increases in intracellular calcium, which were partially sensitive to Ptx, indicating that the effect is mediated in part by Gi family G-proteins (An et al., 1997b; An et al., 1999; Ancellin and Hla, 1999). Additionally, S1P treatment activated SRE in a Gi and Rho-dependent manner, activated PLC, inhibited AC, and induced cell rounding (An et al., 1997b; Van Brocklyn et al., 1999). MEFs from S1P2 null mice showed a significant decrease in Rho activation, but responded normally in assays measuring PLC and AC activity and calcium mobilization (Ishii et al., 2002).

Like S1P2, S1P3 also mediates PLC activation and calcium mobilization that are partially sensitive to Ptx, and AC inhibition and MAP kinase activation, which are completely sensitive to

Ptx (An et al., 1997a; An et al., 1999; Ancellin and Hla, 1999). S1P3 also mediates Rho activation (An et al., 1997a; Paik et al., 2001). Neither S1P2 nor S1P3 knockout mice have an obvious phenotype (Ishii et al., 2001); however, S1P2/S1P3 double null mice had significantly reduced litters and MEFs from these mice did not activate Rho in response to S1P (Ishii et al., 2002; Kono et al., 2004). S1P3 deficient MEFs had a significant reduction in PLC activation and remaining PLC acitivity could be blocked with Ptx. However, in cells over-expressing S1P3, S1P-stimulated PLC activation was Ptx-insensitive (Ishii et al., 2001). Additionally, S1P3 deficient MEFs showed a slight reduction in inhibition of AC, but Rho activation was not affected, indicating that S1P3 plays a minor role in Rho activation (Ishii et al., 2001).

S1P4 mediates activation of PLC, MAP kinases, and Cdc42 via Gi G-proteins (Graler et al., 1998; Van Brocklyn et al., 2000; Kohno et al., 2003). S1P stimulation of CHO cells over-expressing LPA4 results in changes in morphology and cell motility which are mediated by G12 or Gi G-proteins and Rho or Cdc42 (Graler et al., 2003; Kohno et al., 2003). MEFs over-expressing S1P4 activate AC in response to S1P (Ishii et al., 2001; Siehler and Manning, 2002). S1P4 knockout animals have not been reported at this time.

S1P5 over-expression causes inhibition of AC, inhibition of MAP kinase activation, and activation of c-Jun NH_2-terminal kinase (Jnk) (Im et al., 2001; Malek et al., 2001; Niedernberg et al., 2002). siRNA against S1P5 blocked S1P from serving as a survival factor in oligodendrocytes and reduced Akt phosphorylation (Jaillard et al., 2005).

Physiological and Pathophysiological Functions of S1P

S1P is an important regulator of the cardiovascular and immune systems. Studies using animals lacking S1P receptors or sphingosine kinases die during early embryonic development due to vascular immaturity due to S1P's profound effects on proliferation, migration, survival,

and morphology of vascular endothelial cells (Allende et al., 2003; Kono et al., 2004; Krump-Konvalinkova et al., 2005; Mizugishi et al., 2005). Further, S1P is a critical regulator of vascular permeability (Lee et al., 1999; Sanna et al., 2006). S1P's ability to regulate the formation of blood vessels is also important in oncogenic angiogenesis; treatment of tumor bearing mice with FTY720, a functional antagonist of S1P receptors, or Sphingomab™, a monoclonal antibody targeting S1P, potently suppresses tumor growth by inhibiting neovascularization (Chae et al., 2004; LaMontagne et al., 2006; Visentin et al., 2006). In the immune system S1P mediates immune cell trafficking and plays a role in inflammation. S1P signaling is required for lymphocytes to exit lymphoid organs so that they can circulate through the blood stream (Matloubian et al., 2004; Rivera and Chun, 2008). Indeed, FTY720, the S1P receptor functional antagonist, is currently in clinical trials as an immunosuppressive for multiple sclerosis and organ transplants (Takabe et al., 2008). Thus, S1P is a critical regulator of the cardiovascular and immune systems. S1P's role in nervous system physiology will be described in a later section.

As described above, GPCR-mediated lysophospholipid signaling has been implicated in multiple physiological and pathophysiological processes including reproduction, immune responses, tumorigenesis, and development of the vascular and nervous systems (Saba, 2004; Birgbauer and Chun, 2006; Kono et al., 2008; Rivera and Chun, 2008). In all of these processes, LPA and S1P regulate cellular proliferation, apoptosis, differentiation, cellular adhesion, morphogenesis, and migration (Pyne and Pyne, 2000; Pyne and Pyne, 2002; Ye et al., 2002; Spiegel and Milstien, 2003). The following sections will describe the roles of LPA and S1P in neurodevelopment and the role of LPA in ovarian cancer.

LPA and S1P in Neurodevelopment

Mammalian nervous system development begins when the notochord secretes factors to induce adjacent ectoderm to differentiate into neuroectoderm. Neuroectoderm then forms the neural plate, which rolls up to form the neural tube from which the entire nervous system develops. Differentiation of cell populations within the neural tube leads to the formation of the central nervous system (CNS), comprised of neurons and glia, and the peripheral nervous system (PNS), which is derived from neural crest cells. Throughout this process multiple signaling factors are required to regulate cellular proliferation, migration, orientation, differentiation, and morphology. The factors and signaling pathways that control these activities need to be elucidated in order to fully understand the physiology and pathophysiology of the nervous system.

LPA and S1P have both been demonstrated to serve as signaling factors for neural cells. *In vitro* experiments using transformed cell lines such as PC12, NG108-15, and NIE-115, demonstrate that LPA induces neurite retraction (Jalink et al., 1993a; Tigyi et al., 1996a; Tigyi et al., 1996b; Kozma et al., 1997; Kranenburg et al., 1999). LPA1 was initially found in a screen for GPCRs that are highly expressed in the cerebral cortex (Hecht et al., 1996); further, its expression is restricted to neuroblasts of the proliferative region of the ventricular zone and in several other tissues, including myelinating cells, within the developing brain (Saba, 2004). Cell lines derived from the ventricular zone respond to LPA with morphological changes, characterized by cell rounding and process retraction (Fukushima et al., 2002b), electrophysiological changes (Dubin et al., 1999), and increased survival/diminished apoptosis (Weiner and Chun, 1999). Knockout of LPA1 results in a variety of defects including a 50% increase in neonatal lethality that has been attributed to abnormal suckling behavior and

subsequent starvation, possibly a result of malformation of the olfactory bulb and cortex (Contos et al., 2000a). Further, absence of LPA1 signaling results in defective embryonic cortical development and a lack of neurogenesis in the adult dentate gyrus (Kingsbury et al., 2003; Estivill-Torrus et al., 2007; Matas-Rico et al., 2008). Finally, Harrison and colleagues observed behavioral and neurological changes in LPA1 null mice that are reminiscent of several psychiatric diseases (Harrison et al., 2003). Taken together, these data demonstrate that the LPA-LPA1 signaling axis mediates cellular activities critical to neuronal development.

Studies on the role of S1P in development have primarily focused on the vascular system where S1P has profound effects on proliferation, migration, and morphogenesis of vascular endothelial cells and maturation of the mammalian vascular system as a whole (Pyne and Pyne, 2000; Yatomi et al., 2001; Spiegel and Milstien, 2002). In the past 15 years, however, S1P has emerged as an important regulator of neuronal development as well. In *in vitro* studies, S1P affects neural cell survival, proliferation, morphology, and migration. Exposing N1E-115 or PC-12 neural cells to exogenous S1P results in neurite retraction and cell rounding (Postma et al., 1996; Sato et al., 1997). In C6 glioma cells, S1P induces expression of fibroblast growth factor-2 (FGF-2) and early growth response-1 (Egr-1) and activation of extracellular signal-related kinases (ERKs) (Sato et al., 2000). S1P also induces FGF-2 production, MAP kinase activation, and proliferation through a Ptx-sensitive pathway in cerebellar astrocytes (Yamagata et al., 2003; Bassi et al., 2006). Similar pathways appear to be involved in the proliferation of glioma cells (Van Brocklyn et al., 2002). Several groups have demonstrated that S1P receptors are expressed in proliferative areas such as the subventricular zone of the brain in embryonic (E15) and newborn rats and that S1P2 in particular is expressed in the cell bodies and growing axons of differentiating neural precursor cells (Waeber and Chiu, 1999; MacLennan et al., 2001; Harada

et al., 2004). In embryonic rat brain, S1P activates GPCRs that are coupled to Gi family G-proteins (Harada et al., 2004). Further, S1P stimulates proliferation of rat hippocampal neural progenitors through a Gi and MAP kinase dependent pathway and cell rounding/aggregation through the Rho-associated kinase p160ROCK (Harada et al., 2004). Finally, S1P is a powerful chemoattractant for neural progenitor cells (Kimura et al., 2007). Thus, S1P modulates proliferation, survival, morphology, and migration of neural progenitor cells.

Two recent studies demonstrate that LPA and S1P are required for correct formation of the neural tube. In 2005, Mizugishi and colleagues generated knockout mice lacking both SPHK1 and -2 (Mizugishi et al., 2005). Deletion of these enzymes resulted in mice that had undetectable levels of S1P. The lack of S1P caused embryonic lethality (~E12.5), vascular defects, and severe neural tube defects (NTDs). The NTDs included exencephaly and an increase in apoptosis and decrease in proliferation in the neuroepithlial cell layer lining the neural tube (Mizugishi et al., 2005). These data indicate that S1P has pro-growth and anti-apoptotic effects in neural development. In 2006, van Meeteren and colleagues generated ATX knockout mice (van Meeteren et al., 2006). Like the SPHK knockout, the ATX knockout was also embryonic lethal (E9.5) with severe vascular and neural defects. Mice lacking ATX had malformed neural tubes, which failed to close and were kinked and undulated compared with the straight neural tubes found in wild-type animals. Further, ATX knockout animals had asymmetrical neural headfolds, the structures which give rise to the two halves of the cerebrum. The authors noted that ATX is highly enriched in the neural floor plate; ATX knockout therefore results in a local deficiency of LPA, likely causing the observed NTDs (van Meeteren et al., 2006). Taken together, these studies demonstrate critical roles for LPA and S1P in neurodevelopment.

The studies described above utilize several different models to study the effects of lysophospholipids in neural cell types: whole animal models lacking specific lysophospholipid receptors or enzymes required for the synthesis of lysophospholipids; primary cultures from rats and mice; and transformed human or rodent cell lines such as N1E-115, PC-12, or C6 glioma cells. These models have provided significant data about the effects of lysophospholipids in neural development; however, these models have several disadvantages. Animal models provide excellent data about global effects of LPA and S1P, but studies performed in rodents may not accurately reflect what occurs in humans. Primary cultures from animal models are a non-renewable source of cells and small differences in isolation techniques can make it difficult to achieve consistent results.

Cells that are human in origin and self-renewing may offer a good alternative to rodent or transformed tissues. In the past several years, researchers have begun to address some of these problems by using neural cells derived from human embryonic stem cells. Human embryonic stem (hES) cells are cells derived from the cell mass of the blastocyst. These cells are self-renewing and pluripotent, meaning they are immortal and can differentiate to all three germ cell layers and subsequently any cell type found in the body (Mitalipova et al., 2003). In 1998, James Thomson at University of Wisconsin established the first hES cell line (Thomson et al., 1998). Since then researchers have worked to derive various adult cell types from hES cells in the hopes that they can be used for therapeutic purposes and as model systems in which to study various physiological phenomena such as the mechanics of cell fate specification, migration, and proliferation.

In 2001, two groups reported the differentiation of hES cells into neural epithelial progenitors (NEP) (Reubinoff et al., 2001; Zhang et al., 2001). These hES-NEP cells were self-

renewing and multipotent, capable of differentiating into neurons, oligodendrocytes, or astrocytes. Immunohistochemical studies demonstrated that these cells were comparable to the neural epithelial progenitor cells, which form the mammalian neural tube (Mayer-Proschel, 2002). Typically, hES-NEP cells are derived through formation of embryoid bodies after removal of feeder layers. Cells within the embryoid body will differentiate into neural progenitors in the presence of retinoic acid (Reubinoff et al., 2001). However, embryoid bodies have several disadvantages; observation of phenotypic changes is not possible with conventional microscopy and cells within the embryoid body differentiate into multiple cell types. Further, exogenous signaling molecules do not evenly penetrate all the cells within the embryoid body due to its three dimensional nature, creating gradients (Shin et al., 2006). In 2006, Shin and colleagues reported the development of an hES-NEP cell line that grows in adherent monolayers under defined culture conditions (Shin et al., 2006). These hES-NEP cells have several advantages in that they grow under defined conditions in monolayers that can be propagated indefinitely, and retain the ability to differentiate into neuronal or glial lineages. This cell line represents an alternative to the animal models, primary cultures, and transformed cell lines that have been used previously.

In Chapter 3 we establish hES-NEP cells as an *in vitro* model system of lysophospholipid signaling. In this study we demonstrate that hES-NEP cells express functional LPA and S1P receptors coupled to Gi, Gq, and G12-like family G-proteins. Further, both LPA and S1P are shown to enhance proliferation of hES-NEP cells via Gi/o-coupled receptors in an epidermal growth factor (EGF) receptor and ERK1/2-dependent pathway. Additionally, LPA and S1P induce morphological changes through a Rho-associated kinase-dependent pathway. This study confirms that lysophopholipids regulate pathways critical to neural progenitor biology and also

establishes hES-NEP cells as a model system for studying the role of lysophospholipids in neural progenitors.

LPA in Ovarian Cancer

LPA has long been recognized as an important regulator of many of the "hallmarks of cancer" for its ability to regulate cellular proliferation, migration, and survival (Hanahan and Weinberg, 2000; Mills and Moolenaar, 2003). LPA has a demonstrated role in the initiation or progression of ovarian, prostate, breast, melanoma, squamous cell carcinoma of the head and neck, colon, thyroid, and other cancers (Xu et al., 1995b; Eder et al., 2000; Hu et al., 2001; Schulte et al., 2001; Fang et al., 2002; Gschwind et al., 2002; Xie et al., 2002; Shida et al., 2003). LPA receptors are over-expressed in several types of cancer, including colon cancer, ovarian cancer, and prostate cancer (Daaka, 2002; Mills and Moolenaar, 2003; Yang et al., 2005; Lee et al., 2006b). Further, autotaxin, the enzyme primarily responsible for the production of extracellular LPA, is aberrantly expressed in multiple cancers including ovarian cancer, breast cancer, prostate cancer, melanoma, glioma, Hodgkin's lymphoma, and thyroid carcinoma (Stracke et al., 1992; Murata et al., 1994; Umezu-Goto et al., 2002; Kehlen et al., 2004; Baumforth et al., 2005; Kishi et al., 2006; Hoelzinger et al., 2008; Saunders et al., 2008; Gaetano et al., 2009; Liu et al., 2009; Nouh et al., 2009; Zhang et al., 2009). LPA receptors also mediate transactivation of receptor tyrosine kinases, including members of the epidermal growth factor family, which have a well-established role in oncogenesis (van Corven et al., 1989; Cerutis et al., 1997; Daub et al., 1997; Deng et al., 2004; Burgess, 2008). Thus LPA is an important mediator of initiation and progression in multiple forms of cancer.

While LPA has been shown to play a role in multiple types of cancer, it has been particularly linked to ovarian cancer. Ovarian cancer is the fifth leading cause of death from

cancer in women and, after breast cancer, is the second most common gynecological malignancy. In 2008, over 15,000 deaths were attributed to ovarian cancer. Symptoms are largely non-specific and the majority of cases are diagnosed during later stages of the disease, contributing to a low survival rate (Robert S. Porter, 2008). Most ovarian cancer patients present with large volumes of ascites, an accumulation of fluid in the peritoneal cavity that is generated by ovarian cancer cells. The ascitic fluid is rich in growth factors and sufficient to support the growth of ovarian cancer cells (Mills et al., 1988; Mills et al., 1990). In 1995, Xu and colleagues determined that the "ovarian cancer activating factor" in ascitic fluid consisted of multiple forms of LPA (Xu et al., 1995c).

LPA is the predominant growth factor in ovarian cancer cells (Mills et al., 1988; Mills et al., 1990; Mills and Moolenaar, 2003; Umezu-Goto et al., 2004), promoting cell growth (Mills et al., 1988; van Corven et al., 1989; van Corven et al., 1992; Xu et al., 1995a), survival from apoptotic signals (Frankel and Mills, 1996; Levine et al., 1997; Koh et al., 1998; Goetzl et al., 1999b; Weiner and Chun, 1999; Fang et al., 2000b), migration (Sengupta et al., 2003), invasion (Fishman et al., 2001; Sengupta et al., 2007; Wang et al., 2008; Yu et al., 2008; Li et al., 2009), and the production of the growth factors and proteases required for neovascularization and metastasis (Fang et al., 2000a; Sengupta et al., 2007). Ovarian cancer cells constitutively synthesize and release LPA and this production can be stimulated by growth factors such as phorbol esters, laminin and LPA itself (Shen et al., 1998; Eder et al., 2000; Sengupta et al., 2003). Additionally, ovarian cancer cells have higher levels of autotaxin and $sPLA_2$ and lower levels of lipid phosphate phosphatase-1 (LPP-1) compared with non-cancerous cells (Ben-Shlomo et al., 1997; Tanyi et al., 2003a; Tanyi et al., 2003b), thus increasing the amount of LPA available. Peritoneal mesothelial cells also produce LPA, further elevating LPA levels in ascites

and thus promoting growth and spread of the cancer cells (Ren et al., 2006). Finally, decreasing the availability of LPA by over-expressing LPP-3 in ovarian cancer cell lines reduces colony formation, increases apoptosis, and decreases tumor growth both *in vitro* and *in vivo* (Tanyi et al., 2003b). Thus, production and degradation of LPA is aberrant in ovarian cancer cells due to levels and changes in activity of multiple enzymes in the LPA metabolic pathway.

In addition to producing their own LPA, ovarian cancer cells have altered expression of LPA receptors compared to non-transformed cells (Furui et al., 1999; Goetzl et al., 1999a; Fang et al., 2000a). There are currently few specific agonists or antagonists to help determine which LPA receptors are critical to ovarian cancer biology; however, studies comparing the expression of LPA receptors in cancerous and normal tissue and studies utilizing siRNA targeted to specific LPA receptors have helped determine which pathways are important to the initiation and progression of ovarian cancer. The expression of LPA1, LPA2, and LPA3 has been analyzed in studies comparing 3AO, SKOV-3, and OVCAR-3 ovarian cancer cell lines, clinical samples of human ovarian epithelial neoplasms, and normal ovarian epithelial tissue. LPA2 and LPA3 mRNA and protein are consistently expressed at higher levels in ovarian cancer compared with benign tissue. LPA1 is expressed at significantly higher levels in normal ovary than in cancerous ovarian cells (Goetzl et al., 1999a; Fujita et al., 2003; Hu et al., 2003; Wang et al., 2007). Further, expression of LPA2 and LPA3 is correlated with increased ovarian cancer grade (Wang et al., 2007). Further studies will be required to determine the expression profiles of LPA4, LPA5, P2Y5, P2Y10, and GPR87 in ovarian cancer.

Studies using siRNA knockdown or over-expression have demonstrated specific roles for LPA receptors in ovarian cancer cells. LPA2 and LPA3 have been shown to be particularly important in mediating oncogenic pathways in ovarian cancer cells. Studies utilizing siRNA

against specific LPA receptors have shown that LPA2 plays a role in transactivation of the epidermal growth factor receptor (EGFR) and ERK through a Gi G-protein mediated pathway (Jeong et al., 2008). LPA2 and LPA3 also play a role in the production of cytokines, chemokines, and extracellular matrix (ECM) degrading enzymes by ovarian cancer cells. Knockdown of LPA2 and/or LPA3 results in decreased production of interleukin-6 (IL-6), IL-8, vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), urokinase plasminogen activator (uPA), and growth-regulated oncogene α (GROα) (Chou et al., 2005; Lee et al., 2006b; Jeong et al., 2008; Wang et al., 2008; Yu et al., 2008). Knockdown of LPA1, LPA2, or LPA3 also results in an increase of LPA-stimulated migration and invasion (Yu et al., 2008). Over-expression of LPA1, LPA2, or LPA3 enhances LPA-stimulated invasion, migration, and production of IL-6, IL-8, VEGF, and uPA (Sengupta et al., 2003; Fang et al., 2004; Huang et al., 2004; Yu et al., 2008). Conversely, Furui and colleagues showed that over-expression of LPA1 causes an LPA-independent increase in apoptosis and anoikis in A2780 ovarian cancer cells and Jurkat T cells (Furui et al., 1999). In a mouse xenograft model, injection of SKOV-3 ovarian cancer cells over-expressing LPA1, LPA2, or LPA3 results in a greater volume of both primary tumors and ascites compared with mock-transfected SKOV-3 cells (Yu et al., 2008). Further, mice injected with cells over-expressing LPA2 or LPA3 had a decreased survival time (Yu et al., 2008). In addition to changes in receptor expression level, mutations in LPA receptors may also affect responses to LPA in ovarian cancer cells (Contos and Chun, 2000; Huang et al., 2004). These data suggest that changes in LPA receptors are important to ovarian cancer biology.

Additional genetic aberrations in ovarian cancer cells such as amplification or mutation of signaling components downstream of LPA receptors may also contribute to the initiation and progression of ovarian cancer. LPA receptors indirectly activate receptor tyrosine kinases,

including members of the epidermal growth factor (EGF) family which link LPA to mitogenic and survival pathways (Miyamoto et al., 2004; Herrlich et al., 2008; Jeong et al., 2008; Servidei et al., 2008; Kalari et al., 2009). Over-expression of the EGF family member ErbB2/HER2 in epithelial ovarian carcinomas is associated with poor prognosis (Maihle et al., 2002; Serrano-Olvera et al., 2006). Further, LPA and serum have been demonstrated to induce increases in the expression of EGR/ErbB1, ErbB2/HER2, and the EGF receptor family ligands EGF, TGFα, amphiregulin, and heparin-binding EGF (HB-EGF) (Gordon et al., 1995; Deng et al., 2004; Miyamoto et al., 2004). Other downstream mediators, including the phosphatidylinositol-3 kinase/Akt signaling axis, Src, and PKC also have altered expression levels and mutations in ovarian carcinomas and have been shown to sensitize ovarian cancer cells to LPA (Fang et al., 2002; Mills et al., 2002; Umezu-Goto et al., 2004). These alterations trigger feed-forward loops where the increased rate of proliferation leads to even greater mutations which can further enhance growth, survival, and metastasis of ovarian cancer cells (Kobel et al., 2008).

Oncogenesis is a complex process in which the accumulation of multiple independent mutations deregulates the signaling pathways that govern growth, survival, and migration; therefore, understanding the pathways by which ovarian cancer cells mediate these processes is critical to the development of effective therapeutic strategies. The pathways by which LPA exerts its effects in ovarian cancer cells are still not completely delineated. Further, ovarian cancer is a heterogeneous disease with many different signaling pathway alterations occurring in each patient (Shih Ie and Kurman, 2004; Dinh et al., 2008; Kobel et al., 2008). Dozens of ovarian cancer cell lines have been used to study LPA signal transduction cascades; however, little has been done to determine how similar these cell lines are to each other and also how representative they might be of the actual disease state. In Chapter 3 we compare

pharmacological profiles of the signal transduction cascades mediating proliferation in two ovarian cancer cell lines. SKOV-3 and Caov-3 cells are commonly used serous epithelial ovarian cancer cell lines. While both are derived from human ovarian adenocarcinoma, SKOV-3 cells were isolated from malignant ascites, while Caov-3 were derived from a primary tumor site. The two cell lines vary in their tumorigenic properties, particularly in metastatic potential and invasiveness (Gao et al., 2004; Choi et al., 2006; Yao et al., 2007). Our data demonstrate that while both cell lines express LPA1, LPA2, LPA3, and LPA4 and proliferate in response to LPA, they utilize distinct but overlapping combinations of receptors, G-proteins, and signal transduction cascades to mediate LPA-stimulated growth.

Heterotrimeric G-protein activity is an integral component of LPA signaling cascades; therefore proteins that regulate G-protein signaling will play a crucial role in LPA signaling in ovarian cancer cells. The Regulator of G-protein Signaling (RGS) family proteins function to deactivate heterotrimeric G-proteins by accelerating the rate at which they hydrolyze GTP, thus modulating GPCR signaling. RGS proteins have profound effects on the kinetics and magnitude of *in vivo* signaling pathways (Chen et al., 2000; Heximer et al., 2003; Fu et al., 2007). In Chapter 4 we review in detail the role of RGS proteins in cancer biology. RGS proteins are differentially regulated in multiple types of cancer including prostate cancer (Sood et al., 2001; Silva et al., 2003), melanoma (Rangel et al., 2008), renal cell carcinoma (Rae et al., 2000; Furuya et al., 2004), lymphoma (Islam et al., 2003; Han et al., 2006), breast cancer (Smalley et al., 2007a; Wiechec et al., 2008), hepatocellular carcinoma (Chen et al., 2004b; Tsai et al., 2006), thyroid cancer (Tonjes et al., 2004; Nikolova et al., 2008), pancreatic cancer (Hamzah et al., 2008), leukemia (Koga et al., 2004; Schwable et al., 2005), and glioma (Tatenhorst et al., 2004). Further, single nucleotide polymorphisms in RGS encoding genes have been linked to decreased

risk for lung cancer and bladder cancer (Berman et al., 2004; Gu et al., 2006). Specific functions of RGS2, RGS-RhoGEF family RGS proteins, axin, and RGS5 are also discussed.

In Chapter 5, we examine the role of RGS proteins in LPA signaling cascades in ovarian cancer cells. The goal of the study was to determine if endogenous RGS proteins regulated LPA-stimulated Gi/o signaling pathways in SKOV-3 ovarian cancer cells. Gi/o-mediated pathways have previously been implicated regulating EGFR transactivation, MAP kinase activation, proliferation, migration, invasion, and survival (van Corven et al., 1989; Shaw et al., 2002; Moolenaar et al., 2004; Shah et al., 2005; Zhao et al., 2006; Evelyn et al., 2007). We used a well-established mutagenesis strategy to compare signaling activity of Gαi subunits that are wild-type with respect to RGS regulation to Gαi subunits that are insensitive to RGS regulation. LPA signaling in cells that expressed RGS insensitive Gαi subunits was significantly more robust than cells expressing wild-type Gαi subunits assays measuring adenylyl cyclase activity and migration, and, in Chapter 3, proliferation and MAP kinase activation. These data suggest that endogenous RGS proteins normally regulate Gi/o-mediated LPA signaling pathways in SKOV-3 ovarian cancer cells.

In Chapter 6, we examine the expression profiles of RGS proteins in immortalized ovarian surface epithelial (IOSE) cells and SKOV-3, OVCAR-3, and Caov-3 ovarian cancer cell lines. Using reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative PCR, we identify multiple RGS transcripts that could account for the regulation of LPA signaling in SKOV-3 cells as well as two RGS transcripts, RGS4 and RGS6, which are expressed at significantly different levels in non-cancerous and cancerous ovarian cancer cells. Further, we determined the ability of over-expressed RGS2, RGS6, and RGS19 to attenuate LPA signaling of

individually expressed exogenous LPA receptors and endogenous LPA receptors in SKOV-3 and Caov-3 ovarian cancer cells.

Chapter 7 details attempts to identify endogenous RGS proteins that can attenuate or enhance LPA signaling in SKOV-3 ovarian cancer cells. Based on data in Chapter 6 and reports of RGS transcript expression in benign and malignant ovarian tissue in the Oncomine database (www.oncomine.org), we identified RGS proteins that seemed to be likely candidates for regulation of LPA signaling in SKOV-3 cells. The expression of these RGS proteins was manipulated using siRNA to knockdown mRNA transcript expression and transient transfection for over-expression. RGS2, RGS10, RGS12, RGS17, and RGS19 transcripts were reduced by over 50% with siRNA and the effects of RGS knockdown were assessed in assays measuring adenylyl cyclase activity, inositol phosphate accumulation, and migration. None of the RGS proteins tested consistently and significantly altered LPA signaling in SKOV-3 cells, suggesting that multiple RGS proteins may be required.

CHAPTER 2

HUMAN NEURAL PROGENITORS EXPRESS FUNCTIONAL LYSOPHOSPHOLIPID RECEPTORS THAT REGULATE GROWTH AND MORPHOLOGY[1]

[1]Hurst, J.H., Mumaw, J., Machacek, D.W., Sturkie, C., Callihan, P., Stice, S.L., and S.B. Hooks. 2008. *BMC Neuroscience*. 9**:**118

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Abstract

BACKGROUND: Lysophospholipids regulate the morphology and growth of neurons, neural cell lines, and neural progenitors. A stable human neural progenitor cell line is not currently available in which to study the role of lysophospholipids in human neural development. We recently established a stable, adherent human embryonic stem cell-derived neuroepithelial (hES-NEP) cell line which recapitulates morphological and phenotypic features of neural progenitor cells isolated from fetal tissue. The goal of this study was to determine if hES-NEP cells express functional lysophospholipid receptors, and if activation of these receptors mediates cellular responses critical for neural development.

RESULTS: Our results demonstrate that Lysophosphatidic Acid (LPA) and Sphingosine-1-phosphate (S1P) receptors are functionally expressed in hES-NEP cells and are coupled to multiple cellular signaling pathways. We have shown that transcript levels for S1P1 receptor increased significantly in the transition from embryonic stem cell to hES-NEP. hES-NEP cells express LPA and S1P receptors coupled to G i/o G-proteins that inhibit adenylyl cyclase and to G q-like phospholipase C activity. LPA and S1P also induce p44/42 ERK MAP kinase phosphorylation in these cells and stimulate cell proliferation via G i/o coupled receptors in an Epidermal Growth Factor Receptor (EGFR)- and ERK-dependent pathway. In contrast, LPA and S1P stimulate transient cell rounding and aggregation that is independent of EGFR and ERK, but dependent on the Rho effector p160 ROCK.

CONCLUSION: Thus, lysophospholipids regulate neural progenitor growth and morphology through distinct mechanisms. These findings establish human ES cell-derived NEP cells as a model system for studying the role of lysophospholipids in neural progenitors.

Background

We have previously generated a stable neuroepithelial (NEP) cell line derived from human embryonic stem (hES) cells (hES-NEP) that is grown under adherent conditions, is self-renewing, and stably maintains capacity for neuronal or glial differentiation. These hES-NEP cells recapitulate morphological and phenotypic features of neural progenitor cells isolated from fetal tissue (Shin et al., 2006). Such a cell line has potential both as a source for specific neuronal lineages to be used in hES cell neural therapy and as an *in vitro* model system in which to study human NEP cell function and its regulation by signaling mediators such as lysophospholipids. The lysophospholipid signaling mediators Lysophosphatidic Acid (LPA) and Sphingosine-1-phosphate (S1P) are critical regulators of neural development, modulating neural growth, morphogenesis, and differentiation.

Lysophospholipid signaling has been implicated in mediating diverse physiological and pathological responses, including cancer progression, wound healing, angiogenesis, cardiovascular development, and, more recently, neural development (Reviews: (Mills and Moolenaar, 2003; Moolenaar et al., 2004; Chun, 2005; Birgbauer and Chun, 2006)). There is strong evidence that both LPA and S1P are critical in early neural development, as mouse embryos that lack enzymes for S1P or LPA synthesis exhibit severe neural tube defects. Specifically, mice with genetic deletion of sphingosine kinases required for production of S1P developed cranial neural tube defects as a result of increased apoptosis, decreased mitosis and subsequent thinning of the neuroepithelial progenitor cell layer (Mizugishi et al., 2005). These data suggest that S1P mediates anti-apoptotic and pro-growth signaling in normal neuroepithelial development. Similarly, genetic deletion of autotaxin, the enzyme responsible for production of LPA in the brain, yields embryonically lethal mice with neural tube defects. In these embryos,

the neural tube fails to close completely and is kinked (van Meeteren et al., 2006). Further, embryos lacking LPA exhibited asymmetric neural head-fold, reflecting large effusions with high levels of apoptotic cells (Bachner et al., 1999). These studies demonstrate critical and distinct roles of S1P and LPA in early neural development.

LPA and S1P receptors are expressed in neural progenitors, neurons, and oligodendrocytes in the developing and adult brain, and both LPA and S1P are generated by neurons (Fukushima et al., 2000; Birgbauer and Chun, 2006; Kimura et al., 2007). The biological consequences of lysophospholipid signaling in the nervous system are incompletely defined, but evidence for several roles in neural progenitors is emerging. As discussed above, there are clear roles for S1P and LPA in early neural tube development. Further, LPA appears to regulate cortical neurogenesis by promoting morphological changes, survival, and differentiation (Fukushima et al., 2002a; Fukushima et al., 2007). Finally, S1P activity is implicated in mediating migration of neural progenitor cells toward sites of spinal injury (Kimura et al., 2007). Thus, LPA and S1P regulate critical responses in neural progenitor cells that may be exploited to manipulate these cells in traditional pharmacological or cell-based therapeutics.

LPA and S1P bind and activate cell surface G-protein coupled receptors (GPCRs) to regulate cell proliferation, differentiation, and morphological changes, all of which may contribute to their roles in regulating neural progenitor cell function. There are at least five distinct LPA receptors (LPA1-LPA5) and five S1P receptors (S1P1-S1P5) (Anliker and Chun, 2004b). LPA and S1P receptors couple to multiple G-protein pathways to regulate ion channel activity, adenylyl cyclase mediated cyclic AMP (cAMP) production, phospholipase C (PLC) mediated inositol phosphate production and calcium release, activation of the small GTPase Rho, and transactivation of receptor tyrosine kinase receptors (Review: (Ishii et al., 2004)).

Regulation of cell growth and morphology are common effects of lysophospholipids. LPA and S1P have potent proliferative effects in multiple neural cell lines (Daub et al., 1997; Gschwind et al., 2002; Kue et al., 2002). For example, LPA induces proliferation in neurospheres isolated from rat embryonic cortex (Cui and Qiao, 2006), and application of S1P to neural progenitor cells from embryonic rat hippocampus has been shown to stimulate Gi/o pathways which activate Mitogen-Activated Protein (MAP) kinases and DNA synthesis (Harada et al., 2004). The latter observation is consistent with the mechanism for lysophospholipid stimulated proliferation in many cancer cells, in which LPA receptors transactivate the epidermal growth factor receptor (EGFR) pathway, resulting in MAP kinase activation and subsequent proliferation (Daub et al., 1997; Gschwind et al., 2002; Kue et al., 2002).

LPA and S1P also stimulate specific cytoskeletal rearrangements, likely contributing to their roles in axonal pathfinding and migration. Neural cell lines such as NIE-115 cells and PC12 cells undergo rapid and transient neurite retraction in response to LPA and S1P (Jalink et al., 1993a; Sato et al., 1997). LPA induces neurite retraction within minutes, and neurons re-extend neurites after LPA is removed; thus, the retraction is dynamic and may fine tune neurite growth (Fukushima et al., 2002b; Fukushima, 2004). Similar neurite retraction and growth cone collapse occur in response to LPA in differentiating cortical neurons (Fukushima et al., 2002b). Morphological changes also occur in neural progenitor cells, which lack distinct neurites. Both LPA and S1P cause transient aggregation of rat hippocampal neural progenitor cells (Harada et al., 2004), and LPA stimulates cluster contraction, lamellipodia retraction and migration toward the center of the cluster in mouse cortical neuroblasts (Fukushima et al., 2000). LPA stimulates cell rounding of cortical neural progenitors, important in cortical neurogenesis (Fukushima et al., 2000). The mechanisms for these effects is incompletely understood, but in most cases LPA and

S1P induced morphological changes can be partially or completely blocked by pretreatment with inhibitors of the small GTPase Rho or its primary effector in neurons, p160 Rho kinase (ROCK) (Fukushima et al., 2002b; Fukushima, 2004).

The goal of the current study was to define functional lysophospholipid receptor signaling pathways in hES-NEP cells. We have determined that functional LPA and S1P receptors are expressed in hES-NEPs and regulate second messenger pathways, MAP kinase-dependent cell proliferation, and Rho-dependent morphology changes. These results contribute to the molecular characterization of hES-NEP cells, and establish for the first time a human, multipotent, renewable model cell system in which to define the role of LPA and S1P in neural progenitor cell function.

Results

LPA and S1P receptor mRNA transcript expression changes during the transition from ES cells to hES-NEP cells

Expression of transcript encoding all five LPA receptors has been reported in hES cells and in hES cell-derived neurospheres (Dottori et al., 2008), and three S1P receptors (S1P1-3) have also been detected in hES cells (Pebay et al., 2005). As described, the hES-NEP cell line used in this study was derived from the hES cell line, WA09. We performed quantitative RT-PCR to determine expression of transcript of LPA and S1P receptor subtypes in hES-NEP cells, and to determine if receptor expression changed in the transition from embryonic stem cell line to neural epithelial cell line. WA09 ES cells had detectable levels of transcript for all five LPA receptor genes and all five S1P receptor genes; however, in the hESNEP population LPA3 and S1P4 were not expressed at detectable levels after 40 amplifications. Because the RTPCR primer pairs used have been shown to have equivalent amplification efficiency (100% +/- 10%) at the

annealing temperatures used, the relative expression of LPA and S1P receptors can be directly compared within hES-NEP cell RNA. The ΔCT value for each receptor transcript was determined by normalizing with CT values for the endogenous 18s ribosomal RNA. As shown in **Figure 2.1A**, LPA5 receptor transcript expression was significantly lower than LPA1, 2, and 4. Similarly, S1P 1, 2, and 3 transcripts were expressed at significantly higher levels in hES-NEP cells than S1P5. We further determined the fold change in transcript expression of LPA1, 2, 4, and 5 and S1P 1, 2, 3, and 5 in hES-NEP cells relative to their expression in the parent ES cell line WA09. LPA1 receptor transcript expression was increased approximately ten fold while LPA2 expression was decreased approximately five fold in cumulative data representing three experiments, but these changes did not meet criteria for statistical significance. Expression of LPA4 and 5 mRNA transcripts were relatively unchanged between the two populations. S1P1 receptor transcript was dramatically upregulated approximately forty fold in hES-NEP cells relative to the parent ES cell line (**Figure 2.1B**), while significant changes were not observed in expression of S1P 2, 3, and 5 transcript.

NEP cells express functional LPA and S1P receptors

To evaluate expression of GPCRs for LPA and S1P as well as major neurotransmitter classes in hES-NEP cells, we screened agonists of adrenergic, dopamine, muscarinic acetylcholine, LPA, and S1P receptors for activity in assays measuring second messenger production. First, we assessed activity of these compounds in inositol phosphate assays that measure PLC activity. Cells were stimulated with each of the following drugs at a concentration of 10 μM for 30 minutes: clonidine (α2 adrenergic receptor agonist), epinephrine (general adrenergic receptor agonist), quinpirole (D2-like dopamine receptor agonist), bromocriptine (D2-like dopamine receptor agonist), carbachol (general muscarinic acetylcholine receptor agonist), and S1P

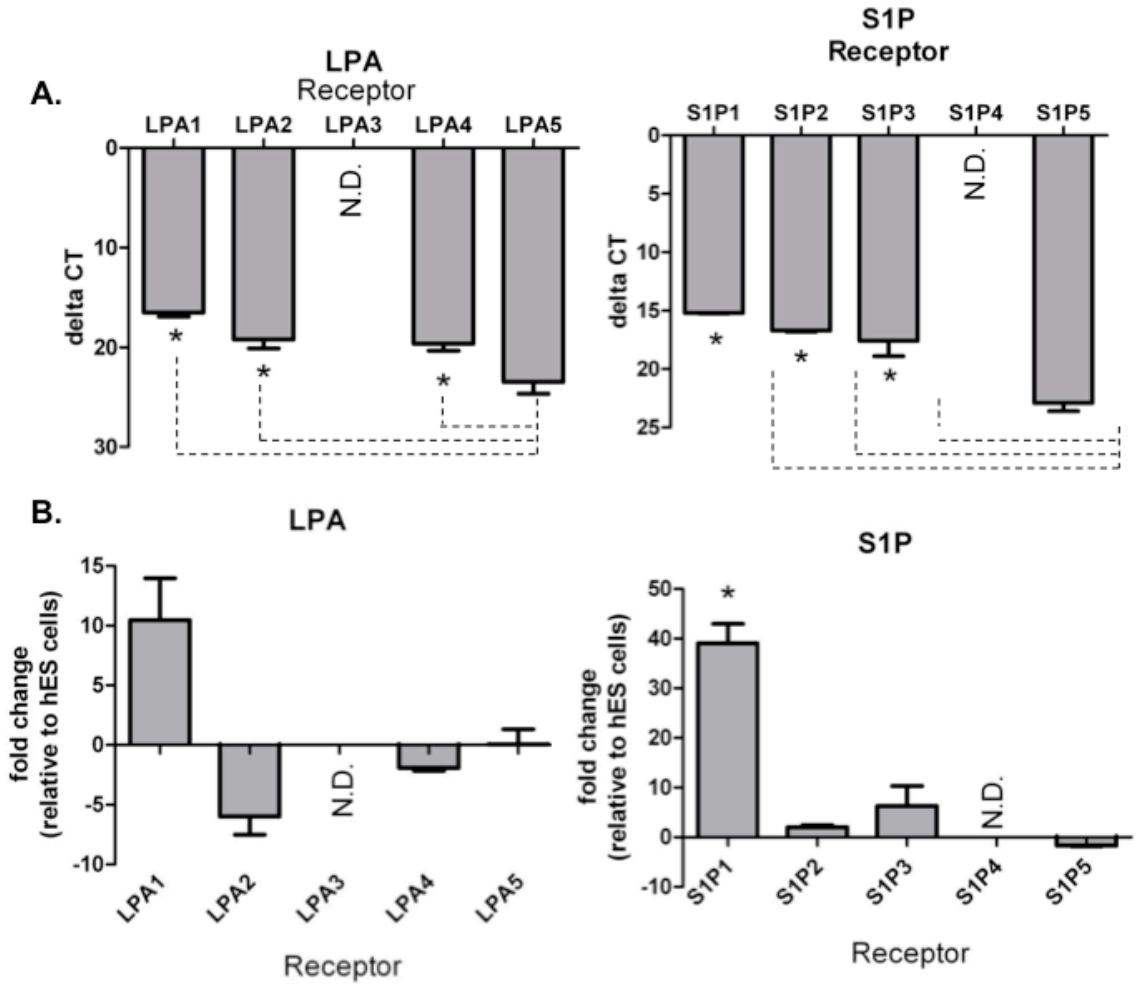

Figure 2.1: LPA and S1P receptor subtype transcript expression in hES-NEP cells.
A Semi-quantitative RT-PCR from hNP cells revealed the relative expression of receptor mRNA. Higher expression is equivalent to a smaller delta CT value (CT value of RNA - CT value of 18s). Statistical significance was measured using a one-way analysis of variance (ANOVA) and Tukey's post- hoc comparisons were used to test the significance between all pairwise comparisons ($p < .05$). Data is represented as the mean ΔCT +/- S.E.M. (CT value of gene - CT value of 18 s) of 3 biological replicates. * in A demonstrates a significant difference (greater expression) than S1P5, * in B demonstrates a significant difference (greater expression) than LPA5. N.D. indicates that the average CT was >35 indicated that the mRNA signal was not detectable. B. Total RNA was isolated from WA09 hES cells and hES-NEP cells, and relative expression of each LPA and S1P receptor transcript was determined using quantitative RT- PCR. Results are reported as fold change in RNA transcript in hES-NEP cells relative to ES cells (details of data analysis in Methods). Data represents three compiled independent experiments and was subjected to ANOVA, tukey post-hoc analysis. Error bars represent standard error; *: $p < 0.0001$.

(general S1P receptor agonist); 18:1 (Oleoyl) LPA (general LPA receptor agonist) was tested at a concentration of 1 μM due to loss of activity at higher concentrations. At these concentrations,

only LPA and S1P stimulated a significant increase in inositol phosphate accumulation compared to vehicle treatment in hES-NEP cells (**Figure 2.2A**). We then generated LPA and S1P dose-response curves in these cells. The EC50 for inositol phosphate accumulation stimulated by either LPA or S1P is approximately 25 nM (**Figure 2.2B, C**). Pre-incubation with 100 ng/mL of the Gi/o selective inhibitor Pertussis toxin (Ptx) for 18 hours did not inhibit S1P stimulated IP accumulation, indicating that this effect is not mediated by Gi/o G-proteins, while Ptx consistently inhibited 30–40% of the LPA stimulated IP accumulation (**Table 2.1, Figure 2.2B, C**). We next determined if hES-NEP cells express functional adrenergic, dopamine, or lysophospholipid receptors coupled to Gs-like increases in cAMP production. hES-NEP cells were treated with the same panel of agonist compounds (although quinpirole, bromocriptine, and carbachol do not activate any known Gs coupled receptors),and none produced a significant increase in cAMP, suggesting there are not functional Gs coupled LPA, S1P, adrenergic, or dopaminergic receptors expressed in hES-NEP cells (data not shown). Finally, the receptor agonists were added to cells following activation of adenylyl cyclase with forskolin to determine if they could decrease cAMP production via Gi/o mediated inhibition of adenylyl cyclase. Adrenergic and dopaminergic receptor agonists had no effect on forskolin-stimulated cAMP levels, and carbachol produced a modest inhibition of cAMP production. In contrast, both LPA and S1P significantly inhibited forskolin-stimulated cAMP accumulation by approximately 50% and 40%, respectively, at 10 μM doses (**Figure 2.3A**). Dose response curves demonstrated that LPA inhibited forskolin-stimulated cAMP accumulation with an EC50 of approximately 10 nM (**Figure 2.3B**), while S1P had an EC50 of approximately 5 nM (**Figure 2.3C**). The activity of both LPA and S1P was completely inhibited by pre-incubation of cells with 100 ng/mL Ptx (**Table 2.1, Figure 2.3B, C**), confirming that this effect is mediated by Gi/o G-proteins.

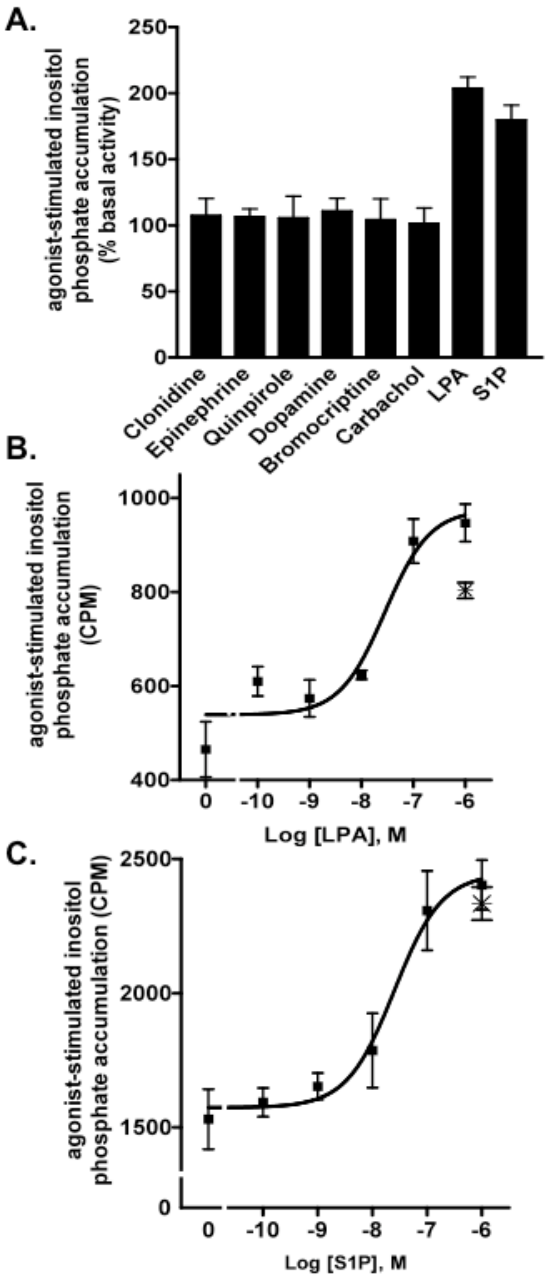

Figure 2.2: hES-NEP cells express functional LPA and S1P receptors coupled to PLC. A) hES-NEP cells were treated with each of the indicated drugs at 10 μM, except for LPA which was assessed at 1 μM, for 30 minutes and assayed for IP levels as described in Methods. Results are reported as percent of basal inositol phosphate accumulation (CPM) with counts for drug treated wells divided by data from vehicle treated wells. B) hES-NEP cells were treated with various concentrations of LPA in the presence (star) or absence (square) of Ptx for 30' and assayed for IP production. C) hES-NEP cells were treated with var- ious concentrations of S1P in the presence (star) or absence (square) of Ptx for 30' and assayed for IP production. Data are consistent with three independent experiments.

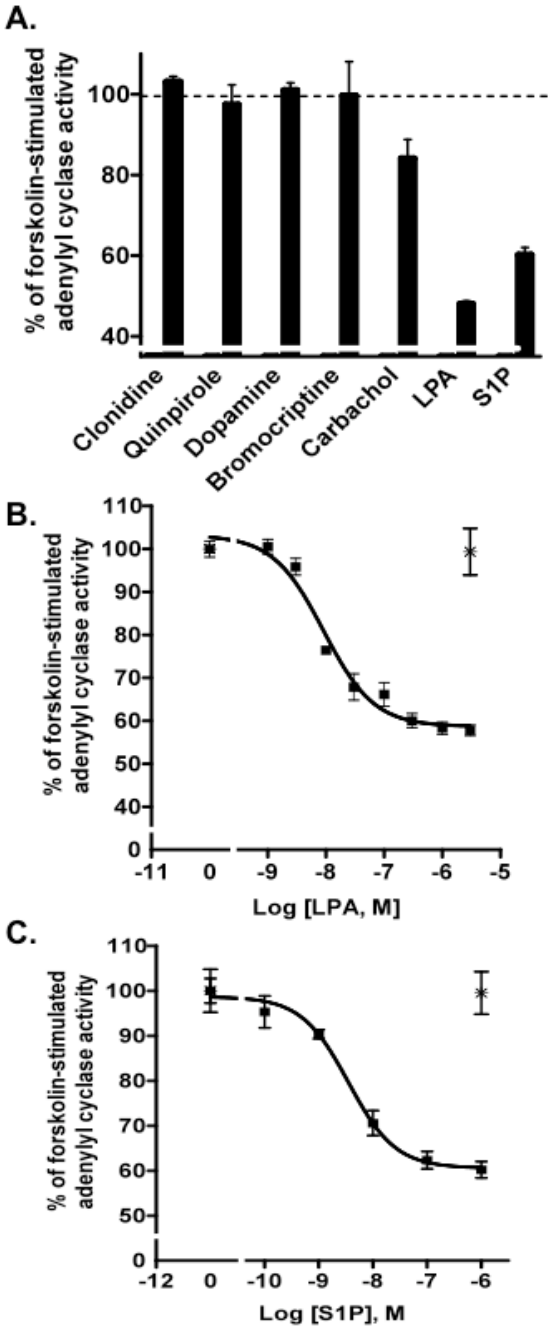

Figure 2.3: hES-NEP cells express functional Gi/o-coupled LPA and S1P receptors.
(A) hES-NEP cells were treated with 50 μM forskolin and 10 μM of each of the indicated drugs for 20', then assayed for cAMP levels as described in Materials and Methods. hES-NEP cells were treated with 50 μM forskolin and various concentrations of LPA (B) or S1P (C) for 20' in the presence (star) or absence (square) of Ptx. Data are consistent with three independent experiments.

LPA and S1P promote growth of hES-NEP cells via Ptx-sensitive G-proteins, EGF receptors, and MAP kinases

To examine the effects of S1P and LPA on cellular growth, we determined the ability of LPA and S1P to stimulate growth of cultured hES-NEP cells over a 36 hour period by determining increases in cell number (**Figure 2.4**). hES-NEP cells were plated in 24-well plates and grown to 50% confluence. Cells were then grown for 36 hours with vehicle, 1 nM, 10 nM, or 100 nM LPA or S1P added to the normal growth media. Cells were not subjected to starve conditions, and therefore continued to grow at a normal basal rate in the absence of added lysophospholipid. Cells under basal growth conditions showed a 60% increase in cell number (increased to 170,000 cells/well at 36 hours, from 108,000 cells/well at time zero). Addition of lysophospholipid resulted in a dose-dependent increase in cell growth from 1 nM to 100 nM LPA (Figure 3.4A) and from 1 nM to 100 nM S1P (**Figure 2.4B**), with S1P showing an apparent higher potency. Cells treated with 100 nM LPA showed a 120% increase in cell number after 36 hours (235,000 cells at 36 hours), and cells treated with 100 nM of S1P showed a similar 130% increase in cell number (252,000 cells at 36 hours), as compared to the 60% increase in control cells. The basal growth rate was approximately linear over the 36 hour experiment (**Figure 2.4B**), and this rate was increased significantly by addition of 100 nM of either LPA (**Figure 2.4B**) or S1P (**Figure 2.4D**) as early as 12 hours. The rate of growth of LPA and S1P treated cells slowed at later time points as these cells approached confluency.

MAP kinases such as p44 and p42 Extracellular signal Regulated Kinases (ERKs) are known to play an important role in neural progenitor cell proliferation (Learish et al., 2000; Li et al., 2001; Deleyrolle et al., 2006), and both LPA and S1P activate the MAP kinase pathway in

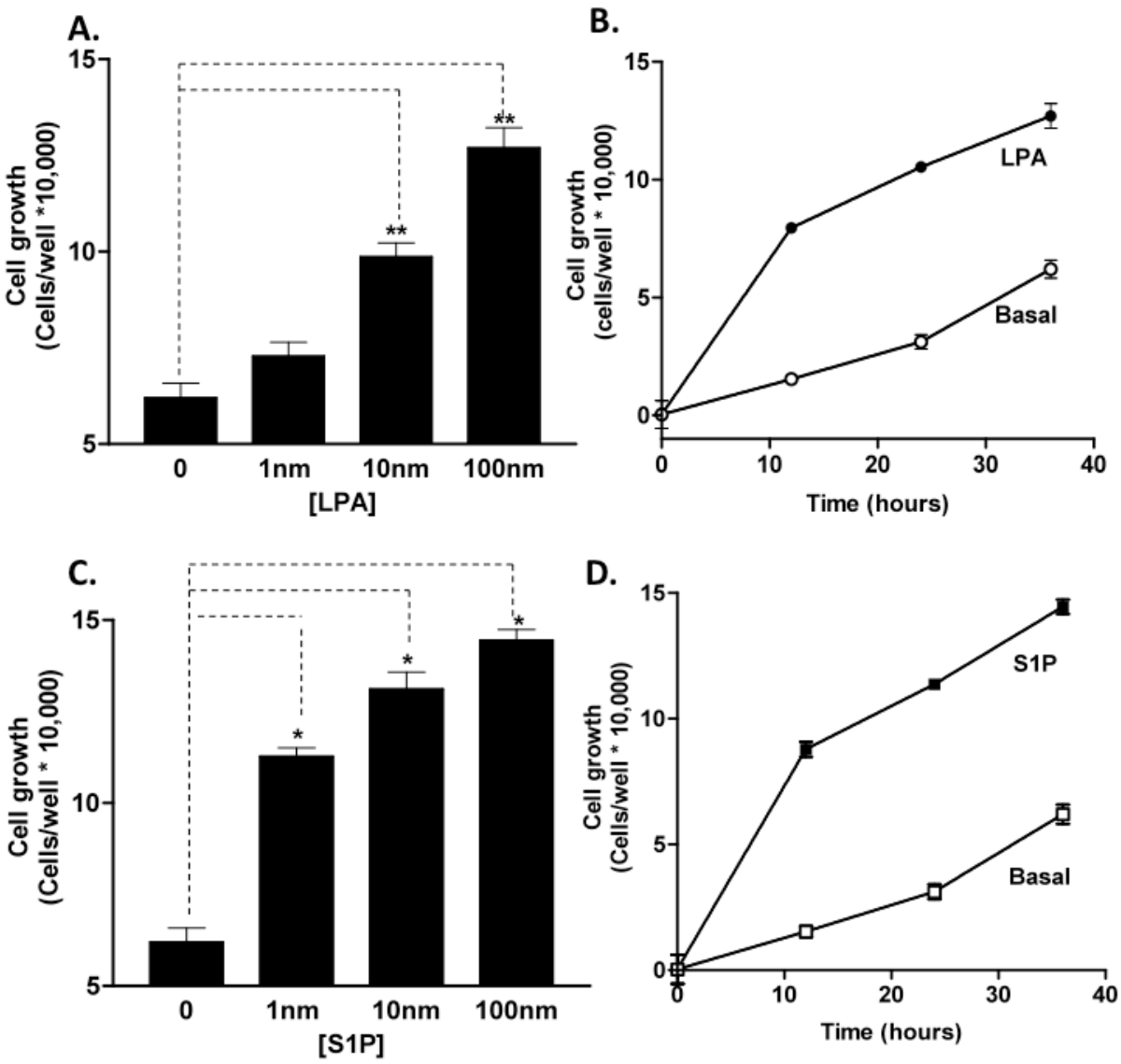

Figure 2.4: LPA and S1P promote growth of hES-NEP cells.
(A, C) hES-NEP cells were plated in 24-well plates and grown to 50% confluence, and the treated with vehicle or 1–100 nM LPA (A) or S1P (C) for 36 hours and then counted. (B, D) Cells were incubated with vehicle or 100 nM LPA (B) or 100 nM S1P (D) and cell growth was determined at 12, 24, and 36 hours. Viable cells were counted and reported as the number of new cells/well. The number of cells at time zero (108,000) has been subtracted from all data shown. Data are representative of two independent experiments analyzed using an unpaired 2-tailed t- test. *: $p < 0.05$; **: $p < 0.01$.

multiple systems (Sato et al., 1999a; Caverzasio et al., 2000; Kim et al., 2000; Van Brocklyn et al., 2002; Harada et al., 2004; Cechin et al., 2005; Kim et al., 2005; Mathieson and Nixon, 2006; Osinde et al., 2007). Further, LPA has been shown to activate MAP kinase pathways through a Gi/o dependent EGF receptor transactivation mechanism (Daub et al., 1997; Gschwind et al., 2002; Kue et al., 2002). To determine which of these pathways is functional in lysophospholipid stimulated growth of hES-NEP cells, the effects of pretreatment with specific pharmacological inhibitors of pathway intermediates were determined: the Gi/o selective inhibitor Ptx (100 ng/mL), the EGF receptor inhibitor AG1478 (2.5 μM), the MAP kinase/ERK Kinase (MEK) inhibitor U0126 (10 μM), the direct ERK inhibitor FR180204 (10 μM), and the p160ROCK inhibitor Y27632 (10 μM). Cells were counted after pre-treatment with inhibitor and again after an 18 hour incubation with LPA (**Figure 2.5B**) or S1P (**Figure 2.5D**). Both LPA and S1P significantly induced increased cell growth over vehicle at this time point. Pre-treatment with Ptx, AG1478, U0126, and FR180204 completely inhibited both basal cell growth and LPA and S1P stimulated growth; however, the p160ROCK inhibitor Y27632 did not significantly affect basal growth or growth stimulated by either LPA or S1P. Further, pre-treatment with the inhibitors did not increase cell staining with Trypan Blue, indicating that these compounds were not cytotoxic at the concentrations used (data not shown). These results suggest that LPA and S1P promote growth of hES-NEP cells through a mechanism dependent on Ptx-sensitive Gi/o G-proteins, EGF receptor, MEK, and ERK, but independent of the Rho-associated kinase p160ROCK.

The data above implicate MAP kinase activation in the ability of LPA and S1P to stimulate cell growth. Thus, we directly tested the ability of LPA and S1P to stimulate phosphorylation of the MAP kinase proteins p44/42 ERK. We performed Western blotting on

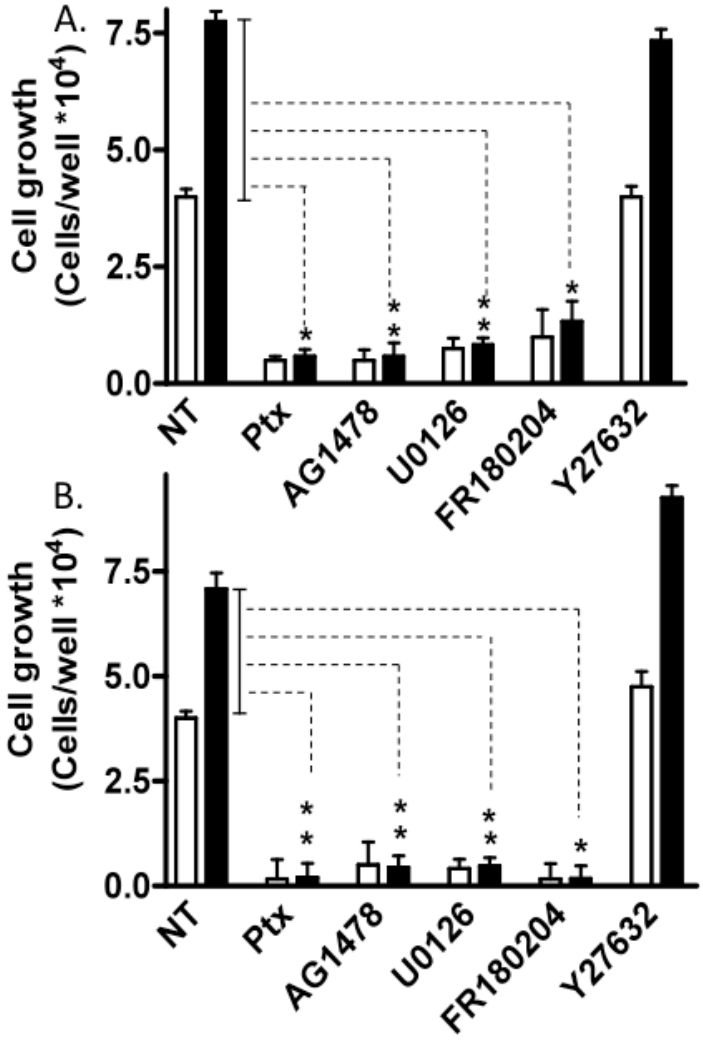

Figure 2.5: LPA and S1P effects of hES-NEP cell growth are mediated by Ptx sensitive G-proteins, EGF receptors, and ERK Map kinases.
hES-NEP cells were pre-treated with 100 ng/mL Ptx, 2.5 μM AG1478, 10 μM U0126, 10 μM FR180204, 10 μM Y27632 or no treatment (NT) overnight and then treated with 100 nM LPA (A), 100 nM S1P (B) or vehicle. Cells were counted after treatment with inhibitors (time zero) and again after incubation with LPA or S1P. Results are reported as the number of new cells per well after LPA or S1P treatment; cell numbers at time zero have been subtracted. Data are representative of two independent experiments analyzed using an unpaired 2-tailed t-test. Comparisons were made between the LPA stimulated fold increase over basal growth in each condition. *: $p < 0.05$; **: $p < 0.01$.

cellular lysates after treating cells with either 1 μM LPA or 100 nM S1P for time points between one and sixty minutes. LPA and S1P each stimulated p44/42 ERK phosphorylation relative to total p44/42 ERK protein, with peak phosphorylation occurring after 5 minutes of stimulation, followed by a later sustained lower level of phosphorylation at 30–60 minutes (**Figure 2.6**). The latter peak was consistently observed in both LPA and S1P treated cells, but did not meet statistical criteria for significance in LPA treated cells.

LPA and S1P induce reversible morphological changes in hES-NEP cells

LPA and S1P mediate morphological changes reflecting cytoskeletal rearrangements in multiple neuronal cell types. We determined the effect of LPA and S1P on hESNEP cell morphology using continuous live cell microscopy. hES-NEP cells were plated and maintained in an environmentally controlled slide incubator system that allows continuous video surveillance of live cells under controlled temperature and atmospheric conditions. After treatment with 1 μM LPA (**Figure 2.7A**) or 100 nM S1P (**Figure 2.7B**), hES-NEP cells became aggregated and rounded, retracting cellular extensions. This morphological change was transient, reaching a peak at approximately 5 hours after treatment and returning to baseline 18 hours after treatment. Addition of vehicle caused no morphological changes under these conditions (data not shown). In contrast to the effects on the proliferative response, overnight pre-treatment of the cells with Ptx, AG1478, or U0126 did not block the ability of LPA (**Figure 2.8A**) or S1P (**Figure 2.8B**) to induce morphological changes, while pre-treatment with Y27632, the inhibitor of p160ROCK, completely prevented cellular aggregation and rounding induced by either lysophospholipid. These data suggest that morphological changes induced by LPA and S1P are mediated by a pathway that does not include Gi/o proteins, EGF receptors, or MEK, but does require the Rho effector p160 ROCK. Notably, Ptx treatment alone caused some cellular aggregation; however,

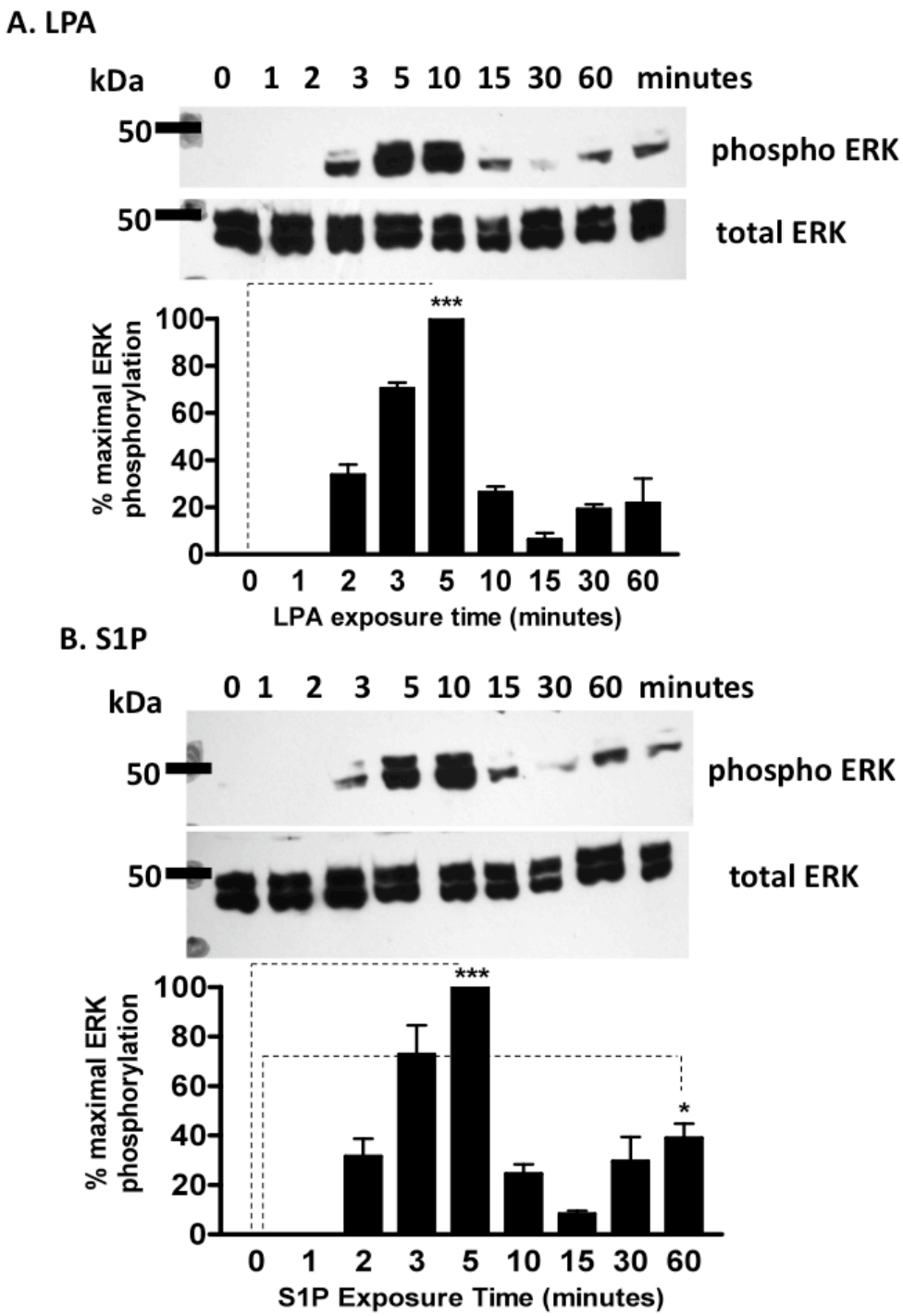

Figure 2.6: LPA and S1P induce ERK phosphorylation in hES-NEP cells.
hES-NEP cells were treated with 1 μM LPA (A) or 100 nM S1P (B) for the indicated amounts of time and then assayed for phosphorylated p44/42 ERK and total p44/42 ERK as described in Materials and Methods. In each case, results are shown as a Western blot image of a representative experiment and a densitometry graph of combined data from two independent experiments analyzed using an unpaired 2-tailed t-test *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

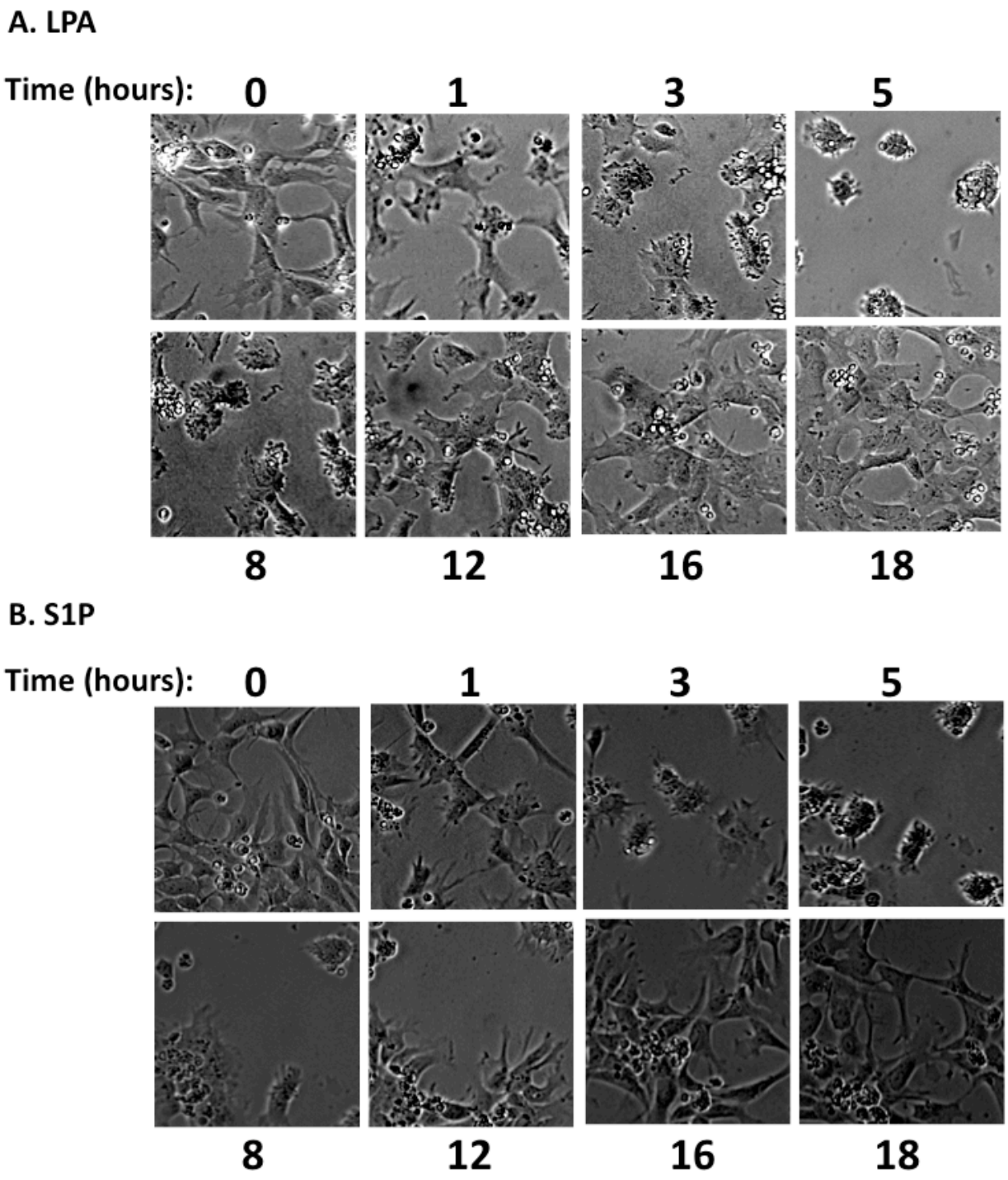

Figure 2.7: LPA and S1P induce reversible morphology changes in hES-NEP cells.
hES-NEP cells were incubated with (A) 1 μM LPA or (B) 100 nM S1P for 18 hours and subjected to continuous video microscopy. Still images of cell morphology changes were recorded as described in Materials and Methods at select time points. Data images are consistent with three independent experiments.

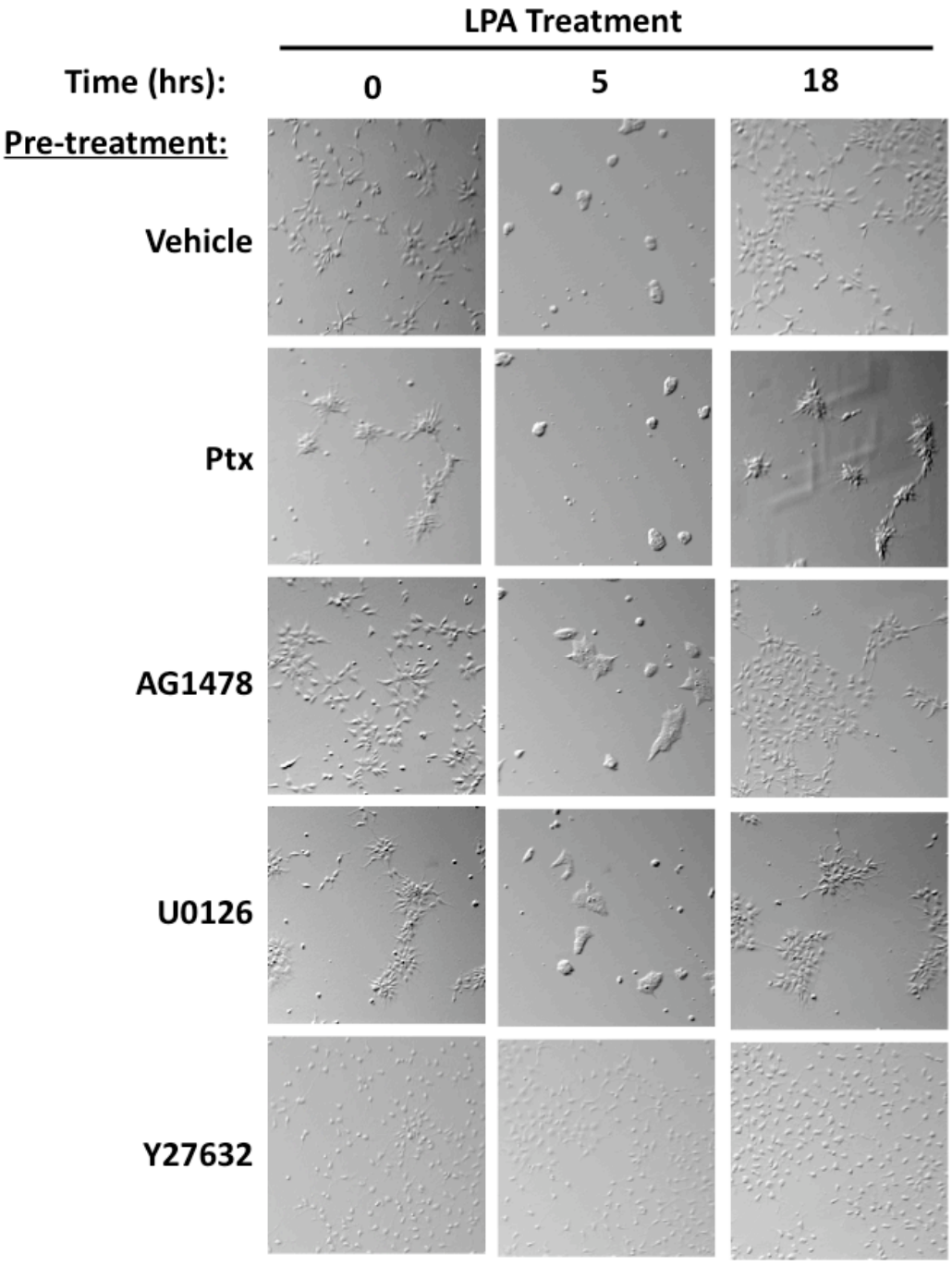

Figure 2.8A: Morphology changes induced by LPA are blocked the p160ROCK inhibitor Y27632. hES-NEP cells were incubated with vehicle, 100 ng/mL Ptx, 2.5 μM AG1478, 10 μM U0126, or 10 μM Y27632 for 18 hours and then treated with 1 μM LPA for 18 hours. Images of cell morphology were captured after treatment with each inhibitor but before addition of LPA (t = 0), after five hours of LPA treatment (t = 5) and after 18 hours of LPA treatment (t = 18). Data images are consistent with three independent experiments.

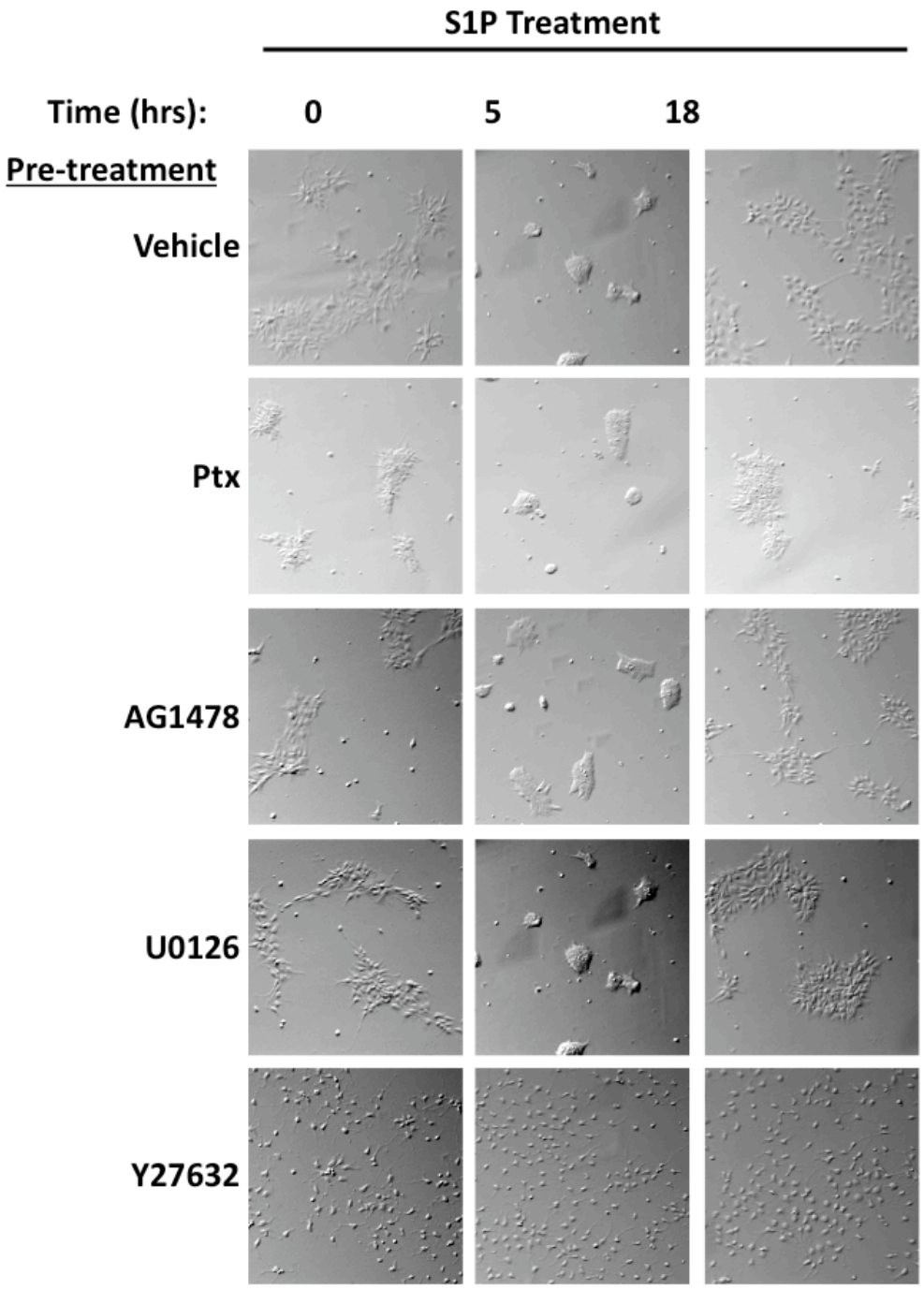

Figure 2.8B: Morphology changes induced by S1P are blocked the p160ROCK inhibitor Y27632. hES-NEP cells were incubated with vehicle, 100 ng/mL Ptx, 2.5 μM AG1478, 10 μM U0126, or 10 μM Y27632 for 18 hours and then treated with 100 nM S1P for 18 hours. Images of cell morphology were captured after treatment with each inhibitor but before addition of S1P (t = 0), after five hours of S1P treatment (t = 5) and after 18 hours of S1P treatment (t = 18). Data images are consistent with three independent experiments.

treatment with either LPA or S1P induced further cell rounding. Further, cells pre-treated with Y27632 had longer, thinner membrane extensions than cells pre-treated with vehicle, consistent with previous observations by Darenfed et al. (Darenfed et al., 2007).

Discussion

Lysophospholipids are hypothesized to be critical regulators of neuronal differentiation, proliferation, and migration during development and following injury. While rodent neural progenitor cells and human transformed cell lines have been used to establish these roles and investigate the pathways responsible, the effects of lysophospholipids in human neural progenitor cells has not been established until now. This study establishes our recently characterized human embryonic neural epithelial progenitor cell line as a valid model system to define the role of LPA and S1P in neural progenitors during human neural development, differentiation, and wound healing.

Our results demonstrate that hES-NEP cells express functional LPA and S1P receptors coupled to Gi/o mediated inhibition of adenylyl cyclase and to a pertussis toxininsensitive PLC pathway, likely mediated by Gq. hES-NEP cells do not express functional Gs coupled receptors for either LPA or S1P. Like the cAMP inhibitory response, the proliferative response was also completely inhibited by Pertussis toxin (Ptx) and is therefore also mediated by Gi/o coupled receptor subtypes. In contrast, the morphological response was not inhibited by Ptx, and so is not mediated by Gi/o coupled receptors. Our data suggest that LPA and S1P morphological responses may be mediated by G12 coupled GPCRs, consistent with the observed Rho dependency, although we cannot rule out a Gq-mediated mechanism. All LPA and S1P receptors except LPA3 and S1P4 were detected in hES-NEP cells. Studies including additional

pharmacologically selective drugs are required to determine the molecular identity of the receptors mediating the observed responses in hES-NEP cells.

Both LPA and S1P stimulate proliferation of many cell types. Studies in multiple cell lines (Daub et al., 1997; Gschwind et al., 2002; Kue et al., 2002; Shah et al., 2005; Zhao et al., 2006) suggest that LPA receptors coupled to Gi/o stimulate cell growth via EGF receptor transactivation and subsequent MAP kinase activation, which directly leads to cell proliferation. While we observed a strong effect of lysophospholipids on cell growth, our data do not distinguish between effects on proliferation versus survival pathways. Future work should directly address the effect of LPA and S1P on apoptosis in these cells. Indeed, LPA does function as a survival factor in many cancer cell types via activation of the PI3 Kinase pathway. Nonetheless, our data are consistent with the proliferative EGF receptor transactivation mechanism described above. The growth responses to LPA and S1P in these cells were completely inhibited by Ptx and inhibitors of EGF receptors and ERK Map kinases, but not by inhibitors of p160 ROCK. Notably, the basal growth of hES-NEP cells was also inhibited by EGFR and MAP kinase inhibitors but not p160 ROCK inhibitor, suggesting that basal growth is mediated by a similar pathway, although not necessarily initiated by LPA or S1P. This also suggests a basal level of ERK MAP kinase activity. Although the data shown in **Figure 2.6** do not show basal ERK phosphorylation due to the short exposure times required to avoid saturation of peak bands for quantification, in longer exposures basal ERK phosphorylation was apparent (data not shown).

The proliferative effect of LPA has been directly demonstrated in rat embryonic neural stem cells (Cui and Qiao, 2006). Cui et al. report a bell-shaped LPA dose response relationship in proliferation assays in which LPA increased thymidine incorporation at concentrations

between 10 nM and 1 μM, but inhibited proliferation at higher concentrations. This biphasic effect of LPA on proliferation is consistent with both our observation that LPA stimulates hES-NEP cell growth between 1 nM and 100 nM, and a recent report in which 10 μM LPA did not stimulate proliferation in human neurospheres (Dottori et al., 2008). Similarly, LPA stimulated production of inositol phosphates reached a maximal level at 1 μM and a reduced activation at higher concentrations.

LPA and S1P effects on morphology of either neurons or neural progenitors are mediated by effects on the actin cytoskeleton and/or microtubules, and effects are typically, but not always, dependent on the small GTPase protein Rho. Rho is known to regulate axonal growth, neuronal differentiation, and neuronal survival, primarily through its well-characterized neuronal effector p160 ROCK (Review: (Schmandke and Strittmatter, 2007)). Rho activation occurs primarily via activation of Rho exchange factors by G proteins of the G12 subfamily, and leads to activation of p160 ROCK which mediates morphological changes by altering cytoskeletal structure. Specifically, p160 ROCK increases actin contractility and stress fiber formation via myosin-II regulatory light chain (MLC) (Schmandke and Strittmatter, 2007) and decreases actin depolymerization via LIM kinases to regulate growth cone collapse (Fukushima, 2004). Alternately, Gi/o pathways can also alter the cytoskeleton through activation of glycogen synthase kinase-3 (GSK-3) (Sayas et al., 2006) or Rac, which promotes cell spreading (Hama et al., 2004; Yanagida et al., 2007).

The effect of LPA on neural cell morphology varies with cell type and distinct morphology changes occur over different time scales. Typically, in neurons or neuronal cell lines that have neurites or growth cones, these retract and cells round in response to LPA within minutes. In NIE-115 and NG108-15 cells, and B103 cells expressing either LPA1 or LPA4, LPA

causes a rapid, transient rounding which initiates at 5 minutes following LPA addition, and cells recover their flattened morphology after 20 minutes, even in the continued presence of LPA (Jalink et al., 1993a; Yanagida et al., 2007). Alternately, in rat hippocampal NP cells both LPA and S1P cause transient aggregation with a maximal response at 3 hours and a return to baseline at 18 hours (Harada et al., 2004; Yanagida et al., 2007). Similarly in B103 cells expressing exogenous LPA4, but not LPA1, LPA stimulated a slow aggregation that peaked at three hours (Yanagida et al., 2007). Like the rapid cell rounding, the slow cell aggregation response is dependent on the Rho effector p160 ROCK, as was the slow cell aggregation observed in this report. In contrast, the known activation time course of p160 Rho kinase is on a scale of minutes, and Rho activation occurs even faster. Thus, even though this response is dependent on Rho/Rho kinase activation, these are not the rate limiting factors in the response. In our experiments, LPA or S1P were added to the media and not washed out throughout the experiment. The long recovery time of shape changes may reflect time course of LPA stability in the media. Consistent with this explanation, when media was changed to remove S1P one hour after addition to cells, morphology changes immediately began to reverse.

Our data clearly implicate Rho-mediated activation of ROCK in mediating LPA and S1P stimulated rounding and aggregation in hES-NEP cells. Inhibition of p160 ROCK completely blocked LPA and S1P stimulated effects, while both phospholipids could still mediate cell aggregation and rounding following inactivation of EGFR, or ERK. Although LPA and S1P still clearly altered cell morphology following treatment with Ptx, Ptx treatment itself induced modest cell aggregation. This effect of Ptx may reflect inhibition of basal Gi/o mediated effects on GSK-3 or Rac as described above.

While the current study describes LPA and S1P effects on proliferation and morphological changes, hES-NEPs are also a promising model cell system in which to study LPA and S1P effects in multiple processes of neural development. There is growing evidence that S1P and LPA regulate neuronal differentiation; however, data from various models report contradictory effects (Fukushima et al., 2000; Harada et al., 2004; Pebay et al., 2005; Cui and Qiao, 2006; Dottori et al., 2008). For example, LPA is reported to increase neuronal differentiation of rat neural progenitors (NP) (Fukushima et al., 2000; Cui and Qiao, 2006) and mouse neurosphere cultures (Fukushima et al., 2000), while more recently LPA was shown to inhibit neuronal differentiation of human ES cell-derived neurosphere cultures (Dottori et al., 2008). These contradictions may reflect bona fide differences in LPA signaling pathways in rodent versus human neural differentiation, or they may be a result of mixed cell populations and the various sources and developmental stages from which the neural stem cells were isolated. For example, significant differences in expression of FGF, Wnt and LIF pathway genes are observed between human neural stem cells derived from hES cells and fetal neural stem cells (Shin et al., 2007). Given these potential differences between neural stem cells from different cell sources, homogeneous multipotent human ES cell-derived neuroepithelial (hES-NEP) cells may be a superior model system in which to elucidate the roles of LPA and S1P cell signaling pathways in neural progenitor cells. Future studies of LPA and S1P effects on differentiation in the homogenous hES-NEP cell system will serve to clarify the effect of lysophospholipids on human neural differentiation.

Conclusion

We have defined LPA and S1P signaling pathways in hES-NEP cells that promote cellular growth and morphological changes by distinct mechanisms. This cell system is superior

to rodent and transformed cell systems in which LPA and S1P effects have been defined by virtue of its human origin, multi-potent status, and non-transformed state. Further, as a stable, homogeneous, adherent, renewable cell line, hES-NEP cells are a convenient model system for future studies defining the functional role of lysophospholipids in proliferation, differentiation, and migration in the developmentally important human neural progenitor cell type.

Methods

Materials

Carbachol, epinephrine, quinpirole, clonidine, bromocriptine, dopamine, and U0126 were purchased from Sigma-Aldrich (St. Louis, MO). Y27632 and AG1478 were purchased from Tocris Bioscience (Ellisville, Missouri). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA) and FR180204 from EMD Biosciences (La Jolla, CA). Oleoyl (18:1) LPA and D-erythrosphingosine-1-phosphate were from Avanti Polar Lipids (Alabaster, AL).

Cell Culture

Commercially available stocks of hES-NEP cells [available as ENStem-A™ (Millipore, Temecula, CA)] were used. These cells were derived from WA09 human ES cells and maintained as described previously (Shin et al., 2006). Briefly, cells were grown on poly-ornithine (20 μg/mL)/laminin (5 μg/mL) (Sigma-Aldrich St. Louis, MO) coated plates in ENStem-A™ Neural Expansion Medium with 2 mM L-Glutamine and 20 ng/mL b-FGF (all from Millipore, Temecula, CA). Cells were passaged approximately every 48 hours and split 1:2 following manual dissociation by trituration. WA09 (WiCel) were cultured in Dulbecco's minimal essential medium/Ham's F12 medium (DMEM/F12), 2 mM L-glutamine, 0.1 mM minimal essential medium (MEM) nonessential amino acids, 50 U/ml penicillin, 50 μg/ml

streptomycin (all from Invitrogen), 4 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN) and 20% KSR (Invitrogen). Cells were cultured on mitomycin-C (Sigma Chemical Co., St. Louis, MO) mitotically inactivated murine embryonic fibroblasts, manually dissociated, and passaged to new feeder layers every 4–5 days (Mitalipova et al., 2003).

Real Time Reverse Transcriptase PCR

RNA was extracted using Qiashredder and RNeasy kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA quality and quantity was verified using a RNA 600 Nano Assay and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc., Foster City, CA) according to manufacturer's protocols. Quantitative RT-PCR (Taqman) assays were chosen for the transcripts from a pre-validated library of human specific QPCR assays, and incorporated into a 384-well Micro-Fluidics Cards. Relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems Inc., Foster City, CA). Expression data for each LPA or S1P receptor was first normalized against endogenous 18S ribosomal RNA within each cDNA, and then the relative expression in hES-NEP was compared to hES cells using the ΔΔCT method of quantification in SDS software (Applied BIosystems Inc., Foster City, CA). Relative fold changes were determined as RQ values for positive changes and -1/RQ values for negative fold changes. ANOVA statistical analysis was performed using Tukey post-hoc analysis.

Inositol Phosphate Assay

Production of Inositol Phosphates (IP) was quantified using established protocols (Hepler et al., 1987). Briefly: To measure IP production by PLC activation, hES-NEP cells were plated in 24-well dishes at ~80% confluency. Cells were labeled with 1 μCi/well [^{3}H] myo-inositol

(American Radiolabeled Chemicals, St. Louis, MO) for 18 hours to label the cellular pool of phosphatidyl inositol. The cells were treated with Oleoyl (18:1) LPA (Avanti Polar Lipids, Alabaster, AL) or D-erythro-sphingosine-1-phosphate (Avanti Polar Lipids, Alabaster, AL) in the presence of 10 mM lithium chloride to inhibit degradation of inositol phosphates for 30 minutes at 37°C. Cells were then lysed in cold formic acid and neutralized with ammonium hydroxide, and the lysates were then loaded onto columns of AG 1-X8 anion exchange resin (Biorad, Hercules, California). The columns were washed with water and dilute ammonium formate to remove unhydrolyzed lipids. The [^{3}H] IPs were then eluted with 1.2 M ammonium formate/0.1 M formic acid, and added to scintillation cocktail for counting. In some experiments, cells were treated with 100 ng/mL pertussis toxin (Ptx) for 18 hours prior to IP assay.

cAMP Assay

We used a modified version established protocols (Hettinger-Smith et al., 1996). hES-NEP cells were plated in 12-well dishes and labeled with 0.6 μCi [^{3}H]-adenine (Perkin Elmer, Waltham, MA) for three hours in the presence or absence of 200 ng/mL Ptx. Assay buffer containing 1 mM isobutylmethylxanthine (IBMX), 50 μM forskolin, and varying concentrations of LPA was added to the cells for 20 minutes at 37°C. Reactions were terminated by aspiration followed by the addition of stop solution containing 1.3 mM cAMP and 2% sodium dodecyl sulfate. [^{14}C]-cAMP stock was added to each well to control for recovery of cAMP, followed by perchloric acid to lyse cells. Lysates were neutralized with KOH and cAMP was isolated using sequential column chromatography over Dowex AG-50-W4 cationic exchange resin (Bio-Rad, Hercules, CA) followed by neutral alumina columns. The resulting eluate was subjected to scintillation counting after the addition of scintillation cocktail.

Cellular Growth

hES-NEP cells were plated in 24-well plates at 50,000 cells per well and grown to reach 50% confluency (approximately 100,000 cells/well). In some experiments, cells were pre-treated with the indicated reagents for 18 hours, triturated to remove them from the plate, and counted using a hemacytometer to determine the number of cells per well. Cells were then treated with LPA, S1P, or vehicle for the indicated amount of time and counted again. Trypan blue exclusion was used to determine cell viability following drug treatment (0.4% (wt/vol) solution of Trypan Blue (Invitrogen, Carlsbad, CA)). Statistical significance of changes in growth was determined using an unpaired, two-tailed T-test.

p44/42 ERK MAP Kinase Phosphorylation

hES-NEP cells were plated in 24-well plates. Prior to the assay, cells were washed one time with ENStem-A™ Neural Expansion Media and allowed to incubate in 250 μL media for 15 minutes at 37°C. LPA or S1P was then applied to the cells for the indicated period of time. The reaction was terminated by aspirating the media and adding 100 μL protein sample buffer. Cells were harvested and lysed in protein sample buffer, separated by SDSPAGE, transferred to nitrocellulose membranes, and immunoblotted using a primary antibody targeted against phospho-ERK or total ERK (Cell Signaling Technology, Danvers, MA) and peroxidase-conjugated secondary antibodies (Bethyl Laboratories, Montgomery, TX). Bands were then visualized using SuperSignal Chemiluminescent substrate (Pierce, Rockford, IL). Densitometry analysis was performed using Total Lab 1D Gel Analysis software. Background bands were not subtracted out and all lanes and bandwidths were of equal size. Densitometry results for phospho-ERK were normalized to total ERK to control for loading, and then normalized to maximal ERK phosphorylation to compare between experiments. Statistical significance of

increases in ERK phosphorylation over basal levels was determined using an unpaired, two-tailed T-test.

Cell Morphology Studies

Continuous video microscopy of hES-NEP cells was performed using the WaferGen Smart Slide System (Wafer- Gen, Incorporated, Freemont, CA). hES-NEP cells were plated on a WaferGen Smart Slide 100 and maintained at 37°C, with the lid at 39°C to prevent condensation. CO_2 was maintained at 5% over the course of the experiment, and negative flow was maintained through systemic purging every two minutes. Images were obtained using a Nikon Eclipse TE2000-S microscope, and captured every two minutes using a Retiga 2000R Fast 1394 camera (QImaging, Canada). Data were processed using Image Pro Plus5.1 version 5.1.0.20 (Media Cybernetics, Inc., Bethesda, MD). To study the effects of pharmacological inhibitors on LPA and S1P stimulated changes in morphology, hES-NEP cells were plated in 6-well plates. Three areas with approximately equal cell densities were identified in each well and an image of each of these areas was captured with a Nikon AZ100 microscope mounted with a Nikon Digital Sight DS-QiMc camera set at 16× magnification. Cells were pre-treated with the indicated compounds for 18 hours. LPA or S1P was then applied for an additional 18 hours. Images of the cells were captured in triplicate after pre-treatment, approximately 5 hours after application of LPA or S1P, and then again 13 hours later.

CHAPTER 3

LYSOPHOSPHATIDIC ACID STIMULATES CELL GROWTH BY DIFFERENT MECHANISMS IN SKOV-3 AND CAOV-3 OVARIAN CANCER CELLS: DISTINCT ROLES FOR Gi AND RHO-DEPENDENT PATHWAYS[1]

[1]Hurst, J.H. and S.B. Hooks. 2009. *Pharmacology*, 2009; 83 (6): 333-47.
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Abstract

BACKGROUND/AIMS: Lysophosphatidic acid (LPA) is an autocrine growth signal critical to the initiation and progression of ovarian cancer. In the current study, we investigated the receptors and signaling cascades responsible for mediating LPA-stimulated cell growth in SKOV-3 and Caov-3 ovarian cancer cell lines.

METHODS: Pharmacological inhibitors of distinct LPA and epidermal growth factor receptors, G proteins and kinases were tested for their effect on LPA-stimulated cell growth, MAP kinase activation and Akt activation in SKOV-3 and Caov-3 cells.

RESULTS: Distinct agonist pharmacological profiles were observed. Saturated and unsaturated LPA species were equally potent in Caov-3 cells, while saturated LPA was less potent than unsaturated LPA in SKOV-3 cells. Further, the LPA1/LPA3 receptor antagonist Ki16425 was more potent in SKOV-3 cells. The effect of LPA on cell growth in both cell lines was dependent on phosphatidylinositol-3 kinases and MAP kinases. However, LPA-stimulated SKOV-3 cell growth required Gi G proteins, while Caov-3 cell growth was dependent on the Rho effector p160 Rho kinase. Finally, we demonstrated that regulator of G protein signaling proteins significantly regulated Gi-dependent LPA-stimulated cell growth in SKOV-3 cells.

CONCLUSIONS: LPA-stimulated cell growth is mediated by distinct but overlapping receptors and signaling pathways in these two model ovarian cancer cell lines.

Introduction

Ovarian cancer is expected to kill over 15,000 women in the US in 2008, making it the most lethal gynecologic cancer and the fifth most lethal cancer overall among women. Less than half of ovarian cancer patients survive five years past their initial diagnosis, due to typically late stage detection of the disease (www.cancer.org). Recent advances in surgical procedures and cytotoxic chemotherapy regimens have had little impact on overall survival (Ashouri and Garcia, 2007); thus, novel therapies that target the signaling pathways responsible for the aberrant growth, migration, and invasion of ovarian cancer cells are needed to dramatically improve patient prognosis. Ovarian cancer has not been linked to a genetic defect in any single protein or pathway. Rather, ovarian cancer is a heterogeneous diseases with subpopulations of patients differentially expressing mutations or gene amplifications in multiple signaling pathways (Dinh et al., 2008), including growth pathways downstream of lysophosphatidic acid (LPA) G-protein coupled receptors and Epidermal Growth factor (EGF) receptor tyrosine kinases, both of which have been explicitly implicated in mediating ovarian cancer initiation and progression (Umezu-Goto et al., 2004; Lafky et al., 2008).

LPA is an autocrine growth signal that is produced by ovarian cancer cells and accumulates to high micromolar concentration in malignant ascites. LPA activates a family of at least five G-protein coupled receptors (GPCRs) to stimulate growth, migration, invasion and survival of ovarian cancer cells (Umezu-Goto et al., 2004). LPA also mediates constitutive and stimulated cleavage and release of heparin bound epidermal growth factor (HB-EGF) via matrix metalloproteinase (MMP) activation, leading to EGF receptor (EGFR) trans-activation and subsequent MAPK activation (Miyamoto et al., 2004). Indeed, LPA stimulated ovarian cancer cell growth has been suggested to require Protein kinase C (PKC)- and MMP-dependent trans-

activation of EGF receptors (van Corven et al., 1989). Alternately, LPA regulation of phosphotidylinositol-3 kinase (PI3K)/Akt survival pathways have also been implicated as mediators of LPA effects on cell growth (Kerbel et al., 2001; Lizcano and Alessi, 2002; Giannini et al., 2003; Lizcano et al., 2003; Wang et al., 2003; El Sheikh et al., 2004). Various components of the potential pathways linking LPA to ovarian cancer cell growth are mutated or amplified in subsets of ovarian cancers and ovarian cancer cell lines. For example, regulators of the PI3K/Akt pathway are amplified or mutated in many ovarian cancer and may correlate with enhanced cell survival (Yuan et al., 2000; Altomare et al., 2004), expression of PKC, which is known to regulate apoptosis, proliferation, and metastasis, correlates with poor prognosis in ovarian cancer (Weichert et al., 2003; Hofmann, 2004), and MMPs are mutated in approximately 40% of ovarian cancers (Landen et al., 2008). Similarly, the enzyme autotoxin that generates LPA is upregulated dramatically in ovarian cancer (Umezu-Goto et al., 2004), and LPA receptors LPA2 and LPA3 are upregulated in 15-50% of ovarian cancers (Fang et al., 2002). Further, expression of the EGF ligand HB-EGF is significantly higher in ovarian cancer patients (Miyamoto et al., 2004), as well as the EGF receptors EGFR/HER1 and ErbB2/HER2, which are over-expressed in 35-70% and 20-30% of primary ovarian cancers, respectively (Scambia et al., 1992; Lafky et al., 2008). Over-expression of either EGF ligand or receptors correlates with poor prognosis. Whether directly activated by EGF or indirectly activated by LPA receptors, EGFR pathways are clearly significant in ovarian cancer, and inhibitors of this pathway are now showing promise in clinical trials (Dinh et al., 2008). However, it is unknown if they are involved in all proliferative responses in ovarian cancer, or if they may only be relevant in a subset of patients, as is the HER2 receptor in breast cancer (Cobleigh et al., 2005; Asgeirsson et al., 2007; Somlo et al., 2008).

Surprisingly, a detailed pharmacological comparison between LPA stimulated growth responses in commonly used ovarian cancer cell lines has not been performed. SKOV-3 and Caov-3 are widely used serous epithelial ovarian cancer cell lines. While SKOV-3 and Caov-3 were both derived from human ovarian adenocarcinomas, SKOV-3 cells were derived from malignant ascites, while Caov-3 cells were derived from a primary adenocarcinoma. These cell lines vary with respect to tumorigenic properties, metastatic potential and invasiveness (Choi et al., 2006). For example, Caov-3 cells form colonies in soft agar and foci in culture to a greater extent than SKOV-3 cells (Yao et al., 2007), and SKOV-3 cells are more invasive than Caov-3 (Choi et al., 2006), but have a lower metastatic potential (Gao et al., 2004). Both SKOV-3 and Caov-3 cells express LPA1, LPA2, LPA3 and LPA4 receptors (Ptaszynska et al., 2008), and both cell types exhibit LPA stimulated cell growth (Hashimoto et al., 2005; Yu et al., 2008), but the pathways linking LPA receptors to the growth response are undefined.

Understanding differences in LPA signaling pathways in SKOV-3 and Caov-3 cells may help explain the differences in behavior of these two cell types in animal models of ovarian cancer. The goal of this study was to compare the pathways required for LPA stimulated growth of SKOV-3 and Caov-3 ovarian cancer cell lines. In this report we present evidence for distinct but overlapping receptors, G-proteins and signal transduction cascades required for LPA-stimulated cell growth in these two ovarian cancer model cell lines.

Materials and Methods

Materials

1-Oleoyl-2-Hydroxy-*sn*-Glycero-3-Phosphate (18:1 LPA), 1-Palmitoyl-2-Hydroxy-*sn*-Glycero-3-Phosphate (16:0 LPA), and 1-Arachidonyl-2-Hydroxy-*sn*-Glycero-3-Phosphate (20:4 LPA) were purchased from Avanti Polar Lipids (Alabaster, AL). Each LPA isoform was

solubilized at a concentration of 10 mM in sterile water containing 1% fatty acid free bovine serum albumin (Sigma Aldrich, St. Louis, MO). Pertussis toxin (Ptx) was from List Biological Laboratories (Ontario, CN). Ki16425 and U0126 were purchased from Sigma Aldrich (St. Louis, MO). LY294002, Y27632, GF109203X, AG825, and AG1478 were purchased from Tocris Bioscience (Ellisville, MO). Human recombinant epidermal growth factor (EGF), FR180204, and GM6001 were from EMD Chemicals, Inc. (Gibbstown, NJ).

Cell Culture

SKOV-3 and Caov-3 human ovarian cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown according to ATCC recommendations. SKOV-3 (HTB-77) cells were maintained in McCoy's 5A Modified medium with 1.5 MM L-glutamine, 2.2 g/L sodium bicarbonate, and 10% fetal bovine serum. Caov-3 (HTB-75) cells were maintained in Dulbecco's Modified Eagle's medium with 10% fetal bovine serum. Both cell lines were incubated at 37 °C in the presence of 5% CO2.

Cell Growth Assay

SKOV-3 or Caov-3 cells were plated in 24-well plates at 40,000 cells/well or 80,000 cells/well, respectively. Twenty-four hours after plating, cells were starved in serum-free media for 18 hours in the presence or absence of inhibitors. Various concentrations of LPA or vehicle were added to the cultures and the cells were incubated at 37 °C for 48 hours. Media containing LPA and/or inhibitors was renewed every 24 hours. Cell number was determined immediately after cells were starved and again after LPA treatment. Cells were removed from the plate by incubating with a 0.1% trypsin/0.04% EDTA solution (Sigma Aldrich) for 15 minutes and resuspended in 750 μL media. The resuspended cells were counted using a hemacytometer. Results were reported as number of cells per milliliter.

p44/42 MAP Kinase/ERK Activation Assay

Cells were plated in 24-well plates and starved 18 hours in serum-free media in the presence or absence of inhibitors. At the time of the assay, either vehicle or LPA was added to the cells which were then incubated at 37 °C for 5 minutes. The reaction was terminated by aspirating the media and harvesting the cells in protein sample buffer. Samples were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted using primary antibodies targeted against phospho-ERK and total ERK (Cell Signaling Technologies, Bedford, MA) and peroxidase-conjugated secondary antibodies (Bethyl Laboratories, Montgomery, TX). Bands were then visualized using Supersignal Chemiluminescent substrate (Pierce, Rockford, IL). Images of the immunoblots were collected using the Alpha Innotech FluorChem™ SP camera system. Densitometry analysis was performed using AlphaEase gel analysis software. Background bands were not subtracted out and all lanes and bandwidths were of equal size.

Akt Activation Assay

Cells were plated in 24-well plates and starved 18 hours in serum-free media in the presence or absence of inhibitors. At the time of the assay, either vehicle or LPA was added to the cells which were then incubated at 37 °C for 5 minutes. The reaction was terminated by aspirating the media and harvesting the cells in protein sample buffer. Samples were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted using primary antibodies targeted against phospho-Akt and GAPDH (Cell Signaling Technologies, Bedford, MA) and peroxidase-conjugated secondary antibodies (Bethyl Laboratories, Montgomery, TX). Bands were then visualized using Supersignal Chemiluminescent substrate (Pierce, Rockford, IL). Images of the immunoblots were collected using the Alpha Innotech FluorChem™ SP

camera system. Densitometry analysis was performed using AlphaEase gel analysis software. Background bands were not subtracted out and all lanes and bandwidths were of equal size.

DNA Constructs and Transfections

Plasmid encoding C352IGαi2 was obtained from UMR cDNA Resource Center (Rolla,MO). RGS-insensitive Gα subunitswere generated with site-directed mutagenesis using a Stratagene QuikChange Site-Directed Mutagenesis System kit according to manufacturers instructions (LaJolla, CA). The G184S mutation was introduced into the Gαi2 C352I construct sequence as previously described (Lan et al., 1998) with the following primers: sense 5′-GTAAAGACCACGAGCATCGTGGAGACA-3′ and antisense 5′-TGTCTCCACGATGCTCGTGGTCTTTAC-3′. Transfections were performed using Fugene 6 transfection reagent according to the manufacturer's instructions at a ratio of 2 μL Fugene 6 reagent to 1 μg plasmid DNA (Roche Diagnostics, Basel, Switzerland). Cells were transfected with up to a total 500 ng/well in 24-well plates. All assays were performed 24-72 hours post-transfection.

Statistics

All statistics were performed using Graphpad Prism software. Statistical significance was determined using a two-tailed unpaired t-test on either raw or normalized data as indicated. Statistical significance is indicated where $P<0.05$ (*), $P<0.005$ (**), and $P<0.0005$ (***).

Results

Pharmacology of LPA stimulated cell growth

SKOV-3 and Caov-3 cells express multiple LPA receptors, and each of these receptors can couple to multiple G-proteins and downstream signaling pathways that have been implicated in various growth-promoting pathways. While completely specific agonists and antagonists are not

yet available for these receptor subtypes, several compounds have been described that have higher affinity for certain receptors. To determine if the receptors responsible for LPA mediated cell growth are the same in Caov-3 and SKOV-3 cells, we determined the potency of three LPA species. 18:1. 20:4, and 16:0 LPA, in cell growth assays (**Figure 3.1 A, B**). While LPA1 and LPA2 have broad specificity among these LPA species, LPA species containing unsaturated fatty acids have been shown to be significantly more potent at LPA3 receptors than LPA containing saturated fatty acids such as 16:0 (Bandoh et al., 1999; Im et al., 2000b). Under normal growth conditions in complete media, SKOV-3 and Caov-3 cells have a doubling time of approximately 36 hours and 48 hours, respectively. Under serum starve conditions growth slows dramatically in both cell lines, with SKOV-3 cells displaying a higher basal growth rate (doubling rate of 72 hours) than Caov-3 cells (data not shown). All three LPA species elicited a strong dose-dependent increase in cell number in both Caov-3 cells (**Figure 3.1B**) and SKOV-3 cells (**Figure 3.1A**) under serum starve conditions. However, while saturated and unsaturated LPA species had nearly identical potencies of approximately 20 nM in Caov-3 cells, the potency of 16:0 LPA was approximately 10 fold lower than the unsaturated LPA species in SKOV-3 cells (**Figure 3.1, Table 3.1**). This suggested that LPA3 receptors play a more significant role in mediating LPA stimulated cell growth in SKOV-3 cells than in Caov-3 cells. To further characterize the receptors responsible for mediating cell growth in response to LPA in these cells, we determined the affinity of a dual selective LPA1/LPA3 receptor antagonist, Ki16425 (**Figure 3.1 C, D**) (Ohta et al., 2003). The IC_{50} of this inhibitor was determined in the presence of a sub-maximal dose of 18:1 LPA in cell growth assays. We found that the IC_{50} of Ki16425 was 0.5 nM in SKOV-3 cells and 3 nM in Caov-3 cells (**Figure 3.1, Table 3.1**), suggesting that LPA1 an/or LPA3 play a larger role in mediating the cell growth response in SKOV-3 cells than in Caov-3.

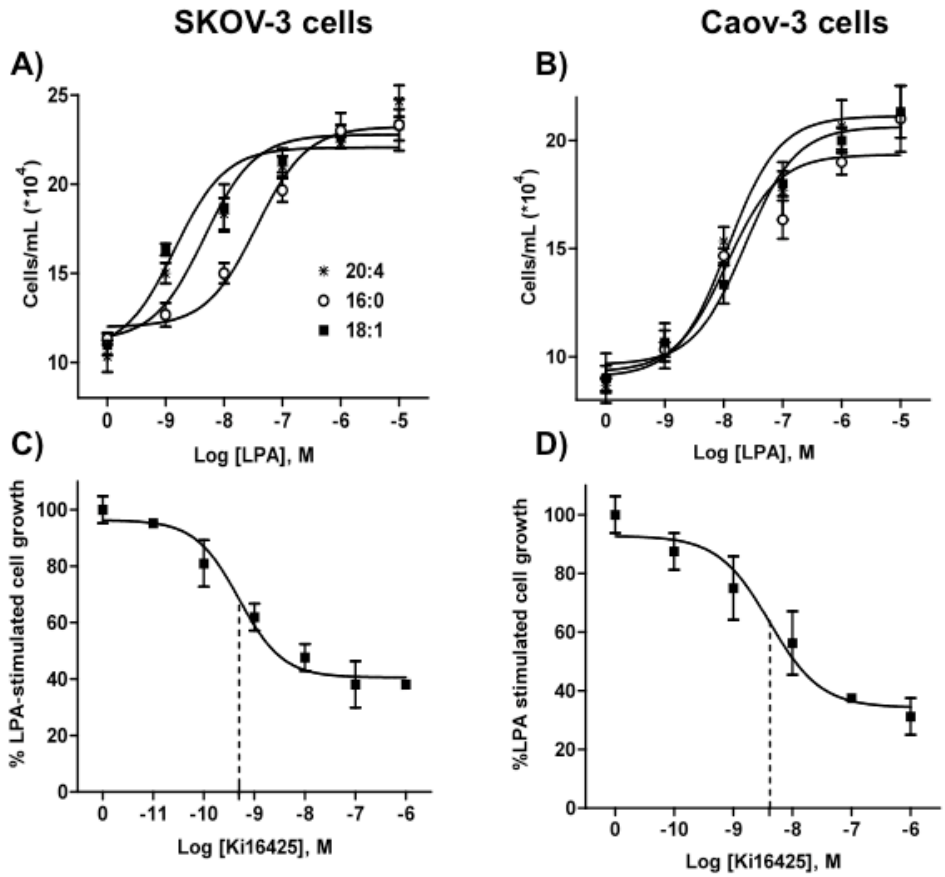

Figure 3.1: Pharmacology of LPA-stimulated cell growth in SKOV-3 and Caov-3 ovarian cancer cell lines.
SKOV-3 (A) and Caov-3 (B) cells were starved in serum-free media then treated with a range of concentrations of 16:0, 18:1, and 20:4 LPA. The number of cells for each condition was determined after the cells were starved and then again 48 hours later. Cell number is reported as number of cells per milliter (cells/mL) of media. SKOV-3 (C) and Caov-3 (D) cells were starved in serum-free media then treated with 100 nM 18:1 LPA and a range of concentrations of Ki16425. The number of cells for each condition was determined after the cells were starved and again after 48 hours of drug treatment. Results are reported as percent inhibition of LPA-stimulated increases in cell number. The data shown is representative of at least three independent experiments.

Effect of pathway inhibitors on LPA stimulated cell growth

The effect of LPA on cell growth could potentially reflect effects on both proliferation and survival, and each of these effects could be mediated by multiple signaling pathways. LPA stimulated cell proliferation had been linked to indirect activation of the ERK 1/2 MAP kinase cascade (Dixon and Brunskill, 1999; Gschwind et al., 2002; Kue et al., 2002). However, Rho-

Table 3.1: Potency of LPA species in cell growth.
Figures in parentheses represent SEM. [a]$p < 0.005$ compared to 20:4 LPA in SKOV-3 cells; [b]$p < 0.0005$ compared to 18:1 LPA in SKOV-3 cells; [c]$p < 0.005$ compared to Ki16425 in Caov-3 cells.

	SKOV-3	Caov-3
log EC_{50}, M		
16:0 LPA	-7.32 (0.08)	-7.49 (0.02)
18:1 LPA	-8.63 (0.11)	-7.49 (0.13)
20:4 LPA	-8.20 (0.13)	-7.43 (0.51)
log IC_{50}, M		
Ki16425	-9.36 (0.15)	-8.46 (0.07)

mediated effects on cellular proliferation have also been described downstream of LPA (Cechin et al., 2005). Further, LPA effects cells. Taken together, our data suggest distinct receptors or combinations of receptors are responsible for mediating cell growth in response to LPA in SKOV-3 cells as compared to Caov-3 ovarian cancer cells, and the LPA3 receptor is implicated in playing a more significant role in SKOV-3 cells.on cell survival have been linked to activation of the PI3K/Akt axis (Baudhuin et al., 2002; Ye et al., 2002; Radeff-Huang et al., 2004; Raj et al., 2004; Hu et al., 2005). Both the activation of ERK cascades and activation of PI3K are typically mediated by Ptx sensitive Gi/o G-proteins, while activation of Rho is typically mediated by Ptx insensitive G12/13 G-proteins (Moolenaar et al., 2004). In order to compare the relative contribution of these pathways to LPA stimulated cell growth in SKOV-3 and Caov-3 cells, we determined the effect of specific inhibitors of Gi/o (pertussis toxin, Ptx, 100 ng/mL), PI3K (LY294002, 10 μM), p160 Rho kinase (Y27632, 10 μM), and ERK MAP kniases (FR180204, 10 μM) in cell growth assays (**Figure 3.2**). We previously determined that each inhibitor used effectively blocks downstream targets at the concentrations used: Ptx was tested in adenylyl cyclase assays, Y27632 was tested in morphology assays, LY294002 was tested in Akt

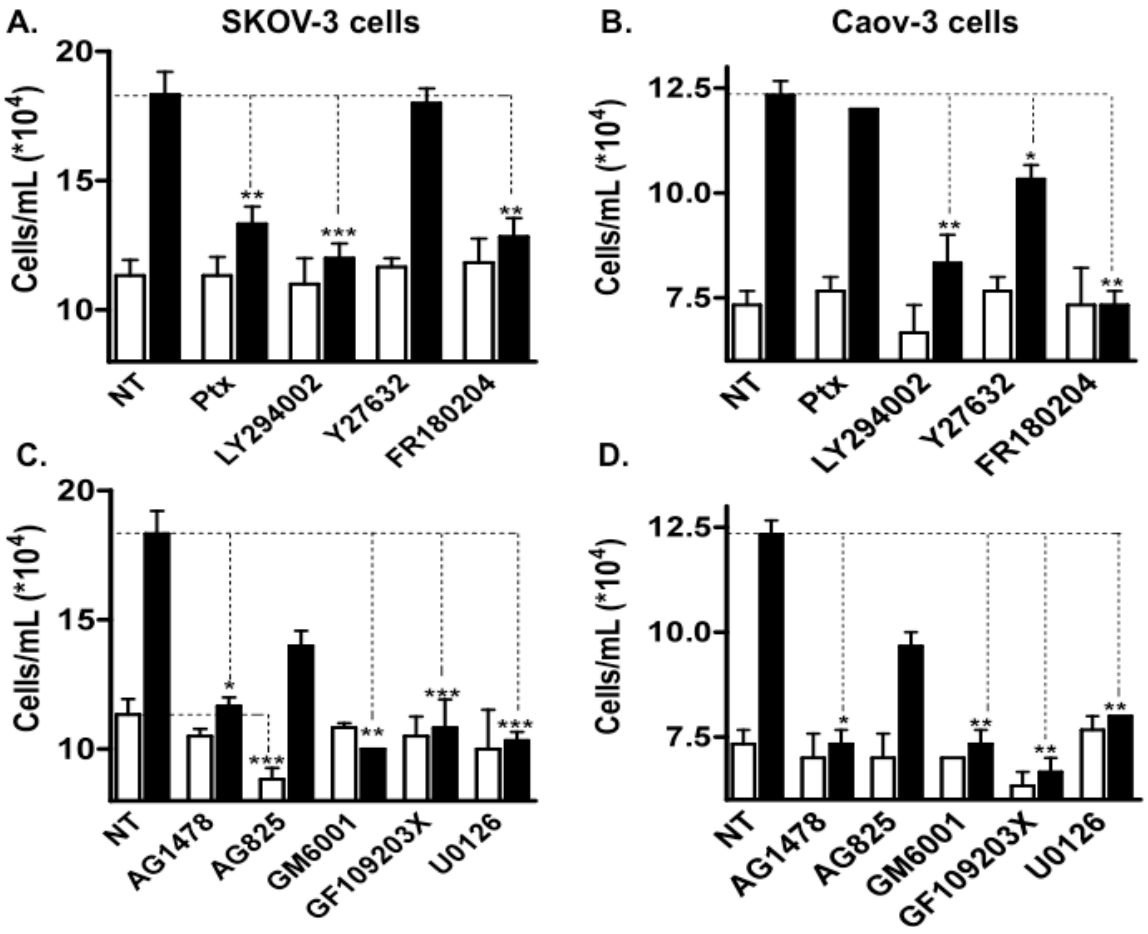

Figure 3.2: Pathways mediating LPA-stimulated cell growth in SKOV-3 and Caov-3 cells. SKOV-3 (A, C) and Caov-3 (B, D) cells were starved in serum-free media containing vehicle or the indicated inhibitors for 18 hours. Cells were then treated with 100 nM 18:1 LPA and the indicated inhibitors for an additional 48 hours. The number of cells in each condition was determined after starvation and again after 48 hours of LPA treatment. (A, B) Cells were treated with vehicle, 100 ng/mL Ptx, 10μM LY294002, 10μM Y27632, or 10μM FR180204. (C, D) Cells were treated with vehicle, 10μM AG1478, 10μM AG825, 10μM GM6001, or 10μM U0126. NT = not treated. (* $P<0.05$, ** $P<0.005$, ***$P<0.0005$)

phosphorylation assays, and FR180204 was tested in ERK-dependent growth assays (data not shown).

Overlapping but distinct patterns of inhibition were observed in the two cell types. Inhibition of either PI3K or ERK 1/2 blocked the ability of LPA to stimulate increases in cell number in SKOV-3 or Caov-3 cells (80% and 67% inhibition, respectively) without significantly

affecting basal cell growth (**Figure 3.2 A, B**). Surprisingly, inactivation of Gi/o with Ptx treatment blocked LPA signaling in SKOV-3 cells, but not Caov-3 cells (**Figure 3.2 A, B**). This suggests that Gi/o coupled receptors are critical for cell growth in SKOV-3 cells, but not Caov-3 cells. In contrast, inhibition of the Rho effector p160 ROCK had no effect on basal or LPA stimulated growth in SKOV-3 cells, but consistently blocked LPA stimulated cell growth in Caov-3 cells by approximately 50% (**Figure 3.2 A, B**). Thus, major distinctions were observed in the roles of Gi/o and Rho dependent cell growth pathways stimulated by LPA in SKOV-3 versus Caov-3 cells.

Previous reports have suggested trans-activation of EGF receptors as a mechanism to couple LPA receptor activation to ERK cascades leading to cell growth (Prenzel et al., 1999; Santiskulvong and Rozengurt, 2003). GPCR stimulated trans-activation of receptor tyrosine kinases is a well established phenomenon that has previously been demonstrated in ovarian cancer cells downstream of LPA, and this mechanism has been implicated in LPA stimulated ERK phosphorylation, proliferation and survival (Fang et al., 2004; Miyamoto et al., 2004; Symowicz et al., 2005). LPA induced trans-activation of EGF receptors in ovarian cancer cells is characterized by LPA stimulated activation of membrane bound MMPs, which cleave heparin bound Epidermal Growth factor (HB-EGF) on the extracellular surface, which can in turn bind and activate EGF receptors (Prenzel et al., 1999; Ye et al., 2002; Giannini et al., 2003; Santiskulvong and Rozengurt, 2003; Miyamoto et al., 2004). EGFR trans-activation by LPA has been shown to be dependent on Gi and Protein Kinase C (PKC), presumably via activation of phospholipase Cβ isoforms. This has most often been observed downstream of Gβγ release following Gi activation, but Gαq could also activate PLC to stimulate this pathway (Bookout et al., 2003; Zhao et al., 2006). Activation of EGF receptors by this mechanism have been shown

to trigger the Ras/Raf cascade that activates p44/42 ERK 1/2 MAP kinases, JAK/STAT cascades, and PI3K/Akt cascades (Dixon and Brunskill, 1999; Laffargue et al., 1999; Kranenburg and Moolenaar, 2001; Yart et al., 2002; Ye et al., 2002; McCubrey et al., 2006).

To compare the role of EGF trans-activation in LPA mediated cell growth in SKOV-3 and Caov-3 cells, we determined the effect of selective inhibition of receptors and mediators implicated in the trans-activation mechanism. Inhibitors of the EGF receptors EGFR/ErbB1 (AG1478, 10 μM) and HER2/ErbB2 (AG825, 10 μM), as well as inhibitors of the downstream mediators PKC (GF109203X, 10 μM), MMPs (GM6001, 10 μM) and MEK 1/2 (U0126, 10 μM were used to determine which intermediates were required to link LPA receptor activation to cellular growth (**Figure 3.2 C, D**). In both cell types, inhibiting the activity of EGFR, MMP, PKC, or MEK 1/2 blocked at least 85% of LPA stimulated cell growth. Strikingly, inhibition of HER2/ErbB2 blocked 80% of the basal growth rate of SKOV-3 cells, suggesting a role for this receptor subtype in mediating the high basal growth rates observed in these cells; however, HER2/ErbB2 inhibition did not affect LPA stimulated cell growth as a percent of basal. Further, HER2/ErbB2 inhibition did not affect the lower rate of basal growth observed in Caov-3 cells (**Figure 3.2 D**). There did appear to be a partial effect of HER2/ErbB2 inhibition of LPA stimulated cell growth in Caov-3 cells, but this effect did not meet criteria for statistical significance ($p=0.057$).

Pharmacology of LPA stimulated ERK phosphorylation

Given the clear role for ERK MAP kinase cascades in mediating LPA stimulated cell growth, we next directly examined the agonist pharmacology of LPA stimulated ERK phosphorylation and the role of EGF trans-activation intermediates in mediating this effect. Similar to our observation on cellular growth, SKOV-3 cells displayed a significantly higher

basal level of ERK phosphorylation, while Caov-3 cells displayed minimal ERK phosphorylation in the absence of LPA under serum starve conditions (**Figure 3.3 A, B**). LPA stimulated an early, transient phosphorylation of p44/42 ERK 1/2 MAP kinase that peaked at five minutes, followed by a weak later peak that is sustained between 45 minutes to an hour in both SKOV-3 and Caov-3 cells (data not shown). The potency of 18:1 LPA was determined with respect to ERK phosphorylation five minutes after LPA addition, and the effect of 18:1 LPA was compared to 16:0 and 20:4 LPA at the intermediate dose of 100 nM. The potency of 18:1 LPA was approximately 20 nM in both cell lines, and in each case the effect of the saturated 16:0 form of LPA on ERK phosphorylation was consistently less than that of the unsaturated forms of LPA (**Figure 3.3 A, B**). This agonist pharmacology corresponds with the rank order potency of LPA stimulated SKOV-3 cell growth, but not in the equipotency of LPA isoforms in LPA stimulated Caov-3 cell growth. Thus, in Caov-3 the rank order potency of LPA stimulated ERK activation is distinct from that of the cell growth responses, suggesting that these responses are mediated by different receptors or combinations of receptors.

Effect of pathway inhibitors on LPA stimulated ERK phosphorylation

We further determined the effect of inhibition of Gi/o G-proteins, EGFR, MEK 1/2, and p160 Rho kinase on LPA stimulated ERK phosphorylation (**Figure 3.3 C, D**). As expected, inhibition of MEK 1/2 kinases completely blocked LPA stimulated ERK phosphorylation in both cell lines and basal ERK phosphorylation in SKOV-3 cells, suggesting that MEK 1/2 kinases are the primary activator of ERK 1/2 under basal and LPA stimulated conditions. Consistent with its effect on cell growth, Ptx inhibition of Gi/o G-proteins completely blocked LPA stimulated ERK phosphorylation in SKOV-3 cells. In Caov-3 cells, Ptx partially but significantly inhibited the

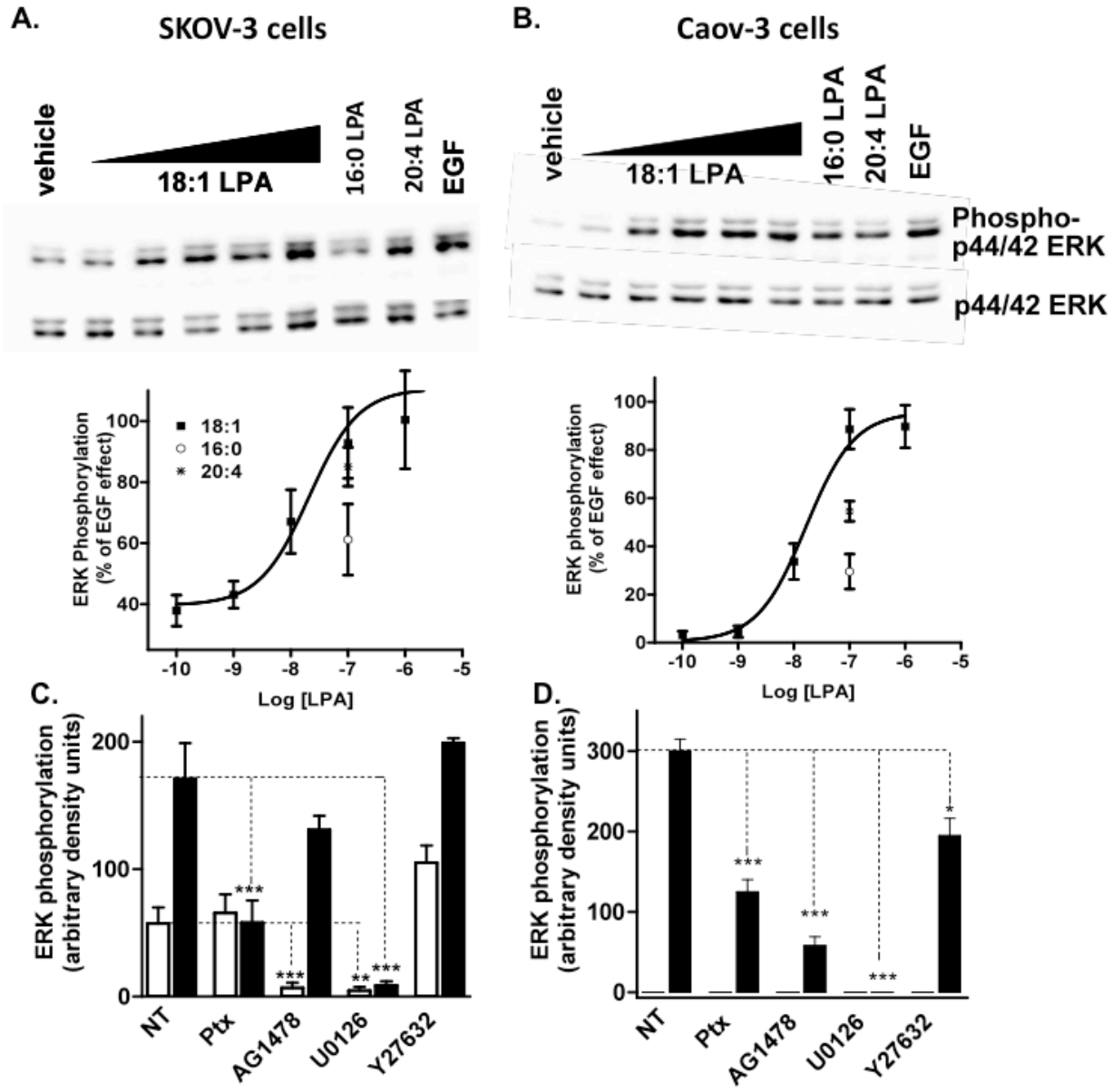

Figure 3.3: Pharmacology of LPA –stimulated activation of p44/42 ERK MAP kinase.
SKOV-3 (A) and Caov-3 (B) cells were incubated with vehicle, various concentrations of 18:1 LPA, 100nM 16:0 LPA, 100nM 20:4 LPA, or 100 ng/mL EGF for 5 minutes at 37 °C and assessed for expression of total ERK and phospho-ERK with Western blotting. ERK phosphorylation was normalized to EGF-stimulated ERK phosphorylation; results are reported as the percent of EGF-stimulated ERK phosphorylation. SKOV-3 (C) and Caov-3 (D) cells were starved in serum-free media for 18 hours in the presence or absence of 100 ng/mL Ptx, 10 μM AG1478, 10 μM U0126, or 10 μM Y27632, then stimulated with 100 nM 18:1 LPA for 5 minutes and assessed for expression of total ERK and phospho-ERK with Western blotting. Results are reported in arbitrary density units. NT = not treated. (*, P<0.05; **, P<0.005, ***, P<0.0005)

ability of LPA to stimulate ERK phosphorylation, in contrast to LPA stimulated cell growth which was completely insensitive to Ptx. This suggests that while LPA can stimulate ERK phosphorylation by both Ptx-sensitive and Ptx-insensitive pathways in Caov-3 cells, only the Ptx-insensitive pathway significantly mediates cell growth under the conditions tested. Inhibition of EGFR in Caov-3 cells inhibited approximately 80% of ERK phosphorylation (**Figure 3.3D**). EGFR inhibition consistently blocked basal ERK phosphorylation in SKOV-3 cells, but did not affect LPA stimulated ERK phosphorylation. These data contrast with the nearly complete block of LPA stimulated cell growth upon EGFR inhibition in SKOV-3 cells, suggesting that although additional Gi/o G-protein dependent pathways can lead to ERK phosphorylation in the absence of active EGFRs, the EGFR dependent pathway is more directly correlated to cell growth. Because inhibition of EGFR completely blocked basal but not LPA stimulated ERK phosphorylation in SKOV-3 cells, it is likely that distinct mechanisms account for basal and LPA stimulated ERK phosphorylation. This is further supported by the observation that Ptx completely inhibited LPA stimulated ERK phosphorylation, but had no effect on basal activity. Finally, inhibition of p160 Rho kinase partially inhibited the effect of LPA on ERK phosphorylation in Caov-3 cells, but had no effect in SKOV-3 cells, consistent with the effect of Rho kinase inhibition on LPA stimulated cell growth. Thus, p160 Rho kinase is required for full activation of ERK kinase in response to LPA in Caov-3 cells, but is not required in SKOV-3 cells.

Pharmacology of LPA stimulated Akt phosphorylation

The ability of the PI3K inhibitor LY294002 to block LPA stimulated cell growth in SKOV-3 and Caov-3 cells implicates the PI3K/Akt survival pathway in regulating and/or mediating this response. To compare the pathways that mediate LPA stimulated Akt activation

and LPA stimulated cell growth, we next wanted to compare the activity of LPA isoforms and pathway inhibitors on this response as above. Unfortunately, pathway mediating LPA stimulated Akt phosphorylation could not be compared between SKOV-3 and Caov-3 cells because SKOV-3 cells demonstrated Akt phosphorlylation that was not increased by further LPA stimulation (data not shown). In contrast, Caov-3 cells had a low, but detectable, level of basal Akt phosphorylation that increased upon stimulation with LPA (**Figure 3.4 A**). Peak LPA stimulated Akt phosphorylation occurred five minutes after LPA exposure and appeared to be sustained at slightly lower levels for up to an hour (data not shown). The potency of 18:1 LPA was determined with respect to Akt phosphorylation five minutes after LPA addition (**Figure 3.4 B**). The EC_{50} of 18:1 LPA stimulated Akt phosphorylation was approximately 300 nM in Caov-3 cells.

Effect of pathway inhibitors on LPA stimulated Akt phosphorylation

We next determined the effect of inhibitors of intermediates in the EGF trans-activation pathway on LPA stimulated Akt phosphorylation in Caov-3 cells. Caov-3 cells were pre-incubated with inhibitors of Gi/o G-proteins, PI3K, EGFR, MEK 1/2, and p160 Rho kinase for 18 hours prior to LPA stimulation (**Figure 3.4 C**). As expected, treatment with the PI3K inhibitor LY294002 lowered basal Akt phosphorylation levels by approximately 60% and completely blocked LPA stimulated Akt phosphorylation, while treatment with Ptx did not have any significant effects on either LPA stimulated or basal Akt phosphorylation. Further, inhibition of EGFR in Caov-3 cells significantly lowered basal Akt phosphorylation and completely blocked LPA stimulated Akt phosphorylation. These effects mirror those seen in Caov-3 cell growth assays following treatment with these inhibitors. The MEK 1/2 inhibitor U0126 did not significantly affect basal or LPA stimulated Akt phosphorylation, suggesting that

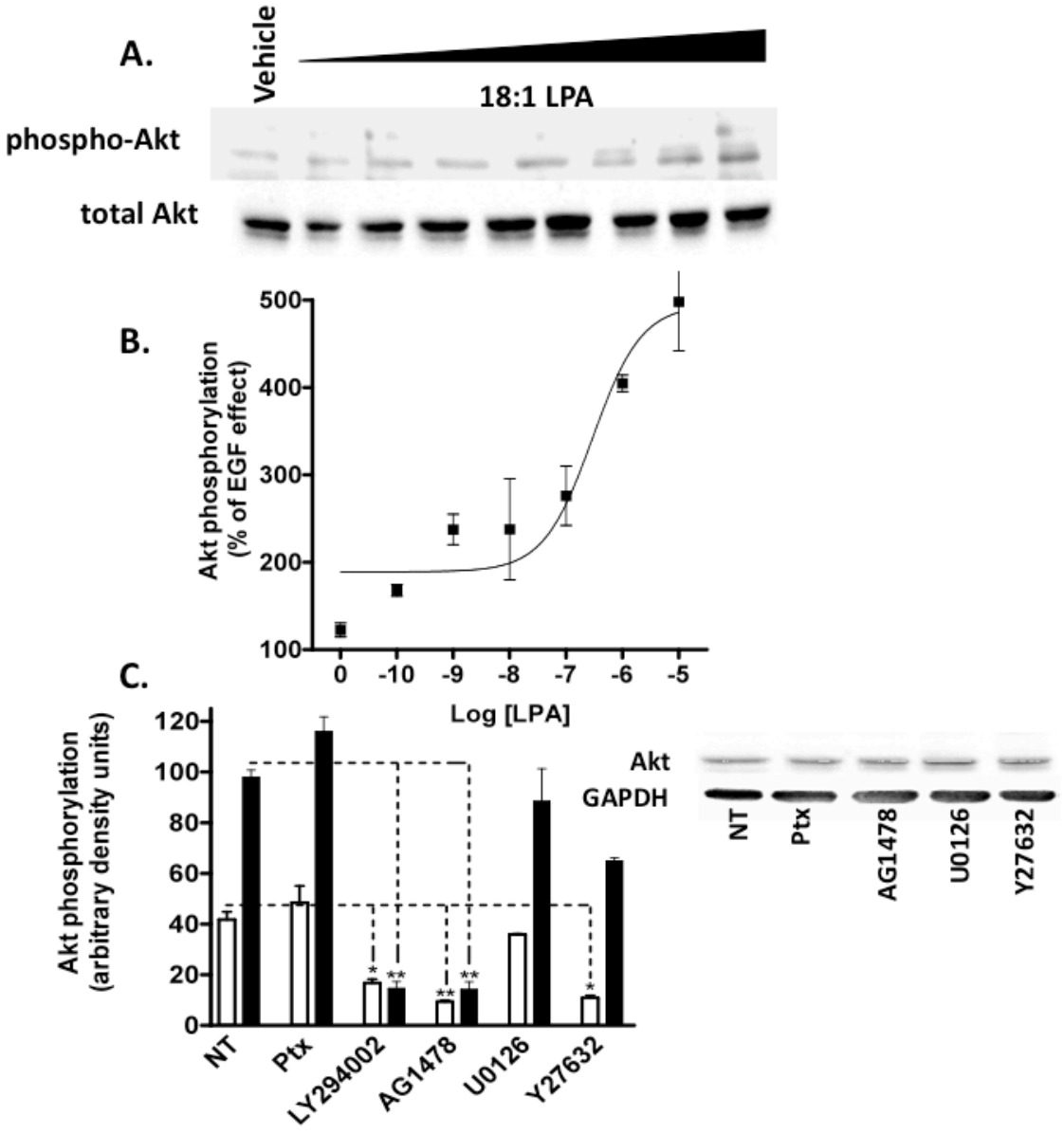

Figure 3.4: Pharmacology of LPA-stimulated Akt phosphorylation in Caov-3 cells.
Caov-3 cells were incubated with vehicle or various concentrations of 18:1 LPA for five minutes at 37 °C and assessed for expression of total Akt and phospho-Akt with Western blotting (A). Akt phosphorylation was normalized to EGF-stimulated phosphorylation; results are reported as the percent of EGF-stimulated Akt phosphorylation (B). Caov-3 (C) cells were starved in serum-free media for 18 hours in the presence or absence of 100 ng/mL Ptx, 10 μM AG1478, 10 μM U0126, or 10 μM Y27632, then stimulated with 100 nM 18:1 LPA for 5 minutes and assessed for phospho-Akt with Western blotting, normalized to GAPDH (bottom). Results are reported in arbitrary density units. NT = not treated. (*, P<0.05; **, P<0.005, ***, P<0.0005)

Akt activation is not downstream of ERK. As expected, inhibition of p160 Rho kinase did not affect LPA stimulated Akt phosphorylation, but surprisingly, did cause a significant decrease in basal Akt phosphorylation. None of the pharmacological inhibitors used had an effect on total Akt levels (**Figure 3.4 C**).

Regulation of Ptx-sensitive cell growth in SKOV-3 cells by RGS proteins

Our data indicate that LPA stimulated cell growth is mediated by distinct G-proteins, suggesting unique regulation of the response in different cell types. The most proximal regulators of G-protein signaling downstream of receptor activation are the Regulator of G-protein Signaling (RGS) proteins, which function to deactivate G-proteins by accelerating the hydrolysis of GTP to GDP (Berman et al., 1996; Chen et al., 1996; Watson et al., 1996). Because RGS proteins are generally selective specific for certain classes of G-proteins, distinct RGS proteins will regulate Ptx-sensitive and Ptx-insensitive G-protein pathways (Hollinger and Hepler, 2002). We have recently shown that regulation of ovarian cancer cell migration is significantly regulated by Gi/o targeted RGS proteins in SKOV-3 ovarian cancer cells (Hurst et al., 2008a). To determine if Ptx-sensitive G-proteins that mediate LPA stimulated cell growth in SKOV-3 cells are also regulated by RGS proteins, we compared the ability of LPA to induce increases in cell number in the presence of wild-type, RGS-sensitive G-proteins versus RGS-insensitive G-proteins, using a Gαi2 G184S point mutation which renders G-proteins resistant to RGS regulation (Lan et al., 1998). In order to directly compare signaling through these two forms of G-proteins, we introduced additional point mutations that rendered the G-proteins insensitive to Ptx, and then deactivated all endogenous Gi/o G-proteins with Ptx. Thus, all signaling was funneled through either the RGS sensitive (RGSwt) or RGS-insensitive (RGSi) Gi/o G-protein. Differences in signaling responses between cells expressing these two forms of

the G-protein reflect regulation by endogenous RGS proteins. We found that while expression of RGS sensitive Gαi2 restored negligible LPA stimulated cell growth in SKOV-3 cells following Ptx deactivation of endogenous Gi/o G-proteins, expression of RGS insensitive Gαi2 resulted in a significantly stronger LPA stimulated cell growth response (**Figure 3.5**). Likewise, LPA stimulated ERK 1/2 phosphorylation was restored by RGS insensitive Gαi2, but not RGS sensitive Gαi2 in SKOV-3 cells (**Figure 3.6**). Thus, endogenous RGS proteins expressed in SKOV-3 cells significantly attenuated LPA stimulated increases in cell growth and ERK phosphorylation via Gαi2. A similar approach to determine the significance of endogenous RGS regulation on the Ptx-insensitive G-proteins that are involved in LPA stimulated cell growth in Caov-3 cells could not be performed because there is not a simple mechanism to eliminate all endogenous Gq or G12 signaling which would allow the direct comparison between a single RGS sensitive and RGS insensitive G-protein subtype. The current study suggests that SKOV-3 and Caov-3 ovarian cancer cells express distinct but overlapping function LPA signaling pathways leading to cell growth, with unique roles for LPA receptors (LPA3), G-proteins (Gαi and Rho), and EGF family receptors (HER2/ErbB2) requirements for LPA stimulated cell growth in SKOV-3 and Caov-3 cells. ERK 1/2 MAP kinases and PI3 kinase are required for LPA stimulated cell growth, consistent with previous studies indicating that LPA activates both MAP kinase and PI3 kinase pathways to induce proliferation and survival, respectively (Dixon and Brunskill, 1999; Kranenburg and Moolenaar, 2001; Yart et al., 2002; Ye et al., 2002). In contrast, key differences between Caov-3 and SKOV-3 cells were also observed. LPA stimulated cell growth in SKOV-3 cells was completely dependent on Ptx sensitive Gi family G-proteins, while the effect of LPA on cell growth was Ptx insensitive in Caov-3 cells. In contrast, the Rho effector p160 Rho kinase did not appear to play a role in SKOV-3 cells, while inhibition

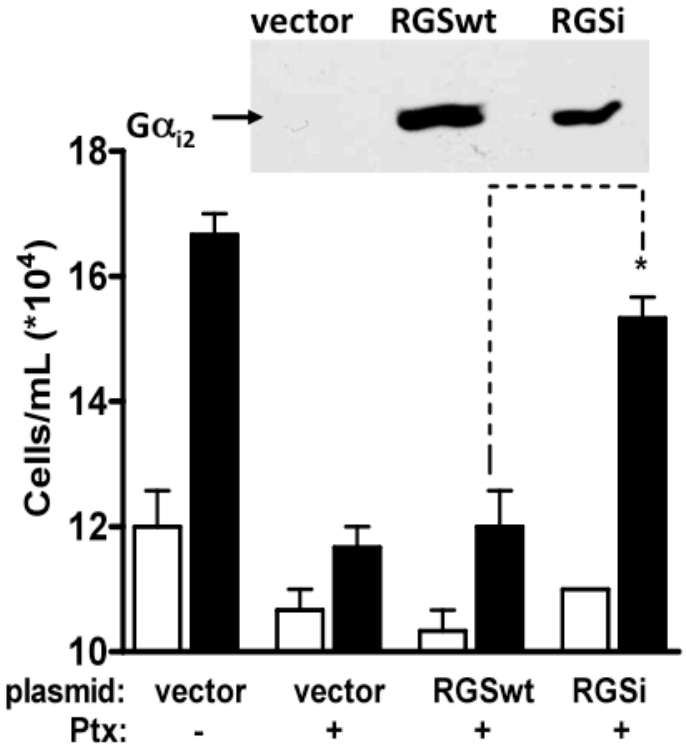

Figure 3.5: RGS protein regulation of LPA-stimulated cell growth in SKOV-3 cells.
SKOV-3 cells were plated in 24-well plates and transfected with empty vector, Gαi2 Ptxi RGSwt, or Gαi2 Ptxi RGSi. Twenty-four hours after transfection, cells were starved with serum-free media and with 100 ng/mL Ptx where indicated. After 18 hours, cells were treated with vehicle (open bars) or 100 nM 18:1 LPA (closed bars) and incubated for 48 hours at 37 °C. Media with LPA and/or Ptx was renewed every 24 hours. The number of cells in each condition was determined after starvation and again after 48 hours of LPA treatment. (*, $P<0.05$) Cells transfected in parallel with cell growth experiments were subjected to Western blotting with Gαi2 antibodies to determine relative expression of the two mutant constructs. The data shown was representative of at least two independent experiments.

of this molecule consistently blocked about half of the LPA stimulated growth response in Caov-3 cells. LPA has been shown to stimulate cell growth in multiple cell lines through Gi and Gq pathways (Kim et al., 2004; Kim et al., 2005; Kim et al., 2006). Rho GTPase, activated downstream of G12 coupled receptors, has more recently been linked to cell growth responses through cell cycle regulators such as p21 and p27 (Budnik et al., 2003; Radeff-Huang et al., 2004; Cechin et al., 2005), or activation of PI3K/Akt (Del Re et al., 2008). LPA receptors LPA1,

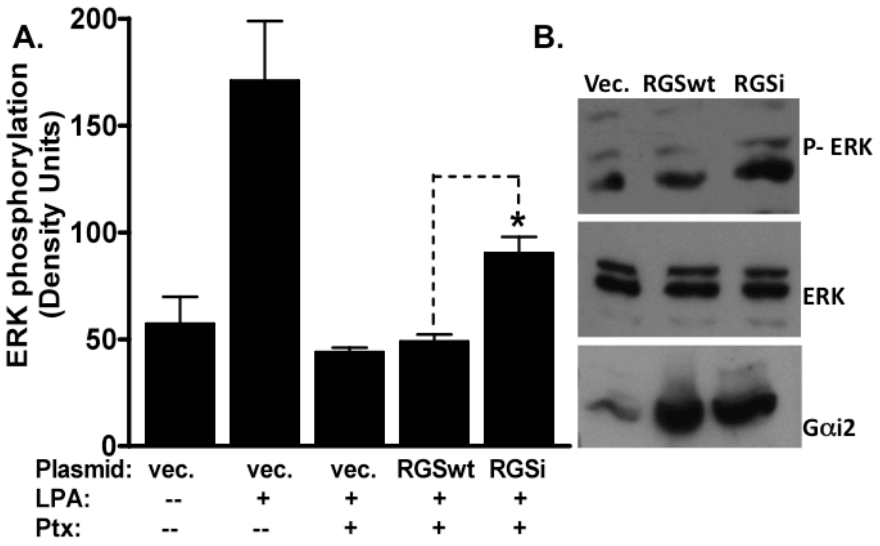

Figure 3.6: RGS protein regulation of LPA-stimulated p44/42 ERK MAP kinase activation in SKOV-3 cells.
SKOV-3 cells were transfected with either empty vector, Ptx-insenitive, RGS-sensitive Gαi2 (RGSwt) or Ptx-insensitive, RGS-insensitive Gαi2 (RGSi) and treated with vehicle or LPA for five minutes with or without prior overnight incubation with Ptx. Cells were lysed and subjected to SDS-PAGE and Western blotting with phospho-ERK, total ERK, GAPDH and Gαi2 antibodies. A. Phospho-ERK immunoreactivity was quantified and normalized to GADPH immunoreactivity as a loading control. *P<0.05 B. Representative blots depict p42/44 ERK phosphorylation in the presence of 100 nM LPA following Ptx treatment, and expression of endogenous (vector), RGS-sensitive (RGSwt), and RGS-insensitive (RGSi) Gαi2.

LPA2, and LPA4 can activate Gαq and Gα12 in addition to Gαi, providing multiple plausible pathways that may account for the Gi-independent cell growth in Caov-3 cells.

Discussion

In both cell lines, trans-activation of EGF receptors and MAP kinase activation was critical to cell growth downstream of LPA; however, the pathways by which LPA stimulates ERK MAP kinase phosphorylation were distinct. LPA stimulated ERK phosphorylation was completel Ptx sensitive and p160 Rho kinase independent in SKOV-3 cells, while in Caov-3 cells, the effect was partially inhibited by both Ptx and an inhibitor of p160 Rho kinase. The effect of Rho kinase inhibition was consistent with the partial Rho-dependency of LPA stimulated cell growth in Caov-3 cells. However, the effect of Ptx on LPA stimulated ERK

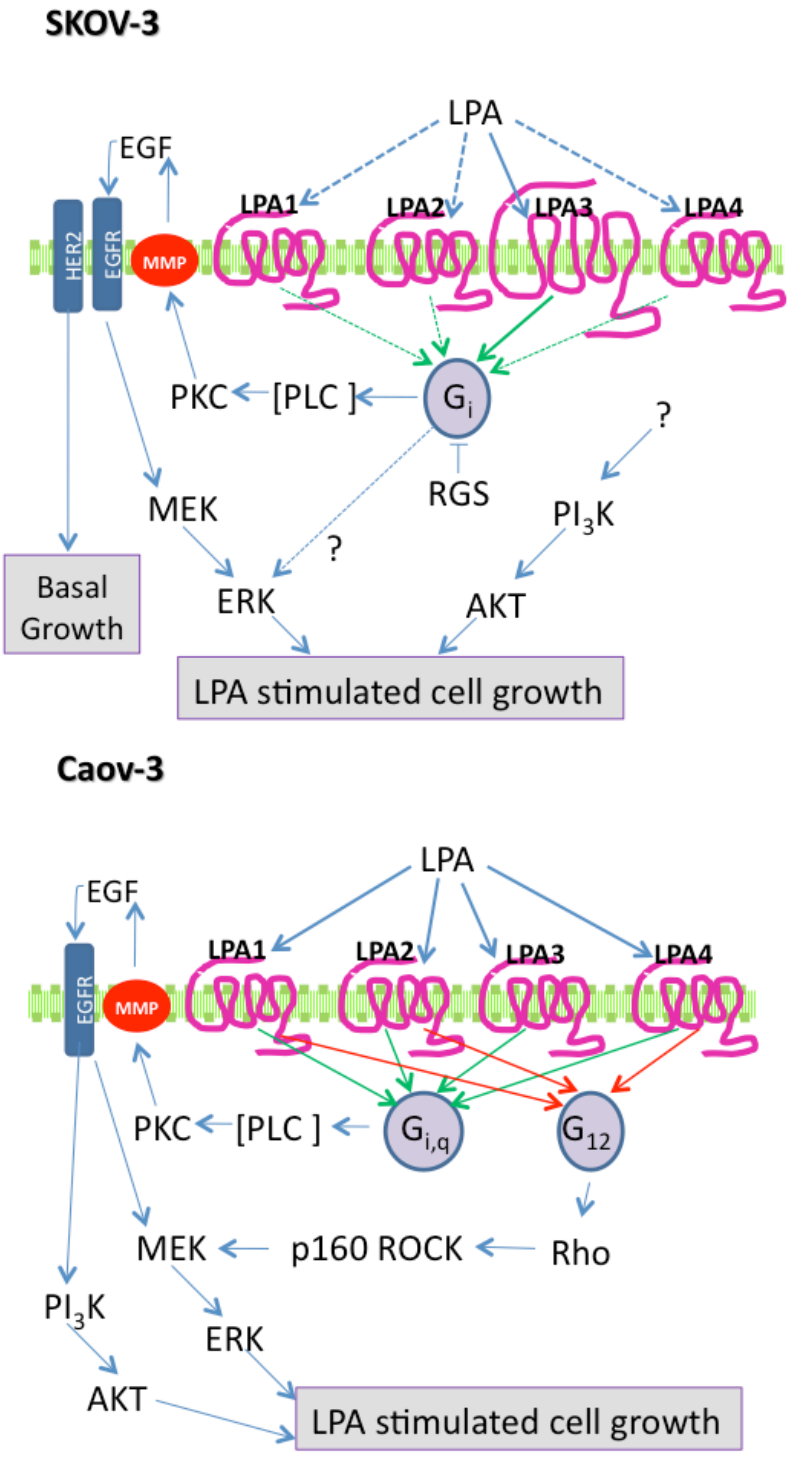

Figure 3.7: Model of cell growth pathways stimulated by LPA in SKOV-3 and Caov-3 ovarian cancer cells. PLC = Phospholipase C.

phosphorylation in Caov-3 cells was unexpected. Partial sensitivity to Ptx suggests a subset of ERK proteins that may be activated by Gi dependent pathways that is distinct from the pool of ERK activated by Gi independent pathways. The latter ERK pool correlates with cell growth. These two pools of ERK may be distinguished by upstream activation of distinct receptors or G-proteins, subcellular localization, or regulation by downstream regulators. EGFR also appears to have distinct effects on growth and ERK phosphorylation. EGFR activity was required for LPA stimulated cell growth but not ERK phosphorylation in SKOV-3 cells, suggesting that LPA stimulates EGF-independent ERK activating pathways in SKOV-3 cells that are not sufficient to stimulate cell growth.

The PI3K/Akt signaling axis, much like the MAP kinase pathway, may be activated via receptor tyrosine kinase receptors and promotes cell survival and cell growth by multiple downstream effectors, including caspase-9, BAD, glycogen synthase kinase-3β, and the regulation of various transcription and pro-apoptotic factors (Datta et al., 1999). Due to the high level of basal Akt phosphorylation in SKOV-3 cells, we were unable to determine the relative potencies of various LPA isoforms, or the effects of pathway inhibitors on LPA stimulated Akt phosphorylation in these cells. Other groups have also noted the high basal Akt phosphorylation in SKOV-3, particularly compared to Caov-3 cells (Tang et al., 2006; Noske et al., 2007). In Caov-3 cells, LPA stimulated Akt phosphorylation in a Ptx insensitive, EGFR dependent manner.

Akt and ERK are regulators of survival and proliferation and promote these activities through trans-activation of EGFR and subsequent activation of PI3K/Akt and MAPK/ERK signaling cascades in cancer (Rozengurt, 2007). LPA has been demonstrated to have differential effects on these pathways depending on cellular context and the strength and duration of the LPA

signal (Rivera and Chun, 2008). Akt promotes cellular growth and survival through the phosphorylation of multiple downstream effectors and transcriptional activators (BAD, caspase-9), and also regulates the cell cycle by altering levels of cyclin D1 and the cyclin D1 kinase glycogen synthase kinase-3β (Vivanco and Sawyers, 2002). Further, PI3K/Akt activates the Raf-MAP kinase cascade in Cos-7 cells (King et al., 1997), but downregulates this pathway in glioblastoma cell lines (Galetic et al., 2003), suggesting context specific roles for Akt. The role for ERK 1/2 in cellular proliferation is well established; a number of growth factors activate p44/42 ERK MAP kinases and substrates for ERK 1/2 such as Elk-1, Ets-2, MNK, and c-myc, which have been linked to regulation of the cell cycle (Roberts and Der, 2007). However, ERK 1/2 also play a role in cell survival via upregulation of the anti-apoptotic protein Bcl-2 (Siddiqa et al., 2008), or deactivation of the pro-apoptotic protein BAD (Bonni et al., 1999; Fang et al., 1999; Scheid et al., 1999). More in depth studies involving pharmacological inhibitors, dominant negative mutants, and analysis of the targets of both the Akt and MAP kinase pathways will be required to determine the contribution of each pathway to LPA stimulated cell growth in SKOV-3 and Caov-3 cells.

In addition to the differences in signaling pathways, our data suggest that different LPA receptor complements may be responsible for mediating LPA stimulated cell growth. LPA receptor transcripts for LPA1, LPA2, LPA3, and LPA4 are detectable in SKOV-3 and Caov-3 cells. When directly compared, LPA3 transcript is equally expressed in SKOV-3 and Caov-3 cells, with LPA2 and LPA3 transcript at higher levels than LPA1 and LPA4 in Caov-3, and LPA1 and LPA2 expressed at higher levels than LPA3 and LP4 in SKOV3 cells (Ptaszynska et al., 2008). However, the relative amounts of LPA receptor transcript are not predictive of the relative contributions of receptor subtypes is distinct signaling pathways. Differences in protein

expression, receptor localization, and G-protein coupling could all affect the ability of specific receptors to modulate pathways leading to increases in cell number. The lack of sensitive and selective LPA receptor antibodies hinders comparison of protein expression and, along with the presence of multiple endogenous receptor subtypes, complicates the interpretation of studies that attempt to knockdown or over-express individual subtypes. Thus, we have employed a classic pharmacological approach using well characterized receptor subtype selective compounds to address the relative contribution of LPA receptor subtypes to LPA stimulated cell growth in these cells. Unsaturated LPA species are more potent LPA3 receptor agonists than saturated LPAs, while LPA1 and LPA2 do not show such selectivity (Bandoh et al., 1999; Im et al., 2000b). In the current study, we demonstrate that saturated LPA (16:0) is less potent than unsaturated LPAs (18:1 and 20:4) in stimulating SKOV-3 cell growth, while all three LPA species are equipotent in Caov-3 cell growth. This suggests a more dominant role of LPA3 receptors in mediating LPA stimulated SKOV-3 cell growth than in Caov-3 cell growth. Consistent with this observation, the semi-selective LPA1/3 antagonist Ki16425 was more potent inhibitor of cell growth in SKOV-3 than in Caov-3 cells. LPA3 does not couple to Gα12, the major pathway by which LPA stimulates Rho activation. It is noteworthy that in Caov-3 cells, LPA stimulated cell growth is less dependent of LPA3 mediated pathways and ore dependent on Rho pathways than in SKOV-3 cells.

Our finding that SKOV-3 and Caov-3 cells rely on distinct G-proteins to mediate cell growth responses suggests fundamental differences in the ways that these responses will be regulated, for example, by RGS proteins. We have demonstrated that endogenous RGS proteins significantly attenuate LPA stimulated cell growth and MAP kinase activation mediated by Gαi. Because many RGS proteins are selective for a class of G-proteins, LPA stimulated cell growth

may be regulated by distinct RGS proteins in SKOV-3 and Caov-3 cells. RGS proteins that can act as inhibitors for particular LPA receptor/G-protein combination would serve as useful investigative and therapeutic tools. Further studies will be needed to identify the specific RGS proteins responsible for this regulation.

In addition to the observed distinctions in pathways mediating and regulating LPA stimulated cell growth in SKOV-3 and Caov-3 cells, differences were also observed in the basal levels of cell growth, ERK activation, and Akt activation. Significantly higher basal activity in the cell growth assay, ERK phosphorylation assay, and Akt phosphorylation assay was observed in SKOV-3 cells as compared to Caov-3. Basal SKOV-3 cellular growth was significantly lowered by selective HER2/ErbB2 inhibitors, suggesting that this receptor subtype contributes to basal activity. Although HER2/ErbB2 is a ligand-less receptor that normally forms heterodimers with other EGF receptor types, high basal autophosphorylation can occur due to homodimerization at high levels of expression (Di Fiore et al., 1987; Lonardo et al., 1990). Indeed, SKOV-3 cells are reported to over-express HER2/ErbB2, while Caov-3 cells do not (Tang et al., 1994), which may further account for the high level of basal cell growth, ERK phosphorylation, and Akt phosphorylation seen in SKOV-3 cells.

In summary, we have identified major distinctions between two commonly studied model ovarian cancer cell lines with respect to receptor pharmacology and signal transduction cascades responsible for mediating LPA stimulated cell growth. This reflects the heterogeneity of signal transduction cascades coupled to LPA receptors in ovarian cancers, and suggests that different model cell lines may more accurately reflect the cell signaling present in distinct populations of ovarian cancers. While altered expression of LPA receptors, EGF receptors, and multiple signaling intermediates connecting these two receptor pathways to the MAP kinase and

PI3K/Akt cascades has been demonstrated in ovarian cancer, expression changes are not uniform across all patients. Thus, distinct cell lines derived from ovarian cancer may represent model systems for distinct disease categories that may correlate to different prognoses and drug responses, as both the LPA and EGF receptor families are being pursued as therapeutic targets in ovarian cancer.

CHAPTER 4

REGULATOR OF G-PROTEIN SIGNALING (RGS) PROTEINS IN CANCER BIOLOGY[1]

[1]Hurst, J.H., and S.B. Hooks. *Biochemical Pharmacology* 2009; 78(10): 1289-97.
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Abstract

The regulator of G-protein signaling (RGS) family is a diverse group of multifunctional proteins that regulate cellular signaling events downstream of G-protein coupled receptors (GPCRs). In recent years, GPCRs have been linked to the initiation and progression of multiple cancers; thus, regulators of GPCR signaling are also likely to be important to the pathophysiology of cancer. This review highlights recent studies detailing changes in RGS transcript expression during oncogenesis, single nucleotide polymorphisms in RGS proteins linked to lung and bladder cancers, and specific roles for RGS proteins in multiple cancer types.

Introduction

Cancer is characterized by the uncontrolled growth of cells through increased proliferation and decreased apoptosis. Additionally, cancer cells can invade adjacent tissues and metastasize to non-adjacent organs and tissues. Uncontrolled growth, invasion, and metastasis are due to changes in cellular signaling pathways, and oncogenic transformation is often the direct result of mutations of the signaling molecules which constitute these pathways. In the past decade, G-protein coupled receptor (GPCR)-stimulated pathways have emerged as critical mediators of oncogenic signaling. GPCRs are a family of cell surface receptors which activate heterotrimeric G-proteins to transduce extracellular signals into the interior of a cell. Regulators of G-protein Signaling (RGS) proteins are a highly diverse family of proteins containing an RGS domain which accelerates the deactivation of heterotrimeric G-proteins, thus modulating signaling initiated by GPCRs. There are over 20 mammalian RGS proteins ranging from small proteins comprised solely of an RGS domain to multi-domain proteins with functions in multiple signaling pathways. These additional domains serve to mediate interactions with other signaling proteins, allowing RGS proteins to serve as signaling scaffolds. This review examines recent studies focused on the involvement of RGS proteins in the initiation and progression of cancer.

G-protein signaling in cancer biology

G-protein coupled receptors (GPCRs) are known to mediate a wide variety of physiological processes, including sensory perception, immune responses, neurotransmission, and cardiovascular activity. Consequently, GPCRs are also linked to many disease states and serve as direct and indirect targets for roughly half of pharmaceuticals currently on the market (Pierce et al., 2002). GPCRs function to mediate ligand-dependent activation of heterotrimeric guanine nucleotide binding proteins (G-proteins). Heterotrimeric G-proteins consist of two

functional signaling units: a guanine nucleotide-binding α-subunit and a βγ-subunit dimer. Upon ligand binding, conformational changes in the receptor activate a heterotrimeric G-protein by promoting the exchange of GDP for GTP in the Gα nucleotide binding site. The active Gα and Gβγ subunits then dissociate and interact with various effector molecules, mediating cellular responses to GPCR activation. The G-protein deactivates when the Gα subunit hydrolyzes GTP to GDP and reassociates with Gβγ. Thus, G-proteins are activated by receptor-stimulated nucleotide exchange and deactivated by GTPase activity.

Several recent reviews have described multiple roles for GPCR signaling in cancer (Li et al., 2005; Dorsam and Gutkind, 2007; Spiegelberg and Hamm, 2007). GPCRs are expressed in cancerous tissues and mediate proliferation, survival from apoptotic signals, invasion, and metastasis and are activated by mitogens including lysophosphatidic acid (LPA), endothelin, thrombin, gastrin releasing peptide (GRP), thyroid stimulating hormone (TSH), cholecystokinin (CCK), angiotensin, stromal cell-derived factor-1 (SDF-1/CXCL12) and prostaglandins (Gutkind, 1998). Many of these GPCR ligands are found in high concentrations in metastatic sites, resulting in autocrine/paracrine activation of their cognate GPCRs (Julius et al., 1989). Further, several of the corresponding GPCRs are over-expressed in cancer cells: LPA receptors in ovarian, breast, colon, and prostate cancer (reviewed in (Mills and Moolenaar, 2003)); endothelin receptors in colon and prostate cancers and melanoma (reviewed in (Bagnato and Rosano, 2008)); TSH receptor in thyroid cancer (Rodien et al., 2003); protease-activated receptor 1 (PAR1) and prostaglandin EP receptors in breast, colon and prostate cancers (Majima et al., 2003; Arora et al., 2007), and CCK and CXCR4 receptors in lung and pancreatic cancers (Kijima et al., 2002; Rozengurt et al., 2002). Finally, constitutively active GPCRs are encoded by cancer-causing viruses like Kaposi's sarcoma-associated herpes virus (Arvanitakis et al., 1997)

and Epstein-Barr virus (Paulsen et al., 2005). Currently, there are not any drugs targeted directly against GPCRs that are used clinically to treat cancer. However, Zhang and colleagues recently used an LPA analogue, α-bromophosphonate LPA (BrP-LPA), to inhibit both LPA receptors and the enzyme responsible for LPA production in breast cancer cells (Zhang et al., 2009). Treatment with BrP-LPA inhibited migration and invasion of MDA-MB-231 breast cancer cells *in vitro*. Further, BrP-LPA induced tumor regression in orthotopic breast tumor xenografts in mice. Reductions in tumor volume and vascularity induced by BrP-LPA was comparable to the effects seen with Taxol (Zhang et al., 2009). These data demonstrate that compounds targeting GPCRs could possibly serve as effective anti-cancer therapeutics.

In addition to canonical GPCR signaling, transactivation of receptor tyrosine kinases (RTKs), in which G-protein activation stimulates activation of RTKs, has recently emerged as a G-protein signaling mechanism that is associated with cancer progression. GPCR-stimulated transactivation has been linked to the hormone therapy refractory forms of prostate (Cao et al., 2006) and breast cancer (Biscardi et al., 2000). Many cancers over-express RTKs, particularly members of the epidermal growth factor family, as well as their cognate ligands (Burgess, 2008). In recent years, RTK-targeted drugs such as Herceptin (trastuzumab) for breast cancer, Iressa (Gefitinib) for lung cancer, and Gleevec (Imatinib mesylate) for myelogenous leukemia, have emerged as cancer therapeutics (Zwick et al., 2002; Bennasroune et al., 2004). Most RTK-targeting drugs work by blocking ligand binding or inhibiting receptor tyrosine kinase or downstream kinase activity (Ciardiello and Tortora, 2001; Mendelsohn, 2001; Baselga and Hammond, 2002). Another possible method of therapeutically modulating RTK acitivity would be through GPCRs. Several models of GPCR-mediated RTK transactivation have been described and can involve both ligand-dependent and –independent mechanisms (Fischer et al.,

2003). Further studies will be required to define specific mechanisms of GPCR-mediated RTK transactivation in order to determine the contribution of GPCRs to RTK signaling and to develop these pathways as therapeutic targets.

In addition to changes in the GPCRs themselves, altered expression and activity of heterotrimeric G-proteins is also known to contribute to tumorigenesis (Vallar et al., 1987; Julius et al., 1989). Heterotrimeric G-protein α-subunits are classified into four families based on homology and effector interactions: Gi, Gs, Gq, and G12. Expression of constitutively active members of all four Gα families induces transformation of rodent fibroblasts (Radhika and Dhanasekaran, 2001). Constitutively active Gi G-proteins, particularly Gαi2, have been found in human endocrine tumors (Lyons et al., 1990). Further, tumor cells expressing constitutively active Gαi2 exhibit faster cell growth and tumor formation, while the expression of dominant negative Gαi2 attenuates cell growth and tumor formation (Hermouet et al., 1996). Growth-promoting hormones such as thyroid stimulating hormone (TSH) and growth hormone releasing hormone (GHRH) activate Gαs-coupled receptors; these pathways are up-regulated in thyroid tumors (Rodien et al., 2003) and pituitary adenomas (Sakai et al., 2008), respectively. Further, Gαs is constitutively active in a subset of pituitary tumors (Landis et al., 1989). G12 is a critical regulator of the cytoskeleton and promotes invasion/migration of prostate, breast, and ovarian cancer cells (reviewed in (Kelly et al., 2007)). G12 was first identified as a transforming gene in a screen of soft tissue sarcoma-derived cDNA library (Chan et al., 1993). Further, over-expression of either wild-type or mutationally activated forms of the protein are capable of transforming NIH 3T3 cells (Xu et al., 1993) and elevated levels of G12/13 G-proteins are found in cancerous tissue compared with matched, non-transformed tissue (reviewed in (Worzfeld et al., 2008)). Finally, over-expression of Gq has been demonstrated to transform NIH3T3 cells

(DeVivo and Iyengar, 1994), and activating mutations of Gαq are associated with uveal melanoma (Onken et al., 2008). These studies demonstrate a role for heterotrimeric G-protein signaling in the cancer progression and metastasis.

RGS proteins regulate G-protein signaling

As GPCRs and G-proteins mediate a wide variety of signals and their activity is finely tuned by multiple regulatory proteins. One critical regulatory point in the G-protein cycle is the deactivation of G-proteins by GTP hydrolysis which is enhanced by GTPase activating proteins (GAPs) (**Figure 4.1**). A group of proteins which function as heterotrimeric G-protein GAPs were identified over a decade ago in yeast, worms, and mammals and termed Regulator of G-protein Signaling (RGS) proteins. Each RGS protein contains a ~120 amino acid domain, termed the RGS box that is responsible for GAP activity. RGS proteins are capable of accelerating GTPase activity up to 1000-fold (Posner et al., 1999) and have been demonstrated to have profound physiological effects. In addition to functioning as GAPs for heterotrimeric G-proteins, RGS domains are also capable of serving as effector antagonists by competitively binding activated Gα subunits or effector enzymes (Roy et al., 2006; Schoeber et al., 2006) or kinetic scaffolds by promoting rapid cycling of Gα subunits between active and inactive states (Zhong et al., 2003; Willard et al., 2007). In the retina, RGS9-1 functions to terminate visual signaling cascades (Chen et al., 2000). In the heart, RGS2 attenuates angiotensin signaling to regulate blood pressure (Heximer et al., 2003). The absence of either of these RGS proteins leads to bradyopsia and hypertension, respectively. Thus, RGS proteins are critical to physiological signal transduction cascades.

RGS proteins are divided into eight subfamilies based upon RGS domain homology and common domain structures (**Figure 4.2**). The distinct combination of domains creates highly

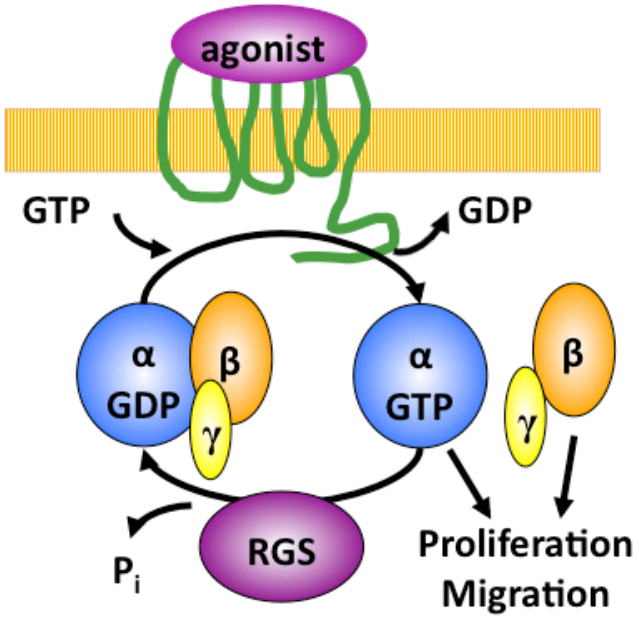

Figure 4.1: RGS proteins accelerate the GTPase activity of heterotrimeric G-protein Gα subunits. In their inactive state, the α subunit of a heterotrimeric G-protein is bound to GDP. Upon ligand activation, conformational changes in the receptor induce the Gα subunit to release GDP and bind GTP. The binding of GTP causes dissociation of the α subunit and the βγ dimer, allowing them to interact with effector molecules and propagate signaling cascades associated with cellular growth, survival, migration, and invasion. G-protein signaling is deactivated when the α subunit hydrolyzes GTP to GDP and the reassociates with the βγ dimer. RGS proteins function to accelerate the GTPase activity of the α subunit, thereby inhibiting downstream activity.

regulated, multifunctional proteins which can carry out complex signaling tasks. The R4 family is the simplest structurally, comprised of only the RGS domain and a small, N-terminal extension involved in receptor selectivity (Zeng et al., 1998). In contrast, the R7, R12, and GEF families contain additional functional domains that dictate subcellular localization, assemble multi-protein complexes, and directly regulate the activity of other signaling molecules. For example, the Dishevelled/egl-10/Pleckstrin (DEP) domain of the R7 family targets these proteins to the plasma membrane or nucleus. R12 family GoLoco domains function as guanine nucleotide dissociation inhibitors, thus preventing activation of Gi family heterotrimeric G-proteins (Kimple et al., 2001). The GEF family contains a Rho guanine nucleotide exchange factor (GEF) domain

which activates the small G-protein Rho, thus linking G12/13 activation with Rho activation. Accessory domains, such as Rac binding domain, Dishevelled homology (DH), Pleckstrin homology (PH), and PSD-95/Discs-large/ZO-1 homology (PDZ), mediate protein-protein interactions, leading to the formation of signaling complexes and providing signaling specificity. For example, RGS12 enhances nerve growth factor-stimulated MAP kinase pathways by binding of Raf and MEK2 (Willard et al., 2007) and the N-terminal domain of RGS2 binds M1 muscarinic acetylcholine receptors to selectively regulate Gq-mediated signaling (Bernstein et al., 2004). Therefore, RGS proteins can serve as inhibitors of G-protein signaling, serve as effectors, or act as scaffold proteins to assemble receptors, G-proteins, and effectors together into a signaling complex. RGS proteins are themselves highly regulated. Post-translational modifications of RGS proteins including phosphorylation, palmitoylation, and sumoylation modulate GAP activity, alter subcellular localization, and influence protein stability and protein-protein interactions (Hollinger and Hepler, 2002). Thus, RGS proteins are complex signaling molecules that are involved in a variety of functions and interactions.

In recent years several approaches have been used to determine the physiological roles of RGS proteins. Many investigators have over-expressed RGS proteins in a given system, but this method is limited in that over-expression of a protein changes the stoichiometry of signaling molecules and may not reflect endogenous specificity. RGS knock-out animals have also become available for the most widely expressed RGS proteins, including RGS2 (Oliveira-Dos-Santos et al., 2000), RGS4 (Grillet et al., 2005), RGS5 (Nisancioglu et al., 2008), and RGS9 (Chen et al., 2000); however, these models are best used to study changes in normal physiological processes. Several groups, including ours, have utilized RGS-insensitive Gα subunits to determine the significance of RGS protein regulation of G-protein signaling pathways (Hurst et al., 2008a), but

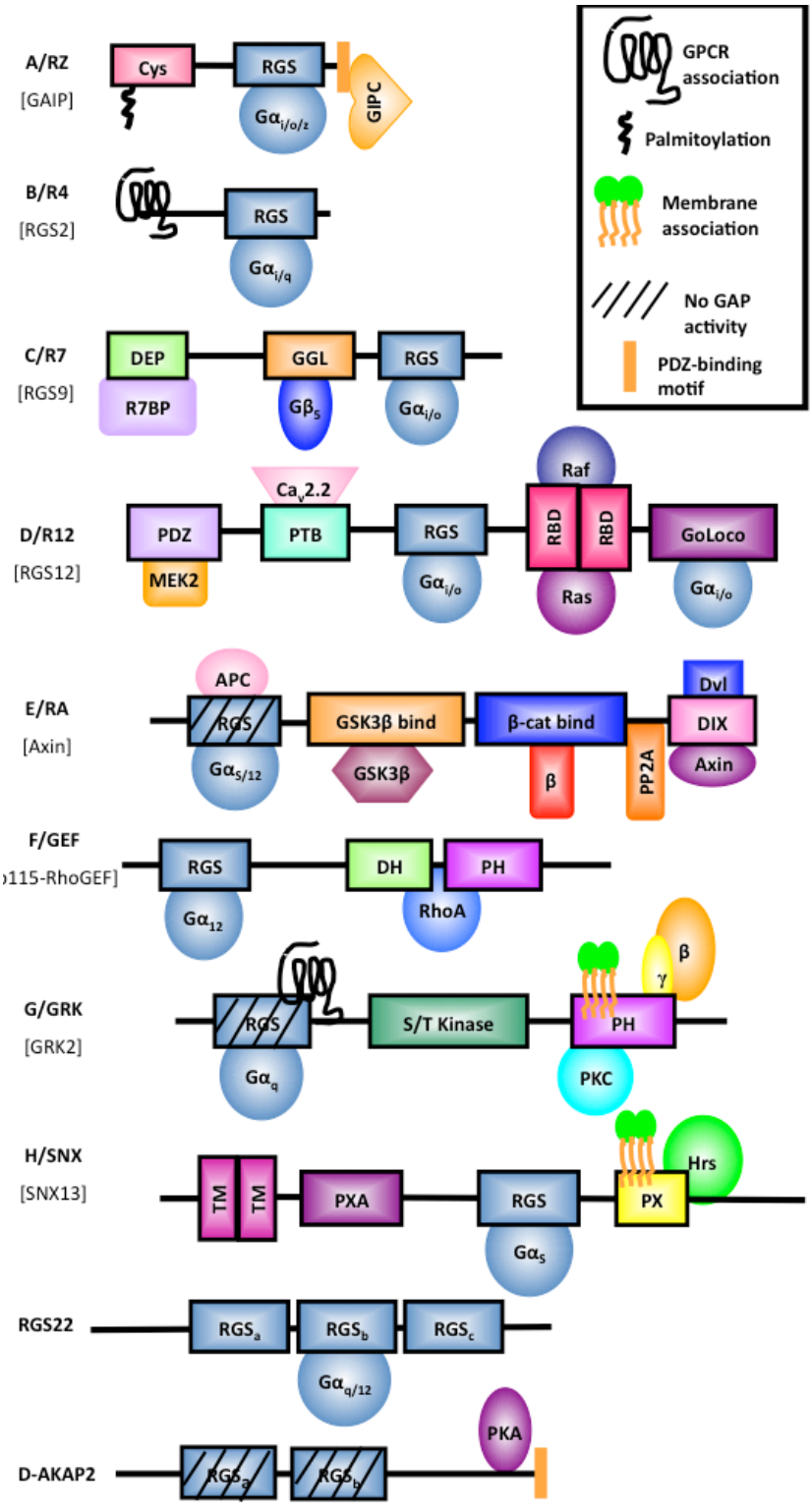

Figure 4.2: RGS subfamilies and common interacting proteins.[1]

this method does not identify specific roles for RGS proteins. While these studies have enhanced our understanding of the physiological role of RGS proteins, much work remains to be done to determine the role of RGS proteins in cancer.

Changes in expression of RGS transcripts and proteins in oncogenesis

The transformation of normal cells into cancerous cells requires concerted changes in the expression of multiple genes. These genetic changes result in the activation of proto-oncogenes and the inactivation of tumor suppressor genes allowing unregulated cell growth. Many recent

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[1]Figure 4.2: RGS subfamilies and common interacting proteins.
RGS proteins are divided into eight subfamilies based on RGS domain homology and accessory domains. Domains outlined in black are part of the RGS protein and common binding partners are shown with no black outline. G-protein specificity of RGS GAP activity is indicated on the Gα subunits. A/RZ family RGS proteins are characterized by an N-terminal cysteine string motif which can be reversibly palmitoylated and is implicated in membrane/protein interactions and intracellular localization. The scaffolding protein GAIP interacting protein, C-terminus (GIPC) binds RGS19 at the C-terminus. The B/R4 family contains the simplest RGS proteins with a short N-terminal region that is required for receptor co-localization. C/R7 family members are characterized by Dishevelled/Egl-10/Pleckstrin (DEP) domains, which bind syntaxin-like proteins such as R7 binding protein (R7BP) to mediate intracellular localization and possibly receptor targeting, and Gγ-like (GGL) domains, which bind $G\beta_5$ subunits. The D/R12 family varies greatly. RGS10 is the smallest, with little more than an RGS domain, while RGS12 and RGS14 have tandem Ras binding domains (RBD) and C-terminal GoLoco motifs (GoLoco), which serve as guanine nucleotide dissociation inhibitors (GDIs) for $G\alpha_{i/o}$ subunits. RGS12 has additional N-terminal motifs, including a PSD-95/Dlg/ZO1 (PDZ) domain, which mediates protein-protein interactions and binds mitogen-activated protein kinases (MEK2), and a phosphotyrosine binding (PTB) domain which has been shown to bind N-type calcium channels ($Ca_v2.2$). Members of the E/RA family are negative regulators of the Wnt signaling pathway. Axin binds Adenomatous polyposis coli (APC) through its RGS domain, β-catenin (β-cat bind), and glycogen synthase kinase-3β (GSK3β bind) to form the β-catenin destruction complex. Other interacting partners include phosphatase PP2A at the C-terminal end of the protein, and Dishevelled (Dsh) at the DIX domain. The DIX domain also mediates axin oligomerization. The F/GEF family consists of three RhoA specific guanine nucleotide exchange factors (GEFs) with canonical Dbl-homology (DH) and Pleckstrin-homology (PH) domains. Leukemia-associated RhoGEF (LARG) and PDZ-RhoGEF also have N-terminal PDZ domains. The G/GRK family consists of the G-protein coupled receptor kinases, each with an N-terminal RGS domain that binds $G\alpha_q$. The serine/threonine kinase domain (S/T kinase) phosphorylates GPCRs to initiate internalization. Three sorting nexins make up the H/SNX family and are characterized by an RGS domain located between phosphatidylinositol-binding (PX) and PX-associated (PXA) domains. The PXA, PX, and transmembrane domains (TM) mediate membrane association and binding to hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) links SNX to the endocytic machinery. SNX13 (aka RGS-PX1) has also been reported to serve as a GAP for $G\alpha_s$, but these findings have not been confirumed. Dual specific-A Kinase Anchoring Protein-2 (D-AKAP2) and RGS22, which both include multiple RGS domains, do not fall under any of the eight families.

studies have attempted to identify proto-oncogenes and tumor suppressors using multiplex gene microarray technology to compare the genetic profiles of matched samples of cancerous and normal tissues. Multiple RGS proteins were identified as differentially expressed genes in a variety of cancers including ovarian cancer (Hurst et al., 2008b), melanoma (Grunebach et al., 2008; Rangel et al., 2008), renal cell carcinoma (Rae et al., 2000; Furuya et al., 2004; Grunebach et al., 2008), lymphoma (Islam et al., 2003; Han et al., 2006), hepatocellular carcinoma (Chen et al., 2004b; Tsai et al., 2006), prostate cancer (Sood et al., 2001; Silva et al., 2003), breast cancer (Smalley et al., 2007b; Wiechec et al., 2008), thyroid cancer (Tonjes et al., 2004; Nikolova et al., 2008), pancreatic cancer (Hamzah et al., 2008), leukemia (Islam et al., 2003; Koga et al., 2004; Schwable et al., 2005),and glioma (Tatenhorst et al., 2004). These changes in RGS expression between normal and cancerous tissues are summarized in **Table 4.1**. Notable changes include significant down-regulation of RGS2 in androgen-independent prostate cancer (Cao et al., 2006) and acute myeloid leukemia (Schwable et al., 2005), axin in metastatic colorectal cancer (Pospisil et al., 2006), and up-regulation of RGS5 in hepatocellular carcinoma (Chen et al., 2004b) and the vasculature of renal cell carcinoma (Furuya et al., 2004).

RGS SNPs in Cancer

Single nucleotide polymorphisms (SNPs) are genetic mutations that occur in translated and untranslated regions such as promoters, either affecting the primary structure or expression levels of the encoded protein. SNPs in genes such as p53 (Whibley et al., 2009) and epidermal growth factor family receptors (Pao and Miller, 2005) are commonly associated with cancer. RGS SNPs have previously been linked to several human diseases including schizophrenia (Chowdari et al., 2008), anxiety and panic disorders (Smoller et al., 2008), celiac disease (Hunt

et al., 2008), and hypertension (Hunt et al., 2008). These studies suggest that genetic variation in RGS proteins may play a significant role in the pathophysiology of multiple human diseases.

Recently, RGS SNPs have also been reported in lung cancer and bladder cancer. A variant allele in the gene encoding PDZ-RhoGEF was found to confer a 40% reduction in the risk of lung cancer in Mexican American males (Gu et al., 2006). The Ser1416Gly mutation is in the C-terminus of PDZ-RhoGEF, the region of the protein that mediates homo- and hetero-oligomerization, which attenuates the ability of the protein to mediate guanine nucleotide exchange/activation of Rho. Further, this mutation reportedly reduces PDZ-RhoGEF activation of serum response element-dependent genes that are activated by transfection of RhoGEF proteins. The reduction in lung cancer risk was apparent in smokers, but not in non-smokers, suggesting a gene/environment interaction. Lung cancer risk varies significantly among different ethnic groups, with Mexican Americans having a lower incidence rate than Caucasians (Lee et al., 1976). Interestingly, the number of Mexican Americans who were homozygous for the protective, PDZ-RhoGEF variant allele was over double the number of Caucasian participants.

SNPs in RGS6 have been linked to a significant decrease in the risk of developing bladder cancer (Berman et al., 2004). Berman and colleagues analyzed the occurrence of 12 non-coding SNPs in genes encoding RGS2, RGS5, RGS6, RGS11, and RGS17, as well as changes in transcript level, alternative splicing events, and protein translation efficiency for each of these alleles. The single most protective allele, a variant of RGS6, was correlated with a 34% decrease in bladder cancer risk and a three-fold greater translation rate. Similarly to the PDZ-RhoGEF variant in lung cancer, this protective effect was most evident in smokers. These reported SNPs suggest that mutations in RGS proteins could have profound effects on the etiology of cancer.

Table 4.1: Changes in RGS transcript expression associated with carcinogenesis.
RGS proteins are emerging as a family of proteins that is linked to the initiation and progression of cancer. These are reports from the literature of changes in RGS transcript expression that have been linked to specific types of cancer. RGS proteins are organized according to sub-family.

Family Member	*Expression in Cancer*
	Family A/RZ
RGS17/RGSZ2	↑ in prostate cancer [99]; ↑ in lung cancer [99]
RGS19/GAIP	↑ in ovarian cancer [53]; regulates wnt/β-catenin signaling [100]; binding partner GIPC down-regulated in primary kidney tumors, colorectal tumors, gastric cancer, and prostate cancer [101]
RGS20/RGSZ1	↑ in melanoma [102]
	Family B/R4
RGS1	↑ in melanoma [55]; ↑ in head and neck squamous cell carcinoma [103]; ↑ in adult T-cell leukemia [70]; ↑ in renal cell carcinoma [54]; ↑ in ovarian cancer [54]; ↑ in cervical cancer [104]; ↑ in mantle cell lymphoma [81]
RGS2	↓ in ovarian cancer [53]; ↑ in breast cancer [65]; ↑ in fibrolamellar carcinoma [105]; ↓ in prostate cancer [17]; ↓ in acute myeloid leukemia [69]; ↑ in mantle cell lymphoma [81]
RGS3	↑ in docetaxel resistant breast cancers [106]; ↑ associated with enhanced glioma cell motility [71]; ↑ in soft tissue sarcomas [107]
RGS4	↑ associated with enhanced glioma cell motility [71]; ↑ in thyroid carcinoma [66]; ↓ in ovarian cancer [53]
RGS5	↑ in hepatocellular carcinoma [61]; ↑ in breast cancer, melanoma, multiple myeloma, ovarian cancer, and acute myeloid leukemia [98]; ↑ in fibrolamellar carcinoma [105]
RGS8	N/A
RGS13	↓ in mantle cell lymphoma [59]; ↑ in B- and T-cell lymphoma [108]
RGS16	↑ in pediatric high hyperdiploid acute lymphoblastic leukemias [109]; ↑ in pineal parenchymal tumors [110]; p53 target gene in colorectal cancer [111]
RGS18	N/A
	Family C/R7
RGS6	↑ in ovarian cancer [53]; SNPs associated with bladder cancer risk [80]
RGS7	N/A
RGS9	N/A
RGS11	Increased expression associated with Oxaliplatin resistance in colorectal cancer [112]
	Family D/R12
RGS10	N/A
RGS12	N/A
RGS14	N/A
	Family E/RA
Axin 1/Axin 2	Mutations associated with gastric cancer [113], renal cell carcinoma [114], intrahepatic cholangiocarcinomas [115], adenoid cystic carcinoma [116], cerebellar medulloblastomas [117], oral squamous cell carcinoma [118]; colorectal cancer [119]; ↓ in colorectal cancer [72]; ↓ in non-small cell lung cancer [120]; ↓ in ovarian endometroid adenocarcinoma [121]; ↓ in breast cancer [122]; ↓in sporadic medulloblastomas [123]
	Family GEF/RF
p115-RhoGEF	N/A
PDZ-RhoGEF	SNP is linked to a reduced risk of lung cancer in Mexican Americans [78]
LARG	N/A

RGS protein function in cancer

In addition to correlative studies demonstrating that changes in RGS gene expression are linked to cancer, there have also been several studies demonstrating functions of RGS proteins in cancer.

RGS2

RGS2 is one of the best characterized RGS genes with respect to cancer. Changes in expression have been linked to breast cancer (Smalley et al., 2007b), prostate cancer (Cao et al., 2006), acute myeloid leukemia (Schwable et al., 2005), ovarian cancer (Hurst et al., 2008b), mantle cell lymphoma (Zhu et al., 2002), and bladder cancer (Berman et al., 2004). Further, the RGS2 gene is localized to chromosome 1q, a region of the genome that is commonly altered in solid tumors (Qin, 2002; Collier and Largaespada, 2007). The following section will discuss the role of RGS2 in prostate cancer and acute myeloid leukemia.

The majority of prostate cancers progress from androgen-dependent to androgen-independent cell growth, making hormone ablation therapy ineffective and reducing treatment options. Cao and colleagues (2006) demonstrated that RGS2 expression is specifically down-regulated in androgen-independent prostate cancer cell lines and tissue samples compared with their androgen-dependent counterparts (Cao et al., 2006), which may result in greater signaling through these GPCR-mediated pathways. Additionally, over-expression of RGS2 decreases AR and MAP kinase activity in androgen-independent prostate cancer cell lines. RGS2 effects on these pathways were only partially blocked by a GAP-deficient mutant, suggesting that non-RGS domain functions of RGS2 may play a role in regulation of androgen-independent signaling (Cao et al., 2006).

Internal tandem duplications (ITD) in the fetal liver tyrosine kinase-3 (Flt3) receptor is one of the most common mutations in acute myeloid leukemia (AML) and is found in over 30% of AML cases (Gilliland and Griffin, 2002). The presence of Flt3-ITD mutants results in an increase in growth factor independent proliferation, clonal growth, and resistance to radiation-induced apoptosis of AML cells. Additionally, Flt3-ITD mutations are associated with increased expression of pro-proliferative genes and decreased expression of pro-differentiation genes. Schwäble and colleagues (2005) showed that the presence of Flt3-ITD mutations decreases RGS2 expression (Schwable et al., 2005). Further, over-expression of RGS2 in Flt3-ITD expressing cells reduces the level of Flt3-dependent phosphorylation of Akt and GSK-3. Finally, RGS2 antagonizes the differentiation block induced by expression of Flt3-ITD mutants, a critical event in transformation of myeloid cells. Thus, RGS2 opposes oncogenic pathways in different forms of cancer.

RGS-RhoGEF Proteins

The small G-protein Rho plays an integral role in many normal physiological and pathophysiological processes and has been demonstrated to mediate actin rearrangements and stress fiber formation, smooth muscle contraction, cell rounding, neurite retraction, gene transcriptional activity, and cell cycle progression (Burridge and Wennerberg, 2004). Rho is required for cellular migration in a variety of cancers, and therefore plays a role in invasion and metastasis (Schmitz et al., 2000). Further, there is evidence linking Rho signaling to cellular proliferation and survival through effects on the cell cycle and transcription (Vega and Ridley, 2008). Like heterotrimeric G-protein α subunits, small monomeric G-proteins are active when bound to GTP and inactive when bound to GDP; they are activated by nucleotide exchange and deactivated by nucleotide hydrolysis. Thus, the activity of Rho and other small monomeric G-

proteins is controlled by a tightly regulated array of guanine nucleotide exchange factors (GEFs), GDP dissociation inhibitors (GDIs), and GAPs. Rho GEFs effectively activate Rho by catalyzing the exchange of GDP for GTP.

RGS-RhoGEFs, including leukemia-associated RhoGEF (LARG), PDZ-RhoGEF, and p115-RhoGEF, contain an RGS domain that binds activated Gα12/13 and a GEF domain that activates Rho by catalyzing the exchange of GDP for GTP. This domain structure allows RGS-RhoGEFs to have a dual role as both RGS proteins and as G-protein stimulated effectors, linking GPCR signaling with downstream Rho activity (Fukuhara et al., 2001). In the past five years, several studies have demonstrated a role for RGS-RhoGEFs in cancer. In 2004, Wang and colleagues demonstrated that LPA and thrombin utilize LARG and PDZ-RhoGEF, respectively, to activate Rho in PC-3 prostate cancer cells (Wang et al., 2004b). As described above, a non-coding SNP in PDZ-RhoGEF was linked to a reduced risk of lung cancer in Mexican Americans (Gu et al., 2006). Additionally, over-expression of p115-RhoGEF, PDZ-RhoGEF, or LARG induces transformation of NIH-3T3 cells (Fukuhara et al., 2001). RGS-RhoGEFs likely undergo complex regulation and participate in multiple signaling interactions. They are capable of associating with receptor tyrosine kinases through their PDZ domains and form hetero- and homo-oligomers via their C-terminal tails, the removal of which enhances GEF activity (Chikumi et al., 2004). RGS-RhoGEF proteins have the potential to be critical regulators of cancer initiation and progression.

Axin

The Wnt signaling cascade regulates proliferation, differentiation, and motility and plays a critical role in development (Salahshor and Woodgett, 2005). Further, aberrant Wnt signaling has been strongly linked to colon cancer, hepatocellular carcinoma, ovarian cancer, prostate

cancer, and melanoma (Nelson and Nusse, 2004). Wnt ligands bind two families of cell surface receptors, Frizzled and low-density-related lipoprotein receptor 5/6, setting off a signaling cascade that controls the stability of the transcriptional regulator and oncogene β-catenin. The accumulation of β-catenin leads to transcription of target genes such as c-jun, c-myc, and cyclin D1. Axin, an atypical RGS protein, serves as a molecular scaffold for a β-catenin destruction complex, binding directly to Adenomatous Polyposis Coli (APC), glycogen synthase kinase-3β (GSK-3β), and β-catenin. This axin-based complex localizes constitutively active GSK-3β such that it can phosphorylate β-catenin, marking it for ubiquitination and subsequent degradation. Binding of Wnt-family ligands to cell surface Frizzled receptors destabilizes the β-catenin destruction complex, preventing the degradation of β-catenin and allowing it to accumulate and translocate to the nucleus where it regulates transcription. Many cancers express mutated forms of APC which are incapable of binding axin (Peifer and Polakis, 2000), resulting in enhanced β-catenin stability and greater β-catenin-dependent transcription. Further, axin participates in the anti-oncogenic TGFβ pathway as a binding partner for SMAD3. Thus, axin is a critical regulator of pathways required for antagonism of β-catenin activity and is a tumor suppressor (Salahshor and Woodgett, 2005).

In colon cancer cells, the Gs-coupled EP2 receptor mediates the mitogenic effect of prostaglandin E2 (PGE2), which induces proliferation and transcription of β-catenin-sensitive genes. Castellone and colleagues (2005) demonstrated that PGE2-stimulated β-catenin-dependent gene transcription is mediated by Gαs. Axin was co-immunoprecipitated with active Gαs, indicating that axin may be an effector of PGE2-stimulated Gαs. Over-expression of the RGS domain of axin almost completely abolished the proliferative response to PGE2. Further, stimulation with PGE2 or expression of constitutively active Gαs was associated with reduced

GSK-3β binding to axin, suggesting that Gαs association with axin may disrupt the β-catenin destruction complex, resulting in greater β-catenin-sensitive gene transcription (Castellone et al., 2005). These data suggest that there are points of intersection between heterotrimeric G-protein signaling and the Wnt signaling pathway that are mediated by the atypical RGS protein axin (**Figure 4.3**).

While axin contains an RGS domain, it does not appear to stimulate GTPase activity of Gα subunits, although it is capable of binding Gαs and Gα12. In MDA-MB 231 breast cancer cells, the axin RGS domain competes with p115-RhoGEF for Gα12. Binding of axin blocks Rho-mediated cell rounding induced by expression of constitutively active Gα12 (Stemmle et al., 2006). As discussed above, G12, p115-RhoGEF, and Rho are known mediators of oncogenesis. It is possible that axin may serve to attenuate the G12/Rho signaling axis via effector antagonism. If axin is mutated or otherwise compromised in cancer cells, its inhibition of Rho signaling would be alleviated, allowing for greater Rho signaling and activation of oncogenic pathways (**Figure 4.3**). Further study will be required to determine the significance of the interaction between axin, G12, and Rho.

RGS5

Angiogenesis is a critical step in the establishment of a solid tumor, allowing the tumor access to growth factors, nutrients, and oxygen (Hanahan and Folkman, 1996); many cancer therapies now target this process. In 2004, two groups identified RGS5 as an up-regulated gene in a microarray screen of hepatocellular carcinoma (Chen et al., 2004b; Furuya et al., 2004). Further, Furuya and colleagues determined that, rather than being expressed in tumor cells themselves, RGS5 is actually found in the pericytes of tumor blood vessels (though not in normal

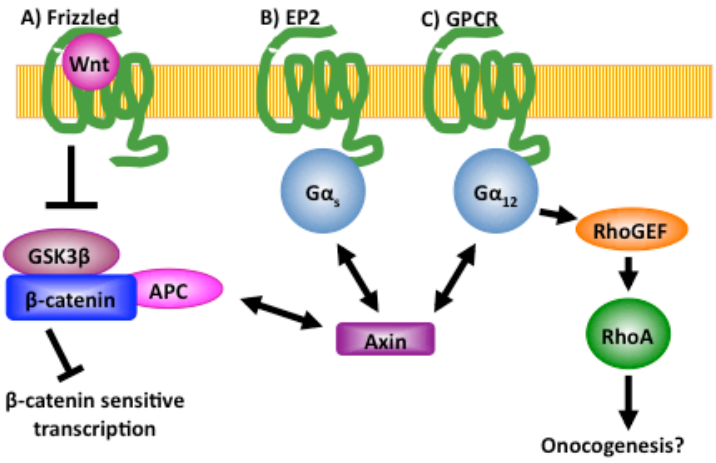

Figure 4.3: Role of axin in Wnt and GPCR signaling cascades.
The atypical RGS protein axin has been reported to play a role in multiple signaling cascades. Blunted arrows indicate inhibition, single-headed arrows indicate activation, and double-headed arrows indicate association. A) In the Wnt pathway, axin blocks β-catenin sensitive transcription by scaffolding its destruction complex. B) In colon cancer cells $G\alpha_s$ binding to axin may disrupt the β-catenin destruction complex, resulting in transcription of β-catenin sensitive genes. C) The RGS domain of axin competes with p115-RhoGEF for $G\alpha_{12}$ in MDA-MB231 breast cancer cells, possibly attenuating the oncogenic G12/Rho signaling axis.

kidney vasculature), indicating that RGS5 may play a role in tumor neovascularization (Furuya et al., 2004).

Tumor vasculature is typically underdeveloped and is characterized by the presence of immature pericytes, tumor hypoxia, and chaotic, leaky vessels. RGS5 expression has been shown to attenuate calcium and ERK signaling downstream of sphingosine-1-phosphate (S1P), angiotensin II, PDGF and endothelin-1, all of which are critical to vascular maturation (reviewed in (Manzur et al., 2009)). In a mouse model of pancreatic islet cancer, RGS5 deletion resulted pericyte maturation and vascular normalization, leading to decreased tumor hypoxia and vessel leakiness compared with wild-type tumor vasculature (Hamzah et al., 2008). The more stable vasculature allows for the growth of larger tumors. At later stages, RGS5 deletion resulted in increased tumor burden and earlier death. While under-developed vasculature causes tumor hypoxia, increased vessel leakiness, and decreased access to nutrients, more chaotic vasculature

also reduces immune system access. Interestingly, though RGS5 deficient mice had an increased tumor burden, they had better response rates to injections of tumor-specific immune cells, indicating that RGS5 attenuation of vascular maturation may protect the tumor from immune attack. Finally, RGS5 has been identified as a broadly expressed tumor antigen, suggesting roles in multiple forms of cancer (Boss et al., 2007).

Conclusions

In the past decade, GPCRs and their cognate ligands have been shown to play a significant role in the initiation and progression of cancer; consequently, it is likely that regulators of GPCRs are also important to the regulation of oncogenic pathways. In this review, we present evidence that the RGS family of proteins play a role in multiple types of cancer. The transcription of over a dozen RGS genes is altered during oncogenesis and mutations in RGS genes have been shown to confer a reduced risk of lung and bladder cancers. Further, specific roles for RGS proteins have been demonstrated in prostate cancer, acute myeloid leukemia, ovarian cancer, colon cancer, and tumor angiogenesis. Further studies will serve to define specific roles of RGS proteins in cancer and lead to a better overall understanding of the signaling pathways regulating oncogenesis.

CHAPTER 5

ENDOGENOUS RGS PROTEINS ATTENUATE Gαi-MEDIATED LYSOPHOSPHATIDIC ACID SIGNALING PATHWAYS IN OVARIAN CANCER CELLS[1]

[1]Hurst, J.H., Henkel, P.A., Brown, A.L., and S.B. Hooks. *Cellular Signalling*, 2008. Feb; 20 (2): 381-9. 2009.

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Abstract

Lysophosphatidic acid is a bioactive phospholipid that is produced by and stimulates ovarian cancer cells, promoting proliferation, migration, invasion, and survival. Effects of LPA are mediated by cell surface G-protein coupled receptors (GPCRs) that activate multiple heterotrimeric G-proteins. G-proteins are deactivated by Regulator of G-protein Signaling (RGS) proteins. This led us to hypothesize that RGS proteins may regulate G-protein signaling pathways initiated by LPA in ovarian cancer cells. To determine the effect of endogenous RGS proteins on LPA signaling in ovarian cancer cells, we compared LPA activity in SKOV-3 ovarian cancer cells expressing Gi subunit constructs that are either insensitive to RGS protein regulation (RGSi) or their RGS wild-type (RGSwt) counterparts. Both forms of the G-protein contained a point mutation rendering them insensitive to inhibition with pertussis toxin, and cells were treated with pertussis toxin prior to experiments to eliminate endogenous Gi/o signaling. The potency and efficacy of LPA-mediated inhibition of forskolin-stimulated adenylyl cyclase activity was enhanced in cells expressing RGSi Gi proteins as compared to RGSwt Gi. We further showed that LPA signaling that is subject to RGS regulation terminates much faster than signaling thru RGS insensitive G-proteins. Finally, LPA-stimulated SKOV-3 cell migration, as measured in a wound-induced migration assay, was enhanced in cells expressing Gαi2 RGSi as compared to cells expressing Gαi2 RGSwt, suggesting that endogenous RGS proteins in ovarian cancer cells normally attenuate this LPA effect. These data establish RGS proteins as novel regulators of LPA signaling in ovarian cancer cells.

Introduction

Lysophosphatidic acid (LPA) is the predominant growth factor that drives the progression of ovarian cancer (Mills et al., 1988; Mills et al., 1990; Mills and Moolenaar, 2003; Umezu-Goto et al., 2004). It mediates multiple aspects of ovarian cancer cell biology including growth promotion (Mills et al., 1988; van Corven et al., 1989; van Corven et al., 1992; Xu et al., 1995a), survival from apoptotic signals (Frankel and Mills, 1996; Levine et al., 1997; Koh et al., 1998; Goetzl et al., 1999b; Weiner and Chun, 1999; Fang et al., 2000b), migration (Sengupta et al., 2003), and production of growth factors and proteases (Sengupta et al., 2007) which are critical for neovascularization and metastasis (Fang et al., 2000a). It has further been shown that lowering extracellular LPA concentrations in ovarian cancer cell cultures by over-expression of degradative enzymes both reduces colony-forming activity and tumor growth and enhances apoptosis (Tanyi et al., 2003b), establishing a direct role for LPA in ovarian cancer cell growth and progression. LPA signaling is mediated by at least five cell surface G-protein coupled receptors (LPA1-5) (Fang et al., 2000a; Tanyi et al., 2003b). These receptors are capable of coupling to multiple G-proteins and all five receptors have been demonstrated to couple to Gi (Fukushima et al., 1998; Contos et al., 2000b; Ishii et al., 2000; Fukushima and Chun, 2001; Lee et al., 2006a; Lee et al., 2007). Gi pathways have been specifically implicated in mediating LPA stimulated proliferation and migration, as these two LPA effects are blocked by treatment with the Gi selective inhibitor pertussis toxin (Ptx) (van Corven et al., 1989; Sengupta et al., 2007).

Regulator of G-protein Signaling (RGS) proteins deactivate heterotrimeric G-proteins by accelerating the rate at which Gα subunits hydrolyze GTP. RGS proteins have been shown to have profound effects on the kinetics and magnitude of multiple in vivo receptor signaling pathways (Chen et al., 2000; Heximer et al., 2003; Fu et al., 2007). RGS insensitive G-protein

mutants are a useful tool to define the effect of endogenous RGS proteins. Originally discovered in a yeast G-protein (DiBello et al., 1998), a single point mutation (G184S) in Gαi has been shown to prevent binding of RGS to the Gα protein, thereby rendering the Gα subunit resistant to RGS regulation, while allowing normal rates of intrinsic GTP hydrolysis, GDP release, and effector activation (Lan et al., 1998).

The goal of this study was to determine the effect of endogenous RGS proteins on LPA-mediated Gi/o signaling in ovarian cancer cells. We used a well characterized and validated strategy (Cavalli et al., 2000; Clark et al., 2003; Clark and Traynor, 2004; Fu et al., 2004; Ikeda and Jeong, 2004; Clark and Traynor, 2005; Clark and Lambert, 2006) to directly compare the signaling of two Gα mutant constructs that were insensitive to Ptx and either wildtype with respect to RGS regulation (Ptxi RGSwt) or insensitive to RGS regulation (Ptxi RGSi) in the presence of Ptx to inhibit endogenous proteins. Using this system, we demonstrate that endogenous RGS proteins attenuate signaling of Gαi-mediated LPA-stimulated inhibition of adenylyl cyclase and cell migration in ovarian cancer cells.

Experimental methods

Cell culture

Human SKOV-3 ovarian cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia) and grown according to ATCC recommendations. Cells were maintained in McCoy's 5A medium with 1.5 mM L-glutamine, 2.2 g/L sodium bicarbonate, and 10% fetal bovine serum at 37 °C in the presence of 5% CO2.

DNA constructs and transfections

Plasmids encoding C351I Gαi1, C352IGαi2, C351I Gαi3, and C351I Gαo and the corresponding C→G mutants of each Gi/o subunit were obtained from UMR cDNA Resource

Center (Rolla, MO). RGS insensitive Gα subunits were generated with site-directed mutagenesis using a Stratagene QuikChange Site-Directed Mutagenesis System kit according to manufacturers instructions (LaJolla, CA). The G184S mutation was introduced into the Gαi2 C352I construct sequence as previously described (Lan et al., 1998) with the following primers: sense 5′-GTAAAGACCACGAGCATCGTGGAGACA-3′ and antisense 5′-TGTCTCCACGATGCTCGTGGTCTTTAC-3′. The G184S mutation was introduced into the C351I Gαo construct using primers covering the corresponding bases. Transfections were performed using Fugene 6 transfection reagent according to the manufacturer's instructions at a ratio of 2 μL Fugene 6 reagent to 1 μg plasmid DNA (Roche Diagnostics, Basel, Switzerland). SKOV-3 cells were transfected with up to a total of 1 μg/well of plasmid DNA in a 12-well plate or 500 ng/well in 24-well plates. All assays were performed 48 hours post-transfection.

Western blotting

Protein expression was determined using standard techniques. Cells were harvested and lysed in protein sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using a primary antibody targeted against Gαi2 (Santa Cruz Biotechnologies, Santa Cruz, CA) and peroxidase-conjugated secondary antibodies (Bethyl Laboratories, Montgomery, TX). Bands were then visualized using SuperSignal Chemiluminescent substrate (Pierce, Rockford, IL). Densitometry analysis was performed using Total Lab 1D Gel Analysis software. Background bands were not subtracted out and all lanes and bandwidths were of equal size.

cAMP assay

We use a modified version of the protocol described in Hettinger-Smith et al. (Hettinger-Smith et al., 1996). SKOV-3 cells were plated in 12-well dishes and labeled with 0.6 μCi [^{3}H]-

adenine (Perkin Elmer, Waltham, MA) for three hours in the presence or absence of 200 ng/mL Ptx. Assay buffer containing 1 mM IBMX, a phosphodiesterase inhibitor, 50 μM forskolin, and varying concentrations of LPA was added to the cells for 10 min (unless other wise noted in time course assay) at 37 °C. Reactions were terminated by aspiration followed by the addition of stop solution containing 1.3 mM cAMP and 2% sodium dodecyl sulfate. [^{14}C]-cAMP stock was added to each well to control for recovery of cAMP, followed by perchloric acid to lyse cells. Lysates were neutralized with KOH and cAMP was isolated using sequential column chromatography over Dowex AG-50-W4 cationic exchange resin (Bio-Rad, Hercules, CA) followed by neutral alumina columns. The resulting eluate was subjected to scintillation counting after the addition of 10 mL scintillation cocktail.

Wound-induced migration assay

Monolayers of SKOV-3 cells were plated in 24-well dishes and transfected with either vector, plasmid encoding Gαi2 Ptxi RGSwt, or Gαi2 Ptxi RGSi. 24 hours later, cells were starved in serum-free media in the presence or absence of 100 ng/mL Ptx for an additional 24 hours prior to wounding. A "wound" was introduced by scraping a single line through the monolayer with a pipette tip, and cells were then treated with 30 μM LPA or vehicle. Images of the wound were captured with a Nikon AZ100 microscope mounted with a Nikon Digital Sight DS-QiMc camera set at 10× magnification at time zero and every 6 hours for 48 hours after the wound was made to compare the speed at which surrounding cells fill the wound. LPA and/or Ptx were also reapplied to cells every 12 hours to prevent depletion. Wound filling was quantified using Nikon NIS Elements BR 2.30 software. Using a "polygonal region of interest" drawing tool, wound edges were traced to create a polygon whose surface area was measured by

the software in pixels squared. Wound closure was measured as the initial area minus the area of the wound at a given time point to yield area covered.

Results

LPA stimulated inhibition of cAMP accumulation can be mediated by each Gαi/o isoform in SKOV-3 cells

In SKOV-3 cells, LPA stimulates a dose-dependent inhibition of forskolin-stimulated cAMP accumulation that is completely inhibited by pre-treatment with Ptx (**Figure 5.1**). Mutation of cysteine residue 351/2 in Gi/o subunits renders the G-protein insensitive to Ptx (Bahia et al., 1998), but previous studies have reported conflicting results regarding the optimal substituting residue for a Ptxi mutant. Massotte and colleagues (Massotte et al., 2002) compared activity of each alternate amino acid substitution and found highest activity in C→I mutants, while Ikeda and Jeong (Ikeda and Jeong, 2004) reported that mutation to glycine yielded better activity than isoleucine (Ikeda and Jeong, 2004). To determine which Ptx mutant was most effective in rescuing LPA-stimulated Gi activity in SKOV-3 cells, we compared the activity of C351G and C351I mutants of Gαi1 for the ability to rescue LPA receptor mediated inhibition of forskolin-stimulated cAMP accumulation in the presence of Ptx. In our assay, the C351I mutant had significantly greater activity than C351G (data not shown).

To determine which Gi/o isoforms could mediate LPA signaling in SKOV-3 cells, cells were transfected with Ptxi mutants (C351/2I) of Gαi1, Gαi2, Gαi3, or Gαo and LPA signaling was assessed following deactivation of endogenous Gi/o proteins with Ptx. All Gi/o Ptxi constructs tested were able to partially rescue the LPA inhibition of cAMP to a similar degree (**Figure 5.1**). Co-expression of Gi and Go subunits increased the degree of rescue (data not

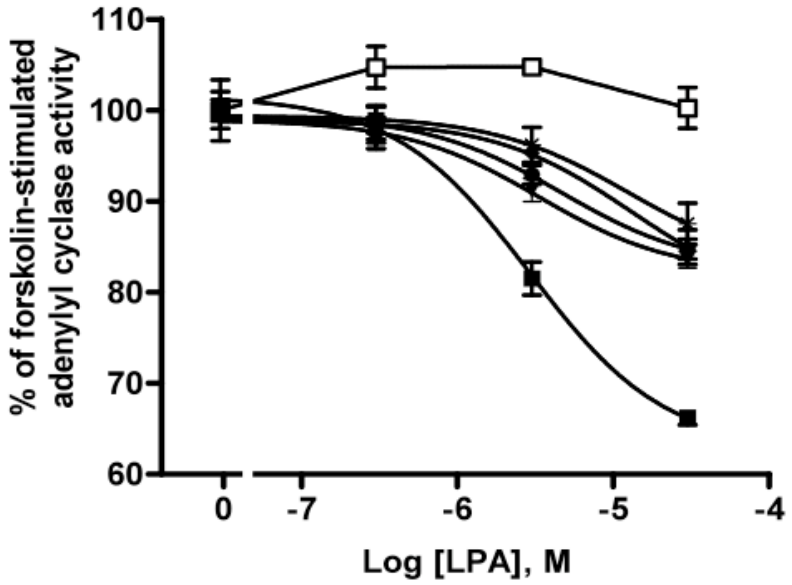

Figure 5.1: All pertussis toxin insensitive mutants of Gi/o partially rescue LPA-stimulated inhibition of forskolin-mediated cAMP accumulation.
SKOV-3 cells were transfected and assayed for cAMP levels as described in Experimental methods. C351I (or equivalent) Ptxi mutations of Gαi1 (▼), Gαi2 (♦), Gαi3 (●), and Gαo (✳) were transfected into SKOV-3 cells and pretreated with Ptx. Vector transfected SKOV-3 cells were assayed in the absence (■) or presence (□) of Ptx pretreatment. Cells were stimulated with 50 μM forskolin and increasing concentrations of LPA. Results are reported as a percent of adenylyl cyclase activity observed in the absence of LPA. Data shown here are representative of three independent experiments.

shown). We continued our studies with Gαi2 because it is widely expressed and can be detected endogenously in SKOV-3 ovarian cancer cells (**Figures 5.3-5.6**).

Endogenous RGS proteins attenuate LPA-mediated inhibition of cAMP levels in SKOV-3 cells

To evaluate the effect of the total complement of RGS proteins expressed in SKOV-3 ovarian cancer cells on Gi/o-mediated LPA signaling, we introduced an additional point mutation in Gαi2 Ptxi to make it insensitive to RGS regulation (Gαi2 Ptxi RGSi) (**Figure 5.2**). This construct was transfected in SKOV-3 cells in parallel with the single Ptxi mutation (Gαi2 Ptxi RGSwt). Cells were then pretreated with Ptx to eliminate all signaling through endogenous Gi/o G-proteins. Thus, all signaling was funneled through the mutant proteins for direct comparison of signaling in the presence and absence of RGS regulation. Transfection conditions

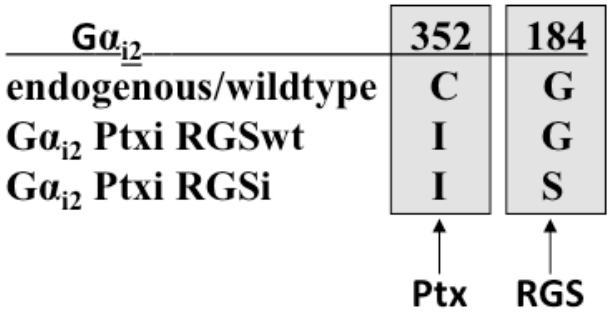

Figure 5.2: Mutagenesis Strategy
Two point mutations of Gαi2 were used in these studies. Residue 352 is a cysteine in the wild-type protein and is the target of ADP-ribosylation and deactivation of the protein by Ptx. This residue is replace by isoleucine in both mutant forms of the protein, rendering the protein insensitive to Ptx (Ptxi). Residue 184 of Gαi2 is a glycine in the wild-type protein, and constitutes a critical contact point between RGS proteins and Gα subunits. This residue is replaced by serine in one of the mutants used, rendering it resistant to regulation by RGS proteins (RGSi).

were optimized to yield equivalent expression of the two Gαi2 mutants as determined by Western blotting and densitometry (**Figure 5.3B**). (Analogous mutations were also generated in Gαo, and these constructs were expressed and evaluated in the following experiments. Data not shown.)

Forty-eight hours after transfection, we determined the ability of the mutants to mediate LPA inhibition of forskolin-stimulated cAMP accumulation. Again, Ptx completely inhibited LPA stimulated effects on cAMP levels in vector transfected cells, demonstrating that all endogenous Gi/o G-protein activity had been blocked. Expression of the mutant Gα subunits did not affect basal cAMP levels or forskolin-stimulated cAMP levels in the absence of LPA. In the presence of Ptx, expression of Gαi2 Ptxi RGSwt mediated a modest level of LPA activity; however, LPA activity was significantly greater with expression of Gαi2 Ptxi RGSi (**Figure 5.3A**). Expression of Gαi2 Ptxi RGSi was equal to or slightly less than expression of its RGSwt counterpart; thus, the increased activity does not reflect higher expression levels. These data

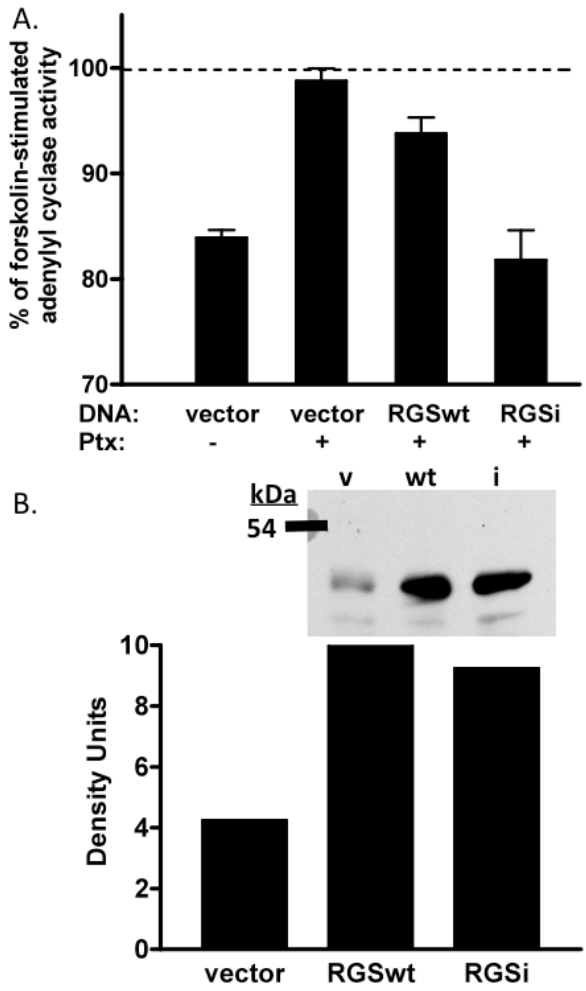

Figure 5.3: Endogenous RGS proteins in SKOV-3 cells inhibit Gαi2-mediated LPA inhibition of adenylyl cyclase.
A) SKOV-3 cells were transfected with empty vector, Gαi2 Ptxi RGSwt, or Gαi2 Ptxi RGSi. Where indicated, cells were pre-treated with Ptx prior to the assay. Cells were treated with 50 μM forskolin and 10 μM LPA for 10' and cAMP was measured as described. Results are reported as percent of adenylyl cyclase activity observed in the absence of LPA. Data shown here are representative of three independent experiments. B) Cells transfected in parallel with the above experiment were subjected to Western blotting with Gαi2 specific antibodies and densitometry was performed as described. Results are reported as arbitrary density units.

demonstrate that LPA-stimulated Gαi2 signaling is higher in the absence of endogenous RGS protein interaction with G-proteins.

We next determined whether endogenous RGS protein interaction with G-proteins affects the potency or efficacy of Gαi2-mediated LPA signaling. Again, equivalent amounts of each

mutant were expressed in SKOV-3 cells (**Figure 5.4B**). Cells were stimulated with a range of LPA concentrations in the presence of forskolin and Ptx to generate dose response curves. In the vector transfected cells, LPA activation of Gi/o was completely blocked, revealing Gαs-like increases in cAMP. Again, Gαi2 Ptxi RGSwt partially recovered LPA-mediated inhibition of cAMP accumulation, but expression of similar levels of Gαi2 Ptxi RGSi resulted in LPA activity that was three times more potent and twice as efficacious as activity mediated by Gαi2 Ptxi RGSwt (**Figure 5.4A**).

The well-defined mechanism of RGS deactivation of G-proteins is to enhance the GTPase activity of Gαi and thereby accelerate the rate of GTP hydrolysis and G-protein deactivation. To determine if RGS proteins regulate the kinetics of Gi activation by LPA, we examined the time course of LPA activation of Gαi2 proteins in the presence and absence of RGS regulation. SKOV-3 cells were transfected with the two Gαi2 mutants as described above and treated with Ptx to eliminate endogenous Gi/o activity. The ability of the G-protein mutants to mediate LPA inhibition of forskolin-stimulated cAMP production was measured at multiple time points ranging from 3 min to 50 min with a single concentration of 10 μM LPA. LPA stimulation of Gi, as indicated by inhibition of cAMP levels, was deactivated in cells expressing Gαi2 Ptxi RGSwt by approximately 10 min, while LPA stimulated Gαi2 Ptxi RGSi was still active at 50 min (**Figure 5.5A**). Taken together, these data indicate that endogenous RGS proteins function to accelerate the deactivation of Gαi2 following activation by LPA, resulting in higher apparent potency and efficacy of LPA-stimulated inhibition of cAMP accumulation.

We also determined the effect of endogenous RGS regulation of Gαo on LPA signaling by comparing LPA activity mediated by Gαo Ptxi RGSwt and Gαo Ptxi RGSi. Similar differences in potency, efficacy, and duration of signaling were observed, suggesting that RGS

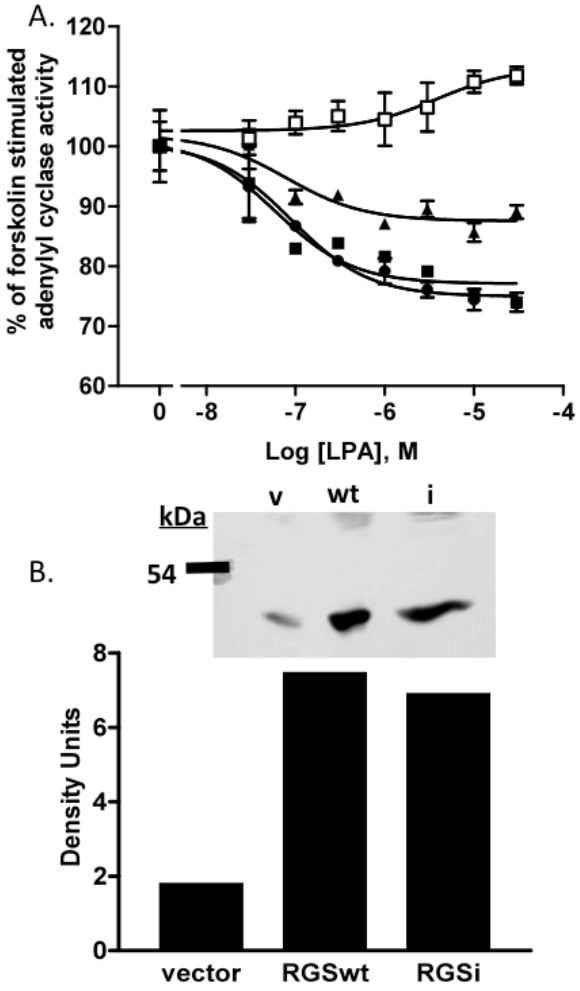

Figure 5.4: Endogenous RGS proteins affect both potency and efficacy of Gαi2-mediated LPA inhibition of adenylyl cyclase.
A) SKOV-3 cells were transfected with empty vector (■, □), Gαi2 Ptxi RGSwt (▲), or Gαi2 Ptxi RGSi (●) and pretreated with Ptx (□, ▲, ●) or vehicle (■) prior to the assay. Cells were treated with a range of LPA concentrations in the presence of 50 μM forskolin and assayed for cAMP levels as described. Results are reported as a percent of
adenylyl cyclase activity observed in the absence of LPA. Data shown here are representative of three independent experiments. B) Cells transfected in parallel with the above experiment were subjected to Western blotting with Gαi2 specific antibodies and densitometry was performed as described. Results are reported in arbitrary density units.

protein endogenously expressed in SKOV-3 ovarian cancer cells are capable of negatively regulating Gαo subunits activated downstream of endogenous LPA receptors; however, no endogenous Gαo was detected in SKOV-3 cells, so the relevance of these observations to signaling in these cells is unclear (data not shown).

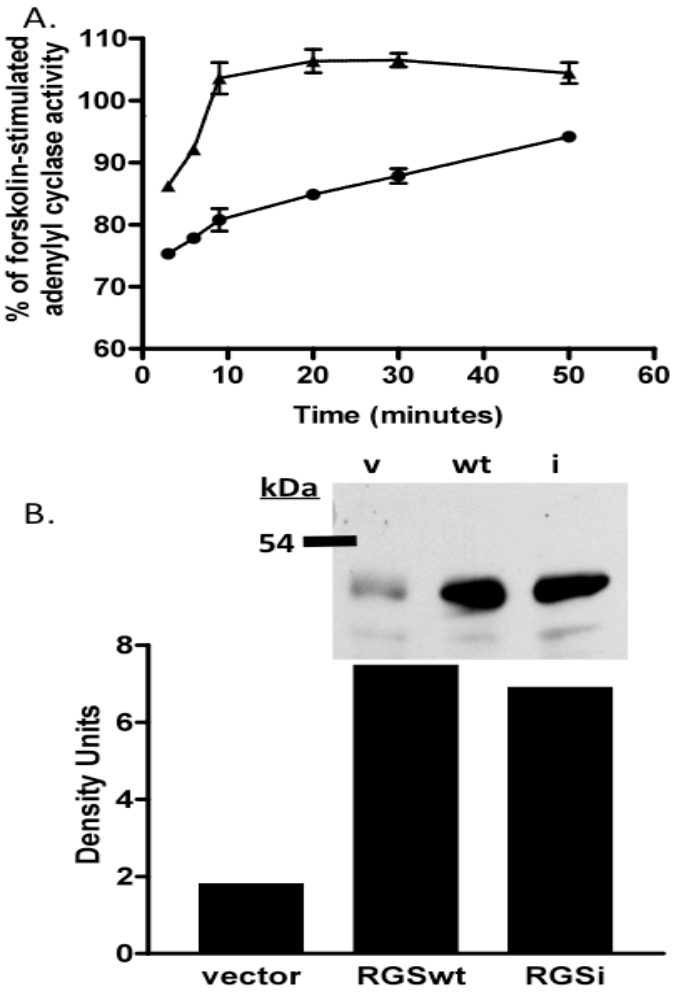

Figure 5.5: RGS proteins affect the duration of LPA-stimulated Gαi2 activity.
A) SKOV-3 cells were transfected with Gαi2 Ptxi RGSwt (▲) or Gαi2 Ptxi RGSi (●). Cells were pretreated with Ptx and treated with 10 μM LPA in the presence of 50 μM forskolin for different time periods. cAMP levels were measured as described. Results are reported as a percent of adenylyl cyclase activity observed in the absence of LPA at each time point. Data shown here are representative of three independent experiments. B) Cells transfected in parallel with the above experiment were subjected to Western blotting with Gαi2 specific antibodies and densitometry was performed as described. Results are reported in arbitrary density units.

Endogenous RGS regulation attenuates LPA-mediated wound closure in SKOV-3 cells

Gi/o pathways have previously been implicated in the regulation of LPA-stimulated cell migration (Panetti et al., 2000; Sengupta et al., 2003). We investigated the role of RGS proteins in regulating LPA stimulated SKOV-3 cell migration. Cells were grown to confluence, serum starved, and scratched to mimic a wound. We then treated cells with vehicle, 30 μM LPA, or

media containing 10% serum in the presence or absence of Ptx pretreatment and quantified cell migration into the wound area using microscopy as described in Experimental Methods. The rate of migration stimulated by LPA was similar to that observed with 10% fetal bovine serum (FBS), with wounds closing within 48 hours, while vehicle treated cells exhibited minimal migration. Ptx blocked migration of both LPA (**Figure 5.6**) and FBS (data not shown) treated cells back down to vehicle treated levels. We next examined the effect of RGS regulation on LPA stimulated migration in SKOV-3 cells using the Gαi2 mutants. In the absence of Ptx, with endogenous Gi/o signaling intact, LPA treated cells transfected with Gαi2 Ptxi RGSwt did not close the wound at a significantly higher rate than vector transfected cells; however, cells transfected with Gαi2 Ptxi RGSi displayed enhanced migration in response to LPA, especially at early time points (**Figure 5.6 A**). In the presence of Ptx, cells transfected with Gαi2 Ptxi RGSwt had a slightly increased migration rate compared with vector transfected cells, demonstrating that signaling through Ptxi mutants was able to recover some of the LPA effect. However, when Gαi2 Ptxi RGSi was expressed, LPA-stimulated migration was increased 3-fold over cells expressing Gαi2 Ptxi RGSwt (**Figure 5.6 B, C**). These data suggest that endogenous RGS proteins attenuate the migratory effect of LPA mediated by Gαi2 in SKOV-3 ovarian cancer cells.

Discussion

The purpose of this study was to determine whether Gi mediated LPA signaling pathways in ovarian cancer cells were sensitive to regulation by endogenous RGS proteins. We have shown that LPA receptors can couple to Gαi2 in SKOV-3 cells, Gαi2 is expressed in SKOV-3 cells, and endogenous RGS proteins expressed in these cells attenuate LPA signaling through Gαi2 by accelerating its deactivation. Specifically, mutagenically blocking the association of endogenous RGS proteins with Gαi2 delayed the deactivation of Gαi2 activity, resulting in more

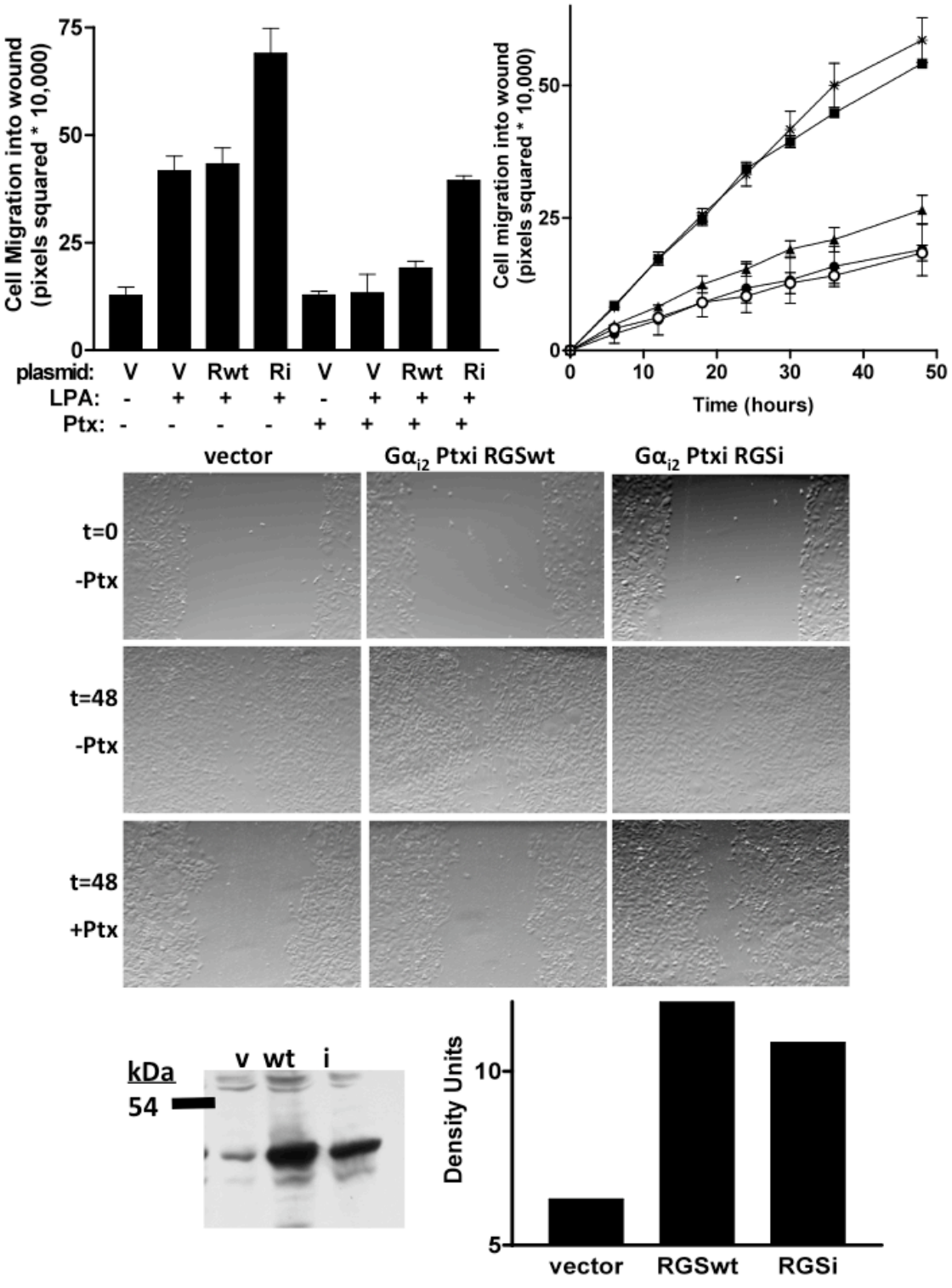

Figure 5.6: Endogenous RGS proteins attenuate LPA-stimulated cell migration.[1]

more robust cell migration responses to LPA. Taken together, these findings establish RGS proteins as novel regulators of LPA signaling in ovarian cancer cells, suggesting that these proteins are potential therapeutic targets.

Ovarian cancer is the most deadly gynecological cancer in the United States (Greenlee et al., 2001). The predominant cellular growth signaling molecule in ovarian cancer cells is LPA. A critical role for LPA in ovarian cancer was first established when Ovarian Cancer Activating Factor (OCAF), a growth factor purified from malignant ascitic fluid, was identified as LPA (Xu et al., 1995c). LPA drives the progression of ovarian cancer by promoting cell division (van Corven et al., 1989; Mills et al., 1990; van Corven et al., 1992; Xu et al., 1995b), initiating metastasis (Fang et al., 2000a; Mills and Moolenaar, 2003), stimulating migration (Sengupta et al., 2003), and allowing cancer cells to survive chemotherapy (Fang et al., 2000a; Tanyi et al., 2003b). LPA1, LPA2, and LPA3 are expressed in multiple ovarian cancer cell lines, including SKOV-3, and expression of LPA2 and LPA3 has been positively correlated with disease potent and efficacious LPA effects on cAMP levels. Further, RGS insensitive Gαi2 mediated

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[1]Figure 5.6: Endogenous RGS proteins attenuate LPA-stimulated cell migration. Monolayers of SKOV-3 cells were transfected with vector, Gαi2 Ptxi RGSwt or Gαi2 Ptxi RGSi, pretreated with Ptx or vehicle. A "wound" was introduced by scraping a single line through the monolayer with a pipette tip and then treated with 30 μM LPA or vehicle for 48 hours. Images were captured every six hours for 48 hours and cell migration was quantified using microscopy as described. Data shown here are representative of three independent experiments. A) Wound surface area covered by migrating cells was determined after 36 hours in the presence or absence of LPA, with or without pretreatment with pertussis toxin (Ptx), in cells transfected with either vector (V), Gαi2 Ptxi RGSwt (Rwt) or Gαi2 Ptxi RGSi (Ri). B) Symbol legend: vector transfected cells treated with vehicle only (○); vector transfected cells treated with 30 μM LPA (⋆); vector transfected cells pretreated with pertussis toxin and treated with LPA (●); Gαi2 Ptxi RGSwt transfected cells treated with LPA and pretreated with Ptx (▲); and Gαi2 Ptxi RGSi transfected cells treated LPA and pretreated with Ptx (■). Wound closure is reported as area of wound filled in (pixels squared) vs. time in hours. Complete time course data observed in the absence of Ptx is provided in supplemental data. C) Columns labels denote which constructs were used to transfect the cells, and row labels indicate the time at which the images were taken (hours) and whether or not cells were pre-treated with Ptx. All images are of cells treated with 30 μM LPA. D) Cells transfected in parallel with the above experiment were subjected to Western blotting with Gαi2 specific antibodies and densitometry was performed as described. Results are reported in arbitrary density units.

progression (Hu et al., 2003), while over-expression of LPA1 induces apoptosis in certain ovarian cancer cell lines (Furui et al., 1999). While the expression of LPA4 and LPA5 has not been reported in specific ovarian cell lines, LPA4 is highly enriched in ovarian tissue (Noguchi et al., 2003). The central mediators of LPA signaling are G-proteins. LPA receptors have been shown in various systems to couple to multiple G-proteins (Noguchi et al., 2003; Anliker and Chun, 2004b): LPA1 and LPA2 to Gq, Gi, and G12; LPA3 to Gq and Gi; LPA4 to Gq, Gi, G12, and Gs; and LPA5 to Gq, Gi, and G12 (Fukushima et al., 1998; Contos et al., 2000b; Ishii et al., 2000; Fukushima and Chun, 2001; Lee et al., 2006a; Lee et al., 2007). Thus, LPA signaling in SKOV-3 cells is likely mediated by multiple receptor and G-protein subtypes. RGS proteins are attractive targets to selectively manipulate these pathways, as most RGS proteins display specificity for a subset of G-proteins, and in many cases RGS proteins only deactivate G-proteins that are coupled to specific receptors (Zeng et al., 1998).

To address the role of endogenous RGS proteins in the regulation of LPA signaling pathways, we used a G-protein double mutant strategy that has been described in detail previously (**Figure 5.2**) (Cavalli et al., 2000; Boutet-Robinet et al., 2003; Clark et al., 2003; Clark and Traynor, 2004; Fu et al., 2004; Ikeda and Jeong, 2004; Clark and Traynor, 2005; Clark and Lambert, 2006). In order to eliminate signaling by all endogenous Gi/o G-proteins so that we could directly compare signaling by RGS sensitive and insensitive versions of a single Gi/o isoform, we employed Gi/o subunits that had been modified to be resistant to Ptx. The interaction of Gi/o subunits with effectors is prevented by treatment with Ptx, which causes ADP-ribosylation of a cysteine residue (351/352) in the C-terminus of the Gα subunit. The mutation of residue 351/352 in Gi/o subunits to any residue other than cysteine renders the G-protein insensitive to Ptx regulation, but not all residues yield a fully active G-protein. Bahia and

colleagues (Bahia et al., 1998) determined that increased hydrophobicity of the mutated residue (i.e. isoleucine) led to greater signaling activity by the G-protein (Bahia et al., 1998); however, other groups have reported that mutations to leucine (Zhang et al., 2006a), glycine (Wise et al., 1999; Jeong and Ikeda, 2000; Clark and Traynor, 2004; Ikeda and Jeong, 2004), and isoleucine (Massotte et al., 2002), all have good activity in a variety of assays and cell types, and Ikeda and Jeong (Ikeda and Jeong, 2004) reported that mutation to glycine is better than isoleucine in their studies measuring Gi/o regulation of N-type Ca2+ channels (Ikeda and Jeong, 2004). The functionality of these mutants is likely dependent on a combination of factors including assay, cell type, and receptor. We determined that the C351I mutant has greater activity than C351G in adenylyl cyclase inhibition assays performed in SKOV-3 cells.

The mutation that renders Gα subunits insensitive to RGS regulation was first discovered in yeast (gpa1sst) and phenotypically mimics the loss of the yeast RGS protein, SST2 (DiBello et al., 1998). Lan and colleagues (Lan et al., 1998) later described the homologous mutation in Gi-family subunits. A single glycine to serine mutation at residue 184 of Gαi, which constitutes a major contact point between Gα and RGS proteins (Tesmer et al., 1997), eliminates binding of Gα to RGS protein and thus eliminates RGS effects on the GTPase activity of Gα. This mutation does not affect either the intrinsic GTPase activity of the Gα subunit, the kinetics of GDP release, or effector activation (Lan et al., 1998). The G→S RGSi mutation was introduced in the C→I Ptxi Gαi mutant background for direct comparison with Ptxi Gαi constructs that retained wild-type RGS regulation. Differences in signaling activity of cells expressing the two different mutant isoforms in the presence of Ptx reveal the effect of RGS regulation. To fairly compare their activity, expression levels should be equivalent. We used a transient expression system, so levels were carefully monitored with Western blotting and densitometry. In all the data

represented, Gαi2 Ptxi RGSwt was expressed at levels as high as or slightly higher than Gαi2 Ptxi RGSi, even though Gαi2 Ptxi RGSi consistently had higher activity. Thus, differences in activity of the two mutant isoforms cannot be attributed to expression levels. Notably, Gαi2 RGSwt Ptxi expressed at levels significantly higher than endogenous Gαi2 levels did not restore signaling to the level observed in the absence of Ptx. This may indicate that endogenous Ptx sensitive signaling reflects contributions by more than one Gi/o subtype, which is consistent with our observation that each Gi/o isoform could partially rescue signaling. Further, the C351I Ptxi mutant may have intrinsically lower activity than the wild-type G-protein.

The presence of the G184S RGS-insensitive mutation in Gαi2 (and Gαo) significantly enhanced LPA mediated inhibition of forskolin-stimulated cAMP accumulation in SKOV-3 cells, reflecting an increase in both potency and efficacy of LPA and consistent with RGS protein attenuation of signaling by wildtype G-proteins. Indeed, studies examining the effect of an RGS-insensitive Gαo on μ-opiod receptor signaling also showed that blockade of RGS regulation increased the potency and efficacy of DAMGO-stimulated inhibition of adenylyl cyclase (Clark et al., 2003). However, it is possible that the introduction of the RGSi mutation in the G-protein could enhance its signaling capacity through a mechanism independent of the RGS mediated acceleration of GTPase activity. Thus, we further characterized the difference in signaling between wild-type and RGSi G-proteins in an activity time course, and found that the rate of deactivation of wild-type G-proteins was much faster than that of RGSi G-proteins. This kinetic effect is consistent with the known ability of RGS proteins to accelerate the deactivation of G-proteins by enhancing GTPase activity, and has also been observed by other groups evaluating signaling from RGSi G-protein mutants (Chen et al., 2004a). Thus, our results strongly suggest that the observed differences in signaling by RGSi and RGSwt G-proteins reflect the GTPase

accelerating activity of endogenous RGS proteins. Future efforts to identify and characterize the specific endogenous RGS proteins in SKOV-3 cells responsible for this regulation are required to formally define the mechanism of this regulation.

LPA has been previously reported to stimulate migration (Zhou et al., 1995; Pietruck et al., 1997; Mills and Moolenaar, 2003; Sengupta et al., 2003; Stahle et al., 2003; Yan et al., 2003; Barber et al., 2004; Bian et al., 2004; Moolenaar et al., 2004; Yamada et al., 2004; Cui and Qiao, 2006; Fisher et al., 2006; Kim et al., 2006; Ren et al., 2006; Hao et al., 2007; Meyer zu Heringdorf and Jakobs, 2007) in many different cell types. We characterized LPA stimulated cell migration in SKOV-3 cells by demonstrating that LPA receptors can couple to Gαi2 to mediate these effects, and that endogenous RGS proteins can attenuate cellular migration mediated by this pathway. The classic wound-induced migration assay used in our study has a longer time course than the commonly used "chamber" method because the distance the cells transverse is large and thus may reflect cell proliferation in addition to migration. Early characterization of the assay demonstrated that migration effects predominate during the early time points of the assay, while proliferation may play a role later in the assay (Kartha and Toback, 1992; Rodriguez et al., 2005). In our experiments, the effects of LPA were completely Ptx sensitive at all time points, and the relative difference betweenGαi2 Ptxi RGSwt and Gαi2 Ptxi RGSi did not appear more pronounced at earlier or later time points. Thus, we cannot distinguish RGS effects on migration and proliferation in this assay.

Our studies demonstrate that RGS proteins expressed in ovarian cancer cells regulate signaling by Gi/o G-proteins activated by LPA. Ptx treatment is widely used to define the role of Gi/o family G-proteins in endogenous signaling systems. Signaling by Ptx sensitive Gi/o proteins is specifically implicated in mediating LPA stimulated proliferation (van Corven et al., 1989),

and increased cellular cAMP levels have been shown to decrease cell proliferation, further implicating Gi mediated cAMP decrease as a growth promoting pathway (Shaw et al., 2002). Finally, studies in adrenal glomerulosa cells (Shah et al., 2005) and human bronchial epithelial cells (Zhao et al., 2006) demonstrated that Gi/o-mediated LPA signaling is implicated in the regulation of epidermal growth factor receptor transactivation and the MAPK pathway, which directly contribute to cell proliferation. Gi/o G-proteins have also been shown to be required for migration and invasion of ovarian cancer cells. In 2003, Sengupta and colleagues demonstrated that ovarian cancer cell migration is inhibited by Ptx treatment (Sengupta et al., 2003), and recently published observations confirm that LPA-stimulated migration of SKOV-3 cells is Ptx sensitive (Evelyn et al., 2007). Gi/o G-proteins regulate the production of second messengers both through the Gα subunit, which inhibits adenylyl cyclase activity to lower cAMP levels in cells, and through the Gβγ subunit, which can activate phospholipase C to increase phosphoinositide hydrolysis and subsequent calcium mobilization and protein kinase C (PKC) activation to regulate diverse pathways including the mitogenic Ras-MAPK cascade, phosphoinositde-3-kinase (PI3K), and PKB/Akt to trigger survival signals (Moolenaar et al., 2004). Some or all of these pathways may contribute to the pathological activity of LPA in ovarian cancer cells.

Conclusions

In summary, our studies of the regulation of LPA signaling in SKOV-3 ovarian cancer cells have demonstrated that both inhibition of cAMP levels and cell migration in response to LPA are mediated by Ptx-sensitive G-proteins, and LPA receptors responsible for these effects can couple to Gαi2, which is endogenously expressed in SKOV-3 cells. Further, we have used RGS insensitive G-proteins to establish a role for endogenous RGS proteins in the regulation of

LPA signaling through Gαi2 in these cells. Detailed comparison of LPA effects on cAMP levels in SKOV-3 cells expressing either RGS sensitive or RGS insensitive G-proteins revealed that endogenous RGS proteins accelerate the termination of LPA signaling and thereby lower the potency and efficacy of LPA effects. Finally, we showed that endogenous RGS proteins attenuate LPA mediated migration of SKOV-3 cells. Taken together, these data establish RGS proteins as novel regulators of LPA signaling in ovarian cancer cells. Future studies should determine which RGS proteins are specifically expressed in ovarian cancer cells and whether these RGS proteins can be linked to regulation of specific signaling pathways that promote the progression of ovarian cancer.

CHAPTER 6

REGULATOR OF G-PROTEIN SIGNALING EXPRESSION AND FUNCTION IN OVARIAN CANCER CELL LINES

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Abstract

Regulator of G-protein signalling (RGS) proteins critically regulate signalling cascades initiated by G-protein coupled receptors (GPCRs) by accelerating the deactivation of heterotrimeric G-proteins. Lysophosphatidic acid (LPA) is the predominant growth factor that drives the progression of ovarian cancer by activating specific GPCRs and G-proteins expressed in ovarian cancer cells. We have recently reported that RGS proteins endogenously expressed in SKOV-3 ovarian cancer cells dramatically attenuate LPA stimulated cell signalling. The goal of this study was twofold: first, to identify candidate RGS proteins expressed in SKOV-3 cells that may account for the reported negative regulation of G-protein signalling, and second, to determine if these RGS protein transcripts are differentially expressed among commonly utilized ovarian cancer cell lines and non-cancerous ovarian cell lines. Reverse transcriptase-PCR was performed to determine transcript expression of 22 major RGS subtypes in RNA isolated from SKOV-3, OVCAR-3 and Caov-3 ovarian cancer cell lines and non-cancerous immortalized ovarian surface epithelial (IOSE) cells. Fifteen RGS transcripts were detected in SKOV-3 cell lines. To compare the relative expression levels in these cell lines, quantitative real time RT-PCR was performed on select transcripts. RGS19/GAIP was expressed at similar levels in all four cell lines, while RGS2 transcript was detected at levels slightly lower in ovarian cancer cells as compared to IOSE cells. RGS4 and RGS6 transcripts were expressed at dramatically different levels in ovarian cancer cell lines as compared to IOSE cells. RGS4 transcript was detected in IOSE at levels several thousand fold higher than its expression level in ovarian cancer cells lines, while RGS6 transcript was expressed fivefold higher in SKOV-3 cells as compared to IOSE cells, and over a thousand fold higher in OVCAR-3 and Caov-3 cells as compared to IOSE cells. Functional studies of RGS 2, 6, and 19/GAIP were performed by measuring their effects on LPA

stimulated production of inositol phosphates. In COS-7 cells expressing individual exogenous LPA receptors, RGS2 and RSG19/GAIP attenuated signalling initiated by LPA1, LPA2, or LPA3, while RGS6 only inhibited signalling initiated by LPA2 receptors. In SKOV-3 ovarian cancer cells, RGS2 but not RGS6 or RGS19/GAIP, inhibited LPA stimulated inositol phosphate production. In contrast, in CAOV-3 cells RGS19/GAIP strongly attenuated LPA signalling. Thus, multiple RGS proteins are expressed at significantly different levels in cells derived from cancerous and normal ovarian cells and at least two candidate RGS transcripts have been identified to account for the reported regulation of LPA signalling pathways in ovarian cancer cells.

Introduction

Lysophosphatidic acid (LPA) is a key mediator of ovarian cancer initiation and progression, functioning as an autocrine activator of proliferation (Mills et al., 1988; Xu et al., 1995b), survival from apoptotic signals (Frankel and Mills, 1996), migration (Sengupta et al., 2003), production of angiogenic growth factors (Sengupta et al., 2007), and production of proteases critical for metastasis (Fang et al., 2000a). LPA is released from ovarian cells and accumulates in ascitic fluid where it is then available to bind and activate a family of cell surface G-protein coupled receptors (GPCRs). Three related, well-characterized "classic" LPA receptors, LPA1, LPA2, and LPA3 (previously named Edg-2, Edg-4, and Edg-7, respectively) are expressed on the surfaces of ovarian cancer cells and mediate responses to LPA (Anliker and Chun, 2004b). Two additional receptors, LPA4 and LPA5, which are related to each other but not to the "classic" LPA receptors have also recently been described (Noguchi et al., 2003; Lee et al., 2006a). Each of these receptors functions by activating heterotrimeric guanine nucleotide binding proteins (G-proteins) in an LPA dependent manner. Thus, the central mediators of signalling by LPA receptors are G-proteins. LPA binding to its receptors stimulates the G-protein to undergo nucleotide exchange, switching from the inactive di-phosphate (GDP) bound form to the active tri-phosphate (GTP) bound form. It is the active GTP bound G-protein that initiates cell signalling cascades to ultimately regulate cancer cell function. G-proteins slowly return to the inactive state by auto-hydrolysis of GTP (Oldham and Hamm, 2006).

Regulator of G-protein signalling (RGS) proteins function to deactivate heterotrimeric G-proteins by dramatically accelerating the rate at which Gα subunits hydrolyze GTP (Chen et al., 2000; Heximer et al., 2003; Fu et al., 2007). RGS proteins have been shown to have profound effects on the kinetics and magnitude of *in vivo* receptor signalling pathways (Chen et al., 2000;

Heximer et al., 2003). Over the past decade, RGS proteins that deactivate G-proteins have been demonstrated to acutely regulate signalling downstream of GPCRs. We recently demonstrated that RGS proteins dramatically regulate LPA signalling in ovarian cancer cells using a mutagenesis strategy to compare signalling activity of Gαi proteins that were either wild-type with respect to RGS regulation or insensitive to RGS regulation. LPA signalling in SKOV-3 ovarian cancer cells was significantly more robust in cells expressing RGS insensitive Gi2-proteins than in cells expressing wild-type RGS sensitive G-proteins with respect to inhibition of adenylyl cyclase and cellular migration. This suggests that endogenous RGS proteins expressed in SKOV-3 ovarian cancer cells normally attenuate signalling of Gαi-mediated LPA-stimulated signalling (Hurst et al., 2008a). The goal of the current study was to identify candidate RGS proteins expressed in SKOV-3 cells that may regulate G-protein signalling and second, to determine if these RGS protein transcripts are differentially expressed among commonly utilized ovarian cancer cell lines and non cancerous ovarian cell lines. We identified multiple RGS protein transcripts in SKOV-3 cells that may account for negative regulation of LPA signalling pathways and determined that two of these RGS transcripts are expressed at dramatically different levels in cell lines derived from normal versus cancerous ovarian cells. We further determined the ability of these RGS proteins to attenuate signalling from individually expressed exogenous LPA receptors and LPA receptors endogenously expressed in SKOV-3 and CAOV-3 ovarian cancer cells.

Methods and Materials

Cell culture

Human OVCAR-3, SKOV-3, Caov-3, HeLa, and COS-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia) and grown according to ATCC

recommendations. Immortalized ovarian surface epithelial cells (IOSE) were a kind gift from Dr. David Puett (University of Georgia).

RNA isolation

RNA was isolated from tissue samples and cultured cells using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Monolayers IOSE, Caov-3, SKOV-3, and OVCAR-3, and HeLa cervical cancer cells were homogenized in 1 ml of Trizol reagent per 10 cm^2 of plate surface area by passing the lysates through a pipette tip several times. RNA preparations were treated with DNase, and the RNA was separated from the DNAse using an RNEasy column (Qiagen). RNA preparations were aliquoted and stored at -80°C.

RT-PCR

The RNA was analyzed for specific transcripts with Superscript One-step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. Primers were designed and analyzed for specificity, secondary structure, and dimerization using Vector NTI Software (Invitrogen). PCR conditions such as annealing temperature were optimized for each primer pair, and plasmid DNA (50 ng) encoding each target RGS sequence was used as a positive control. Reactions included forty cycles of amplification. Products of amplification were resolved by electrophoresis in 1.5 % agarose gels and visualized with ethidium bromide staining.

qRT-PCR

To compare the level of expression of RGS transcript between cell lines**,** DNA was first synthesized from 1ug of total RNA using the High Capacity Reverse Transcriptase cDNA kit (Applied Biosystems) to amplify mRNA. Reactions without reverse transcriptase were also run as a control for DNA content. qPCR reactions were prepared using Applied Biosystem's Taq-man Gene Expression Assays and Universal PCR Mastermix according to the manufacturer's

instructions. Reactions for GADPH were performed in each RNA set as an internal standard. The PCR reaction was carried out in a Biorad iCycler using program parameters provided by Applied Biosystems. Briefly, 50 ng cDNA was provided as template with 2X Universal PCR Mastermix for 50 cycles of 15 second melt at 95°C and 1 minute of anneal/extension at 60°C. The primers in Taqman Gene Expression Assays are designed to use the same annealing temperature. Threshold cycle (CT) values were determined for each transcript using the automated threshold function of the iCycler software, and data are reported as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001), using GAPDH housekeeping gene as internal controls and reporting data as expression in the ovarian cancer cells relative to IOSE cells.

DNA constructs and transfections

Plasmids encoding RGS and receptor proteins were obtained from the UMR cDNA Resource Center (Rolla, MO). Transfections were performed using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions, at a ratio of 2 µl of Fugene 6 reagent to 1 µg plasmid DNA. Inositol phosphate experiments were carried out in 24-well dishes, and were transfected with 500 ng/well total plasmid DNA. COS-7 cells were transfected with 100 ng/well of receptor-encoding DNA, and the amount of RGS DNA per well was determined empirically to yield equivalent expression levels (see results), with the total amount of DNA per well being brought up to 500 ng with vector. ELISA experiments were performed in 96-well dishes, and cells were transfected with vector, receptor, and RGS plasmid DNA in proportional amounts relative to the 24-well dishes up to 100 ng total plasmid DNA/well.

Inositol phosphate assay

Production of inositol phosphates (IP) was quantified using established protocols (Hepler et al., 1987). Briefly: To measure IP production by PLC activation, COS-7 cells were plated in 24-well dishes at 50,000 cells/well. 48 hours prior to the assay, cells were transfected with the appropriate genes. Cells were labeled with 0.5 μCi/well [^{3}H]-myo-inositol (American Radiolabeled Chemicals, St. Louis, MO) for 18 hours to label the cellular pool of phosphatidyl inositol. The cells were treated with Oleoyl (18:1) LPA (Avanti Polar Lipids, Alabaster, AL) in the presence of 10 mM lithium chloride to inhibit degradation of inositol phosphates for 30 minutes at 37°C. Cells were then lysed in cold formic acid and neutralized with ammonium hydroxide, and the lysates were then loaded onto columns of AG 1-X8 anion exchange resin (Biorad, Hercules, California). The columns were washed with water and dilute ammonium formate to remove unhydrolyzed lipids. The [^{3}H]-IPs were then eluted with 1.2 M ammonium formate/0.1 M formic acid, and added to scintillation fluid for counting. In some experiments, cells were treated with 200 ng/ml pertussis toxin (Sigma-Aldrich, St. Louis, MO) for 12 hours prior to IP assay.

Western blotting

Protein expression was determined using standard techniques. Cells were harvested and lysed in protein sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted using HA-epitope polyclonal primary antibodies and peroxidase-conjugated secondary antibodies (Bethyl laboratories, Montgomery, Texas), and visualized using SuperSignal Chemiluminescent substrate (Pierce, Rockford, Illinois).

Cell-surface ELISA

LPA receptor expression constructs used in these experiments contained an N-terminal (extra-cellular) Hemagglutinin (HA) tag that was used to detect receptors expressed at the cell surface. Cells were plated and transfected simultaneously with cells to be assayed in the inositol phosphate assays in 96-well plates. 48 hrs after transfection, on the day the second messenger assays were performed, cells were fixed with freshly prepared 4% paraformaldehyde for 10 minutes at 4ºC. Cells were then washed once with Tris-buffered saline (TBS) and incubated sequentially in: blocking reagent for 1 hour (3% BSA in TBS); primary antibody for 2 hours (Rabbit anti-HA diluted 1:1000 in 1%BSA/TBS); TBS for 5 minutes 3 times; secondary antibody for 1 hour (Goat anti-Rabbit IgG HRP in 1% BSA/TBS); TBS for 5 minutes 3 times; and liquid substrate for 15 minutes [Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Sigma]. The optical density at 405 nm was determined in a plate reader (Victor, Perkin Elmer).

Results

RGS expression in ovarian cell lines

In order to identify candidate RGS proteins that may be responsible for the observed negative regulation of signalling in SKOV-3 cells, we evaluated the expression of transcripts of RGS proteins in SKOV-3 ovarian cancer cells. Further, we compared RGS transcript expression in SKOV-3 cells to two other commonly studied ovarian cancer cell lines, OVCAR-3 and Caov-3, as well as immortalized ovarian surface epithelium (IOSE) cells derived from noncancerous cells. Reverse transcriptase-PCR was performed on total RNA isolated from SKOV-3, Caov-3, OVCAR-3, and IOSE cell lines. High quality RNA was isolated and screened for RGS transcript expression as described in Methods using a panel of primers for 22 distinct RGS transcripts belonging to the five major subfamilies of RGS proteins.

Overall, fifteen RGS transcripts were detected in SKOV-3 cells, and twelve of these (RGS2, 3, 4, 6, 7, 9, 10, 11, 12, 19, and 20) are known to deactivate proteins of the Gi/o family (Hollinger and Hepler, 2002). Distinct patterns of expression were observed across the four ovarian cell lines tested. RGS transcripts segregated into three categories with respect to their expression in ovarian cancer cells. The first, largest category includes twelve RGS transcripts consistently detected in all four cell lines evaluated: RGS2, 3, 7, 9, 10, 11, 12, p115RhoGEF, PDZ-RhoGEF, and LARG. The second category includes five RGS transcripts that could not be detected in any of the ovarian cell lines evaluated, although the primers effectively amplified product in positive controls: RGS 1, 5, 8, 13, and 18. Finally, the most interesting category includes RGS transcripts that were consistently detected in some but not all of the ovarian cell lines. RGS4 was not detected in Caov-3 RNA, but was detected in the other three cell lines, while RGS6 was detected in all three ovarian cancer cell lines, but no amplification product was observed in IOSE RNA. (The double bands observed with RGS6 amplification are expected based on reported splice variants (Chatterjee et al., 2003)). RGS14 transcript was consistently detected in IOSE, Caov-3, and OVCAR-3 cells, but not in SKOV-3. Similarly, RGS16 amplification product was not consistently detected in IOSE and SKOV-3 RNA, but was detected in OVCAR-3 and Caov-3 RNA, and RGS17 transcript was not consistently detected in OVCAR-3 RNA, but was detected in the other three cell lines. A full summary of results is shown in **Table. 6.1** and representative data images (RGS2, 3, 4, 5, 6, 14, 16, and 19) are shown in **Figure 6.1**.

Quantitative comparison of RGS expression

Several RGS primer pairs consistently produced amplification products of different intensity from the different cell lines (e.g. RGS4); however, the conditions used in our RT-PCR

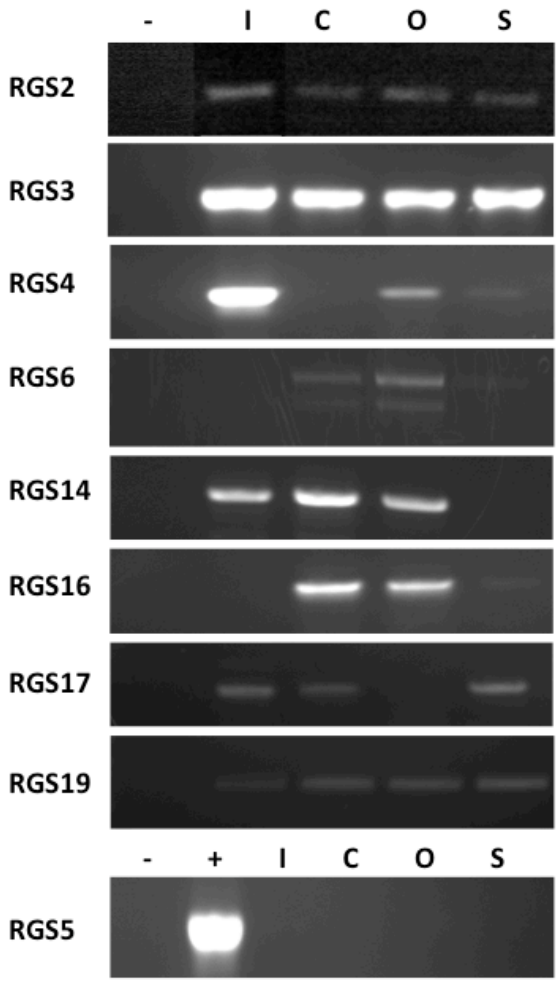

Figure 6.1: RGS protein transcript expression in ovarian cell lines.
RNA was isolated from IOSE cells (I), Caov-3 cells (C), OVCAR-3 cells (O), or SKOV-3 cells (S) using Trizol reagent (Invitrogen) and amplified using Superscript One Step RT-PCR kit (Invitrogen) according to manufacturer's instructions. Positive control reactions (+) contained plasmid DNA encoding the target RGS as template. Negative control reactions (-) contained no RNA or DNA template. Eight representative data images are shown. Results are representative of three separate experiments. Full results are summarized in Table 1.

analysis were highly sensitive to detect low-copy number transcripts (40 cycles of PCR amplification), which limited quantitative comparison between amplification products. Thus, we further evaluated the relative expression of select RGS transcripts between cell lines using real time quantitative RT-PCR. Taqman Gene Expression Assays (Applied Biosystems) were used to compare expression of RGS2, 4, 6, 14, and 19 transcripts to the internal standard GAPDH and to

Table 6.1: Summary of RT-PCR data.
I: IOSE, S: SKOV-3, O: OVCAR-3, C: Caov-3. +: Transcript consistently detected in RNA. -: Transcript not detected in RNA.

	RT-PCR				G-protein target	Primers
RGS protein	**I**	**S**	**O**	**C**		
Family A, RZ						
RGS17	+	+	-	+	i/o	3':TCAGAAGAAGAGCCAGCAGTAC 5':ATGGAGAGTATCCAGGTCCTAGAG
RGS19/GAIP	+	+	+	+	i/o	3':TAGGCCTCGGAGGAGGACTGTGATG 5':CCGCATGAGGCTGAGAAGCAGATC
RGS20/RGSZ	+	+	+	+	i/o/z	3':CAGCAGAGTTCATGAATCGAGG 5':ATGGGATCAGAGCGGATGGAGATG
Family B, R4						
RGS1	-	-	-	-	i/o, q/11	3':TATCTGATTTGAGGAACCTGGG 5':ATGCCAGGAATGTTCTTCTCTG
RGS2	+	+	+	+	q/11>i/o	3': GTCCTGGTAGAATTCTGACTCCAAG 5': GCAAAGTGCTATGTTCTTGGCTGTTC
RGS3	+	+	+	+	i/o, q/11	3':TGAGGGACCAGGGAAGCACAGTCTG 5':TTGCAGGTCTGCCGGCTTCTTG
RGS4	+	+	+	-	i/o, q/11	3':TAAAACTCAGAGCGCACAAAGCGAG 5':AAGGACTTGCAGCTTTGCCCCACTC
RGS5	-	-	-	-	i/o, q/11	3':TCCTCTGGCTTTGGGACAGCAGATC 5':CCTTACTGATGCCACGCAGGAACAAAG
RGS8	-	-	-	-	i/o, q/11	3':CAGAAACTGTTGTTGGACTGCATAG 5':GATGTGCAGAGATGAATCTAAGAGGC
RGS13	-	-	-	-	i/o, q/11	3':TGCAGCTGGACAGAGTGGCAGAGGC 5':ACCACCTGCCTGGAGAGAGCCAAAG
RGS16	-	-	+	+	i/o, q/11	3':ATAACCAAATGGCAACGTCTGATTG 5':GCAGAAACCTGAGTTTCATGAAGACACC
RGS18	-	-	-	-	i/o, q/11	3':AAGCGGGGGACTCATCTTCTTCTGG 5':GCCAAGGACATGAAGAACAAGCTGGG
Family C, R7						
RGS6	-	+	+	+	i/o	3':TCTTCTTGGCCAGCAGCAAATCCTG 5':GCTCAAGGATCCGGGGATCAAAGAG
RGS7	+	+	+	+	i/o	3':TTAGTGCTGGCCCTCAGTGTTGGTG 5':CCAGGGGAATAATTATGGGCAGACC
RGS9	+	+	+	+	i/o	3':TGGCATCGTTGGATGAGAACTGCTC 5':GCCGAGGATGGCATTTCTCCAAAAG
RGS11	+	+	+	+	i/o	3':CTCTTCATCTCCAGCGGGATCCCAG 5':TGCCATCTACCTGGCCAAGAAGAAC
Family D, R12						
RGS10	+	+	+	+	i/o, q/11	3':TTTAGCTGCAGTTTGAGCATCAGGC 5':ATGGAACACATCCACGACAGCGATG
RGS12	+	+	+	+	i/o	3':GCCATCGGTGGAATCTGTGTAAGAG 5':AAACAAAGATCCCTTTCGGAGTCGG
RGS14	+	-	+	+	i/o	3':GTCACAGGTCTGCCGCTTTCCAGTG 5':GAGAGTCTCAGGGCTCCCTCAACTC
Family F, GEF						
p115-RhoGEF	+	+	+	+	12/13	3':CTTCTTGGTCTTGGCAGGCTCGTCC 5':ATCGGGGCTGAGGATGAGGATTTTG
PDZ-RhoGEF	+	+	+	+	12/13	3':AGCAGCATCCATGTCAACATCACTG 5':TGGGCAGCCTGTATGGTGAAAATGAC
LARG	+	+	+	+	12/13	3':GTCAGTCCCATACTACGTTTCTGCC 5':GTTACAGAAAGAACAGGAACGGCTAC

further determine their relative expression in cancer cell lines as compared to IOSE cells (Livak and Schmittgen, 2001). Threshold cycle (CT, the cycle number in which fluorescence signal crosses threshold value) was determined for each transcript in each RNA preparation. The difference between RGS and GAPDH CT values (ΔCT) was determined for each cell line, and the difference between ΔCT values for IOSE and each cancer cell line was then determined (ΔΔCT) (**Table 6.2**). This value was used to calculate positive fold changes in gene expression ($2^{-\Delta\Delta CT}$) and negative fold changes ($-1/2^{-\Delta\Delta CT}$).

The quantitative results are consistent with the results described above. We found that RGS19 was detected at similar levels in each cell line, relative to GAPDH levels. RGS14 was detected in IOSE, Caov-3, and OVCAR-3 cells, albeit at very low levels, with no significant differences between expression levels, and was not detected in SKOV-3 cells. It is noteworthy that the CT values obtained for RGS14 were greater than 40, beyond the manufacturer's suggested reliable range of detection for Taqman expression reagents; however, negative controls lacking reverse transcriptase enzymes or lacking template altogether consistently failed to cross threshold fluorescence signal after 50 cycles of amplification.

Significant differences in the relative expression levels of RGS2, RGS4, and RGS6 transcripts were observed. RGS2 transcript was expressed at slightly but significantly lowers levels in SKOV-3 and OVCAR-3 cells as compared to IOSE cells. Expression was also consistently lower in Caov-3 cells, but the data did not meet criteria for statistical significance due to greater variability (**Figure 6.2A**). Differences in expression of RGS4 and RGS6 were much more dramatic. RGS4 transcript expression was reduced 5000-fold or greater in each of the ovarian cancer cell lines as compared to expression in IOSE cells. Fold changes in expression could not be calculated for RGS4 in Caov-3 cells because RGS4 was not detected in Caov-3

Table 6.2: Summary of quantitative RT-PCR data.
CT: Threshold cycle; SEM: Standard Error of means. Unpaired two-tailed T-test comparing ΔCT values in cancer cell lines to ΔC values in IOSE cells. *: p<0.05, **: p<0.01; ***: p<0.001.

	C_T (SEM)	ΔC_T (SEM)	$\Delta\Delta C_T$ (SEM)
SKOV-3			
GAPDH	17.70 (0.20)		
RGS2	29.05 (0.15)	11.35 (0.05)	*0.35 (4.7e-7)
RGS4	37.25 (0.45)	19.55 (0.25)	***16.13 (0.27)
RGS6	34.35 (0.10)	16.65 (0.30)	*-2.4 (0.7)
RGS14	N.D.	N.A.	N.A.
RGS19	25.83 (0.22)	8.125 (0.425)	-1.2 (0.200)
CAOV-3			
GAPDH	15.05 (0.70)		
RGS2	27.13 (0.37)	12.08 (3.32)	1.075 (0.275)
RGS4	N.D.	N.A.	N.A.
RGS6	23.3 (0.65)	8.25 (0.05)	**-10.85 (0.45)
RGS14	41.70 (1.25)	26.65 (1.95)	1.175 (0.325)
RGS19	24.28 (0.22)	9.22 (0.47)	-.1000 (0.25)
OVCAR-3			
GAPDH	16.35 (0.10)		
RGS2	28.33 (0.02)	11.98 (0.075)	**0.975 (0.25)
RGS4	32.73 (0.02)	16.38 (0.07)	***12.95 (0.50)
RGS6	25.18 (0.42)	8.82 (0.32)	**-10.28 (0.70)
RGS14	44.05 (0.65)	27.70 (0.75)	2.22 (1.52)
RGS19	25.70 (0.45)	9.35 (0.55)	0.025 (0.325)
IOSE			
GAPDH	16.35 (0.30)		
RGS2	27.35 (0.25)	11.00 (0.05)	
RGS4	19.78 (0.78)	3.425 (0.035)	
RGS6	35.45 (0.70)	19.10 (0.40)	
RGS14	41.83 (1.95)	25.48 (2.275)	
RGS19	25.68 (0.07)	9.32 (0.22)	

RNA (consistent with the qualitative RT-PCR results, **Figure 6.1**), denoted by the white bar in **Figure 6.2B**. RGS6 displayed the opposite expression pattern, being expressed at higher levels in cancer cell lines relative to IOSE cells. RGS6 transcript was expressed in SKOV-3 cells at levels approximately 5-fold higher than in IOSE cells, and more than 1000-fold higher in Caov-3 and

OVCAR-3 cells as compared to IOSE cells, also consistent with qualitative results shown above (**Figure 6.2)**.

Expression of RGS2, RGS4, and RGS6 in ovarian cancer cell lines relative to expression in IOSE cells

RNA was prepared from IOSE, SKOV-3, Caov-3, and OVCAR-3 cell lines and real time RT-PCR reactions were performed using Taqman Gene Expression Assays (Applied Biosystems). Positive fold changes in expression were calculated according to the 2-ΔΔCT method, and negative fold changes were calculated as -1/2-ΔΔCT.

RGS regulation of individual exogenously expressed LPA receptors

We next sought to determine the ability of RGS6, RGS2, and RGS19/GAIP to affect the strength of LPA signalling cascades. LPA's effects on Gq and Gi pathways are well documented in multiple systems, and each of the three classic LPA receptors LPA1, LPA2, and LPA3 are known to couple to both Gi and Gq pathways (Fukushima et al., 1998; Contos et al., 2000b; Ishii et al., 2000; Lee et al., 2006a; Lee et al., 2007). We assessed LPA receptor signalling and regulation by RGS proteins in an assay system that reflects activation of both of these G-protein pathways: inositol phosphate production by phospholipase C (PLC) enzymes. Phospholipase C can be activated directly by Gαq subunits (Waldo et al., 1991), or by Gβγ dimers downstream of Gi coupled receptors (Boyer et al., 1992).

First we determined the effect of the RGS proteins on each of the classic Edg family LPA receptors individually expressed in Cos-7 cells, which allow high levels of expression of multiple exogenous proteins via transient transfection. We first characterized LPA-stimulated inositol phosphate production in COS-7 cells expressing each receptor. Although COS-7 cells are known to contain LPA receptors (Hains et al., 2006), in the absence of transfected receptor, we observed

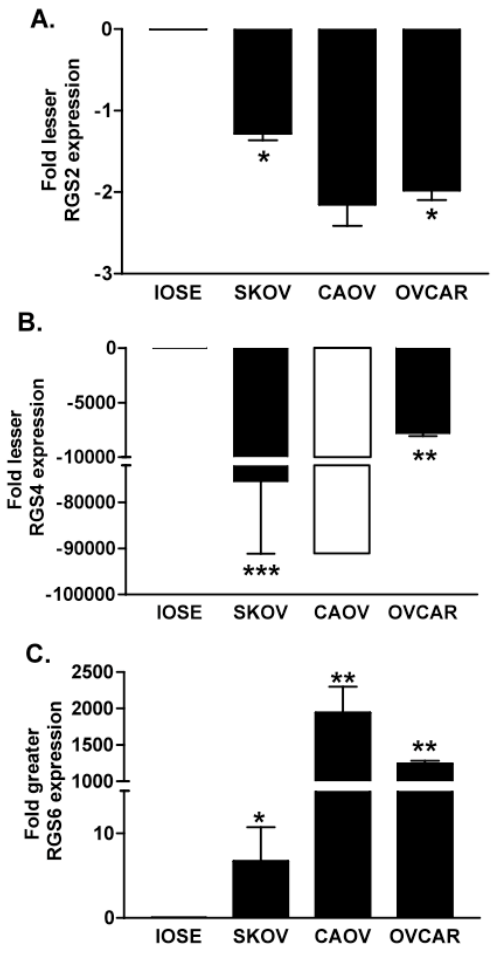

Figure 6.2: Expression of RGS2, RGS4, and RGS6 in ovarian cancer cell lines relative to expression in IOSE cells.
RNA was prepared from IOSE, SKOV-3, Caov-3, and OVCAR-3 cell lines and real time RT-PCR reactions were performed using Taqman Gene Expression Assays (Applied Biosystems). Positive fold changes in expression were calculated according to the $2^{-\Delta\Delta CT}$ method and negative fold changes were calculated as $-1/2^{-\Delta\Delta CT}$. Results represent cumulative data from two independent experiments.

only a modest, inconsistent increase in inositol phosphate production in response to LPA (**Figure 6.3A**). Expression of RGS proteins alone did not significantly alter this endogenous COS-7 LPA response (data not shown). However, transfection of LPA1, LPA2, or LPA3 imparted robust dose-dependent LPA-stimulated inositol phosphate accumulation, with EC50 values of approximately 0.9-1 μM for both LPA1 and LPA3 expressing cells, and approximately 0.1 μM for LPA2 expressing cells (**Figure 6.3A**). To compare the G-proteins mediating the inositol

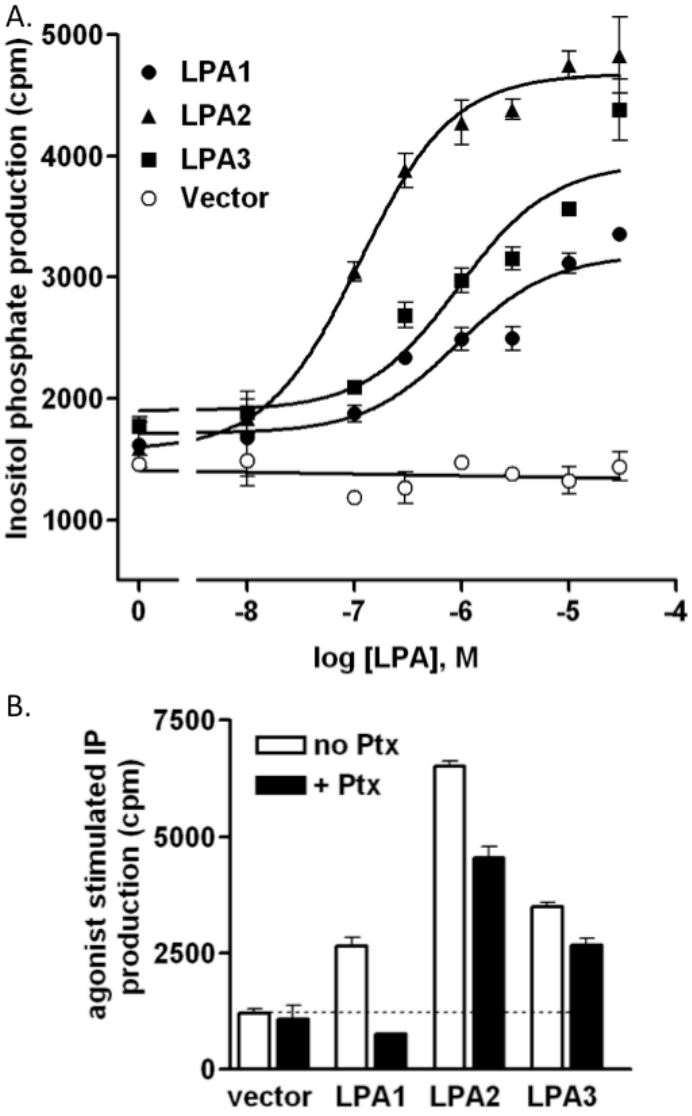

Figure 6.3: Signaling by exogenously expressed LPA receptors in COS-7 cells.
A) LPA receptor activation of inositol phosphate accumulation. LPA dose response curves were determined in inositol phosphate assays in COS-7 cells 48 hours after transient transfection with 100 ng/well of vector (○), LPA1 (●), LPA2 (▲), or LPA3 (■) in 24-well dishes. Cells were labeled with [^{3}H]-myo-inositol and incubated with various concentrations of LPA in the presence of lithium chloride for 30 minutes. Inositol phosphates were isolated by ion exchange chromatography and quantitated with scintillation counting. B) LPA receptor sensitivity to pertussis toxin. Cells exogenously expressing LPA1, LPA2, or LPA3 were treated with 200 ng/ml of pertussis toxin (■) or vehicle (□) for 18 hours, and cells were assayed for inositol phosphate production in the presence of 30 μM LPA. Basal values obtained in the absence of LPA (vehicle controls) have been subtracted from these data. Data shown are representative of three independent experiments.

phosphate response from the three LPA receptors, we treated cells with the Gi specific inhibitor pertussis toxin (Ptx). LPA stimulated inositol phosphate production downstream of LPA1 was completely inhibited by Ptx, suggesting that in this system LPA1 activation of PLC enzymes is mediated by Gi/o type G-proteins. LPA2 and LPA3 dependent activation of PLC activity was inhibited by Ptx by approximately 30% each, suggesting that these receptors predominately utilize Gq-like Ptx insensitive G-proteins to activate PLC in this system (**Figure 6.3B**).

To evaluate the effect of RGS function on LPA signalling pathways downstream of each receptor, we compared activation of inositol phosphate production by LPA in cells expressing exogenous LPA receptor alone versus cells expressing LPA receptor along with RGS6, RGS2, or RGS19/GAIP. Each of the RGS proteins were N-terminally tagged with a 3X HA epitope, so expression levels of the different proteins could be compared directly. The amount of each expression construct required to yield comparable protein expression levels following transient transfection was determined empirically. Each of the RGS proteins was expressed at similarly high levels in COS-7 cells following transient transfection of 50 ng of plasmid DNA encoding RGS19/GAIP, 400 ng of plasmid DNA encoding RGS2 and 200 ng of plasmid encoding RGS6 per well in a 24-well dish (**Figure 6.4A**). [RGS6 protein is found in tissues as a heterodimer with its binding partner Gβ5, which is required for its stable expression (Chen et al., 2003). Thus, Gβ5 was cotransfected (200 ng plasmid DNA/well) with RGS6 in all experiments]. In addition to the predicted inhibitory activity on G-proteins, it is possible that RGS proteins could inhibit exogenous LPA receptor signalling by lowering the amount of LPA receptor expressed on the cell surface. To confirm that any observed RGS effects on LPA signalling were mediated downstream of the receptor, the LPA receptors were each N-terminally epitope tagged to monitor

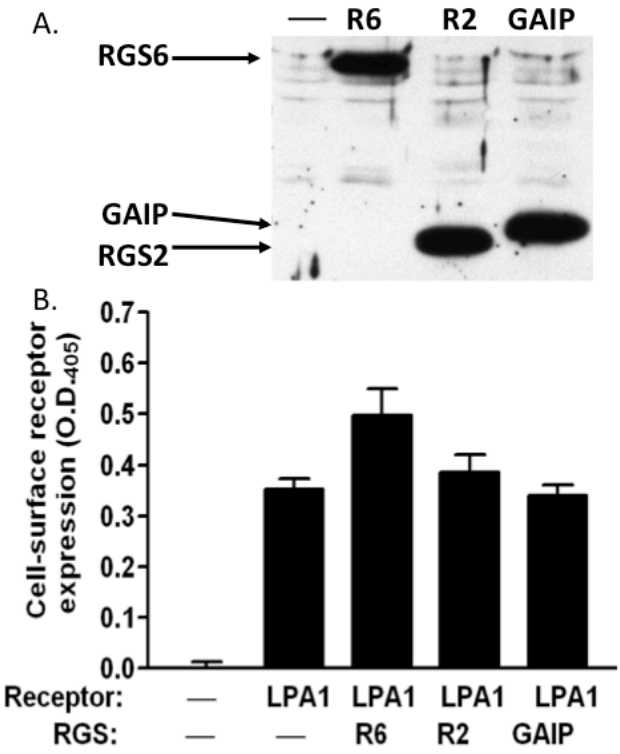

Figure 6.4: Co-expression of exogenous RGS proteins and LPA receptors.
COS-7 cells were transfected with LPA1 receptor alone or along with RGS6, RGS2, or RGS19/GAIP in parallel with the inositol phosphate assay to compare RGS expression and receptor cell- surface expression. A) Expression of RGS proteins in COS-7 cells. Cell lysates were harvested 48 hours after transfection and separated using SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-HA antibody and HRP-conjugated secondary antibody. B) Effect of RGS expression on LPA receptor cell surface expression in COS-7 cells. COS-7 cells were plated in 96-well plates and transfected with 100 ng total plasmid DNA/well at the same proportions as in the inositol phosphate assay. Cells were fixed and incubated with anti-HA antibody and detected as described. Similar results were obtained in experiments with LPA2 and LPA3 co-transfections. Data are representative of at least three independent experiments.

their cell-surface expression. Whole-cell ELISAs were performed with each experiment to ensure that co-expression of RGS proteins did not inhibit LPA receptor expression. In each case, co-expression of RGS proteins either had no effect or slightly increased LPA receptor cell-surface expression, but did not lower LPA receptor expression (**Figure 6.4B**). Expression of RGS proteins alone did not affect ELISA signal (data not shown). LPA-stimulated inositol phosphate signalling in cells expressing LPA1 receptors was completely inhibited by co-expression of RGS2 and partially inhibited by similar expression levels of RGS19/GAIP, but

RGS6 expression had no significant effect on activity (**Figure 6.5A**). RGS2, RGS6, and RGS19/GAIP each significantly inhibited LPA signalling mediated by LPA2 receptor expression, with RGS2 again exerting the strongest effect (**Figure 6.5B**). Finally, in LPA3 expressing cells, RGS6 did not significantly inhibit signalling, while RGS2 and RGS19/GAIP partially inhibited the LPA3 receptor mediated response (**Figure 6.5C**).

Effect of RGS proteins on endogenous LPA receptor signalling in ovarian cancer cells

We next determined if overexpression of individual RGS proteins could also affect signalling mediated by LPA receptors endogenously expressed in SKOV-3 ovarian cancer cells. SKOV-3 cells displayed a robust LPA stimulated inositol phosphate response with an EC50 of approximately 500 nM (**Figure 6.6D**) which was inhibited by pertussis toxin treatment by approximately 70% (**Figure 6.6B**). Expression of RGS2 in SKOV-3 cells strongly inhibited the LPA stimulated inositol phosphate response, while expression of RGS6 and RGS19/GAIP did not (**Figure 6.6C**). LPA dose response curves in SKOV-3 cells transfected with either vector or RGS2 demonstrated that expression of RGS2 in these cells lowered the maximal effect of LPA by approximately 50%, and lowered the potency of LPA by nearly an order of magnitude (**Figure 6.6D**).

We further tested the ability of RGS2, RGS6, and RGS19/GAIP to regulate LPA signalling in Caov-3 cancer cells. Caov-3 cells displayed an LPA-stimulated inositol phosphate response, although with distinctly lower potency (EC50 of approximately 10 μM, **Figure 6.7D**), and with lower sensitivity to pertussis toxin treatment (40% inhibition, **Figure 6.7B**) than in SKOV-3 cells, suggesting that the response in these cells may be mediated by different receptors and/or G-proteins. In Caov-3 cells, significant inhibition of LPA signalling was observed consistently with RGS2 expression (**Figure 6.7C**), although the degree of inhibition varied

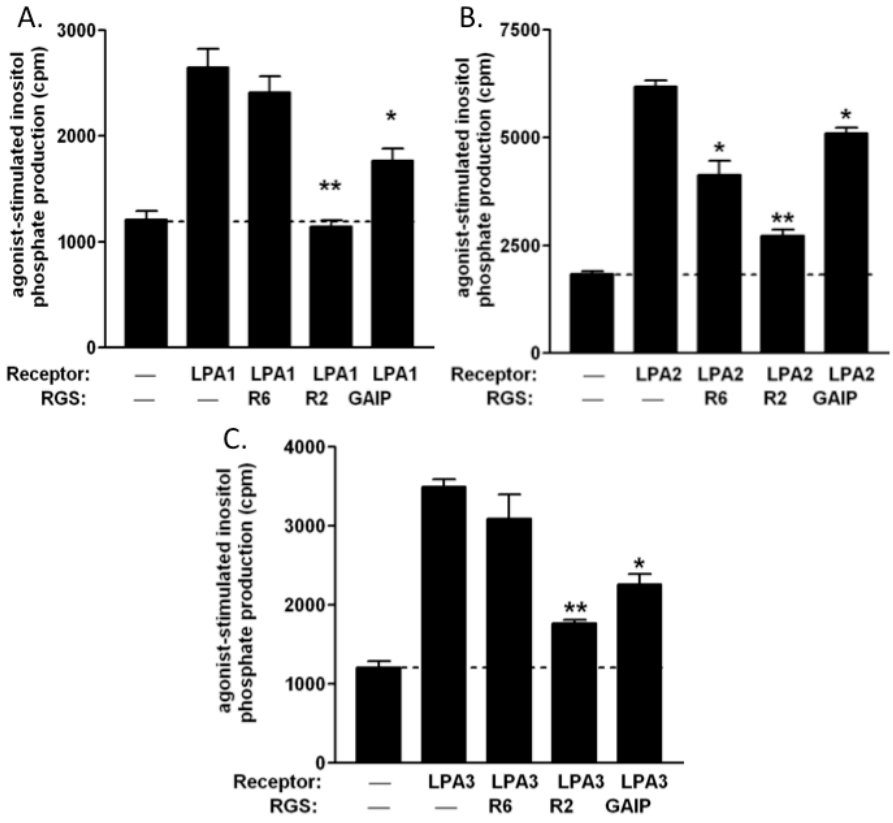

Figure 6.5: The effect of RGS expression on LPA signaling activity in the inositol phosphate assay.
Cos-7 cells were transfected with 100 ng of LPA1 (A), LPA2 (B), or LPA3 (C) and 400 ng of vector, 200 ng/200 ng RGS6/Gβ5, 400 ng RGS2, or 50 ng GAIP. LPA responses were determined in inositol phosphate assays using vehicle or 10 μM LPA as described in Methods. Data are representative of three independent experiments.

between experiments, reflecting the observation that LPA responses fall off sharply at high LPA doses with expression of exogenous RGS2 (**Figure 6.7D**). Significant and consistent inhibition of LPA signalling was observed in the presence of RGS19/GAIP expression in CAOV-3 cells (**Figure 6.7C**), and the dose response to LPA shows 50% lower maximal efficacy in the presence of expressed GAIP, with no significant change in LPA potency (**Figure 6.7D**). Again, RGS6 did not inhibit LPA signalling in CAOV-3 cells.

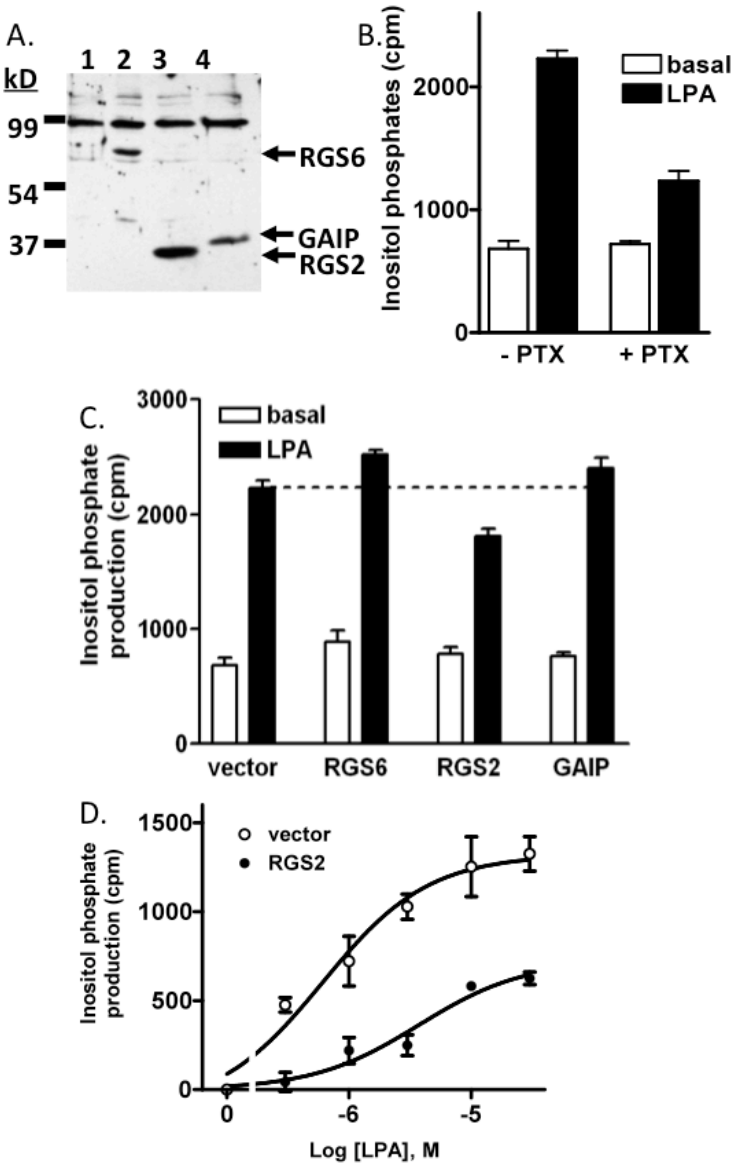

Figure 6.6: RGS regulation of LPA signalling in SKOV-3 cells.
A) RGS expression in SKOV- 3 cells. Cells were plated in 24-well dishes and transfected with: 1) vector; 2) RGS6 (and Gβ5) plasmid DNA; 3) RGS2 plasmid DNA; or 4) RGS19/GAIP plasmid DNA in parallel with inositol phosphate assays. Cell lysates were harvested 48 hours after transfection and separated using SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti- HA antibody and HRP-conjugated secondary antibody. B) Pertussis toxin sensitivity of LPA-stimulated inositol phosphate production in SKOV-3 cells. SKOV-3 cells were treated with 200 ng/ml of pertussis toxin or vehicle for 18 hours, and cells were assayed for inositol phosphate production in the presence of vehicle or 30 μM LPA. C) Effect of RGS protein expression on LPA signaling in SKOV-3 cells. SKOV-3 cells were transfected with vector, RGS6, RGS2, or RGS19/GAIP. LPA responses were determined in inositol phosphate assays using vehicle or 10 μM LPA. D) RGS2 decreases the efficacy of LPA signaling in SKOV-3 cells. SKOV-3 cells were transfected with 400 ng of vector (○) or 400 ng RGS2 (●). LPA responses were determined in inositol phosphate assays using vehicle or various concentrations of LPA. Data are representative of three independent experiments.

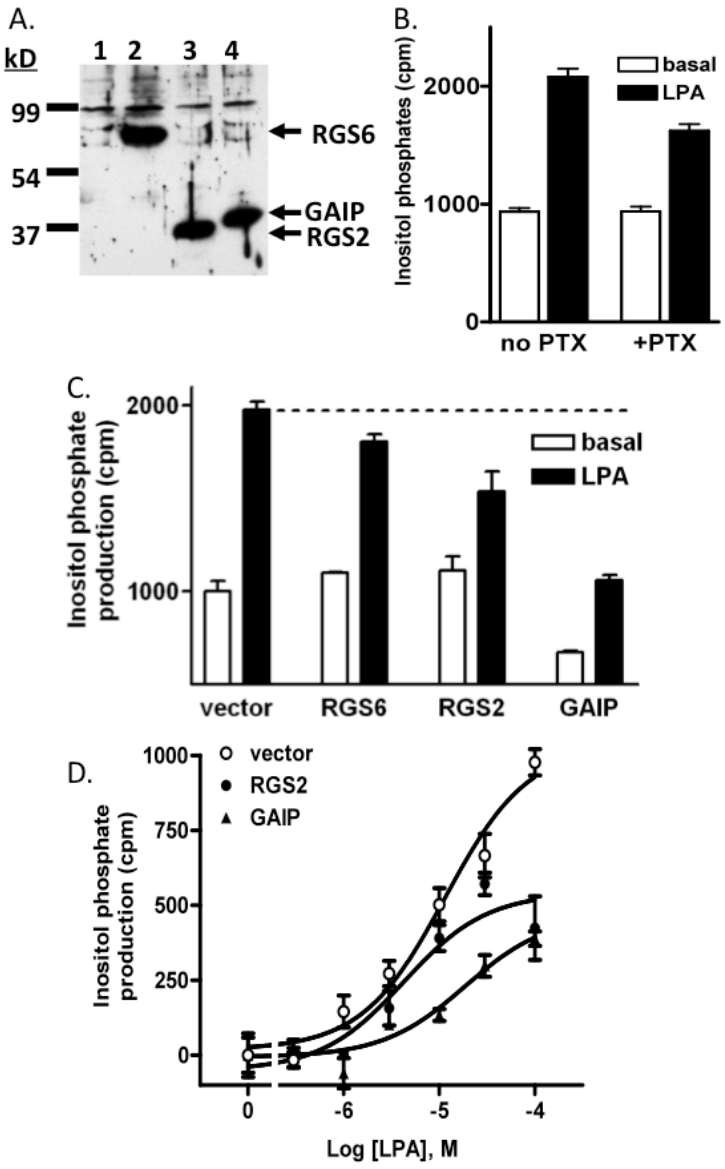

Figure 6.7: RGS regulation of LPA signalling in Caov-3 cells.
A) RGS expression in Caov-3 cells. Cells were plated in 24-well dishes and transfected with: 1) vector; 2) RGS6 (and Gβ5) plasmid DNA; 3) RGS2 plasmid DNA; or 4) RGS19/GAIP plasmid DNA in parallel with inositol phosphate assays. Cell lysates were harvested 48 hours after transfection and separated using SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti- HA antibody and HRP-conjugated secondary antibody. B) Pertussis toxin sensitivity of LPA-stimulated inositol phosphate production in Caov-3 cells. Caov-3 cells were treated with 200 ng/ml of pertussis toxin or vehicle for 18 hours, and cells were assayed for inositol phosphate production in the presence of vehicle or 30 μM LPA. C) Effect of RGS protein expression on LPA signaling in Caov-3 cells. Caov-3 cells were transfected with vector, RGS6, RGS2, or RGS19/GAIP. LPA responses were determined in inositol phosphate assays using vehicle or 10 μM LPA. D) RGS2 and GAIP decrease the efficacy of LPA signaling in Caov-3 cells. Caov-3 cells were transfected with vector (○), RGS2 (●) or GAIP (▲). LPA responses were determined in inositol phosphate assays using vehicle or various concentrations of LPA as described in Methods.

Discussion

In this study, several RGS transcripts were detected in SKOV-3 cells which could account for our recent report that G-protein sensitivity to RGS regulation resulted in diminished LPA signalling capacity in SKOV-3 cells (Hurst et al., 2008a). Further, RGS transcripts were found to be widely expressed in additional ovarian nontransformed and cancer cell lines, and several are differentially expressed among these cell lines. Most notably, RGS4 transcript is expressed at much higher levels in non-transformed ovarian cancer cell than in cancer-derived cell lines, while RGS6 transcript is expressed at much higher levels in ovarian cancer cell lines than in non-transformed ovarian cells.

We chose three representative RGS proteins – RGS2, RGS6, and RGS19/GAIP – to test further for functional regulation of LPA signaling pathways in a defined receptor expression system and in two ovarian cancer cell lines expressing endogenous LPA receptors. RGS2, RGS6 and RGS19/GAIP were each detected in all three of the ovarian cancer cell lines tested. RGS2 and RGS19/GAIP were expressed at similar levels in each cancer cell lines tested (although RGS2 was detected at significantly higher levels in IOSE non-transformed cells), while RGS6 transcript was expressed at much higher levels in Caov-3 and OVCAR-3 than in SKOV-3 cells. RGS2, structurally one of the simplest RGS proteins, is widely expressed, particularly in cardiac tissues, and has been detected in ovary where its expression is increased by stimulation with luteinizing hormone (Ujioka et al., 2000). RGS2 is known to selectively accelerate the GTPase activity of Gq *in vitro* (Heximer et al., 1997), although Gi subunit GAP activity has also been reported in certain systems (Ingi et al., 1998; Heximer et al., 1999; Tosetti et al., 2003). In contrast, RGS19/GAIP, which is widely expressed and found at highest levels in lung, heart and placenta (De Vries et al., 1995), can act as a GAP for both Gi and Gq, but is strongly selective

for Gi G-proteins *in vivo* (Berman et al., 1996; Hepler et al., 1997; Huang et al., 1997). RGS6 is also a Gi/o-specific RGS protein (Hooks et al., 2003). Although RGS6 is expressed at highest levels in heart and brain, RGS6 transcript has been detected in multiple other tissues including ovary (Seki et al., 1999).

To observe potential effect of RGS proteins on either Gi or Gq signalling, we measured LPA effects on PLC activity, which can be regulated by both Gi and Gq as described above. We found that each of these RGS proteins had the capacity to attenuate signalling from LPA receptors in an exogenous expression system, while only RGS2 and RGS19/GAIP significantly inhibited signalling by LPA receptors endogenously expressed in ovarian cancer cell lines. We first determined the role of RGS regulation in a defined overexpression system to determine the effects of RGS proteins on signalling by individual LPA receptors. LPA 1, 2, and 3 appeared to couple to different G-proteins to activate PLC in COS-7 cells: LPA 1 was coupled primarily to Gi/o type G-proteins, while LPA2 and LPA3 were primarily Gq-like Ptx insensitive G-proteins. Based on this observation we predicted that LPA1-mediated signalling would be most strongly regulated by RGS6 and RGS19/GAIP, which are Gi/o selective RGS proteins, while signalling mediated by LPA2 and LPA3 would be most highly regulated by RGS2. However, we observed inhibition of signalling from of all three receptors by RGS2 and RGS19/GAIP expression, while RGS6 selectively inhibited signalling from LPA2, but had no effect on signalling from either LPA1 or LPA3. While the non-selective regulation by RGS2 and RGS19 may be explained by a loss of specificity due to overexpression, the lack of effect of RGS6 on LPA signalling via LPA1 was unexpected. Future studies are required to define the mechanisms of receptor specificity.

RGS2 partially inhibited endogenous LPA signalling in both SKOV-3 and CAOV-3 ovarian cancer cell lines, although differences were observed in the effect of RGS2 expression

on the dose response curve of LPA in the two cell types. In SKOV-3 cells, RGS2 expression appeared to result in an overall lowering of LPA potency and efficacy. In Caov-3 cells, however, LPA effects were only affected by RGS2 expression at high doses of LPA and may reflect non-specific effects. While RGS19/GAIP had no effect on LPA signalling in SKOV-3 cells, we observed strong inhibition of LPA efficacy in Caov-3 cells. Our data do not suggest a significant effect of RGS6 expression on LPA signalling in either SKOV-3 or Caov-3 cells. Ptx inhibition data suggest that the LPA response in SKOV-3 cells is more Gi-dependent than in Caov-3. Surprisingly, the Gq-selective GAP RGS2 had a greater effect in SKOV-3 cells and the Gi-selective GAP RGS19 had a greater effect in CAOV-3 cells. While it is possible that distinct RGS proteins have greater regulatory effects in different cell lines due to specific interaction with unique signalling pathways coupled to LPA receptors in different cells, it is also possible that this reflects a loss of specificity due to overexpression.

While it is tempting to speculate that the observed effects of RSG2 on LPA stimulated inositol phosphate production in SKOV-3 cells may have contributed to the recently reported regulation of LPA stimulated cellular migration and inhibition of cAMP accumulation by endogenous RGS proteins in SKOV-3 cells (Hurst et al., 2008a), such assignment is premature. While LPA stimulated cellular migration and inhibition of cAMP were completely dependent on Ptx sensitive Gi/o G-proteins, in the current study we assayed inositol phosphate accumulation, which was only partially Ptx sensitive and may have been mediated by both Ptx sensitive and Ptx insensitive G-proteins such as Gq. RGS2 preferentially targets Gq G-proteins, but has been reported to deactivate Gi/o G-proteins under certain conditions. Future siRNA knockdown studies should more specifically determine the role of individual RGS proteins on distinct LPA signalling pathways when expressed at endogenous levels.

In summary, our data support a possible role for RGS2 and RGS19 in the regulation of LPA signalling in ovarian cancer cells. In addition to LPA, the gonatropin Luteinizing hormone (Gunthert et al., 2004) and the chemokine Growth-Regulated Oncogene α (GROα) (Lee et al., 2006b) also regulate ovarian cell growth by activating heterotrimeric G-proteins. Thus, RGS proteins expressed in ovarian cancer cells may regulate these pathways as well. Further, we report dramatic differences in expression levels of RGS transcripts in commonly used ovarian cancer model cell lines. SKOV-3, OVCAR-3, and Caov-3 are all derived from human ovarian adenocarcinomas, but vary with respect to metastatic potential and invasiveness (Choi et al., 2006). Differences in the expression of signalling regulators between these cell lines may account for differences in cellular function. Future studies should determine if the differences in RGS expression among ovarian cell lines and tumor tissues contributes to variability in G-protein regulated pathways relevant to cancer progression.

CHAPTER 7

siRNA KNOCKDOWN OF REGULATOR OF G-PROTEIN SIGNALING (RGS) PROTEINS IN SKOV-3 OVARIAN CANCER CELLS

Introduction

Lysophosphatidic Acid (LPA) is the predominant growth factor in ovarian cancer. LPA's effects are mediated by at least five G-protein coupled receptors (GPCRs), which each couple to multiple families of heterotrimeric G-proteins. Gi G-protein mediated pathways have been implicated in LPA-stimulated proliferation, motility, and cytokine production in ovarian cancer cells (van Corven et al., 1989; Sengupta et al., 2003; Sugiyama et al., 2004; Chou et al., 2005; Lee et al., 2006b; Evelyn et al., 2007; Jeong et al., 2008; Hurst and Hooks, 2009). Therefore, proteins capable of regulating the activity of Gi family G-proteins, such as RGS proteins, may be important to the regulation of LPA-stimulated pathways in ovarian cancer cells.

In Chapter 5 we demonstrated that endogenous RGS proteins significantly regulate Gi-mediated LPA signaling in SKOV-3 ovarian cancer cells using a mutagenesis strategy to compare the signaling activity of Gαi2 subunits that are wild-type with respect to RGS regulation with Gαi2 subunits which are insensitive to regulation by RGS proteins (Hurst et al., 2008a). SKOV-3 cells expressing RGS insensitive Gαi2 were significantly more responsive in assays measuring LPA-stimulated inhibition of adenylyl cyclase, migration, extracellular related kinase (ERK) activation, and cell growth compared with SKOV-3 cells expressing Gαi2 subunits that were wild-type with respect to RGS regulation (Chapters 3 and 5) (Hurst et al., 2008a; Hurst and Hooks, 2009). Additionally we performed a screen for RGS transcript expression in three

different ovarian cancer cell lines and immortalized ovarian surface epithelium (IOSE) (Hurst et al., 2009), identifying RGS proteins that are likely to be responsible for the RGS regulation of Gi-mediated LPA signaling observed in SKOV-3 ovarian cancer cells (Chapter 6).

In this study, we identified candidate RGS proteins that could be responsible for the regulation of LPA-stimulated Gi signaling pathways in SKOV-3 ovarian cancer cells and determined the effect of their expression on LPA signaling. To determine if the RGS proteins in question modulate Gi-mediated LPA signaling, we knocked down expression of individual RGS transcripts with siRNA, and compared LPA signaling in RGS siRNA transfected cells to their negative control-transfected counterparts in assays measuring adenylyl cyclase activity, inositol phosphate (IP) accumulation, and wound-induced migration. Expression of RGS2, RGS10, RGS12, and RGS19 was knocked down using siRNA; however, none of the siRNAs tested consistently or significantly altered LPA signaling in SKOV-3 cells. The following sections will detail the experiments performed and offer possible reasons for the lack of effect seen with altered RGS expression as well as suggestions for future studies.

Materials and Methods

Oncomine

Oncomine is a publicly available database summarizing gene chip experiments across tissue types (Rhodes et al., 2007). Oncomine provides data-mining tools to query genes and data sets of interest, and to meta-analyze groups of studies. The database was queried for all RGS proteins. Studies were included if they compared primary ovarian cancers to any of the following: normal ovary, normal ovarian surface epithelium, ovarian cancer tumor grades, ovarian cancer stages, or ovarian cancer omental metastasis. Only studies with $p<0.001$ were included in our analysis.

Cell Culture

Human SKOV-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia) and grown according to ATCC recommendations.

siRNA Constructs and Transfections

We used siRNA from two different manufacturers, Ambion (Austin, TX) and Dharmacon (Layfayette, CO), to knockdown expression of RGS transcripts in SKOV-3 cells. The Ambion *Silencer*® Select Pre-designed siRNAs used for knockdown of RGS transcripts are listed in **Table 7.1**. SKOV-3 cells were simultaneously plated and transfected using siPORT NeoFX transfection reagent (Ambion, Austin, TX) according to the manufacturer's protocol for reverse transfection. A transfection mix containing 10-30 nM siRNA and 2 μL siPORT NeoFX reagent in serum-free medium was added to each well of a 24-well plate, followed by 30,000 cells in normal growth medium. Cells and transfection mix were incubated for 24 hours at 37 °C at which point the media was changed to fresh SKOV-3 growth medium. Assays were performed and samples taken for transcript expression analysis 48 hours after transfection. Cells were transfected in parallel with targeted siRNA and negative control or scrambled siRNAs for each experiment so that non-specific changes in assay results and transcript level caused by transfection could be taken into account. The Dharmacon ON-TARGET*plus*™ SMARTpool siRNAs (Lafayette, CO) used for knockdown of RGS transcripts are listed in **Table 7.1**. SKOV-3 cells were plated at 25,000 cells/well in 24-well plates. Twenty-four hours after plating, cells were transfected with 100nM siRNA and 1 μL Dharmafect 4 transfection reagent according to manufacturer's instructions. Cells and transfection mix were incubated for 24 hours at 37 °C at which point the media was changed to fresh SKOV-3 growth medium. Assays were performed and samples taken for transcript expression analysis 48 hours after transfection. Cells were

Table 7.1: siRNA Constructs used for Knockdown of RGS transcripts
siRNA constructs from Ambion and Dharmacon used to knockdown individual RGS proteins are listed.

Target Gene	Ambion	Dharmacon
RGS2	s224531	L-009887-00
RGS3	S200451, s200452, s200453, s237006, s237007	
RGS10	s11999, s12000	
RGS12	s12001, s12002	
RGS17	S25542, s25543	
RGS19	S20107, s20108	L-008896-00

transfected in parallel with targeted siRNA and negative control siRNAs for each experiment so that non-specific changes in assay results and transcript level caused by transfection could be taken into account.

RNA Isolation

RNA was isolated from tissue samples and cultured cells using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Monolayers of SKOV-3 cells were homogenized in 1 ml of Trizol reagent per well of a 24-well plate by passing the lysates through a pipette tip several times. RNA preparations were treated with DNase, and the RNA was separated from the DNAse using an RNEasy column (Qiagen). RNA preparations were aliquoted and stored at -80°C.

QPCR

To compare the level of expression of RGS transcript between transfection conditions, DNA was first synthesized from 1ug of total RNA using the High Capacity Reverse Transcriptase cDNA kit (Applied Biosystems) to amplify mRNA. RNA samples were taken

from three separate wells for each transfection condition. Reactions without reverse transcriptase were also run as a control for DNA content. qPCR reactions were prepared using Applied Biosystem's Taq-man Gene Expression Assays and Universal PCR Mastermix according to the manufacturer's instructions. Reactions for GADPH were performed in each RNA set as an internal standard. The PCR reaction was carried out in a Biorad iCycler using program parameters provided by Applied Biosystems. Briefly, 50 ng cDNA was provided as template with 2X Universal PCR Mastermix for 50 cycles of 15 second melt at 95°C and 1 minute of anneal/extention at 60°C. The primers in Taq-man Gene Expression Assays are designed to use the same annealing temperature. Threshold cycle (CT) values were determined for each transcript using the automated threshold function of the iCycler software, and data are reported as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), using GAPDH housekeeping gene as internal controls and reporting data as expression in the cells transfected with RGS-targeted siRNA compared with those transfected with scrambled siRNA.

Inositol Phosphate Assay

Production of inositol phosphates (IP) was quantified using established protocols (Hepler et al., 1987). Briefly: To measure IP production by PLC activation, SKOV-3 cells were plated in 24-well dishes at 50,000 cells/well. 48 hours prior to the assay, cells were transfected with the appropriate genes. Cells were labeled with 0.5 μCi/well [^{3}H]-myo-inositol (American Radiolabeled Chemicals, St. Louis, MO) for 18 hours to label the cellular pool of phosphatidyl inositol. The cells were treated with Oleoyl (18:1) LPA (Avanti Polar Lipids, Alabaster, AL) in the presence of 10 mM lithium chloride to inhibit degradation of inositol phosphates for 30 minutes at 37°C. Cells were then lysed in cold formic acid and neutralized with ammonium hydroxide, and the lysates were then loaded onto columns of AG 1-X8 anion exchange resin

(Biorad, Hercules, California). The columns were washed with water and dilute ammonium formate to remove unhydrolyzed lipids. The 3H IPs were then eluted with 1.2 M ammonium formate/0.1 M formic acid, and added to scintillation fluid for counting. In some experiments, cells were treated with 200 ng/ml pertussis toxin (Sigma-Aldrich, St. Louis, MO) for 12 hours prior to IP assay.

cAMP Assay

We use a modified version of the protocol described in Hettinger-Smith et al. (Hettinger-Smith et al., 1996). SKOV-3 cells were plated in 12-well dishes and labeled with 0.6 μCi [^{3}H]-adenine (Perkin Elmer, Waltham, MA) for three hours in the presence or absence of 200 ng/mL Ptx. Assay buffer containing 1 mM IBMX, a phosphodiesterase inhibitor, 50 μM forskolin, and varying concentrations of LPA was added to the cells for 10 min (unless other wise noted in time course assay) at 37 °C. Reactions were terminated by aspiration followed by the addition of stop solution containing 1.3 mM cAMP and 2% sodium dodecyl sulfate. [^{14}C]-cAMP stock was added to each well to control for recovery of cAMP, followed by perchloric acid to lyse cells. Lysates were neutralized with KOH and cAMP was isolated using sequential column chromatography over Dowex AG-50-W4 cationic exchange resin (Bio-Rad, Hercules, CA) followed by neutral alumina columns. The resulting eluate was subjected to scintillation counting after the addition of 10 mL scintillation cocktail.

Wound-induced Migration Assay

Monolayers of SKOV-3 cells were plated in 24-well dishes and transfected with either siRNA or DNA. Twenty-four hours later, cells transfected with siRNA were re-plated in 96-well plates to obtain cells that were sufficiently confluent for the assay. Six hours later, cells were starved in serum-free media for an additional 18 hours prior to wounding. Cells that were

transfected with DNA for over-expression, were plated, starved, and assayed in 24-well plates. A "wound" was introduced by scraping a single line through the monolayer with a pipette tip, and cells were then treated with 1 μM LPA or vehicle. Images of the wound were captured with a Nikon AZ100 microscope mounted with a Nikon Digital Sight DS-QiMc camera set at 10× magnification at time zero and every 6 hours for 48 hours after the wound was made to compare the speed at which surrounding cells fill the wound. LPA was reapplied to cells every 12 hours to prevent depletion. Wound filling was quantified using Nikon NIS Elements BR 2.30 software. Using a "polygonal region of interest" drawing tool, wound edges were traced to create a polygon whose surface area was measured by the software in pixels squared. Wound closure was measured as the initial area minus the area of the wound at a given time point to yield area covered.

Results

The goal of this study was to determine the effect of individual endogenous RGS proteins on the regulation of Gi-mediated LPA signaling in SKOV-3 ovarian cancer cells. We initially identified candidate RGS proteins through an RT-PCR screen of RGS transcripts in IOSE cells and SKOV-3, Caov-3, and OVCAR-3 ovarian cancer cell lines (Hurst et al., 2009). SKOV-3 cells express multiple transcripts for RGS proteins that have demonstrated GAP activity towards Gi-family G-proteins, including RGS2, RGS3, RGS4, RGS6, RGS7, RGS9, RGS11, RGS10, RGS12, RGS17, RGS19, and RGS20 (Hollinger and Hepler, 2002; Hurst et al., 2009).

To determine if the expression of RGS transcripts is altered in clinical samples of ovarian cancer, we queried the Oncomine database. Studies were included if they compared primary ovarian cancers to normal ovary, normal ovarian surface epithelium, ovarian cancer tumor grades, ovarian cancer stages, or ovarian cancer omental metastasis. Only studies with $p<0.001$

Table 7.2: RGS transcript expression in ovarian cancer

The Onocomine database was queried for RGS transcript expression in ovarian cancer. Studies were included if they compared primary ovarian cancers to any of the following: normal ovary, normal ovarian surface epithelium, ovarian cancer tumor grades, ovarian cancer stages, or ovarian cancer omental metastasis. P values are in parentheses. Only studies with $p<0.001$ were included in our analysis.

RGS Transcript	G-protein Specificity	Expression in SKOV-3	Expression Profile in Ovarian Cancer
RGS1	i/o, q/11	No	Three independent studies showing that expression increases with tumor grade (5.4e-6, 7.2e-6, 1.2e-5)
RGS2	q/11>i/o	Yes	Decreased expression compared with normal ovary in: serous adenocarcinoma (6.3e-20); endometrioid carcinoma (22.6e-19); clear cell adenocarcinoma (3.8e-7), mucinous adenocarcinoma (2.16e-6)
RGS3	i/o, q/11	Yes	Increased expression in omental metastasis compared with normal ovary and serous adenocarcinoma (2.7e-5)
RGS4	i/o, q/11	Yes	Decreased expression in ovarian adenocarcinoma compared to normal ovary in: (2.6e-6)
RGS5	i/o, q/11	No	Decreased expression in serous adenocarcinoma compared to normal ovary (3.4e-4); two studies showing that expression decreases with increased tumor grade (8e-4, 9.1e-4)
RGS6	i/o	Yes	Two independent studies showing decreased expression with higher tumor stage (1.6e-6, 2.7e-4)
RGS7	i/o	Yes	Decreased expression compared to normal ovary in serous adenocarcinoma (2e-7); decreased expression in metastases compared with primary site tumor (7.6e-4)
RGS8	i/o, q/11	No	Increased expression with higher cancer stage (1.9e-4)
RGS9	i/o	Yes	N/A
RGS10	i/o, q/11	Yes	Expression increased with higher cancer stage (4.7e-5)
RGS11	i/o	Yes	Expression increased with higher cancer stage (7.4e-4)
RGS12	i/o	Yes	Decreased expression with higher cancer stage (3.5e-6); Decreased expression compared to normal ovary in: serous adenocarcinoma (1e-4), ednometrioid adenocarcinoma (8.9e-4)
RGS13	i/o, q/11	No	Decreased expression with higher tumor grade (8.8e-6)
RGS14	i/o	No	Increased expression in clear cell adenocarcinoma compared to normal ovary (5.9e-4)
RGS16	i/o, q/11	No	Increased expression with higher cancer stage (1.5e-6)
RGS17	i/0	Yes	Decreased expression compared to normal ovary in: serous adenocarcinoma (5.7e-12); endometrioid carcinoma (4.6e-6); mucinous adenocarcinom (2.1e-5); clear cell adnocarcinoma (9.1e-4). Decreases with increased cancer grade (6.2e-4)
RGS18	i/o, q/11	No	N/A
RGS19	i/0	Yes	Two independent studies demonstrating increased expression with in invreased stage (1.1e-4, 3.7e-4); decreased expression in normal ovary compared with serous adenocarcinoma and omental metastasis (1.4e-4)
RGS20	i/o/z	Yes	N/A
PDZ-RhoGEf	12/13	Yes	Increased expression in mucinous adenocarcinoma compared to normal ovary (7.8e-4)
p115-RhoGEF	12/13	Yes	Increased expression compared to normal ovary in: endometrioid adenocarcinoma (2.6e-5); mucinous adenocarcinoma (1.1e-4); serous adenocarcinoma (3.7e-4); clear cell adenocarcinoma (7.6e-4)
LARG	12/13	Yes	Increased expression with higher cancer stage (5.5e-6). Increased expression compared to normal ovary in: serous adenocarcinoma (2.6e-5); mucinous adenocarcinoma (1e-4); clear cell adenocarcinoma (1.9e-4); endometrioid adenocarcinoma (8.2e-4)

were included. These findings are summarized in **Table 7.2**. Generally, expression of transcripts for RGS2, RGS3, RGS10, and RGS19 increased in ovarian carcinomas, while expression of RGS4, RGS12, and RGS17 transcripts decreased. Based on the expression profile of RGS transcripts in SKOV-3 cells and data from Oncomine, we chose to study the effects of RGS2, RGS3, RGS10, RGS12, RGS17, and RGS19 in SKOV-3 cells in assays measuring LPA-stimulated migration, inhibition of adenylyl cyclase, and stimulation of inositol phosphate production.

Knockdown of individual RGS transcripts does not significantly alter LPA-stimulated wound-induced migration

We have previously demonstrated that expression of RGS-insensitive Gαi2 significantly enhances LPA-stimulated migration compared to SKOV-3 cells expressing Gαi2 that is wild-type with respect to RGS regulation (Hurst et al., 2008a). Differences in LPA-signaling between cells expressing the two different Gαi2 constructs were much more pronounced in this assay compared to assays measuring production of second messengers. Further, this assay is pertinent to a cancerous phenotype as enhanced motility and migration is one of the hallmarks of cancer (Hanahan and Weinberg, 2000). We therefore performed our initial screen of RGS-targeted siRNAs in a wound induced migration assay.

SKOV-3 cells were transfected with siRNA targeted against a single RGS transcript or with negative control siRNAs. Wound-induced migration assays were begun 48 hours post-transfection at which time samples were collected for QPCR analysis to determine RGS transcript levels. We were unable to reduce transcript expression of RGS3 after trying five different siRNAs under optimized transfection conditions (data not shown). Knockdown of greater than 50% of transcript was achieved for RGS2, RGS10, RGS12, RGS17, and RGS19.

Knockdown of RGS2 had variable effects on LPA-stimulated migration of SKOV-3 cells. In several of our initial assays, knockdown of RGS2 using an Ambion validated siRNA increased LPA-stimulated migration by as much as 50%, while in another assay a similar level of knockdown decreased migration by ~5%. Because there was only a 50-60% decrease in RGS2 transcript in these assays using the Ambion siRNA, we attempted to knockdown RGS2 using a Dharmacon SMARTpool. The Dharmacon siRNA knocked down over 70% of RGS2 transcript in SKOV-3 cells; however, there still was not a consistent effect on LPA-stimulated migration.

The migration assay shown in **Figure 7.1A** shows a slight decrease in both basal and LPA-stimulated migration; however, if basal migration is subtracted, the level of LPA-stimulated migration is the same for cells transfected with RGS2 siRNA or negative control siRNA. In several assays, RGS2 knockdown enhanced basal migration. These data suggest that the effects of the RGS2 siRNA may be non-specific.

Knockdown of RGS10 also had variable effects on LPA-stimulated migration of SKOV-3 cells. Five experiments were performed with a 70-85% knockdown of RGS10 transcript. In three of the experiments, knockdown of RGS10 enhanced LPA-stimulated migration, by 32%, 47%, and 110%. In two of these experiments, basal migration was enhanced 47% and 74%. The experiment depicted in **Figure 7.1B** shows a significant increase in both basal and LPA-stimulated migration. In three experiments with a similar reduction in RGS10 transcript, there was either no effect on migration, and in one case, a 35% reduction in LPA-stimulated migration. These data demonstrate an inconsistent effect of RGS10 knockdown on LPA-stimulated migration.

siRNA targeting RGS12 and RGS17 did not significantly effect LPA-stimulated migration in SKOV-3 cells. In two experiments, RGS12 was knocked down 50-65%. There was no effect on either basal or LPA-stimulated migration in these experiments (**Figure 7.1C**). RGS17 was knocked down by 60% in two independent experiments, but did not have an effect on either basal or LPA-stimulated migration (**Figure 7.1D**).

RGS19 siRNA did not consistently inhibit or enhance LPA-stimulated migration of SKOV-3 cells. In two experiments with 56% and 66% knockdown, basal migration was enhanced by 112% and 56%, respectively. LPA-stimulated migration was increased 35% and 135%, respectively. Forty percent knockdown of RGS19 transcript resulted in a 23% increase in

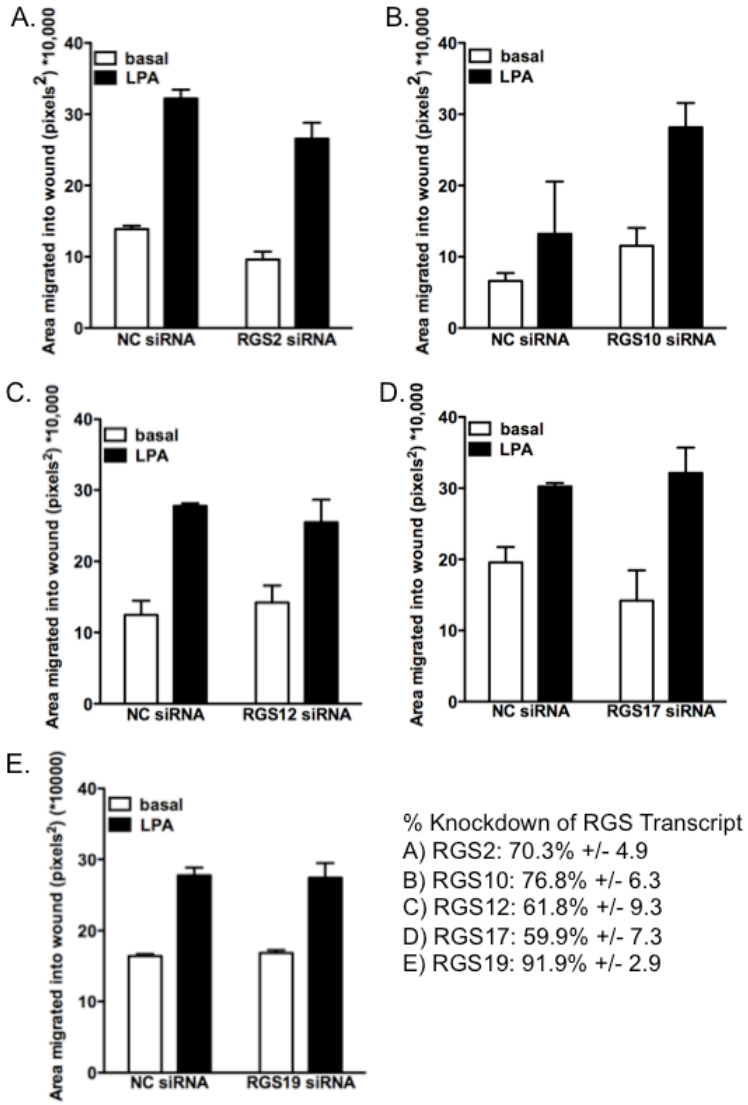

Figure 7.1: Effects of RGS siRNA on LPA-stimulated migration of SKOV-3 ovarian cancer cells
Monolayers of SKOV-3 cells were transfected with siRNA targeting RGS2 (A), RGS10 (B), RGS12 (C), RGS17 (D), or RGS19 (E) transcipts or negative control (NC) siRNA and starved in serum-free media for 18 hours prior to the assay. 48 hours after transfection, a "wound" was introduced by scraping a single line through the monolayer with a pipette tip and then treated with 1 μM LPA or vehicle for 12 hours. Images were captured and cell migration was quantified using microscopy as described. Percent transcript knockdown +/- SEM is indicated in the inset.

basal migration and an 11% increase in LPA-stimulated migration. In three experiments with knockdown of RGS19 ranging from 50% to 70%, there was no effect on either basal or LPA-stimulated migration. Because the knockdown of RGS19 was highly variable and never over

75%, we tried a Dharmacon SMARTPool targeting RGS19. The Dharmacon reagent was highly effective with knockdown of RGS19 consistently over 90%; however, even with 90% knockdown, there was no effect on LPA-stimulated migration (**Figure 7.1E**), suggesting that the previous results were non-specific.

siRNA knockdown of individual RGS transcripts does not significantly alter LPA-stimulated second messenger signaling

Knockdown of RGS2, RGS10, and RGS19 had variable effects on migration, while knockdown of RGS12 and RGS17 did not appear to either enhance or attenuate LPA-stimulated migration (**Figure 7.1**). Therefore, we continued testing siRNA targeted against RGS2, RGS10, and RGS19 in assays measuring second messenger production.

siRNA knockdown of individual RGS transcripts does not significantly alter LPA-stimulated inhibition of adenylyl cyclase activity

SKOV-3 cells were transfected with siRNA targeted against a single RGS transcript or with negative control siRNA and assayed for adenylyl cyclase activity 48 hours after transfection. Knockdown of RGS2, RGS10, or RGS19 did not consistently alter LPA-stimulated inhibition of adenylyl cyclase activity (**Figure 7.2**).

Seven cAMP assays were performed with RGS2 siRNA. In three experiments with RGS2 transcript knocked down 54-63%, LPA-stimulated inhibition of adenylyl cyclase was decreased by 9.9-17%. RGS2 was knocked down 75-85% in four independent experiments. There was a 14% decrease in inhibition of adenylyl cylase activity in the first experiment, no effect in the second experiment, a 15% increase in inhibition in the third experiment, and a 4% increase in inhibition in the fourth experiment (**Figure 7.2A**).

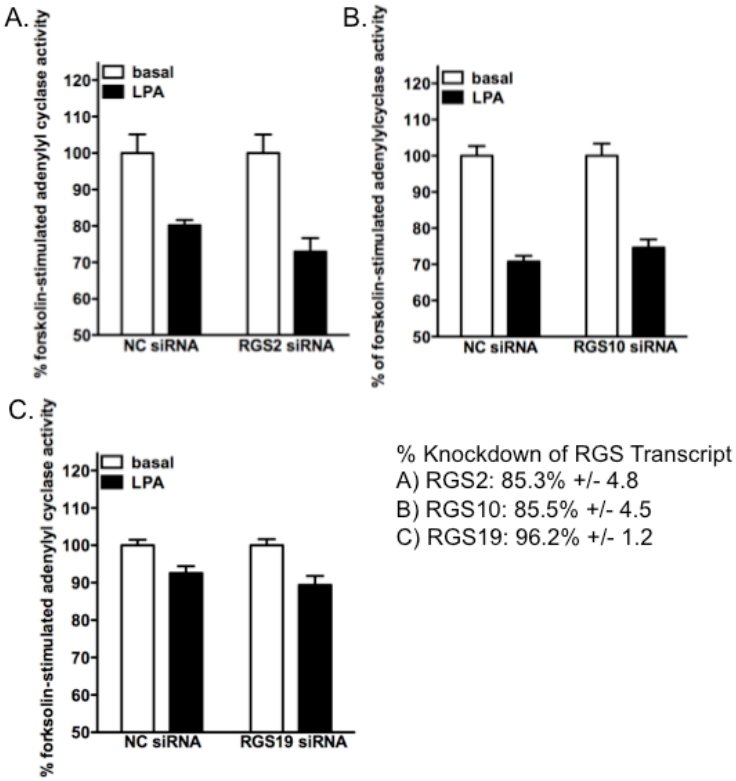

Figure 7.2: Effects of siRNA targeting individual RGS transcripts on LPA-stimulated inhibition of adenylyl cyclase
SKOV-3 cells were transfected with negtive control siRNA (NC), or siRNA targeting RGS2 (A), RGS10 (B), or RGS19 (C). Cells were treated with 1 μM LPA in the presence of 50 μM forskolin for 20 minutes and assayed for cAMP levels as described. Results are reported as a percent of adenylyl cyclase activity observed in the absence of LPA. Percent transcript knockdown +/- SEM is indicated in the inset.

RGS10 was knocked down in four independent experiments. There was not a significant effect on LPA-stimulated inhibition in three of the experiments when RGS10 transcript was decreased by 56-70%. When RGS10 transcript was knocked down 85%, there was a 6% decrease in LPA-stimulated inhibition of adenylyl cyclase (**Figure 7.2B)**.

Effects of RGS19 siRNA on LPA-stimulated inhibition of adenylyl cyclase were highly variable. In two experiments where RGS19 transcript was knocked down 66% and 84%, LPA-stimulated inhibition of adenylyl cyclase activity was completely abrogated. Two more experiments with RGS19 transcript decreased by 40% and 69% did not have any effect on LPA-

stimulated adenylyl cyclase activity. In a final experiment using a Dharmacon SMARTpool, RGS19 was knocked down by 95%; however, LPA-stimulated inhibition of adenylyl cyclase activity only increased by 3% (**Figure 7.2C**).

siRNA knockdown of individual RGS transcripts does not significantly alter LPA-stimulated inositol phosphate (IP) accumulation

SKOV-3 cells were transfected with siRNA targeted against a single RGS transcript or with negative control siRNA and assayed for inositol phosphate (IP) accumulation 48 hours after transfection. Knockdown of RGS2, RGS10, or RGS19 did not significantly alter LPA-stimulated IP accumulation (**Figure 7.3**).

SKOV-3 cells were transfected with RGS2 siRNA in five independent experiments and assayed for LPA-stimulated accumulation of IPs. In two experiments, RGS2 transcript was decreased by 63%. LPA-stimulated IP accumulation was not altered in the first experiment and was decreased ~20% in the second experiment. In a third experiment, RGS2 transcript was knocked down 75%, resulting in a 48% decrease in LPA-stimulated IP accumulation. To achieve greater knockdown, we used a Dharmacon SMARTpool in two more experiments. RGS2 was knocked down 85% in each experiment. In the first experiment, RGS2 siRNA increased both basal and LPA-stimulated IP accumulation by 33% compared with cells treated with negative control siRNA. In the second experiment, shown in **Figure 7.3A**, RGS2 siRNA decreased basal IP accumulation by 7% and increased LPA-stimulated IP accumulation by 13%.

Knockdown of RGS10 consistently increased basal IP accumulation in SKOV-3 cells. In two independent experiments, cells transfected with RGS10 siRNA had a 55-70% decrease in RGS10 transcript expression. In both experiments, basal IP accumulation increased by 35-42% while LPA-stimulated IP accumulation remained unchanged (**Figure 7.3B**).

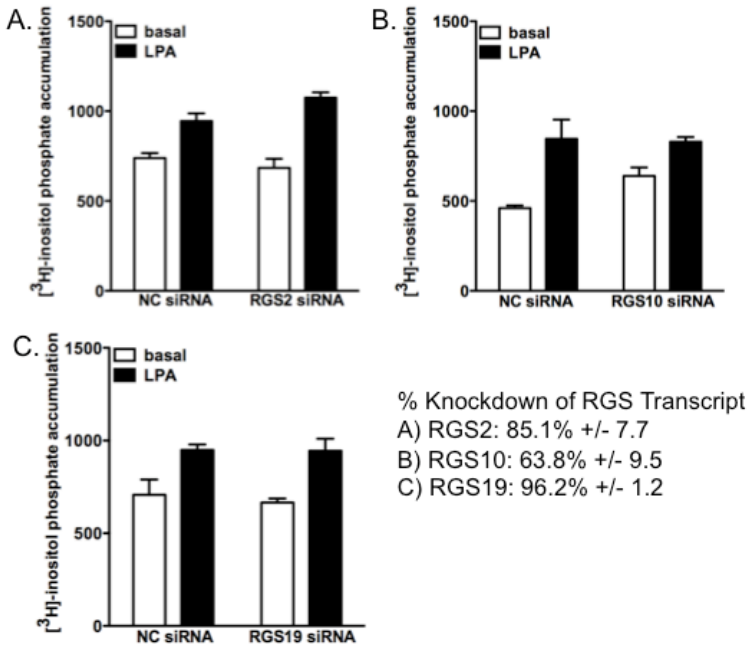

Figure 7.3: Effects of siRNA targeting individual RGS transcripts on LPA-stimulated inositol phosphate accumulation
SKOV-3 cells were transfected with negative control siRNA (NC), or siRNA targeting RGS2 (A), RGS10 (B), or RGS19 (C). Cells were treated with 1 μM LPA in the presence of 50 mM LiCl for 30 minutes and assayed for IP levels as described. Percent transcript knockdown +/- SEM is indicated in the inset.

The effect of RGS19 siRNA on LPA-stimulated IP accumulation was assessed in three independent experiments. A 40% decrease in RGS19 transcript resulted in a 23% increase in basal IP accumulation and an 11% increase in LPA-stimulated IP accumulation. In a second experiment, a 70% decrease in RGS19 transcript increased LPA-stimulated IP accumulation by 20%, but did not alter basal IP accumulation. A final experiment using a Dharmacon SMARTpool had a 95% reduction in RGS19 transcript; however, there was no change in either basal or LPA-stimulated IP accumulation (**Figure 7.3C**).

Discussion

We have previously demonstrated that endogenous RGS proteins regulate Gαi2-mediated LPA signaling in SKOV-3 ovarian cancer cells (Hurst et al., 2008a). Further, we have shown

that several RGS proteins capable of regulating RGS signaling are expressed in SKOV-3 cells (Hurst et al., 2009). The goal of this study was to identify the RGS proteins that regulate Gi G-protein mediated LPA signaling in SKOV-3 ovarian cancer cells. Expression of RGS2, RGS10, RGS12, RGS17, and RGS19 transcripts was knocked down using siRNA and the effects of reduced RGS transcript expression was initially assessed in a wound-induced migration assay. RGS2, RGS10, and RGS19 had variable effects on LPA-stimulated migration, while RGS12 and RGS17 siRNAs did not alter LPA-stimulated migration. Thus, the effects of RGS2, RGS10, and RGS19-targeted siRNAs were determined in assays measuring second messenger production. Knockdown of these RGS transcripts did not significantly or consistently alter LPA responses in assays measuring LPA-stimulated inositol phosphate accumulation or inhibition of adenylyl cyclase.

There are several possible explanations for the lack of effect of RGS-targeted siRNAs. First, reduction in transcript expression does not necessarily cause a reduction in protein levels. Expression of RGS proteins is known to be post-translationally regulated, often through the co-expression of binding partners that stabilize the RGS protein (Chen et al., 2003). Additionally, the degradation pathways controlling the lifetime of RGS proteins are not well understood. Unfortunately, there are few commercially available antibodies for RGS proteins and detection of RGS proteins is difficult as they are not particularly abundant. It is therefore not possible to monitor the levels of endogenous RGS proteins, making it difficult to determine how much of an effective protein knockdown has been achieved.

Another possible explanation for the lack of consistent, significant effects with RGS knockdown is that more than one RGS protein may be responsible for the regulation of Gi-mediated LPA signaling. We have attempted to simultaneously knockdown multiple RGS

proteins; however, most of the siRNAs tested were not as effective in knocking down their RGS transcript targets in combination as they were when used alone. SKOV-3 cells were transfected with all the siRNAs for RGS2, RGS10, RGS12, RGS17, RGS19, and RGS20 and assessed in a migration assay. Interestingly, cells transfected with all of the RGS siRNAs died when treated with LPA, while cells treated with vehicle had the same basal migration rate as cells transfected with negative control siRNA (data not shown).

Finally, it may be necessary to create stable cell lines with reduced RGS expression to create an observable phenotype. In this study we used a transient transfection method with unknown and likely variable transfection efficiency. If a small percentage of cells in the total population are taking up the siRNA constructs, this would be reflected as a reduction in transcript level, but might not be apparent in an assay. Therefore, generating a population in which the majority of cells are transfected will significantly increase the likelihood of a measurable effect.

A proteomic approach may also help identify RGS proteins responsible for regulation of Gi-mediated LPA signaling in ovarian cancer cells. Proteins which co-immunoprecipiate with Gαi subunits in ovarian cancer cells could be compared before and after LPA treatment. Due to the lack of RGS antibodies, RGS proteins would need to be identified with mass spectrometry.

In order to identify the RGS proteins responsible for regulation of Gi-mediated LPA signaling in ovarian cancer cells, it may be necessary to try different genetic and biochemical approaches. RGS protein expression has previously been shown to increase after chronic activation of the pathway it regulates (Rahman et al., 2003). Future studies should examine changes in RGS transcript expression in ovarian cancer cells in response to LPA treatment.

In summary, we tested the effects of siRNA targeted against several different RGS transcripts in assays measuring LPA-stimulated migration, inhibition of adenylyl cyclase, and production of inositol phosphates. While we did not observe any significant effects of reduced RGS transcript expression in these assays, we have identified several possible reasons for the lack of effect and suggested new research directions which may help to determine the role of specific RGS proteins in ovarian cancer cells.

CHAPTER 8

SUMMARY

The goal of this research was to characterize the signaling pathways that mediate the effects of LPA and S1P in two different systems: human embryonic neuroepithelial cells and ovarian cancer cells.

In Chapter 2, we establish hES-NEP cells as a relevant model for lysophospholipid signaling, demonstrating that LPA and S1P promote cellular proliferation and morphology changes through Gi G-protein/EGF and Rho-mediated pathways, respectively. LPA and S1P play critical roles in the development of the mammalian nervous system. Defects in LPA and S1P signaling pathways have been linked to neural tube defects (NTD), thus understanding how their effects are mediated in human neural progenitor cells will provide insight into the pathophysiology of developmental defects.

The hES-NEP model system may prove useful in studies of the effects of toxic agents and pharmaceuticals in the developing nervous system. Fumonisin, a mycotoxin found in corn that has been linked to NTDs, alters sphingolipid metabolism (Gelineau-van Waes et al., 2009). FTY720, an S1P analogue that acts as a functional antagonist at S1P receptors (Mandala et al., 2002), is currently in Phase III clinical trials for the treatment of multiple sclerosis (O'Connor et al., 2009). We are currently investigating the effects of these compounds on proliferation, morphology, and survival of hES-NEP cells.

hES-NEP cells and cell populations derived from hES-NEP cells are also being investigated as cell replacement therapies (Guillaume and Zhang, 2008). Understanding the

signaling pathways that modulate the behavior of these cells will be important to developing useful cell-based therapeutics. There are several reports that LPA and S1P alter differentiation (Cui and Qiao, 2007; Fukushima et al., 2007; Milstien et al., 2007; Dottori et al., 2008) and migration (Kimura et al., 2007; Kimura et al., 2008) of neural progenitors. Future studies should examine the effects of LPA and S1P on differentiation and migration of hES-NEP cells. Correct migration and localization is another critical component of

In addition to regulating neuronal development lysophosphatidic acid signaling is a critical modulator of ovarian cancer. It promotes cellular proliferation, migration, invasion and survival by binding and activation of multiple GPCRs. In Chapter 3 we describe the pathways mediating LPA-stimulated proliferation in two different ovarian cancer cell lines. These data demonstrate that ovarian cancer is a highly heterogeneous disease which implements multiple signaling pathways to promote oncogenesis. Additionally, these data suggest that diversity of ovarian cancer cell lines should be taken into account when attempting to identify signaling pathways for therapeutic targeting.

Signaling mediated by GPCRs has emerged as a critical regulator of oncogenic processes; thus, regulators of GPCR signaling may be important to the pathophysiology of cancer. Chapter 4 reviews the role of RGS proteins in cancer biology, detailing changes in RGS transcript expression between benign and cancerous tissues, mutations in RGS proteins, and specific roles for RGS2, RGS-RhoGEFs, Axin, and RGS5. This review provides evidence that RGS proteins are important in cancer biology.

In Chapters 5 through 7 we explore the role of RGS proteins in ovarian cancer. The data presented in Chapter 5 demonstrates a role for endogenous RGS proteins in the regulation of LPA-stimulated Gi-mediated signaling pathways in ovarian cancer cells. RGS proteins decrease

both the potency and efficacy and shorten the lifetime of LPA-stimulated Gi G-protein signaling in ovarian cancer cells. Further, we demonstrate that LPA-stimulated migration of ovarian cancer cells is mediated by Gi G-proteins and is significantly attenuated by endogenous RGS proteins. These data establish RGS proteins as novel regulators of LPA signaling in ovarian cancer.

Chapter 6 describes the expression patterns of RGS transcripts in benign and malignant ovarian tissue and amongst different ovarian cancer cell lines. These data show that RGS transcript expression varies significantly between benign and malignant tissue and also amongst different ovarian cancer cell lines. Further, we show that over-expression of RGS proteins differentially attenuates LPA signaling from different LPA receptors as well as LPA signaling in different ovarian cancer cell lines. These data suggest a possible role for RGS2 and RGS19 in the regulation of LPA-signaling in ovarian cancer cells. Additionally, these data show changes in the expression of multiple RGS transcripts, suggesting that general changes in the regulation of GPCR signaling may be important to the biology of ovarian cancer cells.

Finally, we used the RGS transcript expression data from Chapter 6 and data from the online database Oncomine, expression in SKOV-3 cells, and G-protein specificity to identify RGS proteins that could regulate Gi-mediated LPA signaling in SKOV-3 ovarian cancer cells. RGS2, RGS3, RGS10, RGS12, RGS17, and RGS19 were identified as candidate RGS proteins. We used a wound-induced migration assay to screen for alterations of LPA activity induced by transfection of siRNAs targeting individual RGS proteins. RGS12 and RGS17 did not alter LPA-stimulated SKOV-3 cell migration, while the effects of siRNA targeting RGS2, RGS10, and RGS19 were variable. siRNAs targeting RGS2, RGS10, and RGS19 were then tested in assays measuring LPA-mediated second messenger production. None of the RGS siRNAs tested

significantly or consistently altered LPA activity in these assays. Interestingly, transfecting SKOV-3 cells with all of the RGS-targeted siRNAs simultaneously caused the cells to die when they were exposed to LPA, suggesting that RGS protein regulation is critical to LPA signaling in SKOV-3 cells. These data demonstrate that regulation of LPA signaling by endogenous RGS proteins may require multiple RGS family members.

Future studies to identify RGS proteins that regulate LPA signaling should involve several different approaches. Genetic approaches including analysis of epigenetic changes in RGS genes during ovarian cancer initiation and progression and LPA-stimulated changes in RGS expression may help determine which RGS proteins are important to ovarian cancer cell biology. Another approach is to use co-immunoprecipitation to determine which RGS proteins are physically bound to Gα G-protein subunits before and after LPA stimulation. Proteomics and mass spectrometry would be used to identify any associated RGS proteins.

Overall, these studies have characterized lysophospholipid signaling pathways in hES-NEP and ovarian cancer cells. Studies of LPA and S1P signaling in hES-NEP cells have established these cells as a model for lysophospholipid signaling in the developing brain which will be useful in determining the effects of toxicological and pharmaceutical agents in developing human neurons. Studies of LPA signaling in ovarian cancer cells identified divergent but overlapping pathways mediating proliferation in two commonly used model ovarian cancer cell lines. Further, these studies establish RGS proteins as novel regulators of LPA signaling in ovarian cancer. Elucidation of the regulation of signaling pathways critical for the initiation and progression of ovarian cancer should help with the rational design of novel therapeutics.

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