

## ABSTRACT

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Inhibition of LPA Signaling Pathways by RGS Protein Overexpression in Ovarian Cancer Cells  
(Under the direction of DR. SHELLEY HOOKS)

Lysophosphatidic acid (LPA) is a signaling molecule that induces survival, metastasis, migration, and proliferation in ovarian cancer cells by binding to G-protein coupled receptors (GPCRs), which in turn activate G-proteins. Regulator of G-Protein Signaling (RGS) proteins deactivate these G-proteins, and therefore stop the LPA signal. RGS proteins are a likely therapeutic target for the cancer causing activities of LPA because there are multiple forms that bind specifically to different G-proteins, therefore potentially regulating specific signals and outcomes. Previous data suggest that RGS proteins play a role in regulating the LPA signal in ovarian cancer cells. By comparing the effects of LPA in RGS sensitive and insensitive cells, we observed differences in cell growth, cell migration, and the production of the second messengers cyclic adenosine monophosphate (cAMP) and inositol phosphate (IP). My current project focuses on overexpressing two distinct RGS proteins and determining their effects on LPA stimulated outcomes of cAMP and cellular migration in SKOV-3 ovarian cancer cells. LPA causes a decrease in the second messenger cAMP, and we have found that overexpression of RGS2 and RGS19 blocks this LPA stimulated inhibition of adenylyl cyclase. Additionally, LPA causes an increase in cellular migration, which is also inhibited by RGS2 and RGS19. Further study will confirm these results by lowering expression of endogenous RGS2 and RGS19 in ovarian cancer cells using siRNA.

INDEX WORDS: LPA, Lysophosphatidic acid, Ovarian cancer, SKOV-3 cells, RGS, Regulator of G-protein signaling, G-protein, cAMP, Migration, RGS2, RGS19

INHIBITION OF LPA SIGNALING PATHWAYS BY RGS PROTEIN OVEREXPRESSION  
IN OVARIAN CANCER CELLS

by

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## TABLE OF CONTENTS

	Page
<b>CHAPTERS</b>	
1 INTRODUCTION.....	1
2 EXPERIMENTAL METHODS.....	3
Cell Culture.....	3
DNA Constructs and Transfections.....	3
Western Blotting.....	3
Wound-induced Migration Assay.....	4
cAMP Assay.....	4
3 RESULTS.....	5
Dose-Dependent Response of LPA in Migration .....	5
Dose-Dependent Response of LPA in cAMP.....	6
Overexpression of RGS Proteins.....	7
RGS 2 Overexpression Regulates Migration into Wound.....	8
RGS19 Overexpression Regulates Migration into Wound.....	10
RGS2 Overexpression Regulates LPA-Stimulated Decrease of cAMP....	11
RGS19 Overexpression Regulates LPA-Stimulated Decrease of cAMP..	12
4 DISCUSSION.....	14
5 CONCLUSION.....	15
WORKS CITED.....	16

## CHAPTER 1 INTRODUCTION

Ovarian Cancer is the most lethal gynecological cancer in the United States (Greenlee, Hill-Harmon et al. 2001). Lysophosphatidic acid (LPA) is known to be a growth factor in ovarian cancer cells (Umezū-Goto, Tanyi et al. 2004), and induces metastasis, survival, growth, migration, and apoptosis in ovarian cancer cells (Ishii, Fukushima et al. 2004). LPA regulates cell function by binding to five different G-Protein Coupled Receptors (GPCRs), which are transmembrane receptors. GPCRs couple to one of four different classes of heterotrimeric G-proteins inside the cell. G-proteins consist of three bound subunits,  $G_\alpha$ ,  $G_\beta$ , and  $G_\gamma$ . When LPA binds to a GPCR, the  $G_\alpha$  and  $G_{\beta\gamma}$  subunits dissociate, as the  $G_\alpha$  subunit exchanges an inactive guanosine diphosphate (GDP) for an active guanosine triphosphate (GTP). While dissociated, the two subunits are able to active different signaling cascades, which in turn cause activities such as migration, proliferation, and changes in second messenger production. Eventually, the  $G_\alpha$  subunit will hydrolyze the GTP, allowing it to bind again to the  $G_{\beta\gamma}$  subunit, and stop the downstream cascade. Regulators of G-Protein Signaling (RGS) proteins more quickly deactivate G-proteins by hydrolyzing GTP back to GDP at a faster rate than the  $G_\alpha$  subunit alone. While there are only four families of G-proteins, there are over 30 RGS isoforms. This potentially allows for more specificity of regulation which is important because not only does LPA induce proliferation and migration of cancer cells, but also apoptotic activities, or programmed cell death (Hurst, Henkel et al. 2008).

It has been shown previously that in SKOV-3 ovarian cancer cells, making G-Proteins insensitive to all isoforms of RGS proteins causes an increase LPA signal strength, suggesting RGS proteins are a possible therapeutic target (Hurst, Henkel et al. 2008). Preliminary data have also suggested that some RGS isoforms are present in different amounts in normal ovarian cells and ovarian cancer cells (Hurst, Mendpara et al. 2008). It has also been shown that some RGS proteins have specific functions within the cell, so that one or a few RGS proteins regulate specific outcomes (Wang, Liu et al. 2002). We have shown that both RGS2 and RGS19 are expressed in SKOV-3 cells. RGS2 expression is decreased in SKOV-3 ovarian cancer cells compared to normal ovarian cells, whereas RGS19 expression is increased in ovarian cancer cells (Hurst, Mendpara et al. 2008). Furthermore, RGS2 preferentially deactivates  $G_q$  G-proteins, whereas RGS19 deactivates  $G_i$  G-proteins. These differences in the proteins could translate to differences in downstream outcomes.

This study will focus specifically on the role of LPA and RGS proteins in wound induced migration assays and cyclic adenosine monophosphate (cAMP) assays. Specifically, RGS2 and RGS19 overexpression will be used in these assays to determine their role in attenuating LPA signaling pathways.

## CHAPTER 2 EXPERIMENTAL METHODS

### *2.1 Cell Culture*

SKOV-3 human ovarian cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). They were grown 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% CO<sub>2</sub>, according to standards.

### *2.2 DNA Constructs and Transfections*

Plasmids encoding multiple RGS proteins were transfected using Fugene 6 transfection reagent according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). They were transfected with at a ratio of 2uL Fugene 6 to 1 ug DNA plasmid. 500 ng DNA was added per well in a 24 well plate. Transfection was performed 48 hours prior to all experiments.

### *2.3 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 primary antibodies (Santa Cruz Biotechnology) and peroxidase-conjugated secondary antibodies (Bethyl Laboratories), and visualized using SuperSignal Chemiluminescent substrate (Pierce).

#### 2.4 Wound-Induced Migration Assay

Cells were plated at 500,000 cells/mL in a 24-well plate. Monolayers of cells were transfected and then starved overnight. A “wound” was introduced by scraping a single line through the monolayer with a pipette tip. The cells were then treated with various concentrations of LPA, from 0 to 10uM, for 48 hours. LPA was replenished every 24 hours to prevent depletion. Images were captured with a Nikon AZ100 microscope mounted with a Nikon Digital Sight DS-QiMc camera every 24 hours for 48 hours. Cell migration was quantified by measuring the area of the wound at each time point using Nikon NIS Elements BR 2.30 software.

#### 2.5 cAMP Assay

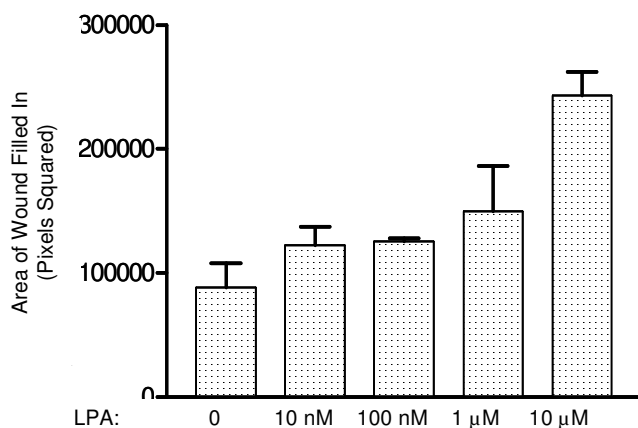
Cells were transfected, then labeled with 0.6  $\mu$ Ci [ $^3$ H]-adenine for three hours in serum free media. Assay buffer containing 1 mM isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, 50  $\mu$ M forskolin, and varying concentrations of LPA were added to the cells for 30 minutes at 37 °C. Reactions were terminated by aspiration and addition of a 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 followed by neutral alumina columns. After 10 mL of Scintisafe was added to the eluate, they were counted using a scintillation counter.



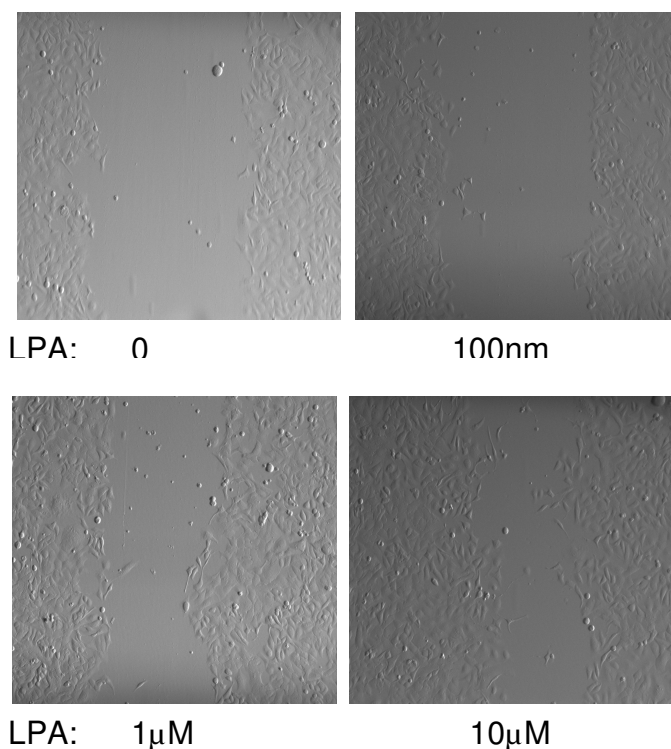
## CHAPTER 3 RESULTS

### 3.1 Dose-Dependent Response of LPA in Migration

We first characterized the activity of LPA in SKOV-3 cells using a classic assay of cellular migration. SKOV-3 cells were grown to confluence and serum starved for 18 hours. A “wound” was made by scratching a pipette tip across the monolayer. The cells were then treated with a range of LPA concentrations, from 0 to 10  $\mu$ M LPA. Area of wound filled in was determined as described in the experimental methods section. After 24 hours, a low level of basal migration was seen when vehicle was added. The cells moved into the wound in a dose-dependent fashion with higher concentrations of LPA causing more migration (Figure 3.1 & 3.2). The effect of LPA was completely inhibited by pre-treatment with Pertussis toxin (Ptx) (data not shown), suggesting that the effect is mediated by Gi/o.



**Figure 3.1** - Wound-induced migration of SKOV-3 cells with a range of LPA concentrations. SKOV-3 cells were grown to confluence and a “wound” was scraped with a pipette tip. They were treated with a range of LPA concentrations, as shown. Pictures were taken every 24 hours and quantified as described in the Experimental Methods section.

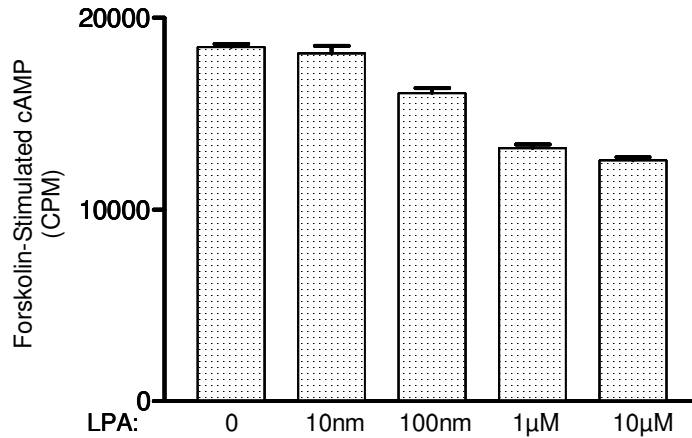


**Figure 3.2** – Wound-induced migration of SKOV-3 cells with a range of LPA concentrations. SKOV-3 cells were grown to confluence and a “wound” was scraped with a pipette tip. They were treated with a range of LPA concentrations, as shown. Pictures were taken every 24 hours and quantified as described in the Experimental Methods section.

### 3.2 Dose-Dependent Response of LPA in cAMP

We also characterized the activity of LPA in regulation of cAMP levels in SKOV-3 cells. Increase of the second messenger cAMP production was stimulated using forskolin, and IBMX was added to block degradation of the cAMP. LPA was added in a range of concentrations, and results were acquired as described in the Experimental Methods section. LPA caused a dose-dependent decrease in cAMP levels, with higher concentrations of LPA more dramatically decreasing cAMP levels (Figure 3.3). With vehicle, levels of cAMP accumulated in the cell were high; with the addition 10 μM LPA, there was a much lower

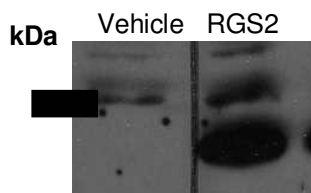
level of cAMP. The effect of LPA was completely inhibited by pre-treatment with Ptx (data not shown), suggesting that the effect is mediated by Gi/o.



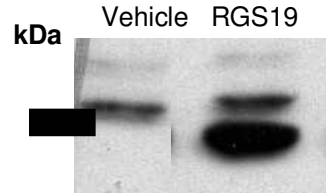
**Figure 3.3** – cAMP accumulation in SKOV-3 cells with a range of LPA concentrations added. SKOV-3 cells were plated in a 24-well plate and labeled. After being treated with forskolin to increase cAMP levels, LPA was added in a range of concentrations. Results are reported as level of forskolin-stimulated cAMP (CPM).

### 3.3 Overexpression of *RGS* Proteins

We next wanted to determine if overexpression of RGS proteins could regulate the ability of LPA to induce signaling in SKOV-3 cells. It is possible to transfect RGS proteins into the SKOV-3 Ovarian Cancer Cells. Plasmids encoding RGS 2 and 19 have been successfully transfected using Fugene 6 reagent (Figure 3.4 & 3.5). Proteins were detected using HA epitope antibody.



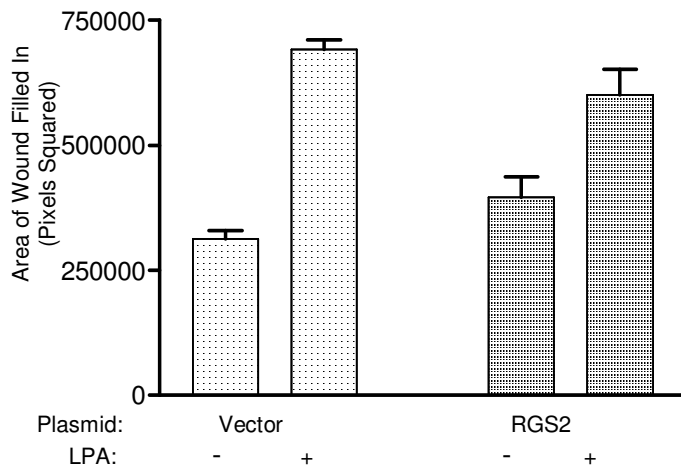
**Figure 3.4** – RGS2 expression with 48 hour transfection



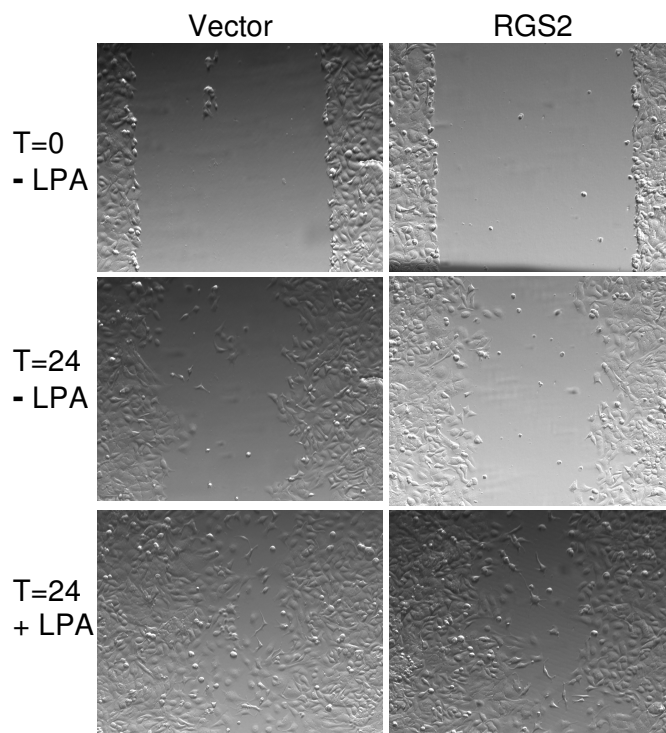
**Figure 3.5** – RGS19 expression with 48 hours transfection

### 3.4 *RGS 2 Overexpression Regulates Migration into Wound*

Next we determined if LPA stimulated migration was regulated by RGS2. SKOV-3 cells were grown to confluence, transfected with either Vector or RGS2 for 48 hours, and serum starved for 18 hours. A “wound” was made by scratching a pipette tip across the monolayer. The cells were then treated with vehicle or 10  $\mu$ M LPA. The area of the wound filled in was determined as described in the experimental methods section. After 24 hours, cells expressing more RGS 2 did not migrate as much into the wound as cells with vehicle, when 10  $\mu$ M LPA was added. Basal levels, with vehicle added, were higher in RGS2 transfected cells. Even with this basal increase, there was still less migration into the wound with 10  $\mu$ M LPA when RGS2 was present (Figure 3.6 & 3.7).



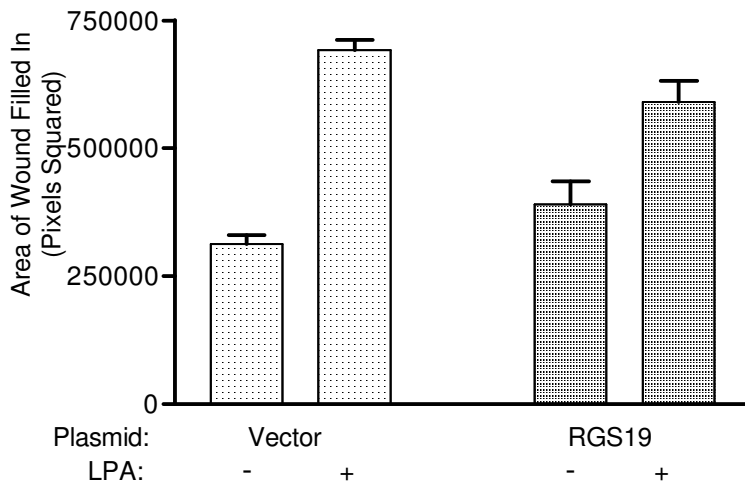
**Figure 3.6** – Wound –induced migration of SKOV-3 cells transfected with vector or RGS2 in the presence of 0 LPA or 10 $\mu$ M LPA. SKOV-3 cells were grown to confluence and transfected with either vector or RGS2. A “wound” was scraped with a pipette tip. They were treated with vehicle or LPA, as shown. Pictures were taken every 24 hours and quantified as described in the Experimental Methods section. Results are reported as the area of the wound filled in (pixels squared).



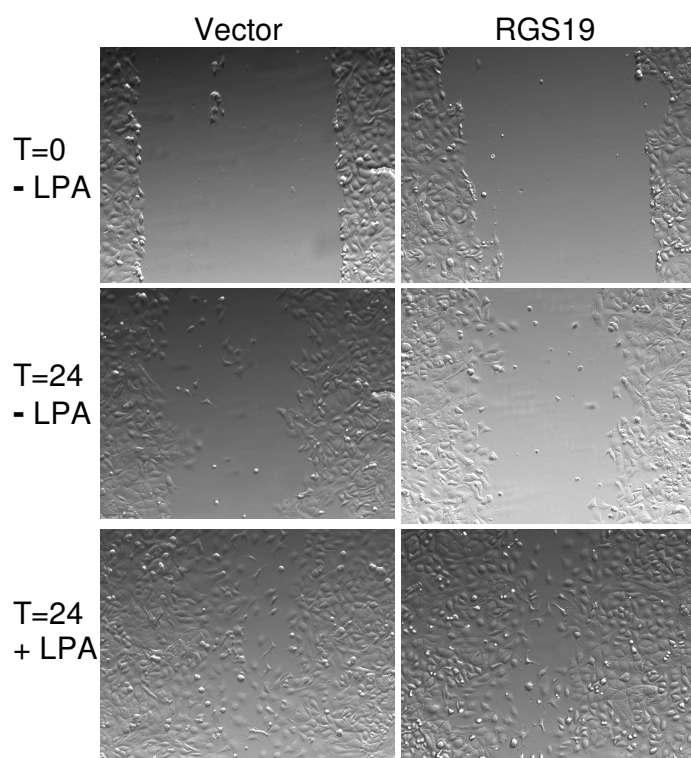
**Figure 3.7** – Wound –induced migration of SKOV-3 cells transfected with vector or RGS 2 in the presence of 0 LPA or 10 $\mu$ M LPA. SKOV-3 cells were grown to confluence and transfected with either vector or RGS2. A “wound” was scraped with a pipette tip. They were treated with vehicle or LPA, as shown. Pictures were taken every 24 hours and quantified as described in the Experimental Methods section. Results are reported as the area of the wound filled in (pixels squared).

### 3.5 RGS19 Overexpression Regulates Migration into Wound

Because migration is  $G_i$  mediated, RGS19 was also tested because it is known to couple to  $G_i$  G-proteins. SKOV-3 cells were grown to confluence, transfected with either Vector or RGS19 for 48 hours, and serum starved for 18 hours. A “wound” was made by scratching a pipette tip across the monolayer. The cells were then treated with vehicle or 10  $\mu$ M LPA. Area of wound filled in was determined as described in the experimental methods section. Similarly, cells expressing exogenous RGS19 did not migrate as far into the wound as cells with vector transfected when stimulated with 10  $\mu$ M LPA. Basal levels, with no LPA added, were higher in RGS19 transfected cells. However, even with this basal increase, cells with an overexpression of RGS19 had less migration into the wound when stimulated by LPA (Figure 3.8 & 3.9).



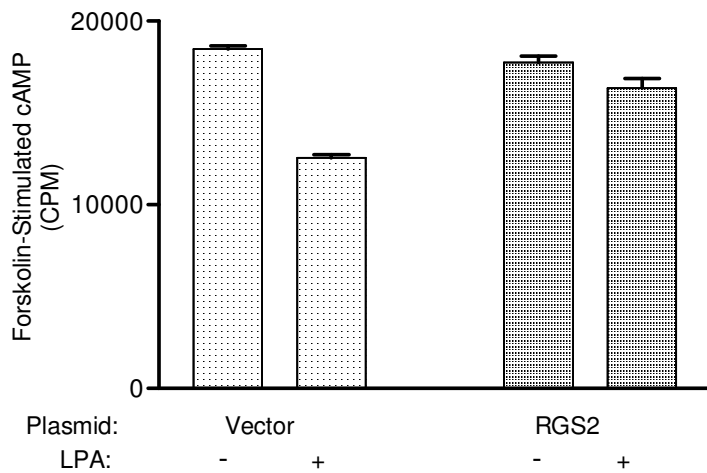
**Figure 3.8** – Wound –induced migration of SKOV-3 cells transfected with vector or RGS19 in the presence of 0 LPA or 10 $\mu$ M LPA. SKOV-3 cells were grown to confluence and transfected with either vector or RGS2. A “wound” was scraped with a pipette tip. They were treated with vehicle or LPA, as shown. Pictures were taken every 24 hours and quantified as described in the Experimental Methods section. Results are reported as the area of the wound filled in (pixels squared).



**Figure 3.9** – Wound –induced migration of SKOV-3 cells transfected with vector or RGS19 in the presence of 0 LPA or 10 $\mu$ M LPA. SKOV-3 cells were grown to confluence and transfected with either vector or RGS19. A “wound” was scraped with a pipette tip. Pictures were taken every 24 hours and quantified as described in the Experimental Methods section.

### 3.6 *RGS2 Overexpression Regulates LPA-Stimulated Decrease of cAMP*

Next we determined the effect of RGS overexpression on LPA stimulated inhibition of the cAMP protein. After the cells were transfected with either vector or RGS2, increase of the second messenger cAMP production was stimulated using forskolin, and IBMX was added to stop degradation of the cAMP. A maximal dose of LPA was added and results were acquired as described in the Experimental Methods section. LPA caused a decrease in the amount of cAMP present in the cells. Overexpression of RGS2 almost completely blocked the effect of LPA on cAMP levels (Figure 3.10). There was little difference in basal levels.



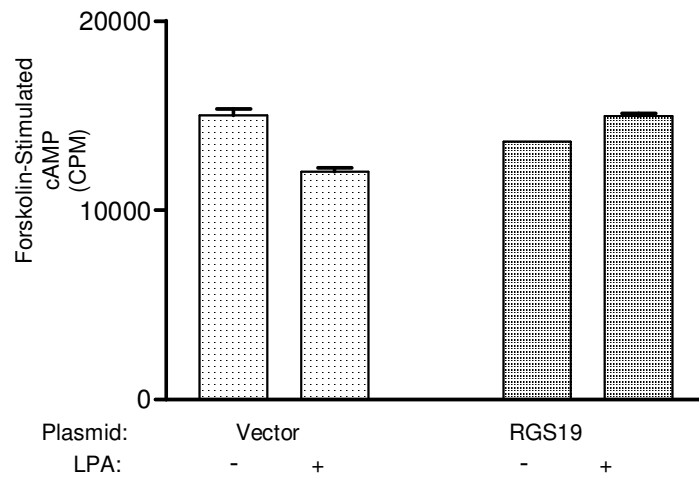
**Figure 3.10** – cAMP accumulation in SKOV-3 cells transfected with vector or RGS2 in the presence of 0 LPA or 10 μM LPA. SKOV-3 cells were plated in a 24-well plate, transfected with either vector or RGS2, and labeled. After being treated with forskolin to increase cAMP levels, LPA was added in a range of concentrations. Results are reported as level of forskolin-stimulated cAMP (CPM).

### 3.7 *RGS19 Overexpression Regulates LPA-Stimulated Decrease of cAMP*

Differences in cAMP levels were also tested with RGS19 overexpression for comparison.

After the cells were transfected with either vector or RGS19, increase of the second messenger cAMP production was stimulated using forskolin, and IBMX was added to stop degradation of the cAMP. LPA was added and results were acquired as described in the Experimental Methods section. Similarly, 1 μM LPA decreased the amount of cAMP. RGS19 overexpression blocked this decrease fully, with little differences in basal activity (Figure 3.11).





**Figure 3.11** – cAMP accumulation in SKOV-3 cells transfected with vector or RGS19 in the presence of 0 LPA or 10 μM LPA. SKOV-3 cells were plated in a 24-well plate, transfected with either vector or RGS19, and labeled. After being treated with forskolin to increase cAMP levels, LPA was added. Results are reported as level of forskolin-stimulated cAMP (CPM).

## CHAPTER 4 DISCUSSION

LPA induces migration in SKOV-3 ovarian cancer cells in a dose-dependent fashion. While cells treated with LPA had increased migration into the wound, RGS 2 and 19 had the effect of blocking some of the migration. LPA also causes a decrease in forskolin-stimulation cAMP production. This too was almost completely stopped with RGS 2 and 19 overexpression, implying that they attenuate this LPA signaling pathway.

Other studies have shown RGS 2 expression is decreased and RGS19 expression is increased in ovarian cancer compared to normal ovarian tissue (Hurst, Mendpara et al. 2008). These results imply that RGS proteins attenuate the cell signaling pathway that leads to migration. Thus, RGS2 and RGS19 have different G-protein specificity and different expression patterns in ovarian cancer. However, they have the same effect on signaling when overexpressed. This could be because the increased amounts of RGS proteins cause a decrease in specificity of binding to G-proteins and therefore downstream outcomes.

Future studies include overexpression of other RGS proteins that are known to be present in different amounts in cancer and normal ovarian cells, and testing specific RGS proteins in different assays, such as proliferation, differentiation, apoptosis, and second messenger signaling. Also, knocking down these same specific RGS proteins will provide a more complete picture of their role in the LPA signaling pathway.

## CHAPTER 5 CONCLUSION

To summarize, this study found that LPA stimulates wound-induced migration in ovarian cancer cells. RGS proteins are able to be transfected into these cells, and are a regulator of LPA signaling in migration and cAMP production of SKOV-3 ovarian cancer cells. Specifically, RGS 2 and 19 have some effect on slowing LPA-induced migration with overexpression, suggesting these could play a role in cancer formation. RGS2 and 19 also regulated the decrease of forskolin-stimulated cAMP caused by LPA. This establishes specific RGS proteins as possible therapeutic targets for ovarian cancer.

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