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CONTRACTING ORGANIZATION: Board of Regents of the University of Oklahoma Health Sciences Center, Oklahoma City OK

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14. ABSTRACT High mortality rate in ovarian cancer is primarily due to lack of an effective targeted therapy. Our project is focused on defining the mechanistic role of UCA1, a long non-coding RNA, in ovarian cancer pathobiology and evaluating the therapeutic potential of targeting UCA1 for therapy ovarian cancer. Results from previous research period have identified the determinant role of UCA1 in orchestrating ovarian cancer epigenome. In the current research period, we further probed this avenue of research to identify any novel druggable targets in UCA1-regulated epigenome. We report here that the silencing of UCA1 relieves the repressing effect of UCA1 on tumor suppressor genes such as TP53BP1, BRCA2, and NEFH. Reduced expression of these tumor-suppressor genes are associated with resistance to cisplatin and PARP-inhibitors. Thus, our results point to the therapeutic potential of targeting UCA1 for overcoming therapy resistance in ovarian cancer. Next, using chromatin isolation by RNA purification (ChIRP) analysis, we have identified the potential epigenetic role of UCA1 in driving ovarian cancer cells towards oncogenic cellular differentiation, metabolic reprogramming, and cellular migration. Further bioinformatic analyses of the ChIRP dataset, coupled with experimental validations, are anticipated to identify the critical genes in cancer epigenome that can be targeted for therapy.					
15. SUBJECT TERMS None listed.					
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1. INTRODUCTION

Ovarian cancer is the 8th most common cancer in women, but ranks 5th in cancer deaths [1,2]. The high mortality rate is largely due to the heterogeneous nature of the disease along with the lack of an efficacious targeted therapy [3,4]. In this context, our pursuit to identify the oncogenic pathways regulated by the *gcp* oncogene - defined by the α -subunit of G α 12 and encoded by the gene GNA12 - unraveled a hitherto unknown role for UCA1, an oncogenic long noncoding RNA (lncRNA) in ovarian cancer genesis and progression. Although lncRNAs were discovered in 2005 [5], their role in cancer pathobiology is being understood only now. Functional characterization of a few of these lncRNAs has shown that the lncRNAs are actively involved in regulating the expression of multiple genes [5,6]. While recent studies have identified the oncogenic role of different lncRNAs in the progression of many different cancers [7-12], their role in ovarian cancer is poorly understood. Our observation that the expression of UCA1 is upregulated by LPA via the *gcp* oncogenes is of great significance in light of the observation that the levels LPA are drastically increased in ovarian cancer patients. Since lncRNAs have been shown to regulate multiple sets of genes involved in a specific cellular response through non-mutually exclusive cytosolic as well as nuclear regulatory mechanisms, UCA1 signaling axis, which is being investigated here, is likely to represent a critical mechanism underlying ovarian cancer genesis, progression, and therapy resistance. With the rationale that defining the upstream and downstream signaling events involving UCA1 could identify novel therapeutic targets, we investigated the mechanistic role of UCA1 in ovarian cancer pathobiology under three specific aims, namely, 1) Define the mechanism by which *gcp*-oncogenes stimulate the expression of UCA1; 2) Define the mechanism by which UCA1 regulates oncogenic signaling; and 3) Analyze the role of UCA1 in ovarian cancer growth in vivo and assess its therapeutic potential using preclinical mouse models. It is anticipated that the successful completion of this project will have a great impact on identifying novel therapeutic targets and development of the new treatment regimens for ovarian cancer. Furthermore, the evaluation of the therapeutic potential of inhibiting UCA1 in combination with cisplatin or epigenetic inhibitors is likely to have a greater impact by expanding therapeutic options that can overcome therapy resistance in ovarian cancer.

2. KEYWORDS:

Ovarian Cancer, Long Non-coding RNA, microRNA, Lysophosphatidic acid, LPA, miRNA, Urothelial Cancer associated-1, UCA1, Polycomb Group Proteins, Tumor-suppressors

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The overarching goals of the proposed studies are to define the mechanism by which LPA stimulates the expression of UCA1 and to assess the therapeutic potential of targeting UCA1 and/or its downstream targets for ovarian cancer therapy. The major goals of the project are 1) Define the mechanism by which *gcp*-oncogenes stimulate the expression of UCA1; 2) Define the mechanism

by which UCA1 regulates oncogenic signaling; and 3) Analyze the role of UCA1 in ovarian cancer growth in vivo and assess its therapeutic potential.

What was accomplished under these goals?

Our accomplishment during this funding period relate the identification of the potential mechanisms by which the UCA1 regulates oncogenic signaling in ovarian cancer (Aim-2). Towards this goal, we have completed the identification of critical tumor-suppressor genes repressed by UCA1 (Aim 2, Sub-task 3) and other key genes regulated by UCA1 (Aim2, Subtask 4). In addition, animal studies proposed under Aim 3 have been initiated (Aim 3, Subtask 2).

Major Activities: The major activities carried out under this period focused mainly on aim 2 sub-tasks 3 and 4. During 2019-2020 funding period, we identified the potential tumor suppressors that could be regulated by UCA1 using an antibody-array platform. In 2020 – 2021 funding period, we validate a panel of these critical genes, whose abrogation by UCA1 could portend poor prognosis. In addition, as proposed under the Sub-task 4 of the aim 2, we carried out chromatin immune-precipitation analysis to identify the genes targeted by UCA that could be utilized for drug discovery.

Specific Aim 2: Define the mechanism by which UCA1 regulates oncogenic signaling in ovarian cancer
Major Task 2: Identification of the UCA1-target genes and proteins involved in ovarian cancer progression and therapy resistance.
Subtask 3: Antibody array analysis to identify the tumor suppressor gene down regulated by UCA1
Subtask 4: ChiRP-Seq analysis to identify the UCA1-targeted gene

Specific Activities: In our 2019- 2020 annual report, we presented our data demonstrating the utility of the PRC1/2-Target Protein Antibody Array (PTP-array) in identifying eighteen tumor suppressor genes that could be epigenetically repressed by UCA1-PRC1/2 complex. In the current funding period, we further validated the antibody array data as follows.

Validation of Antibody Array Analysis. Our results from the PTP-array analysis using lysates OVCAR8, a high grade serous ovarian carcinoma cell line, indicated that the silencing of UCA1 relieved the repression of 18 tumor suppressor genes. Of these genes, deletions or decreased expressions of TP53BP1, BRCA2, and NEFH are known to be associated with poor prognosis and therapy resistance in ovarian cancer [13-17]. Therefore, we tested the expression of these genes in OVCAR8 and OVCAR3 cells independent of array results. To test the expression of UCA1 was knocked down using two different siRNAs. The lysates were processed for the analyses of the expression of TP53BP1, NEFH, or BRCA2. As shown in Figure 1 panel A and B, the silencing of UCA1 led to an increase in the expression of UCA1, thus suggesting the role of UCA1 in suppressing the expression of these PRC1/2 targeted tumor-suppressor genes presumably via PRC1/2.

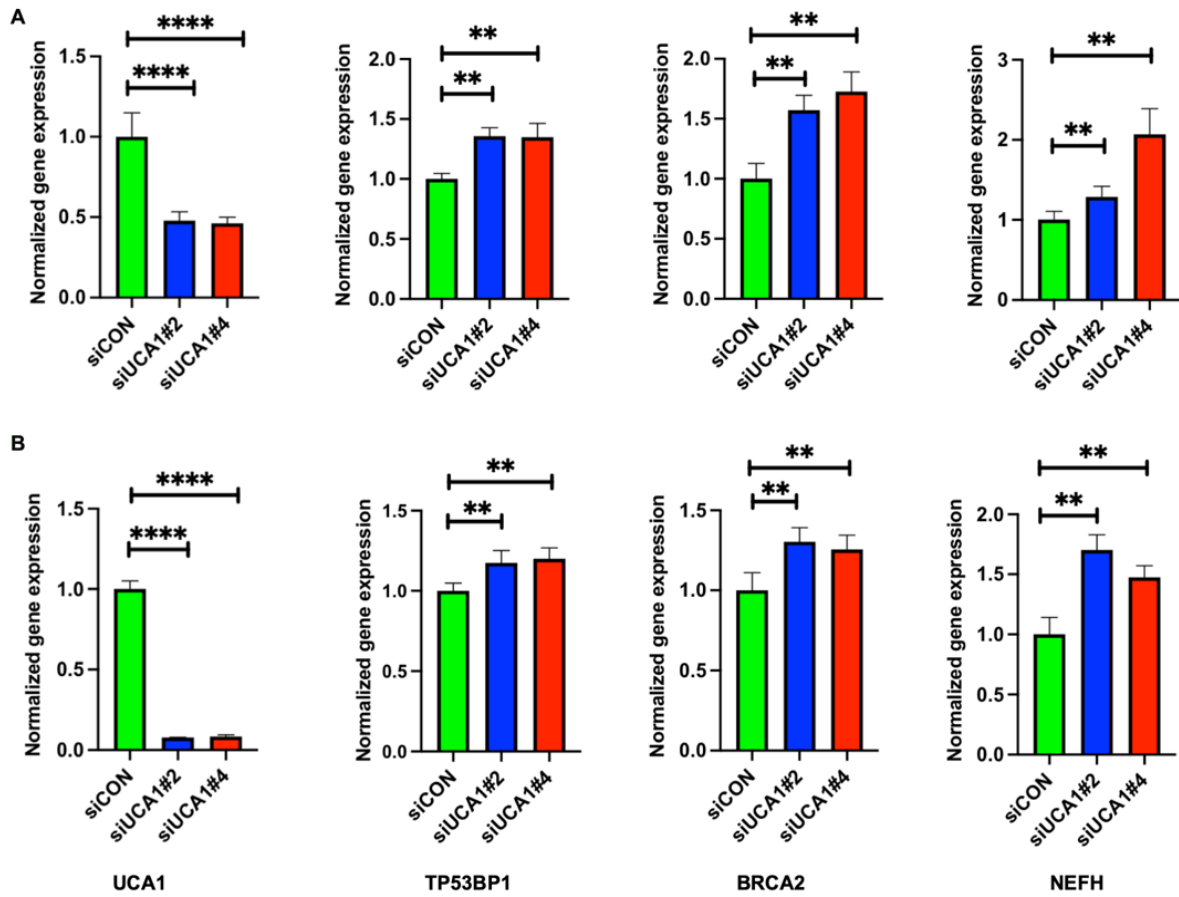
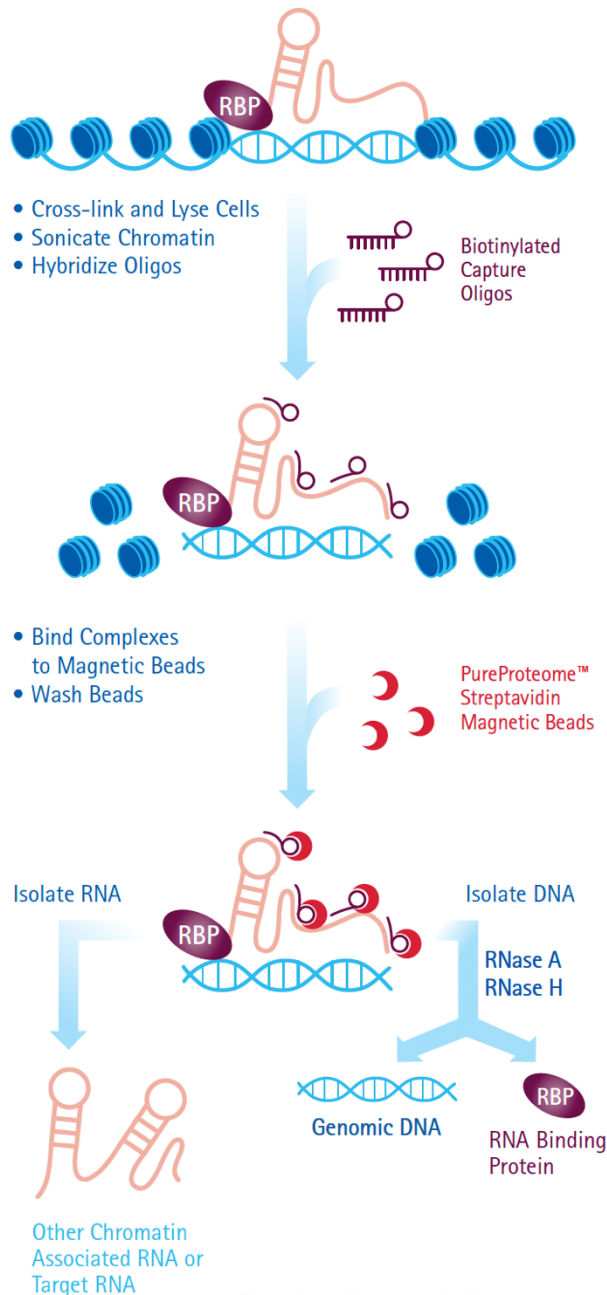


Figure 1. Knockdown of UCA1 Relieves the Repression of Tumor Suppressor Genes. Upregulated expression of representative tumor-suppressor genes upon silencing of UCA1 was validated using OVCAR3 (A) and OVCAR3 (B) cells in which the expression of UCA1 was silenced using two different siRNAs targeting UCA1. Cells transfected with non-targeting scrambled siRNA pool were used as the control (SiCON). After validating the silencing of UCA1 with the respective siRNAs, upregulated genes were validated by monitoring the expression of TP53BP1, BRCA2, and NEFH by qRT-PCR. Statistical significance was determined by Student's *t*-test (***P* < 0.005; ****P* < 0.0005; *****P* < 0.0001).

ChIRP-Seq Analysis to identify UCA1-targeted genes

Thus far, our results have identified the potential role of UCA1 in altering the epigenome of the ovarian cancer cells to accelerate cancer growth and progression. With the ability of lncRNAs such as UCA1 to alter the expression a vast array of genes through epigenetic mechanism, it is of critical interest to identify the epigenomic changes orchestrated by UCA1. With this rationale, we carried out ChIRP analysis using Magna ChIRP RNA Interactome Kit (EMD Millipore Corporation, Burlington, MA). OVCAR8 cells and UCA1-silenced OVCAR8 cells were subjected to Magna

ChIRP assay depicted in Figure 2. OVCAR8 cells were transfected with siRNA using



Lipofectamine RNAiMAX reagent (Invitrogen, Life Technologies, Carlsbad, CA) as recommended by the manufacturer. OVCAR8 cells (2×10^6 cells) were incubated for 24 h with Lipofectamine RNAiMAX reagent (45 μ l) in 750 μ l of Opti-MEM (Invitrogen, Life Technologies, Carlsbad, CA) was incubated for 5 minutes at room temperature. siRNA was added to the Opti-MEM-lipofectamine RNAiMAX solution to a final concentration of 100 nM. After 48 h incubation for gene silencing, 1×10^7 cells were cross-linked with 3% formaldehyde; chromatin was sonicated and hybridized with 3' end Biotinylated UCA1 capture oligos. UCA1-oligo bound RNA, DNA, and protein were pulled down using streptavidin-coated magnetic beads. DNA contained in the UCA1-chromatin was isolated and subjected to NGS library construction and sequenced on an Illumina HiSeq instrument.

Figure 2. Magna ChIRP based analysis of UCA1-Chromatin Interaction. Schematic presentation of the work-flow chart of Magna ChIRP based isolation of UCA1 chromatin-complexes containing DNA, RNA, and protein is presented (<https://www.emdmillipore.com>). “Biotinylated Capture Oligo” is custom-synthesized with UCA1 oligos.

KEGG Pathway Analysis of UCA1-ChIRP enriched genes. The high-density data derived from the ChIRP sequencing is subjected to bioinformatic data analysis.

Initial bioinformatic analysis has identified that UCA1 occupancy can be correlated with 2000 genes in the genome. Reasoning that the functional networking of these genes could provide insight into the mechanism by which UCA1 promotes oncogenic growth of ovarian cancer cells, we carried out pathway and network analyses. Since pathway enrichment analysis places UCA1 to genes involved in many critical pathways involved in cancer. They include genes involved in diverse metabolic pathways, protein synthesis, and pathways associated with cancer (Figure 3). It is of interest to note that the ChIRP enrichment analysis suggests that UCA1 genomic occupancy also

involves the upregulation of the genes listed in the pathway clusters presented here (Figure 3). Further cell culture-based assays will be carried out to validate the genes identified by the ChIRP-enriched pathway analysis (Figure 3).

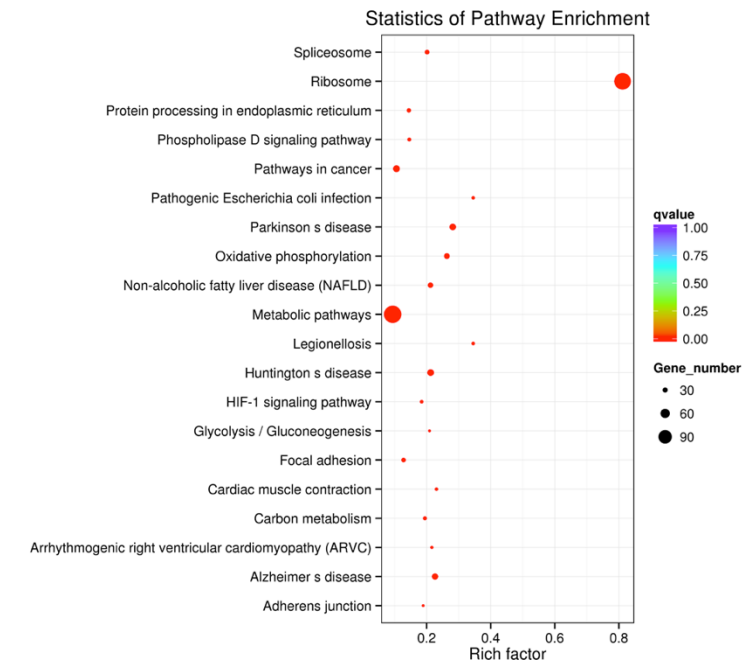


Figure 3. Pathway Enrichment Analysis of UCA1-occupancy sites. Kegg pathway analyses of gene enriched by UCA1-ChiRP cluster them into different groups.

Gene Ontology Enrichment Analysis of UCA1-ChiRP enriched genes. Gene Ontology (GO) enrichment analysis could provide information on the functional relationship among a large set of genes, GO analysis of biological processes (GO: BP), molecular functions (GO:MF) and cellular components (GO:CC) were carried out. In GO: BP ontology, the UCA1-genomic sites were significantly enriched in biological processes

involving cellular development, cell proliferation, and cell motility (Figure 4). In GO:CC ontology, cellular periphery including plasma membrane and cellular projection formed the major categories. In GO:MF ontology, only enriched category appeared to involve cell adhesion molecule binding.

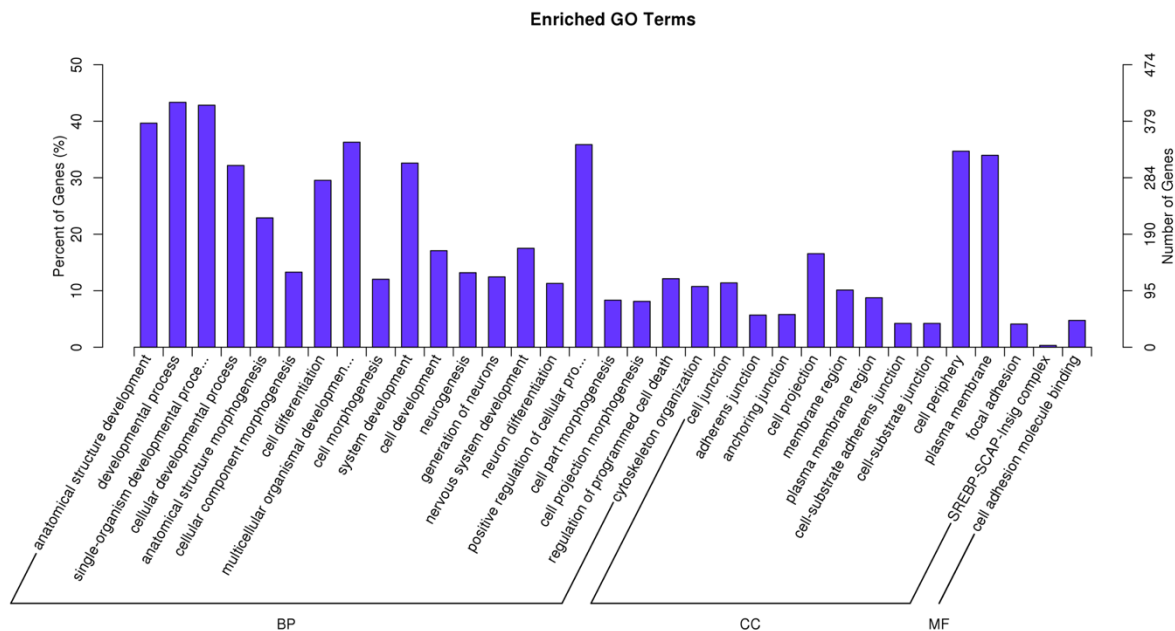


Figure 4. GO Analysis. Gene ontology (GO) enrichment analysis was carried out with UCA1-ChIRP enriched genes. GO term enrichment analyses in terms of the three sub-ontologies: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) are presented.

GO Directed Acyclic Graph Analysis (DAG). Interconnection between the GO terms or nodes and hierarchical relationship in GO could be derived from GO based DAG analysis. Therefore GO-DAG was carried out to map the hierarchical relationship among the GO terms. Such analysis indicated that how the hierarchical relationship among the major GO biological process could lead to cellular phenotypic changes. Likewise, DAG-analysis of GO cellular components (Figure 5)

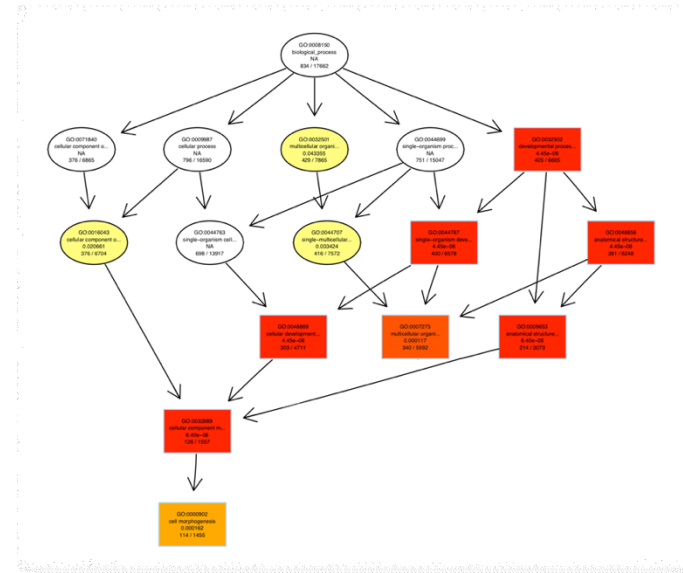
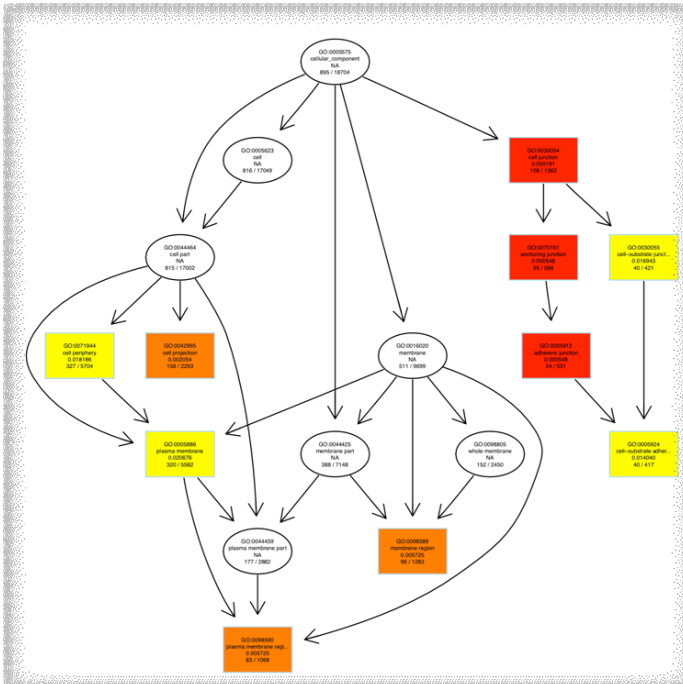


Figure 5. GO-DAG Analysis of UCA1-ChIRP enriched genes in GO: Biological Process (GO: BP). DAG analysis was carried out with GO-BP term enriched by UCA1-ChIRP analysis. The hierarchical structure of the UCA1-ChIRP enriched genomic cluster involved in the biological processes of the cancer cell is presented.



Similarly, a hierarchical structure of the genomic cluster representing cellular component can be derived through GO-DAG analysis. Such an analysis indicated the major hierarchical structure clustering towards cellular components involved in cell adhesion (Figure 6).

Figure 6. GO-DAG Analysis of UCA1-ChIRP enriched genes in GO: Cellular Component (GO:CC). DAG analysis was carried out with GO-CC term enriched by UCA1-ChIRP. Hierarchical structure of the UCA1-ChIRP enriched genomic cluster underlying the cellular components of the cancer cell is presented.

In summary, the UCA1-ChIRP analysis has identified a vast array of genes as potential UCA1 target sites in the chromatin of the

cancer genome. Further analyses of the individual UCA1-ChIRP-identified genes should define the specific roles of these genes in ovarian cancer epigenome and its role in cancer etiology, growth, and therapy resistance.

At present, we are in the process of identifying the candidate driver genes from the UCA1-ChiRP identified genes and validating their role using a panel of high-grade ovarian carcinoma cells.

What opportunities for training and professional development has the project provided?

Under this project, 3 undergraduate and 1 graduate trainee were trained in lncRNA biology and cancer. In addition, this grant is providing training and professional development to two postdoctoral fellows.

How were the results disseminated to communities of interest?

The results are disseminated to basic and clinical researchers via scientific publications and through the presentation of the data at scientific conference venues. Some of the results pertaining to Specific Aim 1, sub-task 3 have been published in two peer reviewed journals [18,19]. The published articles are attached under appendices of this report. In addition, the PI has presented in three international conferences. They are listed under the “PRODUCTS” section of this report.

What do you plan to do during the next reporting period to accomplish the goals?

UCA1-ChiRP analysis has provided a wealth of data that needs to be carefully parsed through and validated. The Bioinformatics Core of the Stephenson Cancer Center is helping us in analyzing the dataset.

Critical animal based in vivo studies will be carried out to assess the therapeutic utility of targeting UCA1, its upstream regulators, or its downstream as an adjuvant therapy in ovarian cancer.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

1. Our studies for the first time have identified unequivocal interactions between UCA1 and PRC family of proteins.
2. We have demonstrated that UCA1 can modulate the expression of genes both in cis and trans manner – affecting both the resident genes in chromosome 19 as well as genes located in other chromosomes.
3. Using antibody array (RPPA), we have identified the target proteins modulated by UCA1
4. Focusing on the tumor suppressor genes targeted by UCA1, we have identified 18 TS genes as the candidate genes repressed by UCA1 to promote tumorigenesis in ovarian cancer.
5. We have identified the potential role of notch-signaling in UCA1 mediated tumorigenic pathway in ovarian cancer.

6. New set of datasets from the ChIRP analysis suggests the potential role of UCA1 in activating multiple oncogenic pathways through physically interacting with specific genomic loci.

What was the impact on other disciplines?

Currently we are developing therapeutic strategies targeting UCA1 using siRNA to UCA one using siRNA-UCA1-enacapsulated targeted nanoparticles. The outcome from these studies could establish a new lncRNA-targeted therapeutic paradigm for ovarian cancer.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Our studies will contribute to the shifting paradigm that defines a role for RNA that are not translated into protein. Such paradigm-shifting studies and their dissemination to the community could promote “blue sky thinking” or “out of the box” thinking in every walk of life.

5. CHANGES/PROBLEMS:

Actual or anticipated problems or delays and actions or plans to resolve them

We were hampered drastically by the COVID-19 lock-down and the recent departure of a postdoctoral associate to his native country due to COVID-19 outbreak. This has seriously delayed the progress of our work by 10 to 12 months. We are resolving this through the recruitment of new research personnel, who has just arrived this month.

Changes that had a significant impact on expenditures.

Due to COVID19 lockdown, research activity in the lab and associated laboratory expenditure has drastically reduced. We were kindly provided with “No Cost Extension” until September 15th, 2022. We are very thankful to the Department of Defense and we are highly confident that we will accomplish all the proposed milestones of the project by then.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications

Published:

1. Identification of GNA12-driven gene signatures and key signaling networks in ovarian cancer. Ha JH, Jayaraman M, Yan M, Dhanasekaran P, Isidoro C, Song YS, Dhanasekaran DN. *Oncol Lett.* 2021 Oct;22(4):719. doi: 10.3892/ol.2021.12980. Epub 2021 Aug 10. PMID: 34429759
Federal support including this grant is acknowledged.
2. GNAi2/gip2-Regulated Transcriptome and Its Therapeutic Significance in Ovarian Cancer. Ha JH, Jayaraman M, Yan M, Dhanasekaran P, Isidoro C, Song YS, Dhanasekaran DN. *Biomolecules.* 2021 Aug 14;11(8):1211. doi: 10.3390/biom11081211. PMID: 34439877
Federal support including this grant is acknowledged.

Under Revision for Resubmission:

3. Ha JH, Radhakrishnan R, Jayaraman M, Yan M, Fung KM, Moxley KM, Sood AK, Isidoro C, Bhattacharya R, Mukherjee P, Song YS, Dhanasekaran DN. G-protein coupled receptor, lncRNA and miRNA signaling nexus in ovarian cancer progression (Under revision for resubmission to *Proc Natl Acad Sci USA*).
4. Jayaraman M, Ha JH, Radhakrishnan R, Song YS, Bhattacharya R, Mukherjee P, Dhanasekaran DN. Transcriptomic analysis of lncRNAs and mRNAs in ovarian cancer (Under revision for resubmission to *Cancers*).

Manuscript Under Preparation:

5. Jayaraman M, Ha JH, Radhakrishnan R, Song YS, Dhanasekaran DN. Regulation of ovarian cancer epigenome by UCA1. Jayaraman M, Ha JH, Radhakrishnan R, Song YS, Dhanasekaran DN. Transcriptomic analysis of lncRNAs and mRNAs in ovarian cancer (Under revision for resubmission to *Cancers*).

Books or other non-periodical, one-time publications.

Commissioned to edit a special issue of “*Onco*” journal (Basel, Switzerland) on “Non-coding RNAs in Cancer Genesis and Progression”.

Other publications, conference papers, and presentations.

1. “LncRNAs and Cancer Epigenome”, December 16, 2020: Cancers 2020 Webinar on Epigenetic and Metabolic Alterations in the Tumor Microenvironment, Virtual Conference, Basel, Switzerland.
2. “LncRNA Regulation of Ovarian Cancer Epigenome” December 28, 2020: Fourth Meeting of the International Society of Precision Cancer Medicine, Virtual Conference, Seoul, S. Korea.
3. “Metabolic and LncRNA Signaling Loop in Tumor Microenvironment”, February 20, 2021: Centennial Conference of the Department of Biochemistry at the Indian Institute of Science, Virtual Conference, Bangalore, India.

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Data or databases

The microarray data that led to the discovery of UCA1 was deposited at the Gene Expression Omni-bus (<https://www.ncbi.nlm.nih.gov/geo/>); accession no. GSE173214.

7. PARTICIPANTS

What individuals have worked on the project?

Name: Dr. Danny Dhanasekaran
Project Role: Principal Investigator
Nearest person month worked: 1
Contribution to Project: Supervised the study.

Name: Dr. Kar-Ming Fung
Project Role: Co-Investigator
Nearest person month worked: 1

Name: Dr. Katherine Moxley
Project Role: Co-Investigator
Nearest person month worked: 1
Contribution to Project: Collaborator

Name: Dr. Ramesh Rajagopal
Project Role: Consultant
Nearest person month worked: 2
Contribution to Project: Need-based consultancy

Following postdocs have replaced the recently left postdocs as reported in 2019-2021 annual report:

Name: Dr. Jihee Ha
Project Role: Postdoctoral Fellow
Nearest person month worked: 2
Contribution to Project: Carried out the studies under the PI's supervision.

Name: Dr. Revathy Nathan
Project Role: Postdoctoral Fellow
Nearest person month worked: 1
Contribution: Carried out the studies under the PI's supervision.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No Change.

What other organizations were involved as partners?

Not applicable.

8. SPECIAL REPORTING REQUIREMENTS

Not Applicable

9. APPENDICES:

PDF files of the following published manuscripts are attached.

1. Identification of GNA12-driven gene signatures and key signaling networks in ovarian cancer. Ha JH, Jayaraman M, Yan M, Dhanasekaran P, Isidoro C, Song YS, Dhanasekaran DN. *Oncol Lett*. 2021 Oct;22(4):719. doi: 10.3892/ol.2021.12980. Epub 2021 Aug 10. PMID: 3442975
2. GNAi2/gip2-Regulated Transcriptome and Its Therapeutic Significance in Ovarian Cancer. Ha JH, Jayaraman M, Yan M, Dhanasekaran P, Isidoro C, Song YS, Dhanasekaran DN. *Biomolecules*. 2021 Aug 14;11(8):1211. doi: 10.3390/biom11081211. PMID: 34439877

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Identification of *GNAI2*-driven gene signatures and key signaling networks in ovarian cancer

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Abstract. With the focus on defining the oncogenic network stimulated by lysophosphatidic acid (LPA) in ovarian cancer, the present study sought to interrogate the oncotranscriptome regulated by the LPA-mediated signaling pathway. LPA, LPA-receptor (LPAR) and LPAR-activated G protein 12 α -subunit, encoded by G protein subunit α 12 (*GNAI2*), all serve an important role in ovarian cancer progression. While the general signaling mechanism regulated by LPA/LPAR/*GNAI2* has previously been characterized, the global transcriptomic network regulated by *GNAI2* in ovarian cancer pathophysiology remains largely unknown. To define the LPA/LPAR/*GNAI2*-orchestrated oncogenic networks in ovarian cancer, transcriptomic and bioinformatical analyses were conducted using SKOV3 cells, in which the expression of *GNAI2* was silenced. Array analysis was performed in Agilent SurePrint G3 Human Comparative Genomic Hybridization 8x60 microarray platform. The array results were validated using Kuramochi cells. Gene and functional enrichment analyses were performed using Database for Annotation, Visualization and Integrated Discovery, Search Tool for Retrieval of Interacting Genes and Cytoscape algorithms. The results indicated a paradigm in

which *GNAI2* drove ovarian cancer progression by upregulating a pro-tumorigenic network with *AKT1*, *VEGFA*, *TGFB1*, *BCL2L1*, *STAT3*, insulin-like growth factor 1 and growth hormone releasing hormone as critical hub and/or bottleneck nodes. Moreover, *GNAI2* downregulated a growth-suppressive network involving proteasome 20S subunit (PSM) β 6, PSM α 6, PSM ATPase 5, ubiquitin conjugating enzyme E2 E1, PSM non-ATPase 10, NDUFA4 mitochondrial complex-associated, NADH:ubiquinone oxidoreductase subunit B8 and anaphase promoting complex subunit 1 as hub or bottleneck nodes. In addition to providing novel insights into the LPA/LPAR/*GNAI2*-regulated oncogenic networks in ovarian cancer, the present study identified several potential nodes in this network that could be assessed for targeted therapy.

Introduction

Ovarian cancer is the seventh most common cancer type in women worldwide, with 250,000 new cases diagnosed worldwide annually, and it lead to 185,000 deaths in 2020 (1). Recent analysis has estimated that the deaths associated with ovarian cancer would be as high as 13,770 by 2021 in the USA alone (2). The high mortality rate in ovarian cancer is associated with a late diagnosis, as well as a lack of an effective targeted therapy (3,4).

The observation that lysophosphatidic acid (LPA) synthesized by cancer cells acts as an endogenous growth factor, and that LPA-mediated signaling pathways serve a tumor-promoting role across numerous cancer types, including ovarian cancer, is clinically significant (5,6). LPA was initially identified as a platelet derived bioactive phospholipid that stimulated the proliferation of fibroblasts involved in wound healing (7). Subsequent studies have shown that LPA stimulates multiple signaling pathways underlying cell proliferation, migration, and survival via specific G-protein coupled receptors and the associated heterotrimeric G proteins (8,9). Of the different G proteins that could be activated by LPA-receptors (LPARs), G protein 12 (G12) has been identified as the major conduit involved in LPA-mediated mitogenic signaling (10-12). In

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Abbreviations: LPA, lysophosphatidic acid; LPAR, lysophosphatidic acid receptor

Key words: ovarian cancer, lysophosphatidic acid, G protein subunit α 12, gene expression, genomics, signaling-network

ovarian cancer, cancer cells synthesize and release LPA into the tumor microenvironment (TME). LPA present in the TME promotes cancer progression and metastasis via the activation of specific LPA-receptors (LPARs) that are present in multicellular components of the TME (13,14). In cancer cells, LPA stimulates an autocrine signaling loop via the activation of cancer cell-bound G-protein coupled LPARs. Although G protein coupled receptors, such as LPARs, have proven to be highly amenable for drug development, targeting LPARs in ovarian cancer has been challenging. High concentrations of LPA in the intraperitoneal ascites surrounding the ovarian cancer tissue and the close proximity of the LPA-synthetic machinery to LPARs on the surface of ovarian cancer cells have impeded LPAR-targeted therapeutic strategies in ovarian cancer. Recent studies from several laboratories, including ours, have reported that the α -subunit of the oncogene G-protein G12, encoded by the gene G protein subunit α 12 (*GNAI2*), is the major conduit involved in transmitting oncogenic signals in numerous cancer types, including ovarian cancer (15–20). It has been revealed that either LPAR-stimulated activation or mutational activation of *GNAI2*, referred to as the *gpc* oncogene, induces the oncogenic proliferation of ovarian cancer cells (15,16).

Tumorigenesis and tumor progression often involve the deregulation of multiple pathways, impacting a cell-wide signaling network rather than an alteration in a single gene or pathway (21). Therefore, we hypothesized that the transcriptomic analysis based on the aggregated expression of genes associated with multiple pathways co-regulated by *GNAI2* could provide additional insights into the LPA/LPAR/*GNAI2*-induced oncogenic signaling network in ovarian cancer. Based on this rationale, the present study aimed to investigate *GNAI2*-orchestrated effects in ovarian cancer pathobiology using micro-array based transcriptomic analysis. Herein, the results from pathway-based bioinformatics analyses are shown in order to define the co-regulatory signaling circuits regulated by *GNAI2* in ovarian cancer. Using SKOV3 cells in which *GNAI2* had been silenced, transcriptomic profiling was conducted to identify the differentially expressed genes (DEGs). Moreover, array results were validated by monitoring the expression levels of representative DEGs via reverse transcription-quantitative (RT-q) PCR analysis in *GNAI2*-silenced-Kuramochi cells. Further Gene Ontology (GO) enrichment and protein-protein interaction (PPI) network analyses were performed using web-based Database for Annotation, Visualization and Integrated Discovery (DAVID) and Search Tool for Retrieval of Interacting Genes (STRING), as well as Cytoscape software applications. In addition to providing a novel insight into the organizational structure of LPA/LPAR/*GNAI2*-driven transcriptomic network in ovarian cancer, the present study has identified specific hub and bottleneck nodes that can be targeted individually or collectively for effective targeted adjuvant therapy for ovarian cancer.

Materials and methods

Cell lines and culture. High grade serous carcinoma cell line Kuramochi and non-serous ovarian carcinoma cell line SKOV3 were obtained from American Type Culture Collection (Manassas, VA) and the cells were authenticated

by short tandem repeat analysis as described (13). Kuramochi cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Cellgro) and SKOV3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Cellgro), both at 37°C in a 5% CO₂ incubator. In both cases, the media were supplemented with 10% FBS (Gemini Bio-Products), 50 U/ml penicillin, 50 μ g/ml streptomycin (Cellgro). For LPA-stimulation studies, 18.1 LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate; cat. no. 85730), was obtained from Avanti Polar Lipids (Alabaster, AL). LPA was dissolved in 10 mM stock solutions in phosphate buffered saline containing 1% BSA and stored at -80°C until use.

Human cell lines and methods used in this study were approved by the Institutional Review Board for the protection of the Human Subjects of the University of Oklahoma (approval no. 9599).

Transfection methods. Silencing of *GNAI2* in SKOV3 cells was carried out as described in our previously publication (16). Briefly, non-target scrambled control shRNA pLKO.1 vector construct (Sigma-Aldrich; Merck KGaA (SHC002) and pLKO.1 vector construct targeting *GNAI2*/G α 12 (RHS3979-98491914; Open Biosystems) were stably transfected into SKOV3 cells using Amaxa Biosystems Nucleofector II, according to the instructions of the manufacturer. The stably transfected NS control and G α -silenced clones were selected with puromycin (2 μ g/ml; MP Biomedicals) and single clones were picked, expanded to obtain stable cell lines. Prior to the array analysis, the silencing of *GNAI2*-expression was ascertained by immunoblot analysis. Silencing of *GNAI2* in Kuramochi cells were carried out using siRNAs targeting *GNAI2* (siGENOME Human *GNAI2* siRNA SMARTpool; cat. no. M-008435-00-0005) and non-targeting scrambled control siRNAs control (siGENOME Non-Targeting siRNA Pool; cat. no. D-001206-13-05) were obtained from Dharmacon/Horizon Discovery. Kuramochi cells were transfected with siRNA using Lipofectamine RNAiMAX reagent (Invitrogen, Life Technologies) as recommended by the manufacturer. Kuramochi cells were seeded in 6-well plates at a density of 1x10⁵ cells per well and incubated for 24 h. Lipofectamine RNAiMAX reagent (9 μ l) in 300 μ l of Opti-MEM (Invitrogen, Life Technologies) was incubated for 5 min at room temperature. siRNA was added to the Opti-MEM-lipofectamine RNAiMAX solution to a final concentration of 100 nM. The mixture was added to the cell culture, and after 48 h incubation for gene silencing, the cells were collected for RT-qPCR studies. Expression of *GNAI2* in the transfectants was monitored by RT-qPCR analysis.

Transcriptomic analysis. Transcriptome profiles were obtained using Agilent SurePrint G3 Human Comparative Genomic Hybridization 8x60 microarray platform. SKOV3-shScr (non-specific scrambled shRNA control) and SKOV3-sh*GNAI2* cells were cultured for 24 h, followed by 16 h of serum starvation. These cells were stimulated with LPA (10 μ M) for 16 h and total RNA was extracted using Qiagen RNeasy mini kit (Qiagen) following the manufacturer's protocol. Agilent QuickAmp labeling kit was used to label RNA samples with Cy3-CTP and hybridized to the array slides following the manufacturer's protocol. The hybridized

array slides were scanned using Agilent SureScan scanner at 2 microns resolution. The spot intensity was extracted using Agilent Feature Extraction version 11.0 software. Further, gene expression analysis was carried out using Agilent GeneSpring GX version 13.0. Differentially expressed genes (DEGs), with a cut-off value of ≥ 5 -fold change compared to control cells, were used for further bioinformatic analyses.

Bioinformatics analysis. Gene ontology Enrichment analysis of the DEGs was carried out using the web-based annotation tool DAVID (<https://david.ncifcrf.gov/home.jsp>) (22). Protein-Protein Interaction Networks Functional Enrichment Analysis was carried out using web-based (<https://string-db.org/>) STRING database (23). The upregulated genes and downregulated genes were analyzed separately with the highest confidence interaction score (0.9) and <10 degree of interaction. Significant modules in the PPI network was analyzed further using Cytoscape software application (24). The hub and bottle neck nodes of the PPI network were identified using the cytoHubba plugin in Cytoscape (25). Multiple algorithms of cytoHubba including Degree, Maximal Clique Centrality and (MCC), maximum neighbourhood component (MNC), Edge Percolated Component (EPC), EcCentricity, Closeness, Betweenness, and Clustering Coefficient were used to identify the hub nodes of the PPI networks (26). BottleNeck algorithm of cytoHubba was used to identify the bottleneck nodes of the network.

RT-qPCR analysis. Total RNA was extracted using Qiagen RNeasy kit (Qiagen) following the manufacturer's instructions. cDNA synthesis was carried out using an iScript™ cDNA Synthesis Kit (Bio-Rad). Real-time quantitative PCR (RT-qPCR) was carried out using the cDNA from the above step using appropriate primers (Table SI) and SoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a BioRad CFX96 Real time PCR detection system. The raw Cq values were normalized against GAPDH, housekeeping gene.

Immunoblot analysis. Antibodies to GNAI2 (sc-409), GAPDH (CB1001), peroxidase-conjugated anti-rabbit IgG (W401B) were obtained from Santa Cruz Biotechnology Inc., Abcam and Promega Corporation, respectively. Immunoblot analysis was carried out according to our previously published methods (12) and developed with a Kodak Image Station 4000 MM.

Statistics. All required statistical analyses were performed using GraphPad Prism by two-tailed unpaired Student's t-test with Welch's correction. Statistics used in bioinformatics such as P-values and False Discovery Rates were calculated using the built-in statistical programs of the respective analytical tools.

Results

Identification of DEGs. Our previous studies have shown that LPA/LPAR stimulates ovarian cancer growth and cell proliferation via the activation of GNAI2, encoded by the gene *GNAI2* or its mutationally activated configuration known as the *gcp* oncogene (15,16). To obtain an understanding of

the transcriptomic network regulated by *GNAI2*, the expression of *GNAI2* was silenced in SKOV3 cells using shRNAs targeting *GNAI2*. These cells were stimulated with LPA and the DEGs in *GNAI2*-silenced cells compared with those in the scrambled shRNA control group were identified using an Agilent array. With a cut-off value of ≥ 5 -fold change, compared with control cells, *GNAI2*-silenced cells had 313 downregulated genes and 293 upregulated genes (Fig. 1A and B). Of the 313 downregulated genes, 145 genes were found to be protein-encoding genes (Table SII). Similarly, among the 293 upregulated genes, 186 genes were found to be protein-encoding genes (Table SIII). Other genes were represented by either long non-coding RNAs or pseudogene transcripts (Tables SIV).

Next, the current study aimed to validate the array results in a cell lines that represent high grade ovarian serous ovarian carcinoma (HGSOC). The expression of *GNAI2* was silenced in Kuramochi cells, a HGSOC cell line, using specific siRNAs targeting *GNAI2*. After determining the efficacy of *GNAI2* silencing in these cells (Fig. 1C), RT-qPCR analysis was conducted to validate the expression levels of the DEGs. Downregulated genes were validated by monitoring the expression levels of the representative growth-promoting genes ankyrin repeat domain 1 (*ANKRD1*), bone marrow stromal cell antigen 2 (*BST2*) and cancer antigen 1 (*CAGE1*), whereas the upregulated genes were validated by monitoring the expression levels of growth-repressive representative genes, namely autophagy-related 16-like 1 (*ATG16L1*), spin-dlin family member 3 (*SPIN3*), thrombopoietin (*THPO*) and tetraspanin 16 (*TSPAN16*). It was found that silencing of *GNAI2* led to the decreased expression of *ANKRD1*, *BST2* and *CAGE1* (Fig. 1D), along with the increased expression of *ATG16L1*, *SPIN3*, *THPO* and *TSPAN16*, thereby validating the array results (Fig. 1E).

GO enrichment analysis of DEGs. It should be noted that the genes downregulated after silencing of *GNAI2* represent the genes whose expression was induced by *GNAI2*, whereas the upregulated genes represent the genes whose expression was repressed by *GNAI2 in situ*. Therefore, defining the functional relationships among the downregulated as well as the upregulated DEGs could provide insights into the mechanism via which *GNAI2* promotes ovarian cancer progression. Since GO enrichment analysis can provide information on the functional relationship among a large set of genes, GO analysis under the three sub-ontologies, namely biological processes (GO:BP), molecular functions (GO:MF) and cellular components (GO:CC), was conducted. GO enrichment analyses of the DEGs were performed using the web-based DAVID analytical tool (22). In GO:BP, the upregulated genes were significantly enriched in BP involving 'cell adhesion', 'proliferation' and 'cell motility' (Table I). These BP were associated with the known oncogenic functions of *GNAI2* in oncogenic cell proliferation and migration. In GO:CC, CC including 'plasma membrane' and 'actin-based cellular projections' formed the major categories, which was consistent with the role of *GNAI2* in actin cytoskeletal reorganization underlying cell invasion (20). In GO:MF, the topmost enriched categories were 'macromolecular interaction', 'chromatin and nucleic acid interaction' and 'transcriptional activation' (Table I), thus

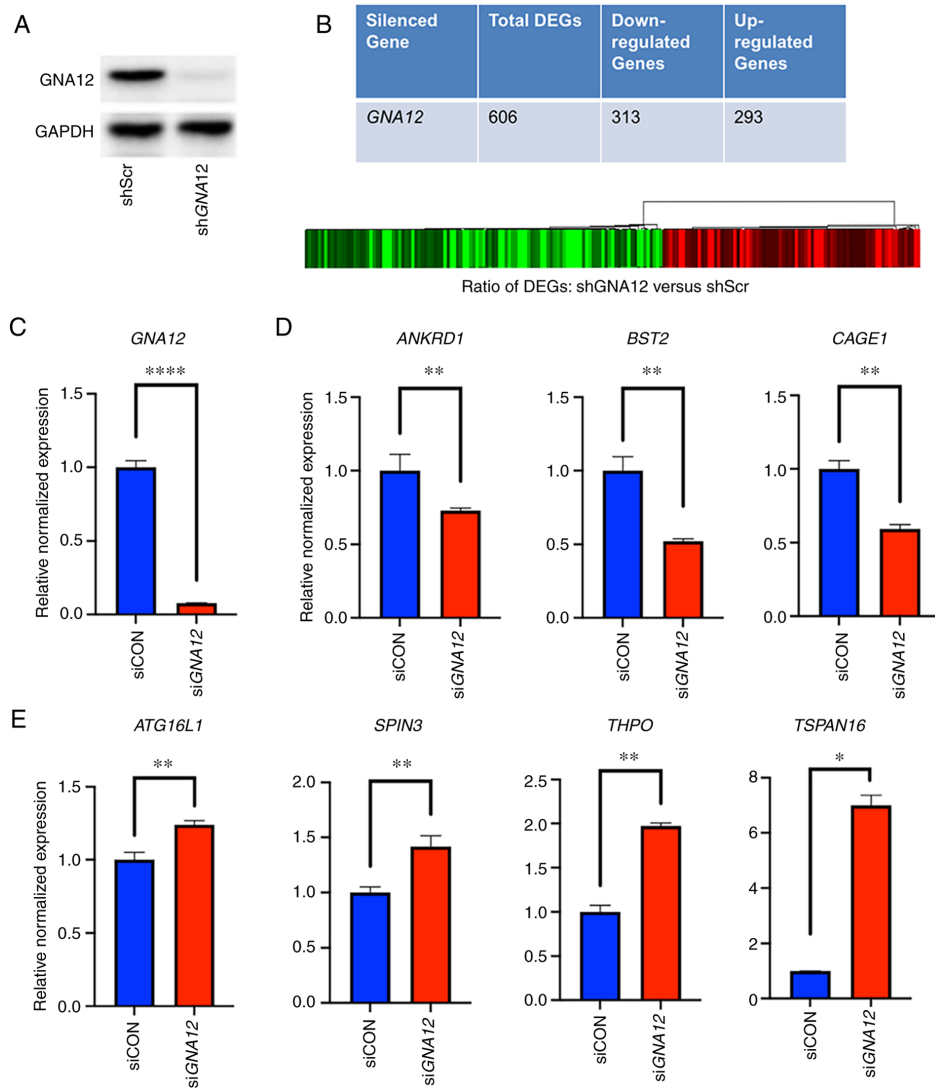


Figure 1. Heatmap of the DEGs and validation. (A) Validation of shRNA-mediated *GNA12*-silencing in SKOV3 cells. Expression of *GNA12* was stably silenced in SKOV3 cells using shGNA12 compared with control cells stably expressing non-targeting scrambled shRNAs. (B) Heatmap of the DEGs; the ratio comparing control cells (scrambled shRNA) and *GNA12*-silenced cells is presented as a heat map. Red, black and green colors represent upregulated, unchanged and downregulated expression, respectively. Total number of DEGs with >5-fold-change compared with control values and the number of down-regulated and upregulated DEGs are presented as a table. (C) Array results were validated by RT-qPCR using siRNA-mediated *GNA12*-silenced Kuramochi cells compared with non-targeting scrambled siRNA controls. (D) Downregulated DEGs were validated by monitoring the expression of the representative genes *ANKRD1*, *BST2* and *CAGE1* by RT-qPCR. (E) Expression of the upregulated DEGs was validated by monitoring the expression of the representative upregulated genes *ATG16L1*, *SPIN3*, *THPO*, and *TSPAN16* by RT-qPCR. * $P < 0.05$, ** $P < 0.005$, **** $P < 0.0005$. DEGs, differentially expressed genes; sh, short hairpin; GNA12, G protein subunit α 12; Scr, scrambled; RT-qPCR, CON, control; ANKRD1, ankyrin repeat domain 1; BST2, bone marrow stromal cell antigen 2; CAGE1, cancer antigen 1; RT-qPCR, reverse transcription-quantitative PCR; ATG16L1, autophagy-related 16-like 1; SPIN3, spindlin family member 3; THPO, thrombopoietin; TSPAN16, tetraspanin 16.

validating the role of *GNA12*-mediated network in molecular interactions leading to oncogenic transcriptional events.

With regards to genes that were downregulated in *GNA12*-silenced cells, GO:BP showed enrichment of categories associated with the overall negative regulation of cellular and BP including different aspects of ‘cell death’ and ‘proteolytic processes’ that can be linked with growth-inhibition (Table II). In GO:CC, the enriched categories included ‘membrane components of the cells’ and components associated with ‘autophagosome membrane’ and ‘extracellular matrix’. This finding was in agreement with the notion that the primary site of action of *GNA12* is closer to cell surface membrane (27). In GO:MF, the enriched categories were associated with ‘protein binding’ and peptidase functions including ‘exopeptidase’,

‘metallopeptidase’ and ‘metalloexopeptidase’ (Table II), which are often associated with programmed cell death (28).

Analysis of PPI networks and pathways. To further investigate the functional interactions among the proteins encoded by the DEGs, PPI network functional enrichment analysis was conducted using the STRING database (23). The upregulated and downregulated genes were analyzed separately, with the highest confidence interaction score (0.9) and <10 degrees of interaction. The PPI network downregulated in *GNA12*-silenced cells was constructed by screening 186 nodes and 306 edges (Fig. 2). The most significant module in the PPI network was determined using Cytoscape software (24). Kyoto Encyclopedia of Genes and Genomes

Table I. GO enrichment analysis of downregulated genes in *GNAI2*-silenced cells.

A, GO: Biological process			
Term	Description	Gene count	P-value
GO:0048518	Positive regulation of Biological Process	45	6.3x10 ²
GO:0048583	Regulation of response to Stimulus	24	8.7x10 ²
GO:0007155	Cell adhesion	21	2.1x10 ²
GO:0008283	Cell proliferation	19	4.9x10 ¹
GO:0048870	Cell motility	15	4.9x10 ¹
B, GO: Cellular component			
Term	Description	Gene count	P-value
GO:0005886	Plasma membrane	45	6.7x10 ²
GO:0071944	Cell periphery	45	9.0x10 ²
GO:0005576	Extracellular region	42	4.8x10 ²
GO:0042995	Cell projection	18	9.3x10 ²
GO:0098862	Cluster of actin-based cell projections	4	7.8x10 ²
C, GO: Molecular function			
Term	Description	Gene count	P-value
GO:0044877	Macromolecular complex binding	15	6.0x10 ²
GO:0001067	Regulatory region nucleic acid binding	10	9.4x10 ²
GO:0000981	Transcription factor activity	9	8.5x10 ²
GO:0003982	Chromatin binding	8	5.7x10 ²
GO:0001228	Transcriptional activator activity	6	7.2x10 ²
GO, Gene Ontology; GNA12, G protein subunit α 12.			

(KEGG) analyses indicated that the major pathways defined by *GNAI2*-dependent genes were pathways involved in 'cancer', 'PI3K/AKT signaling', 'chemotherapy resistance' and 'FoxO signaling' (Table III). Reactome analysis expanded this further into pathways associated with signaling involving 'tyrosine kinases', 'VEGF signaling', 'PI3K/AKT signaling', 'cell surface interactions at the vascular wall', 'cancer associated aberrant signaling by PI3K' and 'signaling by receptor tyrosine kinases' (Table III).

A similar PPI network construction was performed with the genes upregulated after silencing of *GNAI2* by screening a total of 202 nodes and 964 edges from the STRING portal (Fig. 3). KEGG pathway analyses indicated that the network, which was repressed by *GNAI2*, was primarily involved in 'metabolism', 'oxidative phosphorylation', 'proteasomal proteolysis', 'cell cycle arrest' and 'transcriptional misregulation in cancer' (Table IV). Reactome pathways analysis revealed that pathways regulated in this network included 'metabolism of proteins', 'cell cycle checkpoints', 'cellular stress response', 'anaphase-prophase complex (APC/C) mediated degradation of mitotic proteins' and 'ubiquitin-dependent degradation of cyclin D', all of which could be associated with growth-inhibition (Table IV).

Identification of the hub and bottleneck nodes. The network was further analyzed to identify the critical genes that define the hub nodes of the PPI network using the cytoHubba plugin in Cytoscape (25). The multiple algorithms of the cytoHubba, including Degree, MCC, MNC, EPC, EcCentricity, Closeness, Betweenness and Clustering Coefficient, were used to identify the hub nodes of the PPI networks (26). The intersecting genes identified by the different algorithms were tabulated (Table V). Results from this analysis identified *AKT1*, *VEGFA*, *BCL2L1*, *TGFB1* and *STAT3* as the top five hub nodes (Fig. 2, Insert; Table V). In addition to hub nodes, the identification of bottleneck nodes has equal or more importance in PPI networks due to their role as the key 'connector proteins' (29,30). Therefore, the bottleneck nodes of the network were extracted using the BottleNeck algorithm in cytoHubba application of Cytoscape. The results demonstrated that *VEGFA*, *AKT1* and *STAT3* were defined the bottleneck nodes, in addition to *IGF1* and *GHRH* (Fig. 2, Insert).

A similar analysis was conducted to extract the hub nodes and the bottleneck nodes of the upregulated genes. The results identified proteasome 20S subunit (PSM) β 6 (*PSMB6*), PSM α 6 (*PSMA6*), PSM ATPase 5 (*PSMC5*), ubiquitin conjugating enzyme E2 E1 (*UBE2E1*) and PSM non-ATPase 10 (*PSMD10*),

Table II. GO enrichment analysis of upregulated genes in *GNAI2*-silenced cells.

A, GO: Biological process			
Term	Description	Gene count	P-value
GO:0048519	Negative regulation of biological process	46	6.4x10 ²
GO:0048523	Negative regulation of cellular process	45	3.2x10 ²
GO:0006508	Proteolysis	20	7.5x10 ²
GO:0010941	Regulation of cell death	18	8.8x10 ¹
GO:0042981	Regulation of apoptotic process	17	8.7x10 ¹
B, GO: Cellular component			
Term	Description	Gene count	P-value
GO:0016020	Membrane	85	4.9x10 ²
GO:0071944	Cell periphery	51	5.8x10 ²
GO:0005886	Plasma membrane	50	5.9x10 ²
GO:0031012	Extracellular matrix	10	2.3x10 ²
GO:0000421	Autophagosome membrane	3	2.0x10 ²
C, GO: Molecular function			
Term	Description	Gene count	P-value
GO:0005488	Binding	122	9.6x10 ²
GO:0008233	Peptidase	10	9.8x10 ²
GO:0008237	Metallopeptidase	5	6.1x10 ²
GO:0008238	Exopeptidase	4	5.5x10 ²
GO:0008235	Metalloexopeptidase	3	8.9x10 ²
GO, Gene Ontology; <i>GNAI2</i> , G protein subunit α 12.			

the genes involved in proteasomal proteolysis, as the hub nodes (Fig. 3, Insert; Table VI). *PSMA6* and *PSMB6* were also identified as bottleneck nodes, along with *NDUFA4* mitochondrial complex associated (*NDUFA4*), *NADH*:ubiquinone oxidoreductase subunit B8 (*NDUFB8*) and anaphase promoting complex subunit 1 (*ANAPC1*) genes (Fig. 3, Insert; Table VI).

Biological significance of the hub and bottleneck nodes. The biological significance of the hub and bottleneck node genes in relation to ovarian cancer was determined via cBioPortal analysis (31,32). First, the hub and bottleneck genes downregulated in *GNAI2*-silenced cells were examined (Table V). The oncoprint profile of these genes indicated that they were either amplified or upregulated in at least 4-10% of the patients with ovarian cancer (Fig. 4). Functional annotation of these genes, as shown in Table V, indicated that the aberrant increased expression of these genes was associated with cancer growth, progression, metastasis and therapy resistance in ovarian cancer (33-45).

Next, the hub and bottleneck genes upregulated in *GNAI2*-silenced cells were examined (Table VI). These nodes, representing the genes that would have been suppressed by *GNAI2*, were found to be associated with

proteasome-mediated context-specific apoptotic pathways and therapy resistance in numerous cancer types (46-57). Taken together, the functional analyses of the hub and bottleneck genes indicated that the genes downregulated in *GNAI2*-silenced cells coded for pro-tumorigenic proteins, while the genes upregulated upon silencing of *GNAI2* encoded anti-tumorigenic proteins (Fig. 5).

Discussion

Autocrine and paracrine signaling by LPA serve a determinant role in cancer development and progression, and this is evident in the context of ovarian cancer (15,16). Our previous studies have reported that activation of *GNAI2* by LPA/LPAR signaling or mutational activation of *GNAI2* into the *gcp* oncogene serve a critical role in the oncogenic proliferation of ovarian cancer cells (15,16). In the present study, the key signaling pathways and critical genetic nodes that are aberrantly regulated by LPA/LPAR/*GNAI2* signaling in ovarian cancer were identified. In the cellular model used, the genes that were downregulated upon silencing of *GNAI2* represent the genes that would be upregulated by an intact LPA/LPAR/*GNAI2* signaling pathway, whereas the genes that were upregulated upon silencing

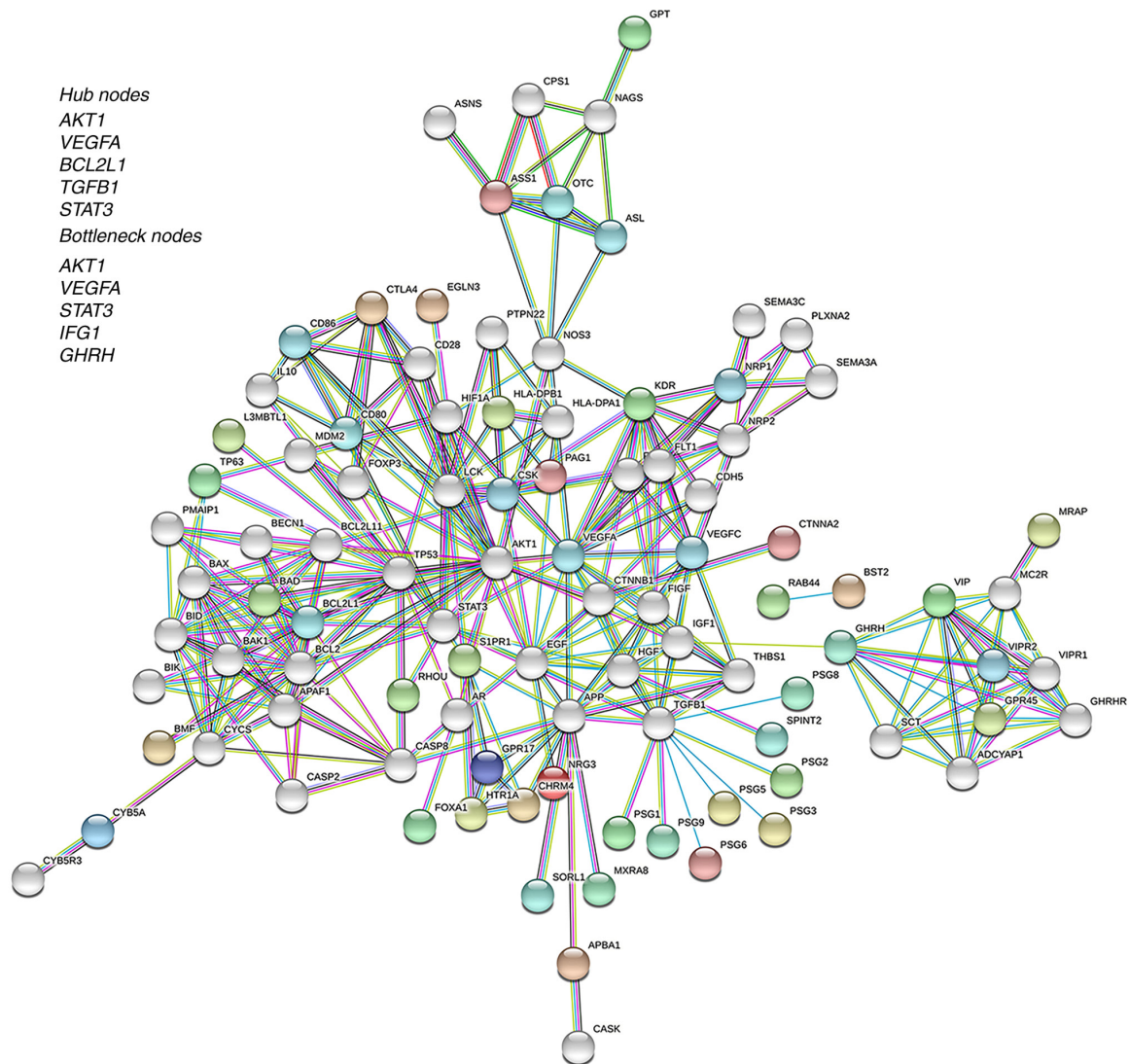


Figure 2. PPI network of downregulated genes. Using the web-based Search Tool for Retrieval of Interacting Genes tool, a PPI network of the genes downregulated in GNA12-silenced cells was constructed. Query proteins and their first shell interactions are denoted by colored nodes. Second shell interactions are in grey. Nodes of similar color identifies the specific cluster of interacting nodes. Predicted functional interactions are indicated by the connecting lines. The colors of the lines represent the types of evidence that were used to predict the PPI associations as follows: Red, known gene fusions; green, gene neighborhood; blue, gene co-occurrence; purple, experimental data; yellow, text-mining; light blue, protein homology; aqua marine, curated database; and black, co-expression. Hub and bottleneck nodes identified by the cytoHubba plugin in Cytoscape application are presented as the inset. PPI, protein-protein interaction; GNA12, G protein subunit α 12; IGF1, insulin-like growth factor 1; GHRH, growth hormone-releasing hormone.

GNA12 represent the genes that would be suppressed by this signaling pathway. The array analyses results indicated that genes that were upregulated by GNA12 (downregulated in GNA12-silenced cells) were mostly pro-tumorigenic, while genes that were downregulated by GNA12 (upregulated in GNA12-silenced cells) were growth-inhibitory. These array results were corroborated by the findings demonstrating that GNA12 silencing decreased the expression of pro-tumorigenic genes, along with a coincident increase in the expression of growth-suppressive genes. *ANKRD1*, *BST1* and *CAGE1* have been previously shown to have oncogenic role in different cancer types, including ovarian cancer. *ANKRD1* has been revealed to promote drug-resistance and epithelial-mesenchymal transition (EMT) in multiple cancer cells, including ovarian cancer cells (58,59). Moreover, *BST1* has been observed to induce EMT in ovarian cancer cells (59,60), while *CAGE1* is known to promote both proliferation and migration in different cancer

cells (61). By contrast, the genes upregulated upon silencing of *GNA12*, have been shown to exert a tumor suppressive role in different cancer types. For example, *SPIN3* has been identified as a tumor suppressor gene that induces apoptosis in human seminoma cancer cells (62). Furthermore, *ATG16L1* has been reported to be involved in promoting autophagic cell death in ovarian cancer cells (63). *THPO*, encoded by *THPO*, has also been observed to induce apoptosis in a context specific manner (64). While the cellular function of *TSPAN16* remains to be fully defined, its weak expression profile in cancer cells has been considered to be indicative of its tumor suppressing potential (65). Collectively, *GNA12* appears to stimulate a pro-tumorigenic network, along with the simultaneous suppression of a growth-inhibitory network.

The synergistic network organization was further clarified by the current results from the GO enrichment analyses. While GO enrichment in CC was in accordance with the known

Table III. Pathway analysis of downregulated genes in *GNA12*-silenced cells.

A, Kyoto Encyclopedia of Genes and Genomes pathway			
Term	Description	Gene count	False discovery rate
hsa05200	Pathways in cancer	28	2.43×10^{11}
hsa04151	PI3K-AKT signaling pathway	18	2.34×10^7
hsa01524	Platinum drug resistance	13	5.69×10^{11}
hsa01521	EGFR tyrosine kinase inhibitor resistance	13	2.35×10^9
hsa04068	FoxO signaling pathway	9	3.60×10^5
B, Reactome pathway			
Term	Description	Gene count	False discovery rate
HSA-9006934	Signaling by receptor tyrosine kinases	24	1.29×10^9
HSA-194138	Signaling by VEGF	12	7.95×10^8
HSA-2219528	PI3K/AKT signaling in cancer	10	9.32×10^7
HSA-202733	Cell surface interactions at the vascular wall	9	1.70×10^4
HSA-2219530	Constitutive signaling by aberrant PI3K in cancer	7	7.17×10^5
GNA12, G protein subunit α 12.			

Table IV. Pathway analysis of upregulated genes in *GNA12*-silenced cells.

A, Kyoto Encyclopedia of Genes and Genomes pathway			
Term	Description	Gene count	False discovery rate
hsa-0110	Metabolic pathways	34	5.57×10^6
hsa00190	Oxidative phosphorylation	26	1.79×10^{22}
hsa03050	Proteasome	24	5.40×10^{29}
hsa04218	Cell cycle	12	2.25×10^5
hsa05202	Transcriptional misregulation in cancer	7	2.32×10^4
B, Reactome pathway			
Term	Description	Gene count	False discovery rate
HSA-392499	Metabolism of proteins	40	7.46×10^5
HSA-69620	Cell cycle checkpoints	33	6.55×10^{24}
HSA-5668541	Cellular responses to stress	35	2.69×10^{21}
HSA-174178	APC/C:Cdh1 mediated degradation of Cdc20 and other APC/C:Cdh1 targeted proteins in late/early mitosis	30	8.83×10^{35}
HSA-75815	Ubiquitin mediated degradation of cyclin D	27	2.49×10^{33}
APC/C, anaphase-promoting complex; Cdh, CDC20 homolog; GNA12, G protein subunit α 12.			

role of GNA12 in transmitting the plasma membrane-located LPA/LPAR signaling from the cell periphery, novel insights could be gained via the analyses of GO BP and MF. The GO:BP enrichment indicated that the pathways upregulated by GNA12, thus downregulated in *GNA12*-silenced cells, were associated with critical BP and MF associated with

cancer progression, metastasis and therapy resistance. In fact, GO:BP, such as 'positive regulation of biological process' (GO:0048518), has already been shown to be associated with chemoresistance in HGSOC (55). Other GO:BPs, such as 'cellular response to stimulus' (GO:0048583), 'cell adhesion' (GO:0007155), 'cell proliferation' (GO:0008283) and 'cell

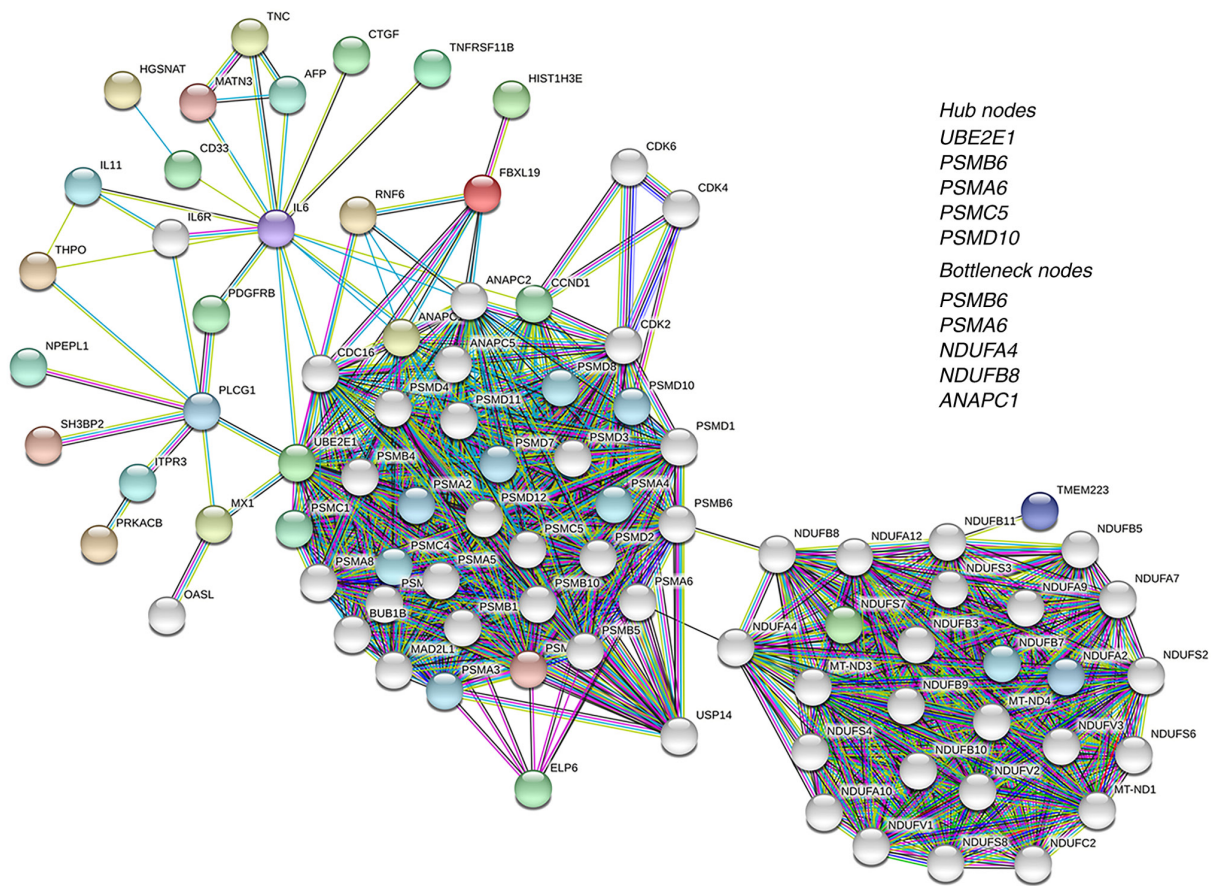


Figure 3. PPI network of upregulated genes. A PPI network of the genes upregulated in GNA12-silenced cells is presented. Colored nodes represent the query proteins and first shell interaction and white nodes denote second shell interactions. Nodes of similar color identifies the specific cluster of interacting nodes. Predicted functional interactions are indicated by the connecting lines. The colors of the lines represent the types of evidence that were used to predict the PPI associations as follows: Red, known gene fusions; green, gene neighborhood; blue, gene co-occurrence; purple, experimental data; yellow, text-mining; light blue, protein homology; aqua marine, curated database; and black, co-expression. Hub and bottleneck nodes of the PPI network derived from the use of cytoHubba plugin in Cytoscape application are presented as the inset. PPI, protein-protein interaction; GNA12, G protein subunit α 12; UBE2E1, ubiquitin conjugating enzyme E2 E1; PSM, proteasome 20S subunit; NDUFA4, NDUFA4 mitochondrial complex-associated; NDUFB8, NADH:ubiquinone oxidoreductase subunit B8; ANAPC1, anaphase promoting complex subunit 1.

motility' (GO:0048870), have been associated with cancer growth, recurrence and therapy resistance in numerous cancer types (56,57,66,67). Together with the indicated functions in GO:MF enrichments, the current novel findings emphasize the oncogenic role of GNA12 in transmitting signals from the LPA/LPAR signaling pathway located in the cellular periphery to a set of highly consequential nuclear events. Another significant analytical suggestion from the results was that genes suppressed by GNA12, as evidenced by their upregulation in GNA12-silenced cells, show GO enrichments in BP and MF associated with 'negative regulation of cell growth processes'. The GO:MF enrichment also suggested the potential role of GNA12 in suppressing proteolysis associated with negative cell proliferative.

Ovarian cancer is characterized by a heterogeneous histopathology with the manifestation of dissimilar genetic and pathway alterations. Major pathways that are aberrantly altered in a range of subtypes include TP53 (68,69), PI3K/AKT (70-72), VEGF (73,74), EGFR (75,76) and FoxO signaling (77). It was significant that KEGG and Reactome pathway analyses of the DEGs upregulated in GNA12-silenced cells directly linked GNA12 to these multiple tumor-promoting pathways. KEGG and Reactome pathways upregulated

in GNA12-silenced cells involved pathways associated with cell cycle check points, including 'APC/C mediated proteasomal degradation of mitotic proteins'. Thus, the pathway analyses present an oncogenic paradigm orchestrated by GNA12 in which mutational or LPA/LPAR activation of GNA12 leads to the stimulation of a pro-tumorigenic network, while concurrently suppressing an anti-tumorigenic network involving anti-mitotic, anti-proliferative and cellular stress pathways. This was further substantiated by the analysis of the hub and bottleneck signaling nodes derived from the PPI networks of the DEGs, especially the ones that were downregulated in GNA12-silenced cells. In line with the oncogenic role of GNA12, these genes were upregulated in distinct subsets of patients with ovarian cancer and were critically involved in ovarian cancer pathobiology. AKT1 gene is frequently upregulated in ovarian cancer and is associated with paclitaxel resistance in patients with ovarian cancer (33). Furthermore, the upregulation of VEGFA has been shown to be associated with distant metastasis and resistance to chemotherapy in patients with ovarian cancer (34-36). Increased expression of TGB1 has been correlated with EMT, tumor growth and metastasis in ovarian cancer (37,38), while overexpression of BCL2L1 is associated with anti-apoptosis effects and

Table V. Hub and bottleneck genes downregulated in *GNA12*-silenced cells.

Genes	Nodes	Function	(Refs.)
<i>AKT</i>	Hub and bottleneck	Amplified in ovarian cancer patients; confers resistance to paclitaxel.	(33)
<i>VEGFA</i>	Hub and bottleneck	Overexpression in ovarian cancer patients; tumor angiogenesis, associated with distant metastasis and resistance to chemotherapy.	(34-36)
<i>STAT3</i>	Hub and bottleneck	Tumor cell growth; survival, growth, stemness and tumor angiogenesis.	(41)
<i>BCL2L1</i>	Hub	Anti-apoptosis; confers platinum resistance.	(39,40)
<i>TGFB1</i>	Hub	Tumor growth, epithelial-mesenchymal transition and metastasis.	(37,38)
<i>IGF1</i>	Bottleneck	Overexpressed in ovarian cancer; tumor cell proliferation; immunosuppressive role.	(42,43)
<i>GHRH</i>	Bottleneck	Endogenous synthesis in ovarian cancer cells; ovarian cancer growth; tumor vascularization.	(44,45)

GNA12, G protein subunit α 12; *IGF1*, insulin-like growth factor 1; *GHRH*, growth hormone-releasing hormone.

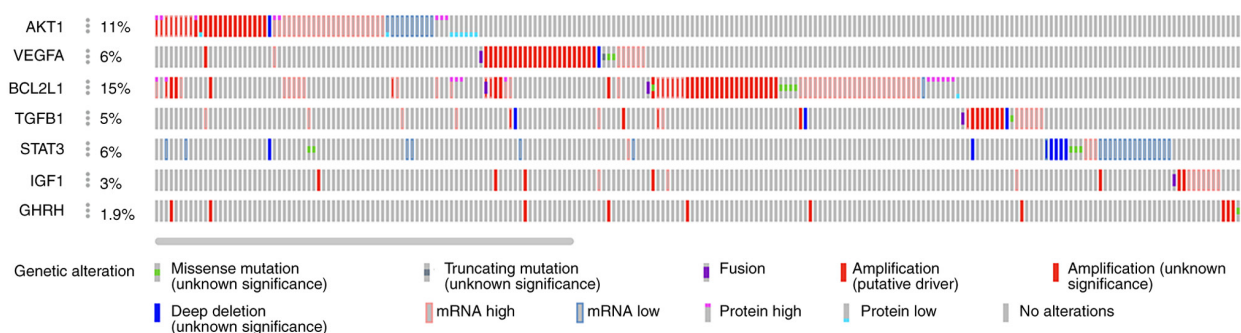


Figure 4. Genomic and expression profile of hub and bottleneck genes in patients with ovarian cancer. Genomic and expression profile of the hub and bottleneck genes in ovarian cancer patients were visualized in OncoPrint at cBioPortal web-portal. *IGF1*, insulin-like growth factor 1; *GHRH*, growth hormone-releasing hormone.

platinum resistance (39,40). In addition, *STAT3* signaling in ovarian cancer has been reported to be associated with tumor cell proliferation, survival, stemness and angiogenesis (41), while the overexpression of *IGF1* has been revealed to stimulate the proliferation of ovarian cancer cells, along with its immunosuppressive role in ovarian cancer (42,43). In a similar manner, *GHRH*, which is endogenously produced in ovarian cancer cells, is involved in ovarian cancer growth and tumor vascularization (44,45). The oncogenic role for these genes has also been shown by the oncoprint profiles of these genes generated using the CBioPortal. In fact, *in silico* data-mining indicated that all of the *GNA12*-regulated hub and bottleneck nodes identified here such as *AKT*, *VEGFA*, *BCL2L1*, *TGFB1*, *IGF1*, and *GHRH* are associated with poor prognosis in ovarian cancer (78-83).

A new paradigm emerging from the current analysis showed the potential role of *GNA12* in suppressing the proteasome pathway. It is of interest to note that the proteasomal proteolytic machinery has been reported to be required for the rapid onset of death receptor-induced apoptosis in a context-specific manner (47,48). In fact, it has been documented that the overexpression of *PSMA6* and *PSMC5* was associated with chemoresistance in prostate cancer and radiation-therapy resistance in lung cancer, respectively (49,50). Moreover, *ANAPC1*, a hub/pathway gene in this network, is part of the APC/C, which is involved in cell cycle arrest at G_1 phase. APC/C is an E3-ubiquitin ligase that regulates cell cycle arrest by

marking cell cycle proteins, such as cyclins, for degradation by proteasomes during cell cycle exit (53,54). *UBE2E1*, which was identified here as the hub and pathway gene, encodes an E2-ubiquitin conjugating enzyme. It has been revealed that *UBE2E1* can complex with polycomb repressive complex 1 (PRC1), the E3 ligase complex responsible for histone H2A ubiquitination and gene silencing (46). With the established role of PRC1 complex in the silencing of tumor suppressor genes (84), it can be considered that *UBE2E1* serves an active role in PRC1-mediated silencing of tumor suppressor genes.

One of the hallmarks of cancer involves metabolic reprogramming in cancer cells, with a shift towards aerobic glycolysis. It is known that along with the glycolytic shift, cancer cells concomitantly suppress mitochondrial oxidative phosphorylation (85). While the mechanism via which cancer cells proactively resort to glycolytic shift is beginning to be understood, the role of an active signaling mechanism involved in suppressing oxidative phosphorylation has thus far remained uncharacterized. In this regard, *NDUFA4* and *NDUFB8*, which have been identified as bottleneck genes in the *GNA12*-suppressed network, are highly relevant. *NDUFA4* and *NDUFB8* are subunits of complex IV and complex I of the mitochondrial electron transport chain, and are essential components involved in mitochondrial oxidative phosphorylation (86-88). The decreased expression of *NDUFA4* has been correlated with the suppression of oxidative phosphorylation and stimulation of glycolysis in renal

Table VI. Hub and bottleneck genes upregulated in *GNAI2*-silenced cells.

Genes	Nodes	Function	(Refs.)
<i>PSMB6</i>	Hub and bottleneck	20S proteasome subunit; context specific apoptosis.	(47,48)
<i>PSMA6</i>	Hub and bottleneck	20S proteasome subunit; context specific apoptosis; confers chemosensitivity.	(49)
<i>PSMC5</i>	Hub	26S proteasome regulatory subunit; confers radiosensitivity.	(50)
<i>PSMD10</i>	Hub	26S proteasome regulatory subunit; oncogene, but involved in context specific apoptosis.	(47,48)
<i>UBE2E1</i>	Hub	PRC1 mediated gene silencing.	(46)
<i>NDUFA4</i>	Bottleneck	Subunit of complex IV of the mitochondrial electron transport chain; reduced expression associated with metabolic reprogramming in renal cell carcinoma.	(84)
<i>NDUFB8</i>	Bottleneck	Accessory subunit of NADH dehydrogenase complex I of the mitochondrial electron transport chain; reduced expression associated with metabolic reprogramming in breast cancer cells.	(91)
<i>ANAPC1</i>	Bottleneck	Cell cycle arrest; mitotic checkpoint regulator.	(53,54)

PRC1, polycomb repressive complex 1; GNA12, G protein subunit α 12; PSM, proteasome 20S subunit; UBE2E1, ubiquitin conjugating enzyme E2E1; NDUFA4, NDUFA4 mitochondrial complex-associated; NDUFB8, NADH:ubiquinone oxidoreductase subunit B8; ANAPC1, anaphase promoting complex subunit 1.

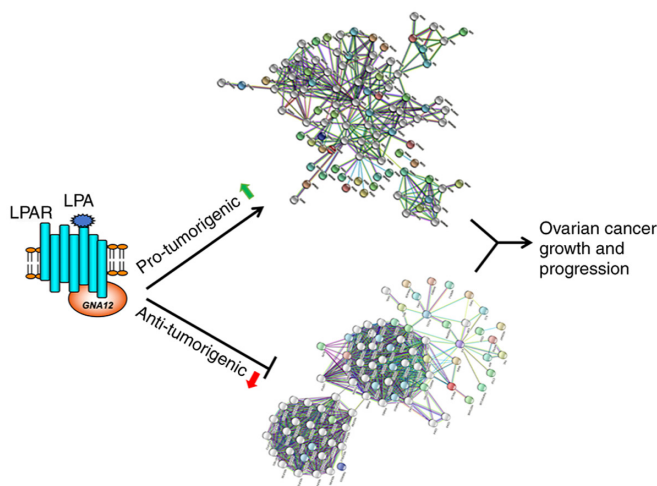


Figure 5. Schematic representation of *GNAI2*-driven genetic networks in ovarian cancer. LPA-LPAR-stimulation or mutational activation of *GNAI2* drove the coordinated regulation of two genetic networks. *GNAI2* activated a pro-growth genetic network with the genetic nodes involved in cell proliferation and simultaneously downregulated a growth-suppressive network defined by pro-apoptotic and growth-suppressive genes. Synergy between these networks promoted aggressive growth and progression of ovarian cancer. LPA, lysophosphatidic acid; LPAR, lysophosphatidic acid receptor; GNA12, G protein subunit α 12.

cell carcinoma (89,90). Similarly, low expression of NDUFB8 has been associated with impaired oxidative phosphorylation, along with a shift towards aerobic glycolysis in breast cancer cells (91). These findings, along with the current results that NDUFA4 and NDUFB8 were identified as bottleneck nodes, revealed the previously uncharacterized signaling node via which the LPA/LPAR/*GNAI2* signaling network could suppress oxidative phosphorylation to promote glycolytic shift in ovarian cancer cells. Thus, taken together, *GNAI2* appears to promote ovarian cell proliferation by suppressing multi-faceted anti-tumorigenic signaling nodes.

In summary, the present results provided novel insights into the mechanism via which *GNAI2*, stimulated by LPA/LPAR or mutational activation, could coordinate the upregulation of a growth promoting signaling network, while simultaneously regulating the downregulation of a growth-suppressive signaling network, to promote ovarian cancer growth. While the dysregulation of numerous different pathways is known in ovarian cancer, the core signal processing unit that connects the signaling nodes into a coordinated oncogenic network remains unknown. The current study demonstrated such a role for the LPA/LPAR/*GNAI2* signaling unit in ovarian cancer. The present study identified the duplex signaling mode of *GNAI2* via which the pro-tumorigenic signaling network was upregulated, along with the simultaneous downregulation of growth-suppressive signaling network in ovarian cancer. It has been realized that the therapeutic targeting of a single pathway may not be an effective treatment strategy for multiple type of cancer. This is especially true in the case of ovarian cancer, due to its subtype and pathway heterogeneity. In this context, the current finding that the LPA/LPAR/*GNAI2* signaling nexus regulated multiple hub and bottleneck nodes of an extensive oncogenic network suggested that this axis may be a potential target for the development of network-targeted combination therapeutic strategies for treating ovarian cancer.

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Availability of data and materials

The microarray data presented in the present study are deposited at the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>; accession no. GSE173214. Oncoprint data supporting the reported results used the Ovarian Serous Cystadenocarcinoma (TCGA, Firehose Legacy) dataset available <https://www.cbiportal.org>.

Authors' contributions

CI, YSS, JHH, MJ and DND conceptualized the study. MY, JHH and MJ developed the methodology. JHH and PD validated the experiments. MY, JHH and PD performed the formal analysis. DND procured resources. MY, MJ, PD and JHH curated and analyzed the data. DND wrote and prepared the original draft. DND, YSS and CI were responsible for writing, revising and editing the manuscript. DND supervised, acquired funds and was project administrator. All authors have read and approved the final version of the manuscript. DND, YSS, MJ, JHH and CI confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Approved by the Institutional Review Board of the University of Oklahoma (approval no. #9599).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Article

GNAI2/gip2-Regulated Transcriptome and Its Therapeutic Significance in Ovarian Cancer

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Abstract: Increased expression of *GNAI2*, which encodes the α -subunit of G-protein i2, has been correlated with the late-stage progression of ovarian cancer. *GNAI2*, also referred to as the proto-oncogene *gip2*, transduces signals from lysophosphatidic acid (LPA)-activated LPA-receptors to oncogenic cellular responses in ovarian cancer cells. To identify the oncogenic program activated by *gip2*, we carried out micro-array-based transcriptomic and bioinformatic analyses using the ovarian cancer cell-line SKOV3, in which the expression of *GNAI2/gip2* was silenced by specific shRNA. A cut-off value of 5-fold change in gene expression ($p < 0.05$) indicated that a total of 264 genes were dependent upon *gip2*-expression with 136 genes coding for functional proteins. Functional annotation of the transcriptome indicated the hitherto unknown role of *gip2* in stimulating the expression of oncogenic/growth-promoting genes such as KDR/VEGFR2, CCL20, and VIP. The array results were further validated in a panel of High-Grade Serous Ovarian Carcinoma (HGSOC) cell lines that included Kuramochi, OVCAR3, and OVCAR8 cells. Gene set enrichment analyses using DAVID, STRING, and Cytoscape applications indicated the potential role of the *gip2*-stimulated transcriptomic network involved in the upregulation of cell proliferation, adhesion, migration, cellular metabolism, and therapy resistance. The results unravel a multi-modular network in which the hub and bottleneck nodes are defined by ACKR3/CXCR7, IL6, VEGFA, CYCS, COX5B, UQCRC1, UQCRC5, and FYN. The identification of these genes as the critical nodes in *GNAI2/gip2* orchestrated onco-transcriptome establishes their role in ovarian cancer pathophysiology. In addition, these results also point to these nodes as potential targets for novel therapeutic strategies.

Keywords: ovarian cancer; *GNAI2*; *gip2*; transcriptome; gene expression; bio-informatics



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1. Introduction

Despite major advances in anticancer drug development research and newer treatment modalities, ovarian cancer lags behind other cancers by failing to show a significantly improved survival rate over the years [1,2]. This is primarily due to the late diagnosis of the disease, which is further compounded by the therapy resistance of the recurrent disease [3,4]. While targeted therapy is emerging as an important deterrent to overcome drug resistance in many cancers including ovarian cancer, it requires a better understanding of the disease mechanism and causative factors involved in disease progression [4,5]. Plasticity of tumor cells, as well as pathway-bypass mechanisms involving the expression

and/or activation of surrogate signaling molecules, were observed to blunt the effectiveness of an otherwise proven targeted therapy [6,7]. In this context, it is of interest to note that the α -subunit of G protein i2, which is encoded by the gene *GNAI2* and often referred to as *gip2* proto-oncogene, shows a biased increased expression in the late stages of ovarian cancer [8]. The role of *gip2* in ovarian cancer pathobiology becomes all the more important considering the fact that *GNAI2* is activated by lysophosphatidic acid (LPA), an endogenous growth factor in ovarian cancer. Such a context-specific expression of *gip2* along with its potent role in activating diverse oncogenic signaling responses such as cell proliferation, EMT, stemness, and invasive metastatic migration necessitates a need to define the global transcriptomic changes associated with the expression of *gip2* so that potential therapeutic nodes can be identified. With this rationale, we sought to gain insight into the onco-transcriptome stimulated by *gip2* in ovarian cancer cells and its functional role in ovarian cancer progression and/or therapy resistance. We report the results from micro-array-based transcriptomic analysis and gene set enrichment analysis on defining *gip2*-dependent transcriptomic network along with the associated hub and bottleneck genes in ovarian cancer cells.

Transcriptome profiling of SKOV3 cells in which the expression of *gip2* was silenced indicated the *gip2*-dependent expression of 264 genes, of which 136 were found to be coding for functional proteins. Many of these genes, totaling 78, are known to be associated with the hallmarks of cancer. The array results were validated by monitoring the expression of *KDR/VEGFR2*, *CCL20*, and *VIP*, as a representative set of pro-tumorigenic genes in the high-grade serous ovarian carcinoma cell lines Kuramochi and OVCAR8. Gene Ontology (GO) enrichment and protein–protein interaction (PPI) network analyses of the dataset from the transcriptome were carried out using the web-based Database for Annotation, Visualization and Integrated Discovery (DAVID), Search Tool for Retrieval of Interacting Genes (STRING), and Cytoscape applications. Gene enrichment analysis indicated the oncogenic role of *gip2* in inducing the expression of genes involved in cell proliferation, adhesion, and migration. PPI network analysis identified the critical pathways regulated by the transcriptome, which include cellular energetics, oncogenic signaling, and therapy resistance. Further network analysis using Cytoscape application identified the potential pro-tumorigenic role of hub and bottleneck genes, namely *CYCS*, *VEGFA*, *IL6*, *UQCRFS1*, *UQCRC1*, *COX5B*, *ACKR3/CXCR7*, and *FYN*, in *gip2*-orchestrated onco-transcriptome. Functional annotation of the hub and bottleneck genes indicated a triplex signaling mode driving ovarian cancer progression. This involves the activation of a network cluster that plays a stimulatory role in cancer cell metabolism, cell proliferation, and invasive migration. In addition to providing new insights into the network organization of *gip2*-stimulated onco-transcriptome in ovarian cancer, the results provide a molecular basis for investigating the therapeutic potential of the hub and bottleneck nodes such as those of *UQCRFS1*, *ACKR3/CXCR7*, and *FYN* for the development of second-line targeted therapy in ovarian cancer.

2. Materials and Methods

2.1. Cell Lines and Culture

High-grade serous carcinoma cell lines OVCAR3 and non-serous ovarian carcinoma cell line SKOV3 were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA), OVCAR8 and Kuramochi cells were procured from the National Cancer Institute (NCI, Bethesda, MD, USA) and Japanese Collection of Research Biosources Cell Bank (JCRB, Osaka, Japan) respectively. Routine authentication of the cell lines was carried out by short tandem repeat analysis as described [9]. Cell-culture conditions and the use of SKOV3 cell lines expressing shRNAs targeting *GNAI2/gip2* and non-targeting scrambled shRNA were previously described [10]. OVCAR3, OVCAR8, and Kuramochi cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Cellgro, Manassas, VA, USA) whereas SKOV3 cells were cultured in Dulbecco's modified Eagle's (DMEM) Medium (Cellgro, Manassas, VA, USA) supplemented with 10% FBS (Gemini Bio-Products,

West Sacramento, CA, USA), 50 U/mL penicillin, 50 µg/mL streptomycin (Cellgro, Manassas, VA, USA) at 37 °C and 5% CO₂. siRNAs targeting *GNAI2/gip2* (siGENOME Human *GNAI2* siRNA SMARTpool; Cat # M-008435-00-0005) and non-targeting scrambled control siRNAs control (siGENOME Non-Targeting siRNA Pool; Cat # D-001206-13-05) were purchased from Dharmacon/Horizon Discovery, Lafayette, CO. *GNAI2/gip2*-specific siRNA and siCon were transfected into Kuramochi, OVCAR3 and OVCAR8 cells using Lipofectamine RNAiMAX (Invitrogen, Life Technologies, Carlsbad, CA, USA) as recommended by the manufacturer. The knockdown of *gip2* in the transfectants was confirmed using RT-PCR. LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) used in the study was prepared as 10 mM stock solution in PBS containing 1% BSA and stored at −80 °C until use.

2.2. Transcriptomic Analysis

Serum-starved stable SKOV3-shScr (nonspecific scrambled shRNA control) and SKOV3-sh*gip2* cells were stimulated with LPA (10 µM) for 16 h. Qiagen RNeasy mini kit (Qiagen, Carlsbad, CA) was used to extract total RNA following the manufacturer's protocol. Agilent SurePrint G3 Human Comparative Genomic Hybridization 8 × 60 microarray platform was employed to generate the transcriptomic profile of these stable cell lines. Complementary RNAs were labeled with Cy3-CTP using the Agilent Quick Amp labeling kit (Agilent, CA) and hybridized to the array slides following the manufacturer's protocol. A total of six samples (3 control and 3 experimental) were used in the microarray platform. Agilent SureScan scanner was used to scan the array slides at 2 microns resolution and the spot intensity extracted using Agilent Feature Extraction version 11.0 software. Delineation of gene expression between the cell lines was established using Agilent GeneSpring GX version 13.0. *gip2*-dependent genes, with ≥ a 5-fold decrease over the control cells, were used for further bioinformatic analyses.

2.3. Bioinformatic Analysis

Multiple web-based enrichment and network analysis were employed to define the pathways and network their interactions. Database for Annotation, Visualization and Integrated Discovery (DAVID) annotation tool (<https://david.ncifcrf.gov/home.jsp>) (accessed on 21 July 2021) was used for the Gene Ontology Enrichment analysis [11]. Protein–protein interaction (PPI) network analysis was carried out employing the web-based Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (<https://string-db.org/>) (accessed on 21 July 2021) with the high confidence interaction score (0.7) and <10 degrees of interaction limit [12]. Cytoscape software application was used to identify the significant modules in the PPI network [13]. The hub and bottleneck nodes of the PPI network were identified using the cytoHubba plugin in Cytoscape [14]. While the hub nodes of the PPI network were identified using Degree, MCC, MNC, EPC, EcCentricity, Closeness, Betweenness, and Clustering Coefficient algorithms, the bottleneck nodes were identified using the BottleNeck algorithm of the cytoHubba plugin [15].

2.4. RT-qPCR Analysis

Total RNA was extracted using Qiagen RNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. cDNA synthesis was carried out using iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, UAS). Real-time quantitative PCR (RT-qPCR) was carried out using the cDNA from the above step using the SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA, USA) in a BioRad CFX96 Real-time PCR detection system. The raw Cq values were normalized against GAPDH, a housekeeping gene. The primers used in this study are shown in Table S1.

2.5. Immunoblot Analysis

Immunoblot Analysis. Antibodies to *GNAI2* (sc-409), GAPDH (CB1001), peroxidase-conjugated anti-rabbit IgG (W401B) were from Santa Cruz Biotechnology Inc (Dallas, TX, USA), Abcam (Cambridge, MA, USA) and Promega Corporation (Madison, WI, USA)

respectively. Immunoblot analysis was carried out using our previously published methods [10].

2.6. Statistics

All gene expression studies were tested by a two-tailed Student's t-test with Welch's correction using GraphPad Prism software (La Jolla, CA, USA). *p* values and False Discovery Rates in the bioinformatic analysis were from built-in statistical analytical tools within respective programs.

3. Results

3.1. Characterization of GNAI2/gip2-Dependent Transcriptome

Our previous studies have shown that LPA-LPAR activation or mutational activation of GNAI2/gip2 stimulates proliferation, EMT, invasive migration, and metabolic reprogramming of ovarian cancer cells [9,16–19]. To gain better insight into the global oncogenic network regulated by gip2, we carried out transcriptome profiling of SKOV3 ovarian cancer cells in which the expression of gip2 was silenced using shRNAs [10]. The cells were stimulated with LPA and the genes that showed a decrease in the gip2-silenced cells compared to the scrambled shRNA control group were identified using Agilent array. With the fold change cut-off value of ≥ 5 compared to control cells, gip2-silenced cells showed a downregulation of 264 genes (Figure 1A; GEO Accession No: GSE173214). Of the downregulated genes, only 135 genes were found to be protein-encoding genes (Table S2). Others were represented by genes encoding uncharacterized transcripts or the ones encoding pseudogenes, anti-sense RNAs, or non-coding RNAs (GEO Accession No: GSE173214). Analysis of the genes through data mining from published literature indicated that 78 of these genes are known to play an oncogenic role in different cancers (Table S3). More interestingly, querying these genes in TCGA ovarian cancer dataset (TCGA, Firehose Legacy) via CBioPortal, indicated that 61 of these genes showed increased expression in ovarian cancer (Table S4) and 40 of these genes showed co-occurrence in their expression profiles (Table S5), thus further validating our results in ovarian cancer patient subgroup. These array results were experimentally validated by monitoring the expression of a representative set of pro-tumorigenic genes in the SKOV3 cell line and high grade serous ovarian carcinoma (HGSOC) cell lines Kuramochi and OVCAR8. Expression of gip2 was silenced using shRNA (SKOV3 cells) or siRNAs specifically targeting gip2 (Kuramochi and OVCAR8 cells). Expression of KDR/VEGFR2, VIP, and CCL20—a representative set of genes from the array results—were monitored by RT-PCR. As shown in Figure 1B–D, silencing of gip2 decreased the expression of all these genes in SKOV3 as well as Kuramochi and OVCAR8 cells, thus validating the array results.

3.2. Gene Ontology Enrichment Analysis of gip2-Dependent Genes

In the gip2-silenced cellular model system used here, the genes that show decreased expression upon the silencing of gip2—the gip2-dependent genes—are in fact defined as the genes that would be stimulated by gip2 in situ in ovarian cancer cells. Consistent with this premise, functional annotation of these genes indicated that at least 50% of the genes (a total of 78 genes out of 136 protein-coding genes) were found to play an oncogenic role in different cancers (Table S2). Reasoning that the functional networking of these genes could provide insight into the mechanism by which gip2 promotes neoplastic growth of ovarian cancer cells, we carried out pathway and network analyses. Since Gene Ontology (GO) enrichment analysis could provide information on the functional relationship among a large set of genes, GO analysis of biological processes (GO:BP), molecular functions (GO:MF) and cellular components (GO:CC) were carried out using DAVID database [11]. In GO:BP ontology, the gip2-dependent genes were significantly enriched in biological processes involving cell adhesion, proliferation, and cell motility (Table 1). In GO:CC ontology, cellular periphery including plasma membrane and membrane region formed the major

categories. In GO:MF ontology, the topmost enriched categories were molecular transducer activity, receptor signaling activity, and lipid and tyrosine kinase binding (Table 1).

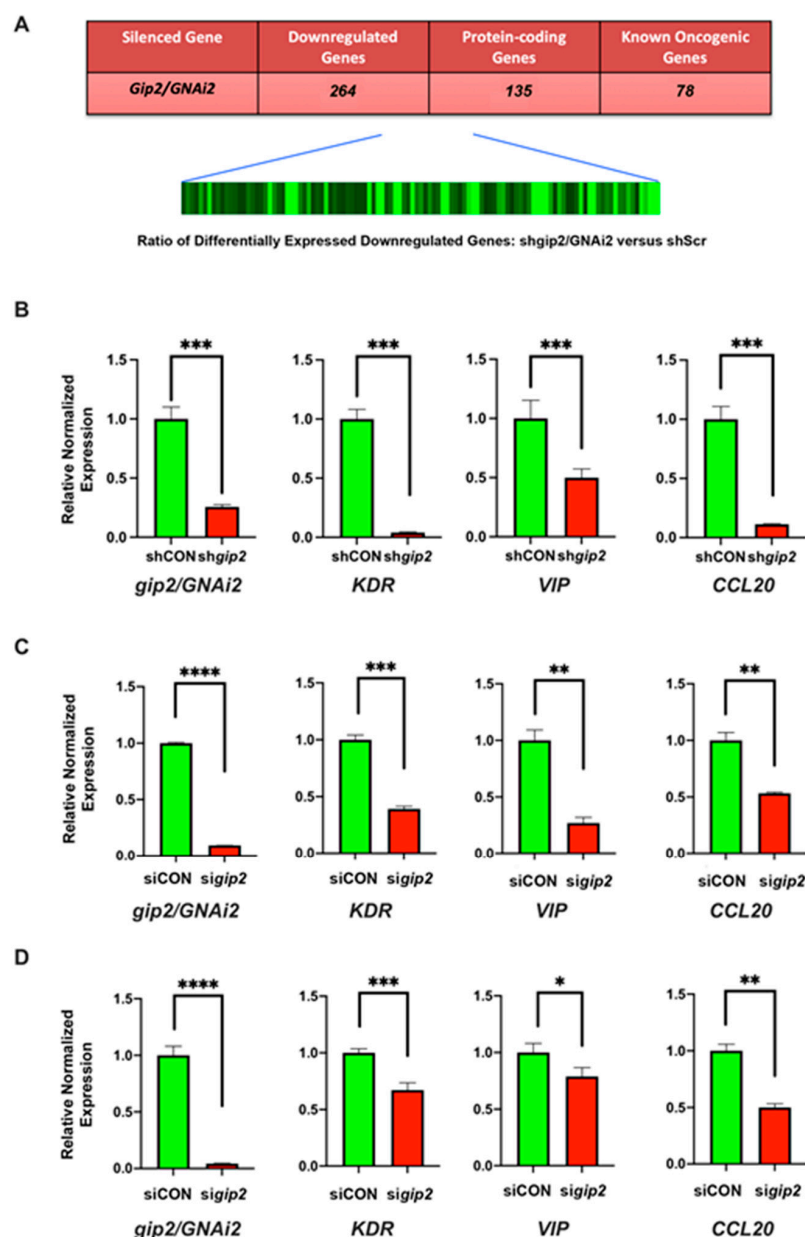


Figure 1. Heatmap of genes downregulated upon silencing of GNAI2/gip2 and validation. (A) Heatmap of genes downregulated upon silencing of GNAI2/gip2. The ratio of comparing control (scrambled shRNA) and gip2-silenced cells is presented as a heatmap. Black and green bands represent unchanged and downregulated expression, respectively. Total number of genes that show > 5-fold reduction in the expression compared to control values is presented as a table insert. Array results were validated by RT-PCR methods using SKOV3 (B), Kuramochi (C) and OVCAR8 (D) cells in which the expression of *gip2* was silenced (*sigip2*) using specific siRNA or siRNA. In SKOV3 cells, cells stably expressing scrambled shRNA (shCON) were compared with cells in which *gip2/GNAI2* was silenced with the stable expression of specific shRNA targeting *gip2/GNAI2* (shgip2). In Kuramochi and OVCAR8 cells, cells transfected with non-targeting scrambled siRNA pool were used as the control group (siCON). Downregulated genes were validated by monitoring the expression of the representative genes KDR, VIP, and CCL20 by RT-PCR. Statistical significance between *gip2*-knockdown and scrambled siRNA cells was determined by Student's *t*-test (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$ ACKR3).

Table 1. Gene ontology enrichment analysis of differentially expressed genes.

Category	Term	Description	Gene Count	p-Value
GO: BP	GO:0050896	Response to stimulus	67	2.9E−2
	GO:0009605	Intracellular signal transduction	25	7.1E−2
	GO:0042127	Regulation of cell proliferation	19	1.3E−1
	GO:0007155	Cell adhesion	17	9.5E−2
	GO:0016477	Cell migration	14	5.2E−2
GO:CC	GO:0005886	Plasma membrane	46	1.7E−2
	GO:0071944	Cell periphery	46	2.5E−2
	GO:0005576	Extracellular region	41	3.1E−2
	GO:0042995	Extracellular space	20	2.8E−3
	GO:0098862	Membrane region	6	8.5E−2
GO:MF	GO:0060089	Molecular transducer activity	18	3.8E−2
	GO:0038023	Signaling receptor activity	18	3.8E−2
	GO:0004888	Transmembrane signaling receptor activity	15	5.2E−2
	GO:0003982	Lipid binding	10	2.5E−2
	GO:0001228	Protein tyrosine kinase binding	3	5.5E−2

Gene ontology (GO) enrichment analysis was carried out with the genes downregulated in SKOV3 cells upon silencing of gip2 using the DAVID gene annotation tool. GO term enrichment analyses in terms of the three sub-ontologies: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) are presented.

3.3. Analysis of PPI Networks and Pathways

To further investigate the functional interactions among the proteins encoded by the gip2-dependent genes, downregulated in gip2-silenced cells, we carried out Protein–Protein Interaction Networks Functional Enrichment Analysis using STRING database [12]. Gip2-dependent genes were analyzed with a high confidence interaction score (0.7) and < 10 degrees of interaction. The PPI network was constructed by screening 185 nodes and 473 edges (Figure 2). The most significant modules in the PPI network were determined using the Cytoscape software application [13].

Using the web-based STRING tool, the PPI network of the genes downregulated in gip2-silenced cells was constructed. Query proteins and their first shell interactions are denoted by colored nodes. Second shell interactions are in white. Key pathway clusters defined by specific nodes are denoted by different colors as follows: Red, metabolic pathway; Blue, Pathways in Cancer; Green, Cytokine–Cytokine Receptor Interactions; Yellow, Oxidative Phosphorylation; Violet, apoptosis; and Aqua Marine, Platinum Resistance. Predicted functional interactions are indicated by the connecting lines. The colors of the lines represent the types of evidence that were used to predict the PPI associations. They are as follows: Red-known gene fusions; Green-gene neighborhood; Blue-gene co-occurrence; Purple-experimental data; Yellow-text-mining; Light Blue-protein homology; Aqua Marine-curated database; and Black-co-expression.

KEGG analyses indicated that the top pathways defined by the gip2-dependent genes were: 1. Metabolic pathways; 2. Oxidative phosphorylation; 3. Pathways in cancer; 4. Platinum resistance; and 5. EGFR-inhibitor resistance (Table 2). Reactome analysis expanded this further into pathways associated with 1. Respiratory electron transport; 2. Apoptosis; 3. Hemostasis; 4. Mitochondrial protein support; and 5. Interleukin-6 signaling (Table 2).

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473 edges (Figure 2). The most significant modules in the PPI network were determined using the Cytoscape software application [13].

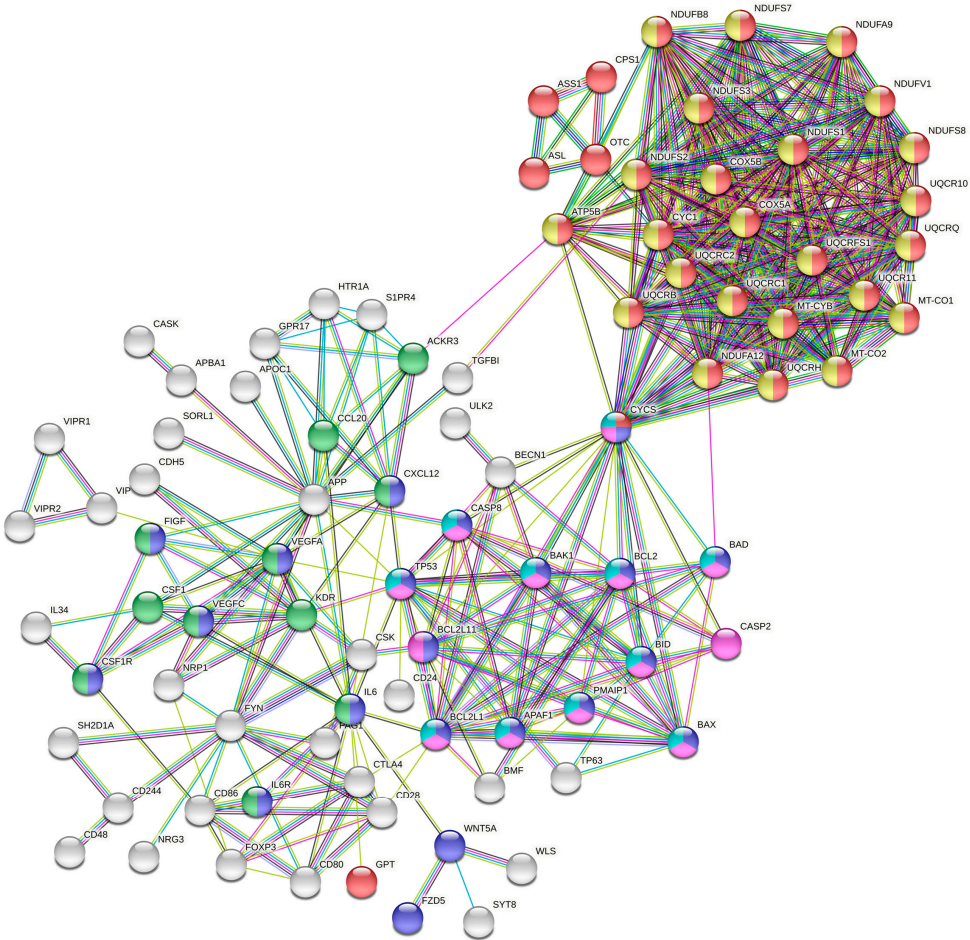


Figure 2. Protein-Protein Interaction Network of gip2-dependent genes.

Table 2. KEGG and Reactome pathway analysis of differentially expressed genes. KEGG and Reactome pathway enrichment analyses were carried out with the genes downregulated in gip2-silenced SKOV3 cells using STRING and Cytoscape applications. Major pathways of the PPI identified in downregulated and upregulated genes along with the gene count and false discovery rate are presented.

Pathway	term	Description	Count	False Discovery Rate
KEGG pathway	hsa00199	Oxidative phosphorylation	31	5.83e−07
KEGG pathway	hsa05200	Pathways in cancer	21	5.77e−07
KEGG pathway	hsa01524	Platinum drug resistance	11	5.45e−09
KEGG pathway	hsa01521	EGFR tyrosine kinase inhibitor resistance	9	5.75e−06
Reactome pathway	hsa109581	TCR Cycle and Respiratory electron transport	14	1.02e−08
Reactome pathway	hsa109582	Apoptosis	14	1.02e−08
Reactome pathway	hsa109582	Apoptosis	14	1.02e−08
Reactome pathway	hsa1268020	Mitochondrial protein import	4	2.76e−02
Reactome pathway	hsa1059683	Interleukin-6 signaling	2	3.69e−02

3.4. Identification of the Hub and Bottleneck Nodes

We probed the network further to identify the critical genes that define the hub nodes of the network using the cytoHubba plugin in Cytoscape [13]. We applied the multiple algorithms of the cytoHubba including Degree, MCC, MNC, EPC, Eccentricity, Closeness,

Betweenness, and Clustering Coefficient to identify the hub nodes of the PPI networks [14]. The intersecting genes identified by the different algorithms were tabulated. Results from this analysis identified CYC5, VEGFA, COX5B, UQCRC1, and IL6 as the top five hub nodes of the network (Table 3). In addition, we identified the bottleneck nodes of the network since they are considered as the “connector” or “choke points” in PPI networks [19,20] using the BottleNeck algorithm in cytoHubba application of Cytoscape. The results identified CYC5, VEGFA, and IL6 along with ACKR3/CXCR7 and FYN as the top five bottleneck node genes of the network (Table 3). Functional annotation of these genes points to the diverse oncogenic roles of these genes in many different cancers including ovarian cancer (Table 3).

Table 3. Hub and bottleneck genes of the PPI network. The top five genes derived from the PPI network using MCC, MNC, Degree, EPC, and EcCentricity algorithms of the CytoHubba plugins in Cytoscape and top five bottleneck genes determined from the BottleNeck algorithm are presented.

Genes.	Nodes	Function	References
CYC5	Hub and Bottleneck	Increased oxidative phosphorylation and pro-survival cellular events	Huttemann et al., 2011
VEGFA	Hub and Bottleneck	Overexpression in ovarian cancer patients; tumor angiogenesis, associated with distant metastasis and resistance to chemotherapy	Guan et al., 2019; Sopo et al., 2019; Li et al., 2020
IL6	Hub and Bottleneck	Ovarian Cancer Growth, stemness, and therapy resistance	Wang et al., 2018; Azar et al., 2020
UQCRC1	Hub	Oncogenic reprogramming of metabolism role in Pancreatic cancer	Kaneko et al., 2003; Ohashi et al., 2004; Owens et al., 2011; Jun et al., 2012
UQCRC1	Hub	Oncogenic reprogramming of metabolism role in Pancreatic cancer	Wang et al., 2020
COX5B	Hub	COX5B-mediated metabolic reprogramming is Associated with poor prognosis in many cancers	Gao et al., 2017; Chu et al., 2020
ACKR3/CXCR7	Bottleneck	Chemokine receptor activated in many cancers to promote invasive metastasis.	Neves et al., 2019; Smit et al., 2020
FYN	Bottleneck	Mediates oncogenic cell proliferation, migration, EMT, and therapy resistance in many cancers.	Saito et al., 2010; Lee et al., 2018; Yu et al., 2020

Gip2-dependent expression of the hub and bottleneck genes were validated by monitoring the expression of IL6 and UQCRC1 in gip2-silenced SKOV3, OVCAR8 and OVCAR3 cells by RT-PCR analysis. As shown in Figure 3, gip2-silencing led to the decreased expressions of both IL6 and UQCRC1 in all the tested cell lines (Figure 3).

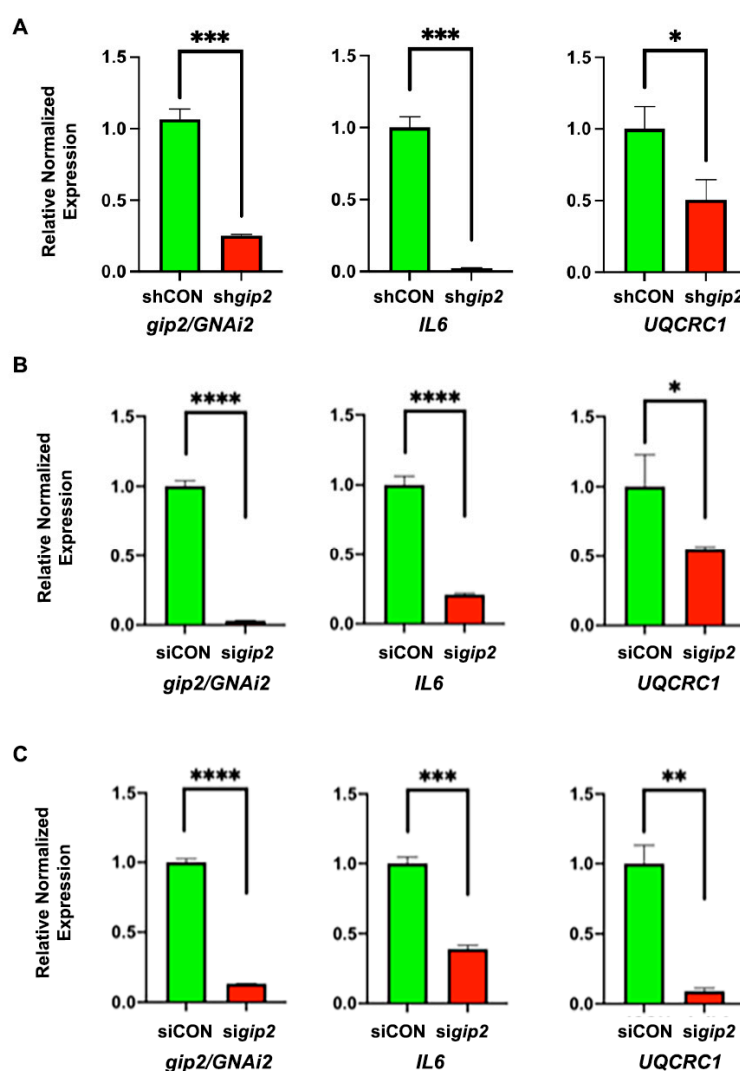


Figure 3. Validation of gip2-dependent hub and bottleneck genes. Downregulation of Hub and Bottleneck (bottleneck) genes upon the loss of gip2 was validated using SKOV3 (A), OVCA8 (B) Hub and Bottleneck (bottleneck) genes upon the loss of gip2 was validated using SKOV3 (A), OVCA8 (B) and OVCA9 (C) cell lines which over the expression of gip2 SKOV3 cells using specific shRNA (SKOV3) or siRNAs (OVCA8 and OVCA9). In the (A) SKOV3 cells and (B) OVCA8 cells expressing non-targeting scrambled shRNA were used as control group (shCON). In (A) SKOV3 and (B) OVCA8 cells transfected with non-targeting scrambled shRNA pool or the used as the control group (shCON). Down-regulated genes were validated by qPCR monitoring the expression of the representative bottleneck gene *IL6* and hub gene *UQCRC1*. Statistical significance was determined by Student's t-test (*P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.0001).

3.5. Significance of the Hub and Bottleneck Nodes

3.5. **Significance of the Hub and Bottleneck Nodes**

Next, we investigated the biological significance of the hub and bottleneck node genes in relation to ovarian cancer through CbioPortal analysis [20,21]. Oncoprint profile of CYCS, VEGFA, IL6, UQCRC1, UQCRCF, COX5B, ACKR3/CXCR7, and FYN indicated that these genes were either amplified or overexpressed in 4–25% of the ovarian cancer patients (Figure 4A). Increased amplification or expression seen with UQCRC1 in ovarian cancer patients (Figure 4A). In the case of amplification or overexpression of IL6, UQCRCF, COX5B, ACKR3/CXCR7, and FYN, increased amplification or overexpression was seen in 4–25% of the ovarian cancer patients (Figure 4A). In the case of amplification or overexpression seen with UQCRCF in ovarian cancer patients, RNAseq data and overall survival plot were obtained through CbioPortal analyses. As shown in Figure 4B, increased expression of UQCRCF (Figure 4B) correlated with the reduced overall survival of ovarian cancer patients (Figure 4C). More intriguingly, increased expression of *gip2* was not observed in UQCRCF1-patients. It is possible that analyses. As shown in Figure 4B, increased expression of UQCRCF1 (Figure 4B) correlated with the reduced overall survival of ovarian cancer patients (Figure 4C). More intriguingly, increased expression of *gip2* was not observed in UQCRCF1-patients. It is possible that the initiating event involved in the expression of UQCRCF1 is the activation of *gip2* rather than an increased expression of *gip2* as in the case of other oncogenes [22].

the initiating event involved in the expression of UQCERS1 is the activation of *gip2* rather than an increased expression of *gip2* as in the case of other oncogenes [22]. Together with the functional annotation of the other hub and bottleneck genes, these results substantiate the potential role of the *gip2*-dependent hub/bottleneck nodal network in ovarian cancer pathophysiology.

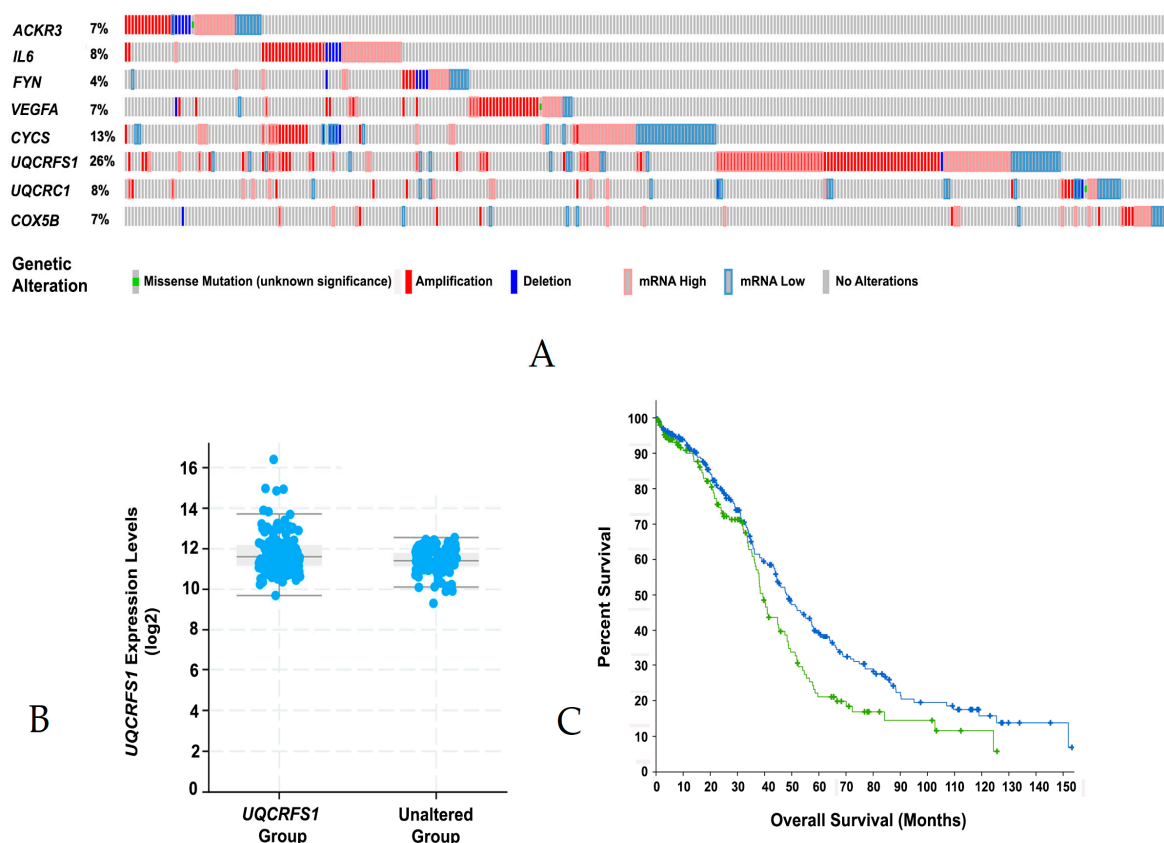


Figure 4. Genomic and expression profile of hub and bottleneck genes in Ovarian cancer patients. Genomic and expression profiles of the hub and bottleneck genes in ovarian cancer patients were visualized in OncoPrint and CBioPortal web portal (A). Plots depicting the expression of the hub node gene UQCERS1 with the *p*-value of 2.54×10^{-8} were obtained from the CBioPortal (B). Overall survival of patients with UQCERS1 amplification or overexpression (Log Rank Test *p*-value of 3.9×10^{-2}) was extracted from the CBioPortal (C).

4. Discussion

4. Discussion

Effective targeted therapy in ovarian cancer has remained elusive primarily due to the heterogeneous subtypes and aberrant signaling pathways associated with the disease [23,24]. While PARP inhibitors and anti-angiogenic agents have provided relief to many patients, the emergence of therapy resistance, especially in late-stage disease, has remained an unsolvable clinical problem [25–28]. This has necessitated the critical need to have a clear understanding of the molecular events associated with different subtypes and stages of the disease so that context-specific therapeutic strategies can be developed. Although recent studies have identified major pathways and genetic risk factors associated with ovarian cancer [29], causative factors involved in sustaining tumor growth in the advanced stages of ovarian cancer are largely unknown. In this regard, the observation that *GNAI2/gip2* shows increased expression in advanced ovarian cancers is quite significant as it suggests the possibility that *gip2* could play a critical, if not unique, role in advanced ovarian cancers. Therefore, we investigated whether *gip2*-dependent transcriptome in ovarian cancer can be probed to identify any novel targets for ovarian cancer therapy. Results from such analysis, presented here, establish the critical role of the *gip2*-dependent transcriptomic network in advanced ovarian cancers. Therefore, we investigated whether *gip2*-dependent transcriptome in ovarian cancer can be probed to identify any novel targets for ovarian cancer therapy. Results from such analysis, presented here, establish the critical role of the *gip2*-dependent transcriptomic network in pathways related to cell survival, proliferation, metastasis, adhesion, and cancer cell metabolism (Table S2 and S3).

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While the individual pathways activated by *gip2* have been cataloged in the past, the extent to which *gip2* is involved in regulating a comprehensive transcriptomic network to facilitate ovarian cancer progression has not been fully understood until now. Our results presented here provide the first evidence to show the synergistic signaling nodes regulated by *gip2* in promoting ovarian cancer growth (Table S2). Functionally, the transcriptomic nodes regulated by *gip2* range from pathways involved in cellular energetics to evading cell death. The genes validated in the HGSOC cells lines OVCAR8 and Kuramochi substantiate this point (Figure 1). *KDR* gene, which encodes vascular endothelial growth factor receptor 2, was shown to promote oncogenic signaling pathways in many different cancers including ovarian cancer [30,31]. Vasoactive Intestinal Peptide, encoded by *VIP*, is known to be involved in autocrine as well as paracrine signaling loop that promotes cancer growth in multiple cancer types [32]. *CCL20* gene encodes the chemokine C-C Motif Chemokine Ligand 20 and it was identified to play a role in metastasis and therapy resistance of ovarian cancer cells [33,34].

In addition to these genes, GO enrichment analysis of the data has identified several novel correlates associated with ovarian cancer growth and progression. Results from GO:CC enrichment analysis are in conformity with the known cellular and signaling locale of GNAI2 in transducing the signals from the membrane-bound LPAR when activated by LPA (Table 1). Similarly, the predicted molecular functions such as molecular transducer activity, signaling receptor activity, transmembrane signaling receptor activity, lipid binding, and protein tyrosine kinase binding, can all be related to the signal-transducing activity of *gip2* (Table 1). More interestingly, GO:BP analysis unravels certain novel aspects of *gip2*-regulated biological processes in ovarian cancer transcriptome. While the role of *gip2* in biological processes such as response to stimulus and intracellular signal transduction has been well characterized, gene enrichment in GO:BPs such as cell proliferation, adhesion, and migration provide a wider glimpse into the transcriptomic landscape activated by *gip2* in ovarian cancer cells. One of the characteristic features of late-stage ovarian cancer is the peritoneal dissemination of ovarian cancer cells, which precedes peritoneal and hematogenous metastasis of ovarian cancer [35]. Biological processes underlying peritoneal dissemination and subsequent metastasis involve cell migration, adhesion, and subsequent tumor angiogenesis [36]. While the molecular components of these pathways have been characterized to a certain extent, the integration of these pathways into a transcriptomic network and the master regulator that modulates the expression of the downstream signaling nodes have remained unknown. Results from the gene enrichment analyses point to such a regulatory role for *gip2* in the transcriptomic reprogramming in ovarian cancer cells.

Organizational features of the *gip2*-orchestrated transcriptomic network are more clearly discernible with the results from the PPI network analysis. Two major clusters can be identified in the PPI network: one that promotes cancer growth through multiple cancer-specific signaling pathways and the other that promotes cancer cell metabolism (Figure 2). The tumorigenic pathways cluster encompasses genes involved in cell proliferation, suppression of apoptosis, and platinum resistance. This cluster is further augmented by signaling circuits involving cytokines, chemokines, and their cognate receptors that are known to play a critical role in cancer cell proliferation, adhesion, invasive migration, and metastasis. The metabolism cluster, on the other hand, primarily includes the genes that encode proteins/enzymes involved in oxidative phosphorylation (Figure 2). Analyses of the hub and bottleneck signaling nodes of the PPI network provide further insight into the *gip2*-regulated transcriptome. The finding that the silencing of *gip2* leads to the reduced expressions of all of these hub and bottleneck genes underscores the critical roles of these nodes in the onco-transcriptome (Figure 3). Functional annotation of these hub and bottleneck genes as well as the Oncoprint analyses of these genes add further support to their tumorigenic roles in ovarian cancer (Table 3; Figure 4A).

The known roles of *VEGFA*, *ACKR3*, *IL6* and *FYN* establish them as the major regu-

factors of cancer pathways cluster in the network. Vascular Endothelial Growth Factor A, encoded by *VEGFA*, was found to be overexpressed in many cancers including ovarian cancer [37–39]. Similarly, autocrine and paracrine signaling pathways stimulated by Interleukin-6, encoded by *IL6*, were shown to be associated with cancer cell proliferation, migration, stemness, and therapy resistance in ovarian cancer [40–41]. Likewise, the signaling pathways activated by CXCR7, a chemokine receptor encoded by *ACKR3*, were shown to be critically involved in the migration and invasive metastasis of multiple cancers [42–43]. Fyn kinase, encoded by *FYN*, stimulates a wide array of oncogenic pathways including cell proliferation, migration, EMT, and therapy resistance pathways including cell proliferation, migration, EMT, and therapy resistance in many cancers [44–46]. Similarly, *CYCS*, *UQCRC1*, and *UQCRC5* genes present themselves as the regulatory nodes in the metabolic cluster of the network. Cytochrome C, encoded by *CYCS*, is a major component of the electron transport chain embedded in mitochondria, and it is tightly associated with the transport vital pathway in normal as well as cancer cells [47]. *UQCRC1* encodes the mitochondrial ubiquinol dehydrogenase I, a core protein in which a partial complex I of the mitochondrial respiratory chain and is part of complex I critically involved in the respiratory chain programming where metabolically varying gene expression [48]. *UQCRC5* is another metabolic gene highly upregulated in cancer [49]. The *UQCRC5* nuclear mitochondrial cytochrome c heme b binding domain full length polypeptide and C-terminal subunit of complex III of the peptide 1 chain is a key subunit of complex III of the electron transport chain and plays a critical role in energy production and oxidative phosphorylation [50–52]. In fact, overexpression of *UQCRC5* has been observed in breast cancer [53], glioblastoma [54], and colorectal cancer [55]. Additionally, *UQCRC5* is also upregulated in various types of cancer [56–58]. The observation that these critical nodes are critical in disorders related to the genetic cluster of hydroxylation of mitochondrial oxidative phosphorylation genes further highlights the significance of the recent findings that mitochondrial oxidative phosphorylation is downregulated in cancer [59–62]. Thus, the comprehensive analysis [55–57] of the comprehensive regulation of the genomic network or regulated by epigenetic factors, the activation of the transcriptional network suppresses cell metabolism, suppression of cell death, angiogenesis, metastasis, and tumor growth leading to aggressive cancer growth and progression (Figure 5).

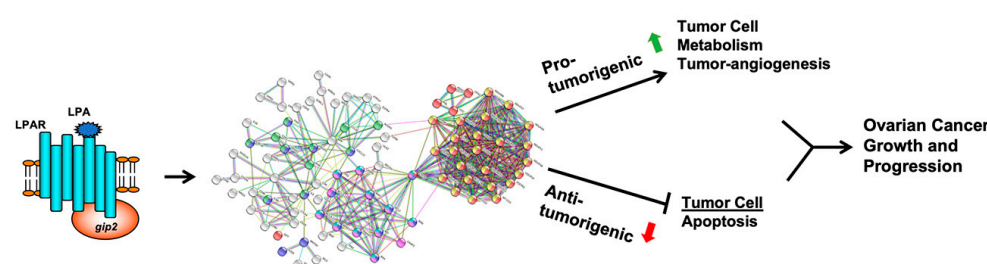


Figure 5. GIP2-regulated onco-transcriptome in ovarian cancer. LPA-LPAR-stimulation or mutational

[†] It should be noted that the limitation of the results is that the transcriptome network depicted here is derived from the data using the single-cell variant deconvolution networks, depicted here that (i) Represent the targets are singulated experimentally in ICSC cell lines (Figures 1 and 6); (2) Represents 50% of the genes validated experimentally in TCGA cell lines (Figures 1 and 9); (3) Represents 50% of ovarian cancer (Table S3); (4) Analysis of these shown to be dysregulated in many cancers including ovarian cancer (Table S3); (5) Analysis of these genes in TCGA ovarian cancer dataset indicated that 61 of these genes showed increased expression in ovarian cancer (Table S4); and (6). Forty of these genes

genes in TCGA ovarian cancer dataset indicated that 61 of these genes showed increased expression in ovarian cancer (Table S4); and (4). Forty of these genes showed co-occurrence in their expression profiles (Table S5) provide external validation of the potential role of these network genes in HGSOC cells and the ovarian cancer patient subgroup. The identification of *CYCS*, *VEGFA*, *IL6*, *UQCRC1*, *UQCRFS1*, *COX5B*, *ACKR3/CXCR7*, and *FYN* as pro-tumorigenic nodes designates them as the novel and potentially druggable targets for effective targeted adjuvant therapy for ovarian cancer. In this context, the role of *UQCRFS1* as a hub node warrants special mention. The query of this gene in cBioPortal indicated that *UQCRFS1* is either amplified or overexpressed in 26% of ovarian cancer patients (Figure 4A). More strikingly, the cBioPortal analysis indicates further that the aberrant expression of *UQCRFS1* could be correlated with the reduced overall survival of ovarian cancer patients (Figure 4B). While the therapeutic potentials of *VEGFA*, *ACKR3*, and *IL6* have been already investigated or exploited [43,58,59], our analysis presented here points to *UQCRFS1* as a, thus far unidentified, potential node for the development of novel therapeutics.

5. Conclusions

In summary, the results presented here provide a paradigm in which *GNAI2/gip2*-dependent transcriptome promotes aggressive cancer growth during advanced stages of ovarian cancer through the gene network that stimulates cell metabolism, invasive metastasis, tumor angiogenesis along with the suppression of cell death. Considering the late-stage expression profile of *GNAI2/gip2* in ovarian cancer, the hub and bottleneck nodes identified here, especially the metabolic signaling nodes such as *UQCRFS1*, should provide newer targets for the development of second-line targeted therapy for advanced ovarian cancers.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/biom11081211/s1>, Table S1: RT-qPCR Primers; Table S2: *Gip2*-dependent Genes Downregulated upon Silencing of *gip2*; Table S3: Oncogenic Profile of *gip2*-dependent Genes; Table S4: Network Gene Alteration Frequency in Ovarian Cancer Patients; Table S5: Co-occurrence of Network Genes in Ovarian Cancer Patients.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the University of Oklahoma (protocol code 9599; 08/27/20).

Data Availability Statement: The microarray data presented in the present study are deposited at the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/> accessed on 21 July 2021); accession no. GSE173214. Oncoprint data supporting the reported results used the Ovarian Serous Cystadenocarcinoma (TCGA, Firehose Legacy) dataset available at <https://www.cbioportal.org> (accessed on 21 July 2021).

Conflicts of Interest: The authors declare no conflict of interest.

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