MicroRNA-30c-2* Expressed in Ovarian Cancer Cells Suppresses Growth Factor–Induced Cellular Proliferation and Downregulates the Oncogene BCL9

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Abstract

MicroRNAs (miRNAs) are small noncoding RNAs that function as master regulators of posttranscriptional gene expression with each miRNA negatively regulating hundreds of genes. Lyosphosphatic acid (LPA) is a mitogenic lipid present within the ovarian tumor microenvironment and induces LPA receptor activation and intracellular signaling cascades like ERK/MAPK, leading to enhanced cellular proliferation. Here, we show that in SKOV-3 and OVCAR-3 cells, LPA stimulation at concentrations ranging from 1 nmol/L to 20 μmol/L for 30 to 60 minutes increases miR-30c-2*, and this effect is mediated through a combination of receptors because knock down of multiple LPA receptors is required for inhibition. The epidermal growth factor and platelet-derived growth factor also increase miR-30c-2* transcript expression, suggesting a broader responsive role for miR-30c-2*. Thus, we investigated the functional role of miR-30c-2* through ectopic expression of synthetic miRNA precursors of mature miRNA or antagonist transfection and observed that microRNA-30c-2* reduces, and the antagonist enhances, cell proliferation and viability in OVCAR-3, cisplatin-insensitive SKOV-3 and chemoresistant HeyA8-MDR cells. Ectopic expression of miR-30c-2* reduces BCL9 mRNA transcript abundance and BCL9 protein. Consistent with this observation, miR-30c-2* ectopic expression also reduced BCL9 luciferase reporter gene expression. In comparison with IOSE cells, all cancer cells examined showed increased BCL9 expression, which is consistent with its role in tumor progression. Taken together, this suggest that growth factor induced proliferation mediates a neutralizing response by significantly increasing miR-30c-2* which reduces BCL9 expression and cell proliferation in SKOV-3 and OVCAR-3 cells, likely as a mechanism to regulate signal transduction downstream. Mol Cancer Res; 9(12); 1732–45. ©2011 AACR.

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs, 18 to 25 nucleotides in length, that negatively regulate gene expression through perfect or imperfect base pairing with the 3′-UTR (untranslated region) of specific target mRNAs, facilitating mRNA degradation, or blocking protein translation, respectively. It is estimated that one miRNA is capable of regulating the expression of several hundred genes through this posttranscriptional process, which ultimately results in protein reduction (1). The mere existence of miRNA in biological systems reveals complex layers of epigenetic regulation govern the outcome of cellular signaling pathway cascades, after the initiation of the primary activating signal and subsequent downregulation of the initiating protein. It also reveals the possibility for an alternative therapeutic strategy for exploitation among disease states, particularly cancer, where multiple pathways are aberrant. However, elucidating the molecular function and systematic influence of individual miRNAs is challenging but has highlighted critical processes regulated through transcript-miRNA interactions (2).

In oncogenesis the deregulation of miRNA results in altered tumor suppressor expression through translational inhibition and/or mRNA target degradation; thus an aberrant class of oncomirs that misregulate gene expression occurs through errors in sequence complementation (3) or a dual role in both processes—inhibition and degradation (4). Among expression profiles of human cancers, miRNA performed significantly better than mRNA gene expression transcriptional profiles to classify types (5), further under-scoring their importance in malignancy and their tissue specificity. In particular, miRNAs are hypothesized to play an important regulatory role in ovarian cancer disease where observations highlighted the deregulation of miRNAs (6). Comparing epithelial ovarian carcinoma with immortalized ovarian surface epithelial cells, the vast majority of miRNA...
changes occur through downregulation (88.6%) by a combination of genomic copy number loss and epigenetic silencing and these differences are sufficient to distinguish malignant versus nonmalignant ovarian epithelium (7).

Lysophosphatidic acid (LPA) is a potent, growth factor-like lipid component of normal circulation that mediates a multitude of cellular responses, including cellular proliferation, through LPA receptor-stimulated activation like ERK/MAPK. LPA accumulates within peritoneal ascites fluid that is abnormally produced in ovarian cancer by the cells (8), whereby it enhances tumorigenicity, aggressiveness (9), and correlates with worsened outcomes (10). Its actions are exerted through a family of transmembrane G protein-coupled receptors that transduce the extracellular LPA signal through G proteins to activate intracellular second-messenger signaling cascades, subsequent protein phosphorylation and then nuclear translocation of transcription factors that regulate gene transcription. There are several known mechanisms that reduce the extracellular LPA signal coming through the LPA receptors, such as clathrin-mediated endocytosis with subsequent receptor trafficking (11) and regulators of G-protein signaling (RGS) proteins that terminate the response (12); however, these do not explain the mechanism terminating ongoing mRNA transcript and protein synthesis which results from transcription factor activation.

Because miRNAs bind to mRNAs and ultimately reduce mRNA translation into protein, we hypothesized that growth factors like LPA must induce specific miRNAs to terminate the original signal that started with the cell surface receptor. Moreover, miRNAs are frequently deregulated in ovarian cancer and because LPA enhances tumorigenicity in this malignancy (10), we also questioned whether LPA elicited a specific miRNA response that is either reduced or eliminated during tumorigenesis, contributing to ovarian malignancy. Because LPA potently induces cell growth, proliferation, viability, and survival (13–16), this effect would most likely be observed through precursor miRNA counter induction to reduce these specific cellular processes.

Although abundant evidence links abnormal LPA production and secretion to pathology, to date there are no reports on miRNA regulation by LPA. Moreover, there is no distinct evidence revealing the mechanism or functional role of miR-30c-2* in ovarian cancer. Because we observed that LPA significantly induces the expression of miR-30c-2* within 60 minutes, we set out to clarify its role. Herein, we observed significant reductions in cell proliferation and viability occur after ectopic expression in several cell lines, including both chemoresistant and chemosensitive ovarian cancer cells. The effects of the minor strand, miR-30c-2*, throughout our analysis seemed to be more potent than the major strand, miR-30c, and this observation is consistent with recent studies suggesting that the minor strands can have major roles in tumor suppression and oncogenes (17). Furthermore, we observed that introduction of either miR-30c-2* leads to a decrease in the mRNA transcript and protein abundance of BCL9, an oncogene that increases cell proliferation (18). Here, we provide evidence that after LPA stimulation or the expression of miR-30c and miR-30c-2* in ovarian cells, the mRNA transcript and oncogenic protein BCL9 is decreased, which likely reduces the LPA growth factor-induced signal, counteracting cellular proliferation and viability.

Materials and Methods

Materials and reagents

LPA (18:1, 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) and VPC31144(s) (N-((1S)-2-hydroxy-1-(phosphonooxy)methyl)ethyl)(9Z)octadec-9-ename, ammonium salt) were purchased from Avanti Polar Lipids Inc. Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) were purchased from Neuromics. OVCAR-3, SKOV-3, and A549 cells were acquired from American Type Culture Collection and maintained in Cellgro RPMI (Mediatech, Inc.) supplemented with 10% FBS (Sigma). The HeyA8-MDR cell line, a taxane-resistant line generated by the long-term exposure to paclitaxel, was a kind gift from Dr. Isaiah J. Fidler (Department of Cancer Biology, University of Texas MD Anderson Cancer Center, Houston, TX) and is verified and presented elsewhere (19). HeyA8-MDR cells are maintained in RPMI 1640 medium with 300 ng/mL paclitaxel with 15% FBS. IOSE cells are maintained in 1:1 mixture of medium 199 and MCDB 105 (Sigma-Aldrich), supplemented with 10% FBS. Cisplatin and paclitaxel were purchased from Sigma-Aldrich.

Whole genome screening of miRNA expression

OVCAR-3 cells were either unstimulated or treated with 5 μmol/L LPA for 1, 6, and 24 hours prior to extraction of small RNA using mirVANA kit (Ambion). The quality of the small RNA was assessed using Agilent chips in gel-dye matrix and running the chips for 35 minutes in the Bioanalyzer 2100 (Agilent Technologies) following the manufacturer’s protocol. Only samples with scores between 9 to 10 were processed further for analysis. The miRNA generated was then reverse transcribed using Megaplex RT Primers, a mixture of miRNA-specific stem-loop primers (Applied Biosystems now by Life Technologies). After addition of TaqMan Universal Master Mix II to the samples, these samples were then carefully pipetted and loaded into TaqMan Array MicroRNA micro fluidic 384-well cards (Applied Biosystems) using centrifugation. The micro fluidic cards were run using real-time PCR and the 7900HT Fast Real-Time PCR System (Applied Biosystems).

siRNA knockdown of LPA receptors

SKOV-3 cells were transfected with Dharmacon SMART pools of siRNA reagents (ThermoScientific/Dharmacon, Inc.) targeting the individual LPA receptors as previously described (14) using Dharmafect reagent (ThermoScientific/Dharmacon) and following the protocol provided by the manufacturer.

Cell viability

OVCAR-3, SKOV-3, or HeyA8 cells were examined for viability as previously described (14, 20) using CellTiter
Blue reagent (Promega). In cisplatin and paclitaxel treatment experiments, the cells were treated with chemotherapy after 24 hours of transfection. The cell viability was examined after 48 hours of drug treatment. Images of individual wells were captured using a 12 megapixel Nikon Coolpix camera (Southern Microscope, Inc.).

**Ectopic expression of synthetic miRNAs or antagonirs**

The OVCAR-3 or SKOV-3 cells were plated $8 \times 10^3$ into 96-wells and 2.5 $\times 10^5$ into 6-wells, and incubated overnight. Cells were transfected with either miR-30c (UUGAAACAUCCUCACUCUCAGC), miR-30c-2* (CUGGGAAGGCGUGUUACUCU), anti-miR-30c, anti-miR-30c-2* constructs or Negative Control (Pre-miR miRNA Precursor Negative Control #1; Ambion) where indicated. The negative control does not target any known miRNA within the human transcriptome. In addition, transfects used either DharmaFECT 2 (ThermoScientific/Dharmacon) or Neo-FX (Ambion) according to the manufacturer’s instructions.

**Quantitative real-time PCR**

miR-30c-2* expression was assessed using quantitative real-time PCR. Cells were transfected as described prior to extraction of small RNA using the mirVANA kit (Ambion). Total and miRNA-specific cDNA was generated using SuperScript III First-Strand Synthesis System from Invitrogen. RT-PCR Taqman primer sets for miR-30c and miR-30c-2* were purchased from Exiqon or Applied Biosystems and protocols provided by the manufacturer were used to assess miRNA levels. In other experiments, mRNA transcript levels were assessed using quantitative real-time PCR and SYBR Green PCR Master (Applied Biosystems) after extracting total RNA using TRI reagent (Ambion). The primers used were based on algorithm-generated sequences from Primer Bank (http://pga.mgh.harvard.edu/primerbank/; ref. 21) or where previously described (10). The primers used were as follows: BCL9 (forward 5’-AGGAGCAGGTATTTTCGCC-3’ and reverse 5’-GGTGTCACTGATGTGGTG-3’), CREM (forward 5’-CTGTTGACATGCCAATATCC-3’ and reverse 5’-GCACAGCCAACACAGTTTTTCAA-3’), and SMPL-1B (forward 5’-AACTCAGTGCTAATCTTCA-3’ and reverse 5’-GCATACAGAAGTGTTGAAG-3’). The reactions were normalized using the housekeeping gene, β2-microglobulin, and calculations were done according to previous methods (22).

**Cell proliferation and cell count assessment with high-throughput screening**

SKOV-3 or OVCAR-3 cells were seeded (8 $\times 10^3$) into 96-wells and allowed to attach overnight prior to transfection with miRNA constructs where indicated. The cells were stained after 48 hours of transfection using the Thermo Scientific ArrayScanVTI HCS Reader (Thermo Fisher Scientific/Celldomics). Graphs presented and the statistical tests were generated using either GraphPad Prism (GraphPad Software, Inc.) or Microsoft Excel (Microsoft Corporation). Experiments are done with a minimum of quintuplicate samples and at least 5 images per well are taken and incorporated in the data analysis.

**Gene expression analysis**

For analysis of miR-30c expression among patient datasets, a publicly-available gene expression dataset (GSE10150, N = 45; ref. 23) was downloaded from the NCBI Entrez Gene Expression Omnibus (GEO) DataSets website (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gds) and analyzed as previously described (10). Box plots using the normalized gene expression were created with GraphPad Prism (GraphPad Software, Inc.).

**Luciferase assay**

Approximately $0.8 \times 10^5$ SKOV-3 cells were seeded in a 12-well plate for 24 hours prior to transfection with 50 ng of target sequence (3’-UTR) expression clone (pEZX-MT01-BCL9 UTR-LUC, GeneCopoeia) and 100 nmol/L of miRNA using Lipofectamine 2000 (Invitrogen). Thirty-six hours later, firefly luciferase activities were measured using the Luciferase Assay System (Promega) according to the manufacturer’s protocol and data was recorded on FB12 luminometer (Zylux corporation). Firefly luciferase activity was then normalized with protein concentration in the same well.

**Immunoblotting**

Approximately $5 \times 10^5$ cells were seeded, transfected and harvested after 48 hours prior to being lysed in radiommunoprecipitation assay buffer. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted using a primary antibody targeted against BCL9 (ab54833, Abcam) and HRP-conjugated secondary antibody (GE Healthcare). Bands were then visualized using SuperSignal Chemiluminescent substrate (Pierce). Densitometry analysis was done using ImageJ software (NIH).

**Statistical analysis**

Statistical differences measuring the significance of experimental data were determined using unpaired Student t test. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ indicate the levels considered significant.

**Results**

Our commencement strategy for investigating the role of miRNAs in proliferation was to identify candidate miRNAs induced upon stimulation with LPA, a mitogenic lipid agonist that mediates its actions through the LPA receptors, in ovarian cancer cells. To initiate the candidate search, we treated OVCAR-3 cells with 5 μmol/L LPA and...
performed whole-genome miRNA screening using TaqMan Array micro fluidic array cards (see methods). The goal was to identify miRNA expression that was significantly altered (Fig. 1A). Although approximately 49% of all miRNAs examined on the array were undetectable in OVCAR-3 cells, there were more than 100 combined changes (>1.5-fold increase) observed in the presence of LPA (Fig. 1B). Twenty-six percent of the observed changes were decreases in miRNA expression, whereas 20% of the miRNAs detected remained unaffected by LPA (Fig. 1C). In contrast, 5% of the total miRNAs increased in expression at 1 hour and the most robust changes were among the miR-30c family whereby miR-30c-2” increased dramatically (Fig. 1A) and the major strand, miR-30c, also increased, although to a lesser extent. As a control, we measured miRNA in unstimulated conditions and observed that OVCAR-3 and SKOV-3 cells used in this study express detectable levels of miR-30c and miR-30c-2" (Table 1).

Next, we verified transfection specificity conditions in SKOV-3 and OVCAR-3 cells (Supplementary Fig. S1), which was necessary to further assess the function of miR-30c-2". Although the changes are quite dramatic (miR-30c Cq value from 19 to 14 and miR-30c-2" from 29 to 18), they may not be indicative of the relative silencing or biological activity of the miRNA, but do confirm the presence of mature miRNA introduced into the cell. Using these SKOV-3s and OVCAR-3s along with immortalized ovarian surface epithelial cells (IOSE) and A549 lung cancer cells, we compared viability after transient transfection (Fig. 2). Both ovarian cancer cell lines significantly (***, P < 0.001) decrease in viability after transfection with miR-30c-2". Although the IOSE or A549 cells showed no similarity in reduction, viability was enhanced in the presence of antagonirs.

Because ectopic expression of miR-30c-2" reduces cellular viability and this assay reflects both total cell number and metabolic rate, it was important to establish whether miR-30c-2" affected cell proliferation. Given that crystal violet staining is an indirect assessment method and manual cell counting may introduce bias, we opted to use a direct nuclear stain and mechanically assess cell proliferation using high-throughput imaging and quantification. We observed that miR-30c-2" significantly reduces the average number of BrdU positive OVCAR-3 cells (Fig. 3A) and the antagomir has the opposite effect (Fig. 3B, P < 0.001 vs. miR-negative target control). Among the cells with BrdU-positive staining, the average fluorescence intensity was lower among those transfected with miR-30c-2" than all other conditions (Fig. 3C, P < 0.001 vs. miR-negative target control). Interestingly, we observed a reduced number of cells after transfection with miR-30c-2" in both SKOV-3s (Fig. 3D, P < 0.01 of mock vs. miR-30c and P < 0.05 of mock vs. miR-30c-2") and OVCAR-3s (Fig. 3E, P < 0.01 of mock vs. miR-30c-2"), but miR-30c was not reproducibly significant. This was confirmed using immunofluorescence and staining the nucleus to observe the number of cells present (Fig. 3F). Through the

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**Figure 1.** LPA enhances microRNA expression in OVCAR-3 cells. OVCAR-3 cells were treated with 5 μmol/L LPA for 1, 6, or 24 hours prior to miRNA extraction and measurement using TaqMan Array MicroRNA micro fluidic cards. A, several miRNAs exhibited increases more than 1,000-fold at 1 hour. Although approximately half of known miRNAs in the human genome are undetectable in OVCAR-3 cells, at each time point increases and decreases were observed, compared with unstimulated controls. The data is presented as a bar graph (B) that indicates the number of miRNAs affected and also as a pie graph (C) to illustrate the percentage of changes.
alteration of proliferation miR-30c-2 may have decreased the plasma membrane integrity and enhanced general permeability, facilitating entry of fluorophores into normally impermeant cells. As a result, more intense staining is seen upon transfection with miR-30c-2 (far right panels).

To further explore the actions of miR-30c-2 on proliferation, we treated the cells with LPA to stimulate cell proliferation. Similar to the previous observations using the whole genome screening approach (Fig. 1), LPA stimulation (0.01–20 μmol/L) increased miR-30c and miR-30c-2 expression in both OVCAR-3 and SKOV-3 cells (Fig. 4A). To determine whether the change in expression was specific for LPA or a result of general growth factor stimulation, we assessed the expression of miR-30c-2 in the presence of 1 nmol/L LPA, EGF or PDGF. The concentrations (1 nmol/L) were deliberately low to reduce agonist bias overwhelming the system. All 3 growth factors increased miR-30c-2 transcription after 60 minutes in SKOV-3 cells (Fig. 4B), although LPA was the only growth factor showing a significant increase between 30 and 60 minutes. We confirmed the increase using VPC3114 (S), a specific agonist of the LPA1/3 receptors (Fig. 4C). All 3 growth factors increased miR-30c after 60 minutes in SKOV-3 cells. Taken together, this suggests that miR-30c-2 is specifically increased after LPA stimulation, which is suggestive that growth factor-responsive downstream events are modulated by these miRNAs.

Although increased miR-30c and miR-30c-2 expression can occur after stimulation by multiple growth factors, we next assessed the mechanism through which LPA (and VPC3114) induces this response. Through sequential knockdown of Edg-family receptors LPA1, LPA2, or LPA3 using siRNA, we observed that individual LPA receptor knockdown does not prevent LPA-mediated increases of miR-30c expression (Fig. 4D). In contrast, knocking down a single receptor appears to magnify the effect of LPA (1 μmol/L) stimulated increase of miRNA expression, particularly with miR-30c (compare siLPA1, siLPA2 or siLPA3 to siNeg Cntrl). Only knockdown of the LPA3 receptor resulted in a statistically significant increase in miR-30c-2 expression.

**Table 1. miRNA expression and Ct values in untreated cells**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>miR-30c-2 Ct</th>
<th>miR-30c Ct</th>
<th>miR-30c-2 Ct</th>
<th>miR-30c Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVCAR-3</td>
<td>28.39 ± 3.42</td>
<td>22.16 ± 0.21</td>
<td>20.25</td>
<td>21.23</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>27.40 ± 2.47</td>
<td>19.78 ± 0.80</td>
<td>24.46</td>
<td>19.23</td>
</tr>
<tr>
<td>Hey-A8 MDR</td>
<td>24.52 ± 0.14</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>A549</td>
<td>30.89 ± 0.28</td>
<td>19.84 ± 0.90</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>IOSE</td>
<td>27.63 ± 0.10</td>
<td>17.38 ± 0.02</td>
<td>N/D</td>
<td>N/D</td>
</tr>
</tbody>
</table>

NOTE: For different cell types, the raw Ct values are presented as the average ± SEM and reflect 3 to 6 repeated experiments done using triplicates. For the 5 micromolar induction with LPA for 1 hour, this value reflects the result produced from the high-throughput microRNA screening experiment done in OVCAR-3 cells.

Abbreviation: N/D, no data.

**Figure 2.** MicroRNA-30c-2 expression reduces viability of OVCAR-3 and SKOV-3 cells. Cells were plated and transfected with the indicated miRNA or antagonim prior to the assessment of any changes in cell viability. *, P < 0.05; **, P < 0.01; and †††, P < 0.001 versus miR-negative control.
We did not include the purinergic-family receptors LPA4 or LPA5 in this analysis since they are expressed at very low levels in SKOV-3 cells (data not shown). Interestingly, knocking down LPA1, LPA2, or LPA3 in the absence of LPA stimulation decreases the expression level of both miR-30c and miR-30c-2* (Fig. 4E). This further suggests a reduced requirement for miR-30c and miR-30c-2* without LPA.

As an alternative approach, we evaluated knocking down combinations of LPA receptors to determine whether combinations would affect miRNA expression in the presence of LPA (1 μmol/L). We observed a significant reduction among both miR-30c and miR-30c-2* expression, compared with siNegative control (Fig. 4F). This suggests that reducing the LPA-initiated signal transduction through available G protein-coupled receptors decreases the miRNA response. The

**Figure 3.** MiR-30c-2* expression reduces cellular proliferation in OVCAR-3 and SKOV-3 cells. A, SKOV-3 cells were plated and transfected to express miR-30c-2* or negative control miRNA. BrdU was pulsed into the cells prior to fixation and staining. Images were acquired using the ArrayScan HCS Reader. The average number of BrdU-positive cells (B) is quantified along with the average fluorescence intensity of positive cells (C). **P < 0.001 versus control by Student t test. The number of cells per microscopic field was also measured by staining with Hoechst and mechanically assessing the number of stained cells per field (N = 5 per experiment and the average number of fields = 8). The results are quantified in SKOV-3 cells (D) and OVCAR-3 cells (E) and visualized (F). **P < 0.01 of mock vs. miR-30c-2* and *, P < 0.05 of mock versus miR-30c-2* by Student t test. Representative images of each condition with color overlay to show staining using Hoechst (blue), plasma membrane permeability dye (green) and mitochondrial transmembrane potential (orange).
SKOV-3 cells were treated with LPA, EGF, or PDGF (all 1 nmol/L) for 30 and 60 minutes prior to the assessment of transcriptional levels. Concentrations of 18:1 LPA for 60 minutes and assessed for changes in the transcription levels. B, to assess the specificity of LPA receptors in SKOV-3 cells (data not shown), which could possibly be due to transcript variants reported among cancer cells. We began these experiments using SKOV-3 cells, a cisplatin-resistant ovarian cancer cell line. We selected based on their reduced chemosensitivity or chemoresistance. Furthermore, the viability reduction is not specific to the chemotherapeutic agent because both a platinum and taxane agent were independently tested. This effect is not indicative of a transient reversion to chemosensitivity because cells without drug treatment, but with ectopic expression of miR-30c-2*

siRNA’s ability to reduce each target was approximately 90% for LPA1 and LPA3 and more than 50% for the LPA2 receptor in SKOV-3 cells (data not shown), which could possibly be due to transcript variants reported among cancer cells (24).

MicroRNA-30c-2* consistently reduced cell proliferation, which is biologically analogous to the goal of chemotherapy. Thus, we questioned whether transfection with miR-30c-2* could prime or synergize with chemotherapy to further reduce viability among chemoresistant ovarian cancer cells. We began these experiments using SKOV-3 cells, a cisplatin insensitive cell line, that requires much higher doses of cisplatin than sensitive cells to effectively eliminate proliferation in culture (25). Treating cells with cisplatin (2.5 μmol/L) shows a clear, visible difference in cell morphology between controls and cells with miR-30c-2* (Fig. 5A). Control cells (top image) and cells transfected with anti-miR-30c-2* (middle image) seem distinct compared with those ectopically expressing miR-30c-2* with cells rounding and blebbing (bottom image; cells near the top left corner and bottom left). MiR-30c-2* nearly eliminates cisplatin-induced cytotoxicity, because alone miR-30c-2* reduces viability below cisplatin-induced levels (Fig. 5B). The average overall viability of cisplatin-treated, miR-30c-2*-transfected cells was approximately half of control cells (***, P < 0.001, control or anti-miR vs. miR-30c-2*, Fig. 5C). This is likely due to the ability of miR-30c-2* to reduce cellular proliferation.

To determine whether the observed differences in the presence of cisplatin was a specific proliferative effect on DNA binding and adduct formation, we assessed viability in HeyA8-MDR cells that are resistant to paclitaxel (25) after ectopic expression of miR-30c-2*. The HeyA8-MDR cells retain a modest response to paclitaxel (Fig. 5D), although miR-30c-2* significantly reduced viability compared to the control and antagonirs (P < 0.001, miR-30c-2* vs. anti-miR or control, Fig. 5E). Taken together, the data show miR-30c-2*

D, E, F: Normalized relative ratio (%) of no LPA

Figure 4. (Continued) Growth factor signaling induces miR-30c and miR-30c-2* expression. A, SKOV-3 or OVCAR-3 cells were treated with the indicated concentrations of 18:1 LPA for 60 minutes and assessed for changes in the transcription levels. B, to assess the specificity of LPA receptor agonists, SKOV-3 cells were treated with LPA, EGF, or PDGF (all 1 nmol/L) for 30 and 60 minutes prior to the assessment of transcriptional levels. C, SKOV-3 cells were transfected and cell viability was measured in the absence or presence of VPC3114(S), an LPA receptor agonist. D, E, F: similar experiments were done using combinations of siRNA against the LPA receptors in the presence of LPA (1 μmol/L, 60 minutes).
In OVCAR-3 cells, similar results were observed with miR-30c-2*. Transient transfection reduced average viability in the presence of both cisplatin and paclitaxel (Fig. 5F, P < 0.05, cisplatin miR-30c-2* vs. control and paclitaxel miR-30c-2* vs. control).* MicroCosm Targets indicates that miR-30c-2* has 1019 predicted and putative targets, which is within the...
range for other miRNAs that varied between 514 (hsa-miR-17; http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) to 1,416 (hsa-miR-340). We sought the identification of a mechanism for miRNA-mediated cell proliferation reduction, which is a considerable challenge. In our approach, we used transcript predictions from MicroCosm and performed extensive qRT-PCR on miR-30c-2* transfected cells. Among transcripts whose gene products regulate proliferation and showed a consistent pattern whereby the miR-30c-2* expression significantly decreased the transcript in SKOV-3, OVCAR-3 and HeyA8-MDR cells, we observed modest reductions in CREM (cAMP responsive element modulator) and significant and consistent reductions is BCL9 (B-cell CLL/lymphoma 9) (Fig. 6A). The sphingomyelin phosphodiesterase SMPDL3B is also shown, but was inconsistent between all 3 cell lines.

To confirm that BCL9 is reduced upon ectopic miR-30c-2* induction, we conducted transient transfections and assessed BCL9 protein levels. Using immunofluorescence we observed a reduction in the intensity of BCL9 compared with negative control (Fig. 6B), whereby the fluorescent intensity of BCL9 was significantly reduced (P < 0.05) with miR-30c-2* (Fig. 6C). Western blotting also confirmed these results by showing a reduction in BCL9 with miR-30c-2* transfection (Fig. 6D). Using a luciferase reporter vector, we confirmed the interaction between miR-30c-2* and BCL9 (Fig. 6E), as predicted. In comparison with immortalized ovarian surface epithelial cells, all of the cancer cell lines we tested had increased BCL9 transcript expression levels (Fig. 6F), underscoring its role as an oncogene involved in proliferation (18). We also compared the reduction in BCL9 in cells treated with the mitogenic agent LPA (1 μmol/L) and observed a similar capability as transient

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**Figure 6.** BCL9 protein abundance and mRNA transcript are reduced upon ectopic expression of miR-30c-2*. A, OVCAR-3, SKOV-3, and HeyA8-MDR cells were transfected with miR-30c-2* before processing samples for quantitative RT-PCR and measuring the transcriptional activity of selected transcripts: CREM (cAMP responsive element modulator), BCL9 (B-cell CLL/lymphoma 9), and SMPDL3B (sphingomyelin phosphodiesterase). Results were normalized to β2-microglobulin and reflect the average of triplicate samples. Results shown are combined from repeated experiments and analyzed by Student’s t test. ***, P < 0.001 of miR-30c-2* versus normalized β2-microglobulin control. B, OVCAR-3 cells expressing miR-30c-2* were assessed for BCL9 expression and the average BCL9 intensity (N = 15) was mechanically quantified (Q). P < 0.05 of control versus miR-30c-2* by Student’s t test. C, Immunoblotting of OVCAR-3 cells confirms the decrease in BCL9 protein upon expression of miR-30c-2*. E, SKOV-3 cells were transfected with the expression clone pEZX-MT01-BCL9 UTR/luc and miRNA as indicated prior to the assessment of luciferase activity. **, P < 0.01 of pEZX-MT01-BCL9 UTR versus expression vector transfected with miR-30c-2*. F, BCL9 was measured in IOSE, A549, OVCAR-3, SKOV-3, and HeyA8-MDR cells using quantitative real-time PCR. The data compare the cancer cells to normalized values from IOSE cells.
transfection of miR-30c-2/C3. This further suggests that LPA elicits this response as a means of counteracting the stimulation of cell proliferation.

Finally, we wanted to know whether a reduction in the relative expression of either miR-30c-2/C3 or miR-30c is relevant in ovarian pathology. For this we downloaded an expression dataset (GSE10150, N = 45; ref. 23) from the NCBI Entrez Gene Expression Omnibus (GEO) DataSets website and assessed miR-30c among the specimens. Although miR-30c-2/C3 was not included in the dataset, we observed a significant range in expression of miR-30c among high-grade serous epithelial ovarian carcinoma (Fig. 6H). This suggests that the expression of miR-30c-2/C3 is even lower (Table 1), although this is speculative because miR-30c-2/C3 was not included in the dataset. In addition, we observed a significant (P < 0.001) reduction in miR-30c expression among specimens isolated from ascites, compared with serous specimens. This is suggestive that miR-30c and/or miR-30c-2/C3 may be reduced in specific microenvironments, like ascites, in ovarian pathology and could prevent regulation of cell proliferation, particularly in the presence of mitogenic agents like LPA. We tested the effect on cell viability after ectopic expression of siBCL9, but observed no change in untreated conditions (Fig. 6I). In contrast, when SKOV-3 cells were treated with increasing doses of cisplatin, the reduction in BCL9 translated to a functional decrease in viability that was significant compared with the control (Fig. 6J).

To further confirm the interaction between miR-30c-2/C3, LPA and BCL9, we conducted a series of experiments using antagomirs. Increasing concentrations of anti-miR-30c-2/C3 at 20, 50, and 100 nmol/L reduced the expression of miR-30c-2/C3 and blunted the expression in the presence of LPA (Fig. 7A), even at higher concentrations of LPA (10 μmol/L; Fig. 7B). The miR-30c-2/C3 antagomir transfection also increased the expression of BCL9 (Fig. 7C). Transient transfection with anti-miR-30c did not show consistent results on the expression of BCL9 (Fig. 7D),

Figure 6. G, SKOV-3 cells either transfected with miR-30c-2/C3 or treated with LPA (1 μmol/L) were collected and processed for immunoblotting and assessment of BCL9 protein. The numbers reflect the level of BCL9 expression relative to miR-negative control. H, the patient dataset, GSE10150, N = 45, was downloaded from the NCBI Entrez Gene Expression Omnibus (GEO) DataSets website and mined for expression levels of miR-30c (miR-30c-2/C3 was not included in the dataset) among patient specimens of ovarian malignancy. The box plot shows the relative variation in miR-30c expression. Average data representing expression of miR-30c in ascites fluid was compared with serous carcinoma and serous high grade using the Student t test and ***, P < 0.001.

I, OVCAR-3 and SKOV-3 cells ectopically expressing either negative control siRNA or siBCL9 were examined for cell viability and no change was observed. J, SKOV-3 cisplatin insensitive cells were treated with cisplatin in the doses indicated after ectopic expression of either siRNA for a negative control or BCL9. * P < 0.05 using the Student t test comparing the same dose of siNeg versus siBCL9.
although anti-miR-30c did reduce miR-30c expression in the presence of LPA (1 μmol/L; Fig. 7E).

**Discussion**

Herein, we present a novel study implicating mitogenic LPA signaling in the generation of a miRNA response, likely as a mechanism to counteract growth factor induction of cellular proliferation. Foremost, we provide evidence showing that miR-30c-2* reduces cellular proliferation in OVCAR-3 and SKOV-3 cell lines. In addition, LPA induces the expression of miR-30c-2* approximately 30 to 60 minutes after receptor-mediated stimulation. The data supports LPA receptor involvement since miR-30c-2*...
expression can by blunted after combination siLPA2 and siLPA1/3 transfection or siRNA against individual LPA receptors in the absence of any additional LPA stimulation.

To our knowledge, this study is the first to assess LPA-mediated effects on miRNA; furthermore, it investigates the molecular function of miR-30c-2" in an ovarian cancer cell model, with previous limited studies focusing on this miRNA in a cardiac model and the regulation of p53 and tissue connective growth factor (26, 27). The interpretation of our data of miR-30c and miR-30c-2" function in ovarian cancer, is that these miRNA may be simple compensatory mechanisms to reduce cell proliferation after growth factor stimulation, which can initiate the cell cycle, particularly if cells are in G1 phase.

Although there are more than 1,000 predicted targets that could be affected by each miRNA, we observed an ability of miR-30c-2" to significantly reduce transcriptional levels of CREM and BCL9, the latter of which is an oncogene involved in cell proliferation (18). Our data show reduction in both BCL9 transcript and BCL9 protein levels upon ectopic expression of miR-30c-2", suggesting the likelihood of BCL9 acting as a direct target. Furthermore, the bioinformatic database microRNA.org predicts the direct binding between multiple miR-30 family members and BCL9. Recent work suggests the BCL9 is an oncogenic component of the Wnt pathway with the capability to enhance tumor progression (EMT transitioning, migration, invasion, and metastatic potential) and cell proliferation through regulation of cyclin D1 expression, while knocking down BCL9 increases survival outcomes among xenograft mouse models of multiple myeloma and colon cancer (18). Interestingly, knocking out CREM in germ cells results in a 10-fold increase in apoptotic cells (28), likely due to an inability of CREM to participate in regulating the cell cycle (29).

Our unpublished results suggest that miR-30c-2" is capable of modulating APOBEC3B transcripts that are induced by LPA (10). Although G protein-coupled receptors have mechanisms to downregulate signal transduction, including receptor internalization and trafficking to the lysosomes, our data suggest that the mitogenic LPA signal is also regulated at the transcriptional level. Receptor signal transduction leads to protein phosphorylation and nuclear translocation where transcription factors are activated and bind to DNA to initiate transcription necessary for the cell cycle and proliferation. Reducing the intracellular transcriptional outcomes resulting from the LPA-mediated signal may account for the counter signal and a mechanism to reduce cell proliferation initiated by mitogenic growth factors.

In contrast, without the ability to downregulate transcription an LPA or other growth factor signal could be magnified and potentiated. Speculative extrapolation of the observation that miR-30c expression is decreased in ascites could indicate that cells in ascites are more responsive to growth-factor enhancement of cell proliferation due to autocrine and paracrine signaling coupled with decreased expression of miRNAs, like miR-30c-2", that counteract proliferative signals and halt transcription of factors promoting proliferation. The impact of LPA signal amplification on the system could have enormous effects on tumor aggressiveness (9) and outcomes (10).

Recent work identified that miR-30 family members, including miR-30c, can suppress p53 expression (26). Although this result is highly consistent with the observation that LPA reduces the nuclear and cytoplasmic abundance of p53 through receptor-mediated signaling in lung and hepatocellular carcinoma (30), it is not directly relevant to ovarian cancer because p53 is nonfunctional. The majority of ovarian tumors contain mutant p53 alleles in their genome and most of these occur through a point mutation creating missense codons, which affects the DNA-binding domain of the p53 protein (31). Furthermore, we and others, have observed an overabundance of mutant, nonfunctional p53 protein present in OVCAR-3 cells (data not shown).

Although the function of miR-30c or miR-30c-2" in tumorigenesis has not been explored, more recent literature has investigated miR-30c in rat cardiac cells, rat ventricular myocytes and rat fibroblasts where it regulates connective tissue growth factor and p53 (26, 27). Using predictive algorithms, bioinformatic programs like MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), suggest miR-30c is capable of regulating 1,056 gene transcripts, implying that while these observations have significant merit, they are merely scratching the surface of miR-30c's capabilities. In addition, reports have yielded incongruent observations about the expression of miR-30c and hypothesized a "direct involvement" in chemoresistance based on the pattern of expression (32). This publication indicated that miR-30c expression is downregulated in chemoresistant ovarian cancer cells, which compared parental A2780 cells against A2780 variants resistant to cisplatin or paclitaxel (in the presence or absence of cyclosporine; ref. 32). Another report indicated that miR-30c was expressed 1.41-fold higher among stage III ovarian cancer specimens from patients resistant to platinum-based chemotherapy (n = 12) versus patients with chemosensitivity (n = 25) to platinum agents (33).

The gene for miR-30c-2" is located at chromosome 6q13 and its transcription produces a noncoding gene product, which will develop into the stem-loop structure, hsa-mir-30c-2. The stem-loop will become 2 products: the mature major sequence, miR-30c, and the passenger sequence, miR-30c-2". The intriguing connection between chromosome 6 and ovarian malignancy is that it is often deleted in primary cancers and may functionally suppress neoplasia in epithelial ovarian cancer cell lines that overexpress it (34). On the basis of the assembly of the RNA-induced silencing complex (RISC), the 2 mRNA strands are not equally eligible to populate RISC; rather, only one of the 2 strands is selected for and then loaded into RISC, which is determined by helicase initiating the duplex unwinding at the easier end (35). As the names suggest in this case, miR-30c is unequally loaded into RISC for translational repression of its targets, which means that miR-30c-2" is more often degraded than incorporated, although it suggests little about its biological actions or molecular effects.
It is difficult for any study to individually assess all of the predicted transcripts regulated by miRNAs. We were prepared to assess 10% of the predicted targets by quantitative real-time PCR, if necessary, and were surprised by the relatively little positive data resulting from the predictions. It is not clear whether this is a result of mathematical algorithms or the aberrant nature of cancer cells. We investigated the named genes within predictions and found only more than 40 to be bona-fide or predicted oncogenes. In addition, the prediction of 1,019 is based on bioinformatic algorithms but may not reflect a precise number of genes regulated by the miRNAs.

Throughout this study, we observed some striking differences in the response to miRNAs between the ovarian cancer cell lines, SKOV-3 and OVCAR-3. For example, the relative abundance of miR-30c and miR-30c-2' between these cells varies along with the responses induced by synthetic precursors. The detailed meaning of this is unclear, but speculative theories could include alterations in miRNA processing, RISC incorporation, miRNA degradation, and pathway alterations. These questions will be addressed in future studies. The passenger strand, miR-30c-2', repeatedly achieved robust effects compared with the major strand, which could reflect miRNA processing by these cells. If any mechanistic alterations in processing are responsible, when and why do these occur? Furthermore, if they exist, do these changes occur prior to malignant transformation and are they causative of ovarian pathology? There is much to learn about miRNA regulation in the cell and more research is required to fully comprehend what role these have in oncology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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