MiR-106a represses the RB tumor suppressor p130 to regulate cellular proliferation and differentiation in high-grade serous ovarian carcinoma.

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Abstract: The degree of differentiation in human cancers generally reflects the degree of malignancy, with the most undifferentiated cancer being also the highest grade and the most aggressive. High-grade serous ovarian cancers (HGSOC) are poorly differentiated and fast-growing malignancies. The molecular mechanisms underlying the poor differentiation of HGSOC has not been completely characterized. Evidence suggests that microRNAs (miRs) are dysregulated in HGSOC. Therefore, we focused on those miRs that are relevant to tumor differentiation. Expression profiling of miRs in HGSOC, indicated miR-106a and its family members were significantly upregulated. Upregulation of miR-106a was further validated by real-time RT-PCR and miR in situ hybridization in a large cohort of HGSOC specimens. Overexpression of miR-106a in benign and malignant ovarian cells significantly increased the cellular proliferation rate and expanded the side-population fraction. In particular, SKOV3 cells with miR-106a overexpression had significantly higher tumor initial/stem cell population (CD24 and CD133 positive cells) than control SKOV3 cells. Among many miR-106a predicated target genes, p130 (RBL2), an RB tumor suppressor family member, was not only confirmed as a specific target of miR-106a but also related to tumor growth and differentiation. The importance of mir-106a and RBL2 was further demonstrated in vivo, in which, SKOV3 cells overexpressing miR-106a formed poorly differentiated carcinomas and had reduced RBL2 levels. To our knowledge, this is the first study of miR-106a mediating proliferation and tumor differentiation in HGSOC.

Implications: The current study suggests that the RB tumor suppressor pathway is a critical regulator of growth and differentiation in HGSOC.

Keywords: MiR-106a, RBL2, ovarian serous carcinoma, differentiation
INTRODUCTION

Ovarian cancer is a deadly disease. According to the American Cancer Society, approximately 21,000 women develop ovarian cancer and approximately 15,000 women die from this disease in the United States in 2011. Despite great efforts in clinical and medical research, the overall survival rate of ovarian cancer has not changed in the past 50 years. (1) Histologic and molecular heterogeneity has complicated our understanding of the tumor biology, behavior and limited the treatment options for this deadly disease. For example, high grade serous ovarian carcinomas (HGSOC), accounting for up to 60% of ovarian epithelial carcinoma, are genetically and histologically heterogeneous tumors. According to The Cancer Genomic Atlas (TCGA), HGSOC can be further divided into 4 subtypes defined as Differentiated, Immunoreactive, Mesenchymal and Proliferative based on the different molecular signatures from global gene and miRNA profiles. (2)

The degree of differentiation of human cancers is used to score the degree of malignancy, with the most undifferentiated cancer being the highest grade and most aggressive. Although the exact mechanisms in controlling HGSOC differentiation are probably complex and remain to be fully characterized, the TCGA study indicates that a subset of gene and microRNAs are associated with tumor differentiation. (2) MicroRNAs are a group of small non-coding RNAs that are significantly dysregulated in ovarian epithelial carcinoma. (3) Furthermore, the microRNA signature can be used for tumor classification and for grading differentiation. (4) In addition to the findings of altered microRNA expression in association with aggressive tumor growth in HGSOC, (3) one of the major functional roles of microRNAs in ovarian carcinogenesis is likely to be associated with tumor stem/initiation cell regulation and tumor differentiation. (5-8) In our global microRNA profiling analysis in early and late stages of HGSOC, we found that miR-106a and its family members were significantly upregulated. MiR-106 family members, as the key players in stem cell self-renewal, are highly induced during early stages of cell reprogramming. (9, 10) Most importantly, Estrogen receptor alpha (ERα) target protein c-MYC binds the miR-106a-363
promoter in an estrogen-dependent manner and upregulates miR-106a in breast cancer cell lines. (11) miR-106a is known to function in tumor-initiating cells and regulate tumor differentiation through the RB pathway and cell cycle and FoxO signaling in many solid carcinomas. (10-12)

In this study, we want to determine if miR-106a overexpression is associated with tumorigenesis of HGSOC. We investigated the functional and oncogenic roles of miR-106a both in vivo and in vitro. We characterized that RBL2, a tumor suppressor gene commonly downregulated in HGSOC, is the specific target gene of miR-106a. Our findings suggested that miR-106a can specifically repress expression of the retinoblastoma family member RBL2 and miR-106a overexpression results in rapid tumor growth and poor differentiation.
MATERIALS AND METHODS

Patients and tissue samples

This retrospective study included 117 cases of high-grade serous carcinoma (HG-PSC) and 30 samples of fallopian tube tissue. All cases were collected from Northwestern Memorial Hospital between 2007 and 2012. Fresh-frozen tissue from 30 cases of HGSOC and matched normal fallopian tube tissue were also collected for the study. The study was approved by the Northwestern University institutional review board. The archived tissue sections were collected and prepared for tissue microarray (TMA) as previously described. (13)

Cell lines and cell cultures

Normal OSE cell line T29 and normal FTSE cell line FTE187 were generated and originally tested by Dr. Liu’s lab. The nature of these cell lines were described in previous publications. (14, 15) T29 and FTE187 cells were maintained in cell culture medium consisting of 1:1 Medium199 (Sigma-Aldrich, St. Louis, MO) and MCDB105 medium (Sigma-Aldrich) with 10% heat-inactivated fetal bovine serum (FBS; USA Scientific, Ocala, FL) and 10 ng/ml epidermal growth factor (Sigma-Aldrich). Ovarian cancer cell lines SKOV3, HEY, OV-90 and OVCAR-3 were purchased (American Type Culture Collection, Manassas, VA). SKOV3 cells were cultured in McCoy’s 5A modified medium (Gibco, Invitrogen, Carlsbad, CA). HEY, OV-90 and OVCAR-3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Invitrogen). All media contained 10% FBS (Gibco, Invitrogen), 100 µg/ml penicillin, and 100 µg/ml streptomycin. All cells were cultured in a humidified atmosphere of 5% CO2 at 37°C.

Antibodies, histological and immunohistochemical staining analysis

Antibodies used for immunohistochemical staining included anti-RBL2 (sc-317, Santa Cruz Biotechnology), anti-p53(Dako, Carpinteria, CA, USA), anti- Ki-67 (cell proliferation marker; Neomarkers, Fremont, CA, USA), anti-CD133(HPA004922, Sigma-Aldrich, MO),...
anti-CD24-PE(ebioscience, San Diego, CA, USA). Formalin-fixed and paraffin-embedded tissues were sectioned at 4 μm. Tissue slides were deparaffinized in xylene and rehydrated in a graded series of ethanol. Antigen retrieval was performed by heat-induced epitope retrieval as previously described.(16) All immunohistochemical staining procedures were performed on a Ventana Nexus automated system (Tucson, AZ). Slides were blocked with 1.5% normal goat serum for 30 minutes at room temperature and then incubated overnight at 4°C with primary antibodies (RBL2, p53, Ki67 and CD133) in a humid chamber. Staining was detected with I-View DAB detection system.

**MicroRNA in situ hybridization**

In situ hybridization was performed as previously described,(17) Formalin-fixed and paraffin-embedded tissues were sectioned at 4 μm. Hybridization was performed with DIG-labeled, locked nucleic acid (LNA)-based probes specific for human miR-106a and U6 (Exiqon, Woburn, MA). Hybridizations were performed overnight at 55°C after the addition of 50 nM of miR-106a and 20 nM of U6 (Exiqon) diluted by hybridization solution (BIOCHAIN Institute Inc, Hayward, CA). After stringency wash (50% formamide and 2×SSC) at hybridization temperature, slides were blocked with 2% normal goat serum for 60 minutes at room temperature and then incubated with anti-DIG antibody (1:800; Roche Diagnostics, Indianapolis, IN) overnight at 4°C. Alkaline phosphatase activity was detected using BM Purple AP Substrate (Roche Diagnostics, Indianapolis, IN). In situ hybridization results were quantified according to the intensity of staining and were normalized by U6.

**RNA Isolation and Quantitative RT-PCR**

Total RNA was isolated using the mirVana™ RNA Isolation kit following the manufacturer’s instructions (Ambion, Austin, TX) and cDNA was synthesized by reverse transcription of 1μg of total RNA with M-MLV Reverse Transcriptase(Promega, Madison, WI). MirVana qRT-PCR primers and the mirVana qRT-PCR Detection Kit (Ambion) were used to test miR-106a and miR-106b expression. SYBR Green
real-time RT-PCR (Applied Biosystems) was used to detect the expression of RBL2. Small nuclear RNA U6 was used as an internal control.

**Lentiviral vector construction, viral production and infection**

To generate a miR-106a expression vector, a 522-bp fragment containing pre-miR-106a was cloned into pGIPZ lentiviral vector. RBL2 shRNA in pGIPZ was purchased from Open Biosystems (Huntsville, AL). Lentivirus expressing miR-106a or shRBL2 was produced in HEK293T cells packaged by pMD2G and psPAX2. For stable infection, 1×10^5 cells were plated in six-well plates along with 2 mL of medium without antibiotics. After overnight incubation, the medium was removed and replaced with 1 mL per well of Opti-MEM Reduced-Serum Medium containing lentivirus 8 µg/mL polybrene. Next, 50 µL of concentrated lentiviral particles were added to each well. Twenty-four hours later, fresh medium containing 2 µg/mL puromycin was added to each well. Fresh medium containing puromycin was replaced every 3 to 4 days. Single colonies were obtained after 3 weeks of puromycin selection.

**Oligonucleotide transfection**

MiRIDIAN miR-106a Inhibitor (Dharmacon, Inc.; Lafayette, CO) was transfected 50 nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfection efficiency was monitored using BLOCK-iT Fluorescent Oligo (Invitrogen). Forty-eight hours after transfection, Cells were harvested and analyzed for miR-106a and RBL2 expression. The harvested transfected cells were also plated for cellular proliferation assay.

**Anchorage-Independent Colony Formation**

Anchorage independent cell growth in soft agar was performed in triplicate with cells suspended in 3 ml of a low density (0.75 × 10^4 cells) culture medium containing 0.3% agar (USB Corporation, OH) and seeded onto a base layer of 3 ml of 0.6% agar bed in 60-mm tissue culture dishes. After 2 to 4 weeks (based upon different cell lines), colonies were stained, photographed, and scored.
Matrigel invasion assay

Matrigel-coated chambers (BD Biosciences) containing 8 μm pores were used for the assays. Briefly, 2.5 ×10^5 cells were seeded into the upper chambers (coated in Matrigel) in certain mediums containing 0.5% FBS. The lower chamber of the transwell was filled with culture media containing 10% FBS as a chemo-attractant. The chambers were incubated at 37°C for 16–72 hours depending on the different cell lines. Noninvaded cells on the top of the transwell were scraped off with a cotton swab. Successfully translocated cells were fixed by 10% formalin, stained with 0.1% crystal violet for 30 minutes, and counted under a light microscope.

Cellular proliferation assay

The WST-1 (Roche) was used for cell proliferation assay according to the manufacturer’s instructions. Cells were seeded in 96-well plates in triplicate at densities of 2×10^3 cells per well. Cell proliferation was monitored at different times (1–5 days). After incubation at designated times, 10 μl of WST-1 was added to each well and then incubated for 4 h at 37°C. Absorbance of the samples was measured by an ELISA plate reader with a test wavelength at 450 nm and a reference wavelength at 630 nm.

3′-UTR of RBL2 vector construction and luciferase reporter assay

The 3′-UTR of RBL2 containing two putative miR-106a binding sites (513bp) was amplified by polymerase chain reaction (PCR) and cloned into psiCHECK2 vector (Promega, Madison, WI) using the XhoI and NotI sites. The psiCHECK2-RBL2-Mut construct containing the mutations located at the miR-106a binding sites was generated by overlap extension PCR and cloned into the psiCHECK2 again.(18) For the luciferase assay, T29-Vector and T29-miR-06a cells were seeded at 1 × 10^4 cells/well in a 96-well plate 24 h before transfection. Cells were transfected with 100ng of psiCHECK2 control, psiCHECK2-RBL2, and psiCHECK2-RBL2-Mut. Twenty-four hours after transfection, luciferase activity
was measured using the Dual-Glo Luciferase Assay System (Promega). Renilla luciferase activity was normalized to corresponding firefly luciferase activity and plotted as a percentage.

**Western blotting**

Cells were lysed and protein concentration was determined using the BCA assay kit (Thermo Scientific, Rockford, IL). Protein samples were separated by SDS-PAGE and electro-transferred onto polyvinylidene fluoride membrane. The membrane was incubated with specific primary antibodies. Rabbit rabbit anti-pRb2/p130 (sc-317, Santa Cruz Biotechnology), mouse anti-p21 (sc-6246, Santa Cruz Biotechnology) and mouse anti-β-actin (Sigma-Aldrich, MO) were tested. Proteins of interest were detected with horseradish peroxidase-conjugated secondary antibodies and developed using the ECL-enhanced luminol reagent (PerkinElmer Inc.).

**Flow cytometry analysis**

For Side population analysis, cells (106/mL) were incubated in pre-warmed DMEM containing 2% FBS (Gibco, Invitrogen). Cells were stained with 5μg/ml Hoechst 33342 alone or in combination with 50 μM verapamil (Sigma). The cell wells were mixed and placed in the 37°C water bath for exactly 90 minutes. The tubes were mixed several times during incubation. At the end of incubation, cells were centrifuged at 4°C and resuspended in cold PBS with 2% FBS. Propidium iodide (Sigma) solution was added to a final concentration of 2μg/ml and cells were then analyzed in FACS with UV laser (BD Biosciences, San Jose, CA). To evaluate cell cycle distribution, 1 × 10^6 cells were fixed in 4ml of cold 70% ethanol at −20 °C overnight. After centrifugation at 1000×g for 10min, cell pellets were incubated with 0.5 ml of PBS containing 100μg/ml RNase and 5μg/ml propidium iodide at 37 °C for 30 min. Cell cycle distribution was analyzed by measuring DNA content using FACS. For the CD133 staining and FACS analysis, 2× 10^6 cells were incubated with CD133-PE(MACS, Miltenyi Biotech) for 30 minutes at 4°C in the dark. After the washing steps, labeled cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA).

**Nude mice xenografts**
Female nude mice (6-8 weeks old) were purchased from NCI. For xenograft experiments, equal numbers 
(5×10^6) of SKOV3-Vector and SKOV3-miR-106a cells were harvested and resuspended in 0.1 ml of PBS 
for subcutaneous injection. Six mice were used for each group and each mouse received two injections. 
Tumor volume was calculated with the use of the following formula: tumor volume (in mm^3) = \( a \times b^2 \times 0.52 \), 
where \( a \) is the longest diameter, \( b \) is the shortest diameter, and 0.52 is a constant to calculate the volume of 
an ellipsoid. Tumor sizes were measured weekly, and mice were observed up to 5 weeks. The mouse was 
euthanized when a tumor reached 1.5 cm in diameter. All tumors for each group were excised, fixed in 10\% 
formalin and subjected to routine histologic examination and immunostaining of RBL2 and Ki67.

**Statistical analysis**

All data is presented as means and standard errors in triplicate. Student’s t test was used for comparisons 
between two groups of experiments, and one-way ANOVA was used for comparisons among three or more 
groups. P < 0.05 was considered statistically significant.
RESULTS

MiR-106a and its family members are overexpressed in high-grade ovarian serous carcinoma

MicroRNA dysregulation is common in HGSOC and many microRNAs are associated with the tumorigenesis of HGSOC. (19) However, microRNA expression analyses from most studies were obtained from snap-frozen tissue samples. The tumor and stromal ratio cannot be well characterized. To get a better view of microRNA expression in relatively pure normal and tumor epithelia, we collected tissue samples by microdissection from normal fallopian tube, STIC (serous tubal intraepithelial carcinoma) and HGSOC (invasive high grade serous ovarian carcinoma) in five cases and obtained global microRNA expression using the MTG platform.(17) We found that miR-106 and its family members were significantly overexpressed in HGSOC (S Table 1). microRNA profile analysis in five cases revealed that HGSOC showed more than a 4 fold increase in miR-106a expression over control FT (Figure 1C, S Table 1).

To confirm miR-106a overexpression in STIC and HGSOC, we conducted microRNA in situ hybridization for miR-106a (Exiquon, see Methods) in a large cohort of HGSOC (n=117) and control FT (N=30). As illustrated in Figure 1A, both STIC and invasive HGSOC showed high levels of miR-106a expression, while normal fallopian tube epithelial cells showed lower miR-106a expression. By a semiquantitative analysis, we found miR-106a expression was significantly higher in HGSOC (0.68±0.0532) than in normal FT (0.46±0.1109) (P<0.01, Figure 1B). However, miR-106a expression detected by microRNA in situ hybridization is at best for a semiquantitation, and may not reflect the actual level of miR-106a expression.

MiR-106a overexpression promotes ovarian cancer cell proliferation in vitro

To investigate the oncogenic properties of miR-106a in HGSOC, we prepared and established cell lines with stable miR-106a overexpression in normal fallopian tube (FTE187), ovarian surface (T29) epithelial cell lines and ovarian cancer (SKOV3 and HEY) cell lines (S Figure 1A). We found that introducing
miR-106a overexpression resulted in a significant increase of anchorage independent growth in both normal (T29) and malignant (SKOV3) cell lines (S Figure 1B, p<0.05) in soft agar assay. MiR-106a overexpression could also slightly enhance tumor cell migration in Matrigel (S Figure 1C). MiR-106a overexpression significantly enhanced cell proliferation in normal fallopian tube epithelial cells (FTE187) (Figure 2A). MiR-106a overexpression could also promote cell proliferation in ovarian cancer cell lines (Figure 2A). Cell cycle analysis by flow cytometry showed that miR-106a overexpression led to an increase in the percentage of cells at S and G2/M phase for both FTE187 and SKOV3 cells (Figure 2B and 2C).

SKOV3 cells with miR-106a overexpression slightly enhanced invasion through Matrigel (S Figure 1), but did not promote apoptosis based on Annexin V - PI Staining (data not shown). When treated with different doses of paclitaxel and cisplatin, SKOV3 cell lines with miR-106a overexpression were significantly more resistant to chemotherapy (S Figure 2). The findings suggest that miR-106a can promote cell proliferation and confer chemoresistance.

**MiR-106a increases the yield of putative cancer stem cells**

We next measured the proportion of cell population in cells with and without miR-106a overexpression, respectively. SP (side population) cells were quantitated by Hoechst 33342 staining and flow cytometry in FTE187 and SKOV3 (Figure 3). Benign and malignant ovarian cancer cells that were transduced with miR-106a showed more than 2-fold increase in the size of SP, when compared to cells transfected with the control vectors. The findings can be repeated in another two cell lines (S Figure 4). To further evaluate whether miR-106 overexpression promotes the formation of ovarian tumor initiating cells, we examined the ratio of ALDH1, CD133 and CD24 positive and negative cell populations by flow-cytometry analysis. CD133 is considered as stem cell marker.(20) As shown in Figure 3B and 3D, both normal (FTE187) and malignant (SKOV3) ovarian cell lines with miR-106a overexpression had slightly increased frequency of CD133 positive cells. CD24 expression level was also increased from 3.98% to 17.9% in SKOV3-miR-106a cells compared to SKOV3-Vector cells (Figure 3E). No significant change of ALDH1
by miR-106a overexpression was seen (data not shown). Our findings suggested that miR-106a may promote ovarian cancer stem cell formation.

**MiR-106a specifically represses RBL2 expression in ovarian cancer**

Previous studies revealed that miR-106 and its family member can specifically repress CDKN1A (p21) expression through targeting at its 3’UTR. Repression of p21 may partially explain the mitogenic role of miR-106a in ovarian cancer. However, little is known about the role of miR-106a in tumor differentiation. We found that RBL2 is one of the predicted target genes of miR-106a. RBL2 is a member of the RB gene family, and is involved in cell cycle regulation, tumor differentiation and stem cell self-renewal. Previous studies from global gene expression showed that higher expression of miR-106a or its family members is associated with lower RBL2 expression. To investigate whether RBL2 is the specific target of miR-106a, we examined and characterized the molecular interaction between miR-106a and RBL2. Among 4 ovarian cancer cell lines, three of them (HEY, OVCAR3 and OV-90) showed high levels of endogenous miR-106a expression and OV-90 had the highest miR-106a (Figure 4A).

By computer software analyses (PicTar, TargetScan and miRBase), we found that RBL2 had two very conservative sites complementary to miR-106a ‘seed’ sequence (Figure 4B). The luciferase expression constructs containing 1.35kb of wild type and mutant (replacement of 4 nucleotides in ‘seed’ sequence of miR-106a binding sites) 3’UTR sequence of RBL2 cDNA were respectively generated. Co-transfection of miR-106a with wild type RBL2 3’UTR construct resulted in at least 2 fold reduction (p<0.05) in luciferase activity in T29 cells, but no change of luciferase expression was noted when co-transfected with mutant RBL2 3’UTR construct (Figure 4C). RT-PCR and Western blot analyses showed that transient transfection of miR-106a (Dharmacon, see Methods) in OV-90 cells resulted in a significant reduction of RBL2 expression at mRNA (not shown) and protein level (Figure 4D). As a control, p21 expression was also determined. These results indicate that RBL2 can be specifically repressed by miR-106a (Figure 4C and 4D).
To investigate whether miR-106a mediated RBL2 downregulation was associated with cell proliferation, we prepared ovarian cancer cell lines with stable miR-106a overexpression and constant inhibition of RBL2 expression (shRBL2), respectively (Figure 4E). We found that introducing miR-106a overexpression or inhibiting RBL2 expression significantly enhanced HEY cell proliferation (Figure 4E). This finding could be reproduced in another ovarian cancer cell line OV-90 (data not shown). Anti-miR-106a treatment in OV-90 cells restored RBL2 expression and prohibited tumor cell growth (Figure 4F). These findings suggest that miR-106a enhances cell proliferation by negatively regulating the tumor suppressor gene RBL2.

**MiR-106a overexpression enhances tumor growth and dedifferentiation in xenografts**

To examine whether miR-106a overexpression promotes ovarian cancer growth in vivo, we inoculated ovarian cancer cell line SKOV3 with and without miR-106a overexpression in nude mice (n=8 for each group). Tumor size began to show significant difference three weeks after implantation, and reached a peak tumor size of 870 mm³ for SKOV3-miR-106a, and only 220 mm³ for SKOV3-Vector (p<0.01, Figure 5A and 5B). In addition to difference in tumor size, the two groups also differed in the degree of tumor differentiation. In tumor sections of SKOV3-vector, over 60% of the tumor showed glandular differentiation, characterized by well-formed epithelial glands with an orientated cell border and moderate nuclear atypia (Figure 5C, S Figure 5). Immunostaining for Ki-67 showed a proliferative index of 40% (Figure 5D) and a low rate of tumor necrosis (data not shown). Immunostaining revealed a strong and diffuse immunoreactivity for RBL2 in almost all tumor cells (Figure 5C and 5D). In contrast, tumors formed by SKOV3 cells with miR-106a overexpression showed a mostly solid growth pattern (glandular and solid ratio of 1:4). SKOV3-miR-106a cells tended to be larger with a higher nuclear to cytoplasmic ratio and were more poorly differentiated than the SKOV3-vector cells (Figure 5C). The Ki-67 index in SKOV3-miR-106a tumors was nearly 60% (Figure 5D) with brisk mitoses and extensive tumor necrosis. As expected, a lower immunoreactivity for RBL2 (relative immunointensity was 1.5 in comparison to 2.7
for SKOV3-vector) (Figure 5C). Immunostaining revealed a significant increase of CD133 and CD24 positive cells in SKOV3-miR-106a tumor (Figure 5C and 5D, S Figure 3). In summary, we found that the xenografts of SKOV3-miR-106a showed larger tumor masses with high proliferation index, increased tumor stem cell population and poor differentiation. These findings suggested that miR-106a overexpression is associated with an aggressive ovarian cancer growth and dedifferentiation through negative regulation of tumor suppressor gene RBL2.

Expressions of MiR-106a and its target genes in high grade serous ovarian carcinoma

MiR-106a and its family members were found to be significantly overexpressed in HGSOC by our chip-based microRNA profiling analysis in formalin-fixed and paraffin-embedded tissue sections (Figure 1). Previous study showed that RBL2 is downregulated in ovarian carcinoma (24). We confirmed that RBL2 is specifically targeted by miR-106a (Figure 4). To further investigate whether miR-106a, miR-106b mediated downregulation of RBL2 occurs in HGSOC, we examined their expressions in 30 HGSOC tumor tissues and 8 normal fallopian tube (FT) tissues by Real-time RT-PCR with RNA loading controls of U6 and β-Actin. MiR-106a and miR-106b expressions were significantly higher in HGSOC (32.44±10.22 and 36.18±11.79, respectively) than in FT (8.10±0.87 and 12.05±1.69, respectively (Figure 6A and 6B)). In comparison, FT had relatively higher levels of RBL2 expression (14.04±2.41) than HGSOC (3.81±1.00) (P<0.01) (Figure 6C). Further case-matched analysis revealed that the expression of miR-106a and b was negatively correlated with that of RBL2 (r=-0.12—0.23) (Figure 6D). All these findings support that miR-106a/b overexpression and RBL2 downregulation in HGSOC are the crucial molecular events that are associated with tumor dedifferentiation.
DISCUSSION

Altered microRNA expression is commonly observed in ovarian cancer. (2, 25) MicroRNAs that are associated with specific phenotypes of ovarian cancer have been studied extensively, including DNA damage response (miR-182), (17) epithelial mesenchymal transition (miR-200), (26) p53 dysfunction (miR-34), (27) cell proliferation and initiation (let-7), (28) tumor survival (miR-214), (29) and so on. Here we demonstrated that miR-106 is overexpressed in ovarian cancer and leads to the downregulation of tumor suppressor RBL2. Thus, many microRNAs are involved in the various aspects of ovarian carcinogenesis. MiR-106 functions as an Onco-miR and has been found to be associated with carcinogenesis in many carcinomas. (24, 30-33) MiR-106a and family members were significantly overexpressed in ovarian carcinoma (S Table 1). (34-36) In this study, we found that miR-106a was significantly overexpressed in both early and late stage of high grade serous ovarian carcinoma (HGSOC) (Figure 1). Tumors with miR-106a overexpression exhibited a higher proliferation rate and larger tumor size than the control tumors (Figure 2 and 5). SKOV3 cells with miR-106a overexpression showed more paclitaxel resistance than the control tumor cells (S Figure 2). This is consistent with a recent study, in which miR-106a overexpression is significantly associated with paclitaxel resistance both in ovarian cancer cell lines and in primary human ovarian cancer (37). Moreover, upregulated miR-106a expression enriched the population of tumor- initiating cells, as reflected by the expanded side population (SP) and CD133+ cell population (Figure 3 and S Figure 3). In the xenografts of nude mice, we noted that tumors formed from SKOV3 cells with miR-106a overexpression were not only larger in size, but were also more poorly differentiated, and showed a significantly higher proliferation index than tumors from control SKOV3 cells (Figure 5). All these findings prompted us to search for the specific target genes of miR-106a that are associated with ovarian cancer. As illustrated in Figures 4 and 5, we found that RBL2 is specifically repressed by miR-106a, and that its dysregulation is closely related to the aggressiveness of ovarian cancer both in vitro and in vivo.
It appeared that *miR-106a* overexpression can conferred two prominent oncogenic features to ovarian cancer cells, the enhancement of cell proliferation (Figure 2, 3, 5 and S Figure 1) and the promotion of tumor-initiating cell expansion and dedifferentiation (Figure 4, 5 and S Figure 5). The mitogenic function of *miR-106a* can be best explained by its repression of cell cycle checkpoint protein p21 (Figure 4D), as reported in previous studies, (22) and of tumor suppressor *RBL2* identified in this study (Figure 4E). Therefore, *miR-106a* and its family members may serve as good targets in reducing tumor growth.

This is the first study to show that SKOV3 cells with *miR-106* overexpression produces poorly differentiated or dedifferentiated carcinomas in xenografts of nude mice (Figure 5). It is emerging that *miR-106* and its family members regulate stem cell differentiation.(11, 23) For example, the expression of *miR-106b*~25* cluster is high in self-renewing adult NSPCs and but is low when cells are stimulated to undergo differentiation.(10) *MiR-106* clusters are one of a few miRNAs that are highly induced in cells that regained pluripotency.(9) In transgenic mice, overexpression of the *miR-106* cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells by targeting *rbl2*. (38) All these findings suggest that *miR-106* plays an important role in controlling cell proliferation and differentiation. However, the role of *miR-106a* in tumor cell differentiation has not been well characterized or studied. In this study, we demonstrated that *miR-106* promotes cell proliferation and inhibits differentiation.

Regulation of *miR-106* cluster in ovarian cancer remains unclear. Recent study by Thangavel et al (39) in breast cancer revealed that activation of RB pathway suppresses *miR-106* cluster expression, while knockdown RB expression or RB deficient cells can rescue or enhance *miR-106* cluster expression. These findings provide another layer of evidence that *miR-106a* and its family members are critical in regulation of RB function and loss of RB pathway in most ovarian cancer may be responsible for *miR-106a* overexpression.

*RBL2*, a member of the retinoblastoma family of proteins, is significantly downregulated in ovarian cancer. (24) *RBL2* is capable of repressing *E2F4* target genes as a part of the DREAM repressor complex.
complex.(40) P130 (RBL2/RB2), a major E2F component and pocket protein, actively regulates the ES cell proliferation(41) and likely function through regulation of DNA methyltransferase (Dnmt) expression.(42) The RBL2/p130-E2F4 protein complex inhibits the transcription of multiple genes that are required for cell cycle progression and induces cell cycle arrest at G0/G1, which is required for induction of cell differentiation in cells of many tissues in vivo and various cell lineages in vitro(43). The functional role of RBL2 characterized so far may best explain the tumor phenotype identified in xenografts of SKOV3 tumor in this study. It is not surprising that well differentiated SKVO3 tumors (mostly consisting of well-formed glandular formation) have high RBL2 expression and poorly differentiated tumors (solid and highly mitotic activity) have low RBL2 expression (Figure 5). Based on these findings, we propose that miR-106 mediated downregulation of RBL2 may represent one of the major molecular events contributing to the aggressiveness of HGSOC.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

Experimental design (JJW, ZL), conducting experiments (ZL, EG, XX, XZ, RD, JJW), providing research materials and methods (CS, PL, JL, BK), data analysis (JJW, ZL) writing manuscript (JJW, ZL).
LEGENDS FOR FIGURES

Figure 1 Expression analysis of miR-106a in ovarian carcinoma. A. photomicrographs illustrate consecutive sections of normal fallopian tube epithelium (arrow), serous tubal intraepithelial carcinoma (STIC, arrow head) and invasive high grade serous carcinoma (double arrow heads) by hematoxylin/eosin stain (H/E, left panel), Immunostain for P53 (middle panel) and microRNA in situ hybridization for miR-106a (right panel). Strong immunoreactivity for P53 represents the accumulation of the mutant P53 in STIC and invasive high grade serous carcinoma. B. The histo-bars represent the mean values of relative miR-106a expression in 117 HGSOC and 30 FT (normalized by RNA loading control of U6) by microRNA in situ hybridization. C. The relative miR-106a and miR-106b expression detected by microRNA profiling analysis (n=5) in normal fallopian tube (light gray), STIC (dark gray) and high grade serous ovarian carcinoma (HGSOC, black). **: P<0.01.

Figure 2 MiR-106a promotes normal and malignant ovarian/fallopian tube epithelial cell proliferation. A. Growth curves illustrate the significant differences in cell proliferation from day 2 to day 5 in normal fallopian tube secretory epithelial cell line (FTE187, left panel) and ovarian cancer cell line (SKOV3, right panel) with and without miR-106a overexpression (see Materials and Methods). B and C. Cell cycle analysis by the cell flow cytometer reveals that a significant increase of S phase cell population in FTE187 and in SKOV3 with miR-106a overexpression (C) in comparison to those without miR-106a overexpression (B) (p<0.05).

Figure 3 miR-106a overexpression increases stem cell-like (side-population) population. The side-population, major population of normal (FTE187, 3A) and malignant (SKOV3, 3C) cell lines were analyzed by flow cytometer (see Materials and Methods). Circled areas represent the side populations. Overall, a 2-4 fold increase of side population cells were observed in four cell lines with miR-106a overexpression. CD133 and CD24 expression were counted by flow cytometry in FTE187 (3B) and in
SKOV3 (3D, 3E). Cells with miR-106a overexpression significantly increase CD24 and CD133 positive cell population in comparison to those without miR-106a overexpression (p<0.05).

**Figure 4** Tumor suppressor gene RBL2 is specifically targeted by miR-106a. A. RT-PCR analysis reveals an inverse correlation of endogenous RBL2 with miR-106a expression in normal (FTE187, T29) and malignant (SKOV3, HEY, OV-90 and OVCAR3) cell lines. U6 is used as an RNA loading control. B. Two predicted binding sites of the miR-106a family in RBL2 3’UTR (untranslation region) and their sequence. C. Histobars illustrate the relative luciferase expression in LE293 cells cotransfected with wild type and mutant miR-106a binding sites with (gray bars) and without (black bars) miR-106a overexpression. Vector stands for luciferase transfection without RBL2 3’UTR. D. Western blot analysis reveals that introducing stable miR-106a overexpression in FTE187 cells results in significant reduction of RBL2 and P21 (well know target gene of miR-106a, used as positive control). MiR-106a and U6 expression are shown below. Histobars represent the average levels of RBL2 and P21 expression with and without miR-106a expression. *: p<0.05; **: p<0.01. E. Blocking RBL2 expression promotes cell proliferation as did with miR-106a overexpression. F. MiR-106a targeted gene expression was further validated by blocking miR-106a expression with anti-miR-106a in OV-90 cells; Blocking miR-106a expression inhibits cell proliferation compared to control cells.

**Figure 5** miR-106a overexpression leads to faster growth and poor differentiation of tumor mass in xenografts of nude mice. A. Photographs illustrate an example of larger tumor masses in xenografts of SKOV3 cell lines with miR-106a overexpression, in comparison to the tumors without MiR-106a overexpression. B. Growth curves for tumor volumes in xenografts of nude mice with SKOV3 cell line with and without miR-106a overexpression (each consists of 8 mice), measured from day 14 to day 35. C. Photomicrographs illustrate the histology (upper panel), Ki-67, RBL2, CD133 and CD24 expression (by immunohistochemistry stain, middle and lower panels) in xenografts of SKOV3 cell lines with (miR-106a (+)) and without (miR-106a (-)). SKOV3 tumors without miR-106a overexpression show well
differentiated tumor growth, characterized by mostly glandular growth pattern, low Ki-67 index (low cell proliferation rate) and high level of RBL2 expression. While SKOV3 tumors with miR-106a overexpression show poorly differentiated and solid growth patterns, high Ki-67 index and lower RBL2 expression. D. The statistical analysis of mean (wide histobars) and standard errors (small T-bars) between tumors with (T) and without miR-106 (C) overexpression in the order of tumor volume, % of glandular component, ki-67 index, RBL2, CD133 and CD24 expression. *: p<0.05; **: p<0.01.

Figure 6  miR-106a, miR-106b and RBL2 expression in high grade serous ovarian carcinomas and fallopian tube. Real-time RT-PCR analysis of miR-106a (6A), miR-106b (6B) and RBL2 (6C) expression in individual panels and combined comparison (6D). The relative expression of each gene was normalized by either U6 (for microRNA) or actin (for RBL2).
Table 1  Clinical characteristic of 117 Ovarian Carcinomas

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REFERENCES

10.1101/gad.1640608. PubMed PMID: 18381893; PubMed Central PMCID: PMC2279201.


Figure 1

(A) H/E, TP53, and miR-106a imaging.

(B) Relative miR-106a/U6 expression in HGSOC and FT.

(C) miR-106a and miR-106b expression in HGSOC, STIC, and FT.
Figure 2

A

FTE187-Vector
FTE187-miR-106a

Relative Cell Proliferation (OD)

Days

B

FTE187 Vector
G0/G1=58.5%
S=31.3%
G2/M=10.2%

DNA content

Events

SKOV3 Vector
G0/G1=62.8%
S=26.4%
G2/M=10.8%

DNA content

Events

C

FTE187 miR-106a
G0/G1=46%
S=38.9%
G2/M=15.1%

DNA content

Events

SKOV3 miR-106a
G0/G1=54.7%
S=36.4%
G2/M=9.9%

DNA content

Events
Figure 4
Molecular Cancer Research

MiR-106a represses the RB tumor suppressor p130 to regulate cellular proliferation and differentiation in high-grade serous ovarian carcinoma.

Zhaojian Liu, Elizabeth Gersbach, Xiyu Zhang, et al.

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