Original Paper

A Double-Negative Feedback Interaction between MicroRNA-29b and DNMT3A/3B Contributes to Ovarian Cancer Progression

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Key Words

Micro-RNAs • DNA methyltransferase(DNMT) • Epigenetics • Epithelial Ovarian Cancer(EOC)

Abstract

Background: Epigenetic abnormalities are increasingly observed in multiple malignancies, including epithelial ovarian cancer (EOC), and their effects can be significantly counteracted by tumor-suppressor microRNAs, namely epi-miRNAs. Here, we investigated the role of miR-29b, a well-established epi-miRNA, in the DNA methylation regulation of EOC cells. **Methods:** The correlation between miR-29b and DNMT3A/3B expression was evaluated by RT-qPCR, western blotting and immunohistochemical analysis. The functional roles of miR-29b and DNMT3A/3B were tested by anti-miRs and microRNA precursors. A luciferase reporter assay was employed to detect the direct binding of miR-29b to DNMT3A/3B 3' UTRs. Co-IP was utilized for investigating Id-1 binding activity. **Results:** miR-29b was negatively correlated with DNMT3A/3B expression at the cellular/histological levels. miR-29b silencing was correlated with increased DNMT3A/3B levels, whereas miR-29b over-expression caused DNMT3A/3B down-regulation. Luciferase reporter assays confirmed that the miR-29b-mediated downregulation of DNMT3A/3B occurred through the direct targeting of their mRNAs' 3'-UTRs, whereas BGS assays found that DNMT3A/3B knockdown increased miR-29b expression via CpG island promoter hypomethylation, thus suggesting a crucial crosstalk between miR-29b and DNMT3A/3B via a double-negative feedback loop. Co-IP assay confirmed direct binding between DNMT3A and Id-1. **Conclusion:** Taken together, our study sheds light on a novel epigenetic circuitry regulating EOC progression and may provide novel options for miR-29b-based epi-therapeutic approaches for future EOC treatment.

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Introduction

In addition to genetic regulation, gene expression can be regulated by epigenetic modifications that do not affect the underlying genetic sequences [1]. To date, a number of epigenetic modifications have been implicated in tumorigenesis, including DNA methylation, post-translational modification of histones, and the most recently discovered epi-microRNAs, which are currently considered key players in human cancer pathogenesis [2]. Among those epigenetic mechanisms, DNA methylation has been a prominent focus in cancer research, and it represents one of the hottest fields of current epigenetic research. Being one of the most common defects in epigenetic regulation, DNA methylation is frequently observed in cancerous onset and progression [3]. This process is delicately orchestrated by DNA methyltransferases, namely DNMT1, DNMT3A and DNMT3B, among which DNMT1 primarily maintains existing DNA methylation patterns, whereas DNMT3A and DNMT3B are de novo methyltransferases and are essential for introducing methyl groups onto CG sites that were unmethylated on the parental template strands of DNA during development [4-6]. In particular, DNA hypermethylation at promoters of tumor-suppressor genes has been given great attention and has thus been hotly discussed and prominently reported. DNA hypermethylation of tumor-suppressor genes will lead to reduced gene expression, consequently providing a selective advantage to cancer cell development.

MicroRNAs (miRNAs) are noncoding endogenous RNAs, approximately 22 nt in length, that primarily function as post-transcriptional regulators of gene expression by translation inhibition or mRNA degradation through partial or complete base pairing with the 3'-UTRs of the target mRNAs [7]. In addition to their roles in diverse physiological and pathological processes, including cell differentiation, cell cycle control, and apoptosis, miRNAs have been reported as potential biomarkers of malignant tumors, including EOC [8-12]. Furthermore, increasing evidence has shown that miRNAs are involved in modulating DNA methylation patterns. Some miRNAs regulate the expression of epigenetic machinery, and the expression levels of miRNAs may be affected by DNA methylation [13]. Among the 2000 plus miRNAs that have been identified, miR-29b is a commonly recognized tumor-suppressor miRNA that regulates a number of important genes that mediate carcinogenesis and tumor development in various cancer types, including EOC [14-17]. miR-29b has been found to play essential roles in mediating cancer cell motility, invasion, proliferation, dysregulated metabolism, and stemness maintenance [18-21]. However, the interaction between miR-29b and DNA methylation is still largely unexplored, and evidence for a role of miR-29b in regulating DNA methylation is mainly limited to the correlation found between DNA hyper-methylation and dysregulated miR-29b levels [22-24]. The molecular mechanisms directly linking miR-29b and DNA methylation by DNMTs have not been elucidated. In the present study, we aimed to explore the interaction between miR-29b and DNMT3s and its underlying molecular mechanism in EOC cells, which will broaden our theoretical understanding of human ovarian cancer and provide novel possibilities for future EOC treatment approaches.

Materials and Methods

Cell lines and human tissue specimens

The human ovarian cancer cell lines SKOV3 and A2780 (both obtained from ATCC, Manassas, VA, USA) were maintained in high-glucose DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere. Human ovarian carcinomas and matched normal ovarian tissue samples were collected from patients at The First Affiliated Hospital of Xi'an Jiaotong University, PR China. This study was approved by the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University, China. Written consent was obtained from each study participant enrolled.

Bioinformatics analysis

Potential targets and binding sites of miR-29b were predicted using several online programs, including miRanda (<http://www.microrna.org/microrna/home.do>), miRBase (<http://www.mirbase.org/index.shtml>), miRWalk (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html>), and TargetScan (<http://www.targetscan.org/>); DNMT3A and DNMT3B were predicted to be downstream target genes of miR-29b by all of these software packages, and this was further validated by experiments.

Cell transfection

The control mimics, miR-29b mimics, control inhibitors, and miR-29b inhibitors were all purchased from RiboBio (Guangzhou, China). DNMT3A/3B-targeted siRNAs and their negative controls were purchased from Invitrogen (USA). Ovarian cancer cells were seeded onto 6-well plates until 50%-60% confluency and transiently transfected with 60 nM control/miR-29b mimics or with 120 nM control/miR-29b inhibitors or 100 nM control/DNMT3A/DNMT3B siRNAs using X-treme GENE siRNA Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. The cells were collected for further experiments 48 h after transfection.

Quantitative real-time PCR

qPCR was performed as depicted before [19]. Briefly, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized with a PrimeScript RT reagent kit (Perfect Real Time; Takara, Dalian, China). qPCR was then performed using SYBR Premix Ex Taq™ II (Takara) on a CFX96 real-time PCR System (Bio-Rad, Hercules, CA, USA). β-actin was used as an internal control for results normalization. The miR-29b levels were detected using a TaqMan microRNA kit (Applied Biosystems) and normalized to small nuclear RNA (Rnu6), which served as a control. The data were expressed as Log 2-fold changes relative to miRs/U6 snRNA levels. The primers for miR-29b and U6 reverse transcription and amplification were designed by and purchased from RiboBio Co., Ltd. (Guangzhou, China). All primers used are listed in Table 1.

Western blot analysis

Cell lysates were collected using mammalian protein extraction reagent (Pierce, Rockford, IL, USA) with protease inhibitors (Roche, Indianapolis, IN, USA). The protein concentration of each sample was determined using a BCA-200 protein assay kit (Pierce, Rockford, IL, USA). The proteins were resolved on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blocked in blocking buffer (5% non-fat milk in TBST). Mouse anti-human DNMT3A (ab13888, Abcam, Cambridge, UK), mouse anti-human DNMT3B (ab13604, Abcam, Cambridge, UK), and mouse anti-human β-actin (#3700S, CST, MA, USA) were diluted to 1:800, 1:800, and 1:2000, respectively, and were incubated with the membranes overnight at 4°C. After the membranes were washed with TBST, the blots were visualized with anti-rabbit or anti-mouse IgG conjugated with peroxidase (HRP) and ECL reagents (Pierce, Rockford, IL, USA).

Drug treatment

5-Aza-2'-deoxycytidine (5-Aza-dC, Sigma Chemical Co., St Louis, MO, USA) was stored at -20°C at a concentration of 20 mM and freshly dissolved into culture medium before use. In brief, 5,000 cells/well were seeded on 96-well plates and routinely cultured for 24 h. For AZA treatment, AZA was continuously administered to the cells by replacing the medium containing 1 μM AZA

Table 1. Primers list

Genes	Primer Sequence (5'-3')	Experimental use
DNMT3A	F: GCCCATTCGATCTGGTGATT R: GCGGTAGAACTCAAAGAAGAG	Real-time PCR
DNMT3B	F: AAGAGTTGGGCATAAAGGTAGG R: CCTCAGTCGTTACGTATT	Real-time PCR
DNMT3A 3'-UTR	F: ATTGCTAGCCGAAAAGGGTTGGACATCAT R: TTAGTCGACGCCGAGGGAGTCTCCTTTTA	Real-time PCR
DNMT3B 3'-UTR	F: AAGGTCCCAAGGACCTAAATTACAGAGCCCTTAAT R: AATGTATTCCATTTTCAAACACTATGATGGGAGT	Real-time PCR
beta-actin	F: TCCCTGGAGAAGAGCTACGA R: AGCACTGTGTTGGCGTACAG Mature chain: UAGCACCAUUGAAUACAGUGUU	Real-time PCR
miR-29b	F: ACATCCAGCTGGGTAGCACCATTGAAATC R: TGGTGTGCTGGAGTCG Mature Chain: CCCAGUGUUUAGACUACUGUUC	Real-time PCR
miR-199b	F: CCCAGTGTTTAGACTATCTGTTC R: TGGTGTGCTGGAGTCG	Real-time PCR
U6	F: CTCGCTTCGGCAGCACA R: TGGTGTGCTGGAGTCG	Real-time PCR

every 24 h for 3 consecutive days. For TSA treatment, 300 nM TSA was added for 2 consecutive days. For both AZA and TSA treatments, AZA was added for the first 48 h, after which TSA was added for the next 24 h. Medium containing DMSO was used as a positive control.

Bisulfite genomic sequencing (BGS)

Genomic DNA was extracted from A2780 and SKOV3 cells using the phenol-chloroform approach. Bisulfite treatment was conducted using a CpGenome Universal DNA Modification Kit (Millipore, Boston, USA) according to the manufacturer's instructions. The PCR products were gel-purified and subcloned into a pMD19-T vector system for subsequent bisulfite sequencing (Takara, Dalian, China). At least five colonies were sequenced to evaluate the methylation degree of each CpG site. The primers are listed in Table 1.

Luciferase reporter assay

The DNMT3A and DNMT3B 3' UTRs containing the predicted miR-29b target sequence were amplified from genomic DNA (SKOV3 and A2780 cells) and cloned into the pGL3 firefly luciferase control vector (Promega, Madison, WI) at the XhoI restriction site directly downstream of the reporter gene. To generate DNMT3A and DNMT3B 3' UTRs with a mutant target sequence, transversion mutations of 7 nucleotides were made at the miR-29b seed region complementary sites as shown in Figure 2C. Inhibition of the luciferase reporter gene levels by miR-29b was assessed in SKOV3 and A2780 cells. Briefly, EOC cells were seeded onto 24-well plates and cultured until 70%–80% confluency; the cells were then co-transfected with either miR-29b or control mimics at a 120 nM final concentration or with 200 ng of a pGL3 reporter construct containing wild-type or miR-29b with the mutated DNMT3A and DNMT3B 3' UTRs using the X-treme GENE HP DNA Transfection Reagent according to instructions (Roche, Indianapolis, IN, USA). Relative firefly luciferase activity, normalized with Renilla luciferase, was then measured 48 h after the transfection with a dual-luciferase reporter gene assay system (Promega, Madison, WI, USA), and the results were manifested as the percentage change over the respective control.

Cell proliferation

Cell proliferation was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Amresco, Solon, OH, USA) assay. In brief, 5,000 cells/well were seeded on 96-well plates. After 72 h of routine culture, the cells were incubated in 20 µl MTT solution at a concentration of 5 mg/ml for 4 h at 37°C and then lysed in 150 µl of DMSO for 10 min at room temperature. The absorbance in each well at 570 nm was assessed using a microplate reader.

Immunohistochemistry assays

IHC assays were performed on single serial sections prepared from surgical samples. The slides were probed with a primary antibody against DNMT3A (1:50; ab13888, Abcam, Cambridge, UK), DNMT3B (1:50; ab13604, Abcam, Cambridge, UK) and then incubated with HRP-conjugated IgG (1:500; Invitrogen, Carlsbad, CA, USA). The proteins were visualized in situ with 3,3'-diaminobenzidine. High-power fields (400×) were photographed using an Eclipse 80i (Nikon, Tokyo, Japan) microscope.

Co-immunoprecipitation

When cultured A2780 cells had reached approximately 80% confluence, the cell layer was washed with ice-cold 1×PBS. Nuclear extracts were collected using the NE-PER nuclear and cytoplasmic extraction kit. Then, salts were removed from the nuclear extracts using the Pierce Slide-A-Lyzer MINI Dialysis Unit (Pierce Biotechnology, Rockford, IL). The supernatant was collected, and total protein (300 µg) was prepared for later immunoprecipitation. The supernatant was incubated at 4°C for 2 h with 1 µg of Mouse anti-human DNMT3A (ab13888, Abcam, Cambridge, UK) or mouse anti-human DNMT3B (ab13604, Abcam, Cambridge, UK) antibody and 40 µl of 25% protein A/G agarose slurry. The protein A/G agarose was centrifuged at 5000 rpm and washed 3–4 times with ice-cold lysis buffer. Proteins were eluted with 20 µl of SDS loading buffer by boiling for 5 min and subjected to immunoblot analysis with Rabbit anti-human Id-1 (SC-488, Santa Cruz, CA, USA) antibody.

Statistical analysis

All of the experiments were performed at least in triplicate. Each experiment was independently performed at least 3 times. Data were presented as the mean $\pm$ standard deviation (SD) and analyzed using GraphPad Prism 5 software. Statistical significance was assessed using a two-tailed unpaired Student's *t*-test. When a *P* value was less than 0.05, the differences were considered as statistically significant.

Results

miR-29b is inversely correlated with DNMT3A/3B in EOC

In our previous study, we have identified the differential expression of miR-29b among normal ovarian and EOC cell lines [19]. Among the four EOC cell lines tested, A2780 (with highest endogenous miR-29b level) and SKOV3 (with lowest endogenous miR-29b level) were selected as model cells in our present study (Fig. 1A). To explore the potential interaction between miR-29b and DNMT3s, we first examined the relative expressions of miR-29b and DNMT3A/3B in EOC at both cellular and histological levels. qPCR assays found that SKOV3 cells with low endogenous miR-29b expression showed relatively higher mRNA levels of DNMT3A and DNMT3B, whereas A2780 cells with high endogenous miR-29b expression showed relatively lower mRNA levels of DNMT3A and DNMT3B, indicating an inverse correlation between miR-29b and DNMT3s in ovarian cancer cells (Fig. 1B). Fifteen EOC tissues were picked up. qPCR assays were performed to detect the miR-29b expression, and the DNMT3A/3B levels were assessed by IHC in all EOC epithelia that were picked up. The results from scatter diagram found a negative correlation between miR-29b and DNMT3A/3B in EOC tissues (Fig. 1C). Typical IHC photos from both miR-29b^{high} and miR-29b^{low} groups were demonstrated in Figure 1D. Collectively, these results indicated an inverse correlation between the expression of miR-29b and DNMT3A/3B in ovarian cancer.

Fig. 1. miR-29b inversely correlated with DNMT3A/3B in EOC. A) and B) q-PCR results reveal a negative correlation between miR-29b and DNMT3A/3B in both SKOV3 and A2780 cells; C) Scatter diagram showing miR-29b expression by qPCR and DNMT3A/3B expressions by IHC and their correlations (miR-29b vs. DNMT3A: $r^2=0.28$, $P=0.04$; miR-29b vs. DNMT3B: $r^2=0.59$, $P=0.001$) in 15 tissues of EOC epithelia; D) IHC results indicate a negative correlation between miR-29b and DNMT3A/3B in EOC epithelia. Data are presented as means $\pm$ SEM, $n=3$. * $p<0.05$ versus control.

Fig. 2. miR-29b directly targets and thus negatively regulates DNMT3A/3B. A) and B) qPCR(A) and Western(B) results show that DNMT3A and DNMT3B expressions were significantly decreased by miR-29b over-expression and greatly increased by miR-29b inhibition at both the mRNA and protein levels in both SKOV3 and A2780 cells; C) and D) Schematic representation of different AKT2/AKT3 3'UTR luciferase reporters used in the transfection experiments are depicted; E) SKOV3/A2780 cells were co-transfected with either miR-29b or control mimics and 200 ng pGL3 reporter construct containing wild type or miR-29b site mutated 3'UTR of DNMT3A/3B. The relative firefly luciferase activities normalized with renilla luciferase were measured 48 hours post-transfection and results are plotted as percentage change over respective controls. Data are presented as means $\pm$ SEM. * p < 0.05 versus control.

miR-29b directly targets and thus negatively regulates DNMT3A/3B

The inverse correlation between miR-29b and DNMT3A/3B triggered our interest to explore the underlying mechanism. We assumed that miR-29b might play its part in epigenetic regulation by directly targeting DNMTs and thereby negatively regulating their expressions. To confirm our hypothesis, we employed micro-RNA mimics and inhibitors to specifically over-express and knock down the endogenous expressions of miR-29b in SKOV3 and A2780 cells, respectively. As shown in Figure 2A and 2B, the expression of both DNMT3A and DNMT3B were significantly decreased by being transfected with miR-29b mimics and were greatly increased with miR-29b inhibitors at both the mRNA and protein levels, indicating that miR-29b plays a role in negatively regulating DNMT3A and DNMT3B expression in both selected EOC cell lines. We then employed bioinformatics software (including miRanda, Targetscan, miRBase, and miRWalk) for a prediction of miR-29b downstream targets related to DNMT3s. By analyzing the 3'-UTR sequences of DNMT3A and DNMT3B as well as the mature chain sequence of miR-29b, we found that the "seed region" in the miR-29b mature

Fig. 3. DNMT3A/3B suppressed miR-29b in a DNA methylation-dependent manner. **A)** Effect of 5-Aza-CdR and TSA on miR-29b expression in EOC cells was tested. Treatment of 5'-AZA (1 μ M), TSA (300 nM), and combination of AZA and TSA all led to increase in miR-29b levels in both SKOV3 and A2780 cells. Results for miR-199b was used as a positive control; **B)** DNMT3A or DNMT3B knockdown as well as double knockdown of DNMT3A and DNMT3B via target siRNAs transfection led to increased in miR-29b levels in both SKOV3 and A2780 cells; **C)** WB results showing the effects of target siRNAs in knocking down DNMT3A and DNMT3B; **D)** Mapping of the methylation status of individual CpG sites in the miR-29b promoter by BGS assay in BGC-control, BGC-siDNMT3A/3B cells, as well as BGC-siDNMT3A+siDNMT3B cells. The regions spanning the CpG island with 8 CpG sites were analyzed. Each blue circle represents a methylated CpG site; while each white circle represents an unmethylated CpG site. Each row represents bisulfite sequencing of miR-29b promoter for a single analyzed random clone. **E)** methylated percentage diagram in BGC-control, BGC-siDNMT3A/3B cells, as well as BGC-siDNMT3A+siDNMT3B cells. Data are presented as means $\pm$ SEM. * $p < 0.05$ versus control.

chain is fully complementary with and thus potentially binds to the DNMT3A and DNMT3B 3'UTR sequences (Fig. 2C). This observation raises the possibility that miR-29b might negatively regulate DNMT3A and DNMT3B expressions by directly binding to their 3'UTR sequences. Finally, 3'UTR luciferase reporter assays confirmed that miR-29b directly binds to the 3'UTRs of both DNMT3A and DNMT3B. In brief, ovarian cancer cells were transfected with control or miR-29b mimics together with a luciferase construct containing either the wild-type DNMT3A/3B 3'UTR or a mutant DNMT3A/3B 3'UTR (Fig. 2D). Only transfection of the wild-type DNMT3A and DNMT3B 3'UTRs significantly decreased luciferase expression, and this suppressive effect of miR-29b was later abolished by mutating the miR-29b site in the DNMT3A and DNMT3B 3'UTRs (Fig. 2E). Together, these results demonstrated that miR-29b directly binds to its complementary sequence motifs in the DNMT3A and DNMT3B 3'UTRs, thus negatively regulating their expression.

DNMT3A/3B suppressed miR-29b in a DNA methylation-dependent manner

Because miR-29b is an important regulator in EOC, we next intended to explore the regulatory mechanism of miR-29b expression. In fact, aberrant hypermethylation is frequently observed in carcinogenesis and is critical for miRNA down-regulation[25, 26]. We thus raised the hypothesis that the downregulation of miR-29b may be attributed

Fig. 4. DNMT3A, but not DNMT3B, interacts with Id-1, a transcription factor related to EOC progression. A) Validation of the Dnmt3A/Id-1 interaction in A2780 cells by using immunoprecipitation method; B) MTT assay results reveal that both DNMT3A and DNMT3B siRNAs transfection led to a decrease in absorbance (OD value) at 570 nm in A2780 cells; C) A schematic showing interactions among miR-29b, DNMT3A, DNMT3B, Id-1 and epithelial ovarian cancer is provided. Data are presented as means $\pm$ SEM. * $p < 0.05$ versus control.

to promoter hypermethylation in ovarian cancer cells. To test our hypothesis, we first examined the expression of miR-29b in 5'-AZA- and/or TSA-treated EOC cells. We found that there was a 2.6-fold increase in themiR-29b levels after treatment with 5'-AZA, a 2-fold increase after treatment with TSA and a 2.4-fold increase after treatment with both 5'-AZA and TSA in SKOV3 cells, whereas there was a 3.5-fold increase after treatment with 5'-AZA, a 2-fold increase after treatment with TSA and a 3-fold increase after treatment with both 5'-AZA and TSA in A2780 cells (Fig. 3A), indicating that miR-29b expression in EOC cells might be regulated by DNA methylation. Because miR-199b has been demonstrated to be epigenetically inactivated in EOC, we used it as a positive control here [27]. Further, the down-regulation of DNMT3A/3B and the double knock-down of both DNMT3A and DNMT3B by transfection with target siRNAs led to increased miR-29b levels in both SKOV3 and A2780 cells, suggesting an inverse correlation between miR-29b and DNMT3A/3B in EOC cells (Fig. 3B). Considering the fact that DNMT3A and DNMT3B are direct downstream targets of miR-29b, we further raised the hypothesis that a negative feedback loop may exist between miR-29b and DNMT3A/3B. To test this hypothesis, the methylation status of CpG islands in the miR-29b promoter region was evaluated by a BGS assay in the DNMT3A/3B-knockdown EOC cells. First, WB assays were performed to test the knock-down effects of target siRNAs (Fig. 3C). Then, as shown in Figure 3D, 8 individual CpG sites within the CpG island regions were sequenced to identify methylated cytosine residues. miR-29b promoter methylation in DNMT3A/DNMT3B-knock-downas well as DNMT3A+DNMT3B double knock-down A2780 cells accounted for 30%, 33.75%, and 23.75%, with its SKOV3 cells accounting for 46.25%, 42.5%, and 35%, respectively, all of which were significantly lower than the frequency of control A2780 cells(56.25%) and SKOV3 cells(60%)(Fig. 3D and 3E). These results indicated that the transcriptional silencing of miR-29b in EOC cells is associated with CpG island hypermethylation. Collectively, the hypothesis of a negative feedback interaction was confirmed between miR-29b and DNMT3Aand between miR-29b and DNMT3B, which also indicates an important molecular mechanism underlying miR-29b down-regulation in EOC.

DNMT3A, but not DNMT3B, interacts with Id-1, a transcription factor related to EOC progression

Over the past decade, it has been found that interactions between Dnmts and transcription factors could target specific-sites of Dnmt promoter regions, which then promotes the methylation of these promoters. However, only a few observations are available reporting the interactions between transcription factors and de novo Dnmts. In our previous study, we identified the negative regulation of Id-1, a transcription factor promoting EOC progression, by miR-29b. Intriguingly, previous data from transcription arrays indicated a putative interaction between DNMT3A and Id-1, which aroused our interest in further exploring the interaction between Id-1 and DNMT3A/3B. By performing Dnmt3A-immunoprecipitation from chromatin, Id-1 was co-immunoprecipitated, suggesting that the Dnmt3A/Id-1 interaction occurred in EOC cells. However, Id-1 failed to be co-immunoprecipitated in Dnmt3B-immunoprecipitation, indicating that no direct interaction was found between Dnmt3B and Id-1 (Fig. 4A). Moreover, MTT assay results showed that DNMT3A and DNMT3B also influenced ovarian cancer cell proliferation. Specifically, the down-expression of DNMT3A and DNMT3B via the target siRNAs transfection led to a decrease in the absorbance (OD value) at 570 nm in both SKOV3 and A2780 cells, suggesting that DNMT3A and DNMT3B have a role in enhancing EOC cell proliferation (Fig. 4B). Similarly, we have also demonstrated the role of miR-29b and Id-1 in manipulating EOC growth and motility in early studies [19, 28]. Collectively, these results reveal a mutual interaction among miR-29b, DNMT3a/3B and Id-1 that promotes the progression of EOC (Fig. 4C).

Discussion

An increasing body of evidence indicates that miRNAs could act as oncogenes or tumor-suppressor genes to regulate multiple cellular events in both tumor onset and progression [29, 30]. Among the currently identified 2,000-odd microRNAs, miR-29b is a well-documented tumor-suppressor miRNA (or namely epi-miRNA) that is found dysregulated in various human cancers, including non-small-cell lung cancer, breast cancer, and prostate cancer [30-34]. In the current study, we showed that miR-29b negatively regulates the de novo methyltransferases DNMT3A and DNMT3B by directly targeting their 3'UTR sequences, which adds a novel role for miR-29b as an epigenetic regulator in EOC development. Of note, our data are in agreement with previous reports demonstrating that miR-29b is capable of regulating DNMTs in lung cancer lines, which suggests that the epi-miRNA miR-29b's regulation of DNMTs may be a common feature in cancer cells [35]. miRNAs are well known for repressing target genes by forming complementary base-pairs with specific sequences in 3'UTRs of mRNAs. The mechanism of posttranscriptional regulation has been found to be related to the degree of complementarity between miRNAs and their targeted mRNAs. Specifically, complete or perfect pairing between miRNAs and target mRNAs usually induces target mRNA cleavage, whereas partial pairing most often results in translational repression and mRNA decay through deadenylation pathways [36]. There is also evidence showing that the extent of base-pairing also affects stability of the miRNA itself, which later causes a positive or negative feedback for miRNA's repression of target genes [37]. In our study, miR-29b affects not only translation but also gene transcription of DNMT3A/3B, which may have contributed to the perfect matching between miR-29b and DNMT3A/3B 3' UTRs. In fact, more than one pairing site with miR-29b has been found in both DNMT3A and DNMT3B 3'UTRs.

The role of epigenetic alterations in the pathogenesis of malignancies, including EOC, has aroused increasing interest. DNA methylation is manipulated by DNMTs. Unlike DNMT1, which preferentially maintains established DNA methylation patterns after replication, DNMT3A and DNMT3B play a critical role in the de novo DNA methylation process [38]. DNMT dysregulation has been frequently reported in malignant tumors, including leukemia, prostate cancer, and gastric cancer [39-41]. However, elucidation of the role of dysregulated

DNMTs in ovarian carcinogenesis is in its infancy. Employing bioinformatics-based predictions, we found that miR-29b was involved in the regulation of DNMT3A and DNMT3B expressions. Further evidence for this regulation was provided by reporter gene assays and rescue experiments. We also observed that treatment of SKOV3 and A2780 cells with 5-aza-dC resulted in an increase in miR-29b levels, suggesting that miR-29b expression in EOC cells might be regulated by DNA methylation. Later, a BGS assay confirmed that the transcriptional silencing of miR-29b in EOC cells is associated with CpG island hypermethylation. Together, these results revealed a double-negative feedback loop between miR-29b and DNMT3A/3B, which consequently strengthened the anti-cancer function of miR-29b and highlighted a potential therapeutic implication of miR-29b as well as DNMT3s inhibitors in future EOC treatment.

The transcription factor Id-1 has been found to be upregulated in EOC and has been associated with dedifferentiated, proliferating and invasive cell phenotypes, advanced tumor stage, and poor prognosis [42, 43]. In our previous study, we have revealed miR-29b's direct targeting and negative regulation of Id-1 and Id-1's role in EOC progression [28]. Here, we further revealed the mutual interaction between DNMT3A and Id-1, thus confirming that the miR-29b-DNMT3A/3B-Id-1 axis regulates the activity of aberrant methylation patterns, and epigenetic alterations may be early events in the malignant transformation of cells. The description of the direct interaction of Dnmt3A with transcription factors also provides a possible explanation for the hypermethylation of target genes and the mechanisms employed by transcriptional factors in the manipulation of gene expression. Admittedly, the specific molecular mechanisms underlying the DNMT3A/3B-Id-1 interaction and its role in EOC development call for further exploration in future studies.

In summary, the data obtained in the present study indicated that with a complementary structure, miR-29b combines and thus negatively regulates DNMT3A and DNMT3B. DNMT3A and DNMT3B also suppress miR-29b through DNA methylation. The mutual negative feedback between miR-29b and both DNMT3A and DNMT3B raises the possibility that miR-29b and DNMT3s inhibitors may be potent options for future clinical treatments of ovarian cancer.

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Disclosure Statement

The authors declare that no potential conflicts of interest exist.

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