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ARTICLE

miR-9 Regulation of BRCA1 and Ovarian Cancer Sensitivity to Cisplatin and PARP Inhibition

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Background Expression of BRCA1 is commonly decreased in sporadic ovarian cancer, and this is associated with platinum sensitivity and favorable prognosis. However, multiple mechanisms underlying low BRCA1 expression are not fully understood.

Methods A bioinformatics-driven microRNA (miR) library screening was used to identify miRs that regulate BRCA1 expression. The effects of miR-9 on cisplatin (cDDP) and PARP inhibitor sensitivity were measured in ovarian cancer cells and C13* xenograft mice (n = 6 per group). The roles of miR-9 on prognosis were assessed in a cohort of ovarian cancer patients (n = 113) with Kaplan–Meier and Cox proportional hazards analyses. All statistical tests were two-sided.

Results Reverse miR library screening revealed that miR-9 reduced the normalized luciferase activity to 60.3% (95% confidence interval [CI] = 52.0% to 68.5%; $P < .001$). miR-9 bound directly to the 3′-UTR of BRCA1 and downregulated BRCA1 expression in ovarian cancer cells. Treatment with miR-9 agomiR sensitized BRCA1-proficient C13* xenograft tumors to cisplatin and AG014699. In serous ovarian cancer, higher levels of miR-9 were inversely correlated with BRCA1 expression (Spearman rank correlation: $R^2 = 0.379$; $P = .003$). Patients with higher levels of miR-9 had better chemotherapy response, platinum sensitivity, and longer progression-free survival (PFS) (high vs low miR-9 expression: median PFS = 26.4 months, 95% CI = 13.8 to 39.0 months vs median PFS = 15.4 months, 95% CI = 6.8 to 23.9 months, $P = .01$).

Conclusions miR-9 mediates the downregulation of BRCA1 and impedes DNA damage repair in ovarian cancer. miR-9 may improve chemotherapeutic efficacy by increasing the sensitivity of cancer cells to DNA damage and may impact ovarian cancer therapy.

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Epithelial ovarian cancer (EOC) is the leading cause of gynecological cancer related deaths in the developed world, with approximately 64 500 anticipated deaths from the disease in 2011 (1). Although the initial response of ovarian carcinomas to standard therapy (surgical debulking and platinum-based chemotherapy) is often promising, platinum-resistant relapse usually occurs and patients succumb to their disease.

Women with a germ-line mutation of *BRCA1* have an increased risk of developing ovarian cancer (2) but also have an improved chemotherapy response and a better prognosis (3,4). This phenomenon is because BRCA1 is a key component of the error-free homologous-recombination (HR) double-strand DNA repair pathway (5,6). Recent data suggest that many sporadic EOC (SEOC) patients display "BRCAness," or dysfunction of BRCA1. According to previous studies, dysregulation of BRCA1 has been attributed to somatic mutation or aberrant methylation of the *BRCA1* promoter. But somatic BRCA1 mutations are uncommon in SEOCs (approximately 9% of cases) (7–9), and *BRCA1* promoter methylation has been reported in only 5% to 15% of SEOC cases (10–12). Thus, it seems likely that BRCA1 is dysregulated by other mechanisms because somatic mutation and promoter methylation, even combined, cannot account for the reduction of BRCA1 expression in SEOCs.

MicroRNAs (miRs) can post-transcriptionally repress target gene expression by binding to the 3′-UTR of messenger RNA (mRNA). Their regulatory potential is vast, with more than 60% of protein coding genes being computationally predicted as miR targets (13). We hypothesize here that the downregulation of the BRCA1 protein is achieved through the action of miR overexpressed in ovarian cancer.

Methods

In Silico Prediction and Luciferase Assay for miR Targeting of *BRCA1* 3′-UTR

Six algorithms were used to predict potential miR targeting of 3′-UTR of *BRCA1*: Pictar (http://pictar.mdc-berlin.de/), Targetscan

(http://www.targetscan.org/), DIANA-microT (http://diana.cslab.ece.ntua.gr/microT/), microCosm Targ (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets), MiRanda (http://www.microrna.org/microrna/home.do) and RNA22 (http://cbcsrv.watson.ibm.com/rna22.html). To reduce the number of false positives, only the miR that were predicted by at least four algorithms were subsequently validated through luciferase reporter assay (details are described in the Supplementary Methods, available online).

Tissue Samples

With the approval and support of the Ethics or Institutional Review Board of Tongji Hospital, serous ovarian cancer patients staged IIIC or IV (International Federation of Gynecology and Obstetrics staging) were gathered. All patients underwent debulking and subsequent platinum/taxane-based chemotherapy. Informed consent was obtained from all patients. Progression-free survival (PFS) was calculated from time of surgery to time of progression or recurrence. Platinum resistance or platinum sensitivity was defined as relapse or progression within 6 months or after 6 months from the last platinum-based chemotherapy, respectively. Primary therapy response was defined as response evaluation criteria in solid tumors (RECIST). All samples were used to construct the tissue microarrays. Then, miR-9 expression was determined by in situ hybridization with the miRCURY LNA microRNA ISH Optimization Kits (FFPE) (Exiqon, Vedbaek, Denmark) following the manufacturer's recommendations, and BRCA1 expression was detected by immunohistochemical staining (details are described in the Supplementary Methods, available online).

Tumor Xenograft Studies

We subcutaneously injected 5×10^6 C13* cells resuspended in 50 μL of phosphate-buffered saline into the right flank of 4 week old BALB/C athymic mice (HFK bioscience, Beijing). Mice were housed and maintained under specific pathogen-free conditions. All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of Tongji Hospital. When mice had palpable tumors, the mice were randomly assigned, to avoid treatment bias, to treatment groups (n = 6 mice per group). AG014699 (20 mg/kg) was given intraperitoneally daily for 28 consecutive days; cDDP (5 mg/kg) was given intraperitoneally every 4 days for 28 days. For agomiR treatment, agomiR-9 or agomiR-NC (RiboBio, Guangdong, China) was directly injected intratumorally at the dose of 1 nmol (diluted in 20 μL phosphate-buffered saline) per mouse every 4 days for 7 times. Tumor volumes were calculated as length × (square of width)/2. After the initial treatment, the tumor size was determined every day. Mice were killed by cervical dislocation under anesthesia. Investigators were blinded to the treatment groups.

Statistical Analysis

A two-sided Student *t* test was used to compare the differences between two groups of cell experiments in vitro. Analysis of variance was used to compare the differences among multiple groups. BRCA1 and miR-9 expression values were first analyzed as continuous variables, and Spearman rank correlation was used to determine the correlation between BRCA1 and miR-9 expression. Mann–Whitney *U* test was used to determine the differences in BRCA1 and miR-9 expression between platinum-sensitive or -resistant groups. Differences in patient characteristics between different BRCA1 and miR-9 groups were tested with Pearson χ^2 test. Differences in PFS between different groups were examined with Kaplan–Meier curves with the log-rank test and Cox proportional hazards analysis. A two-sided *P* less than .05 was considered statistically significant. All statistical analyses were done using SPSS 17.0 (SPSS Inc, Chicago, IL).

Results

miR-9 Targeting of *BRCA1* in Ovarian Cancer Cells

Intrinsic BRCA1 levels were associated with cDDP sensitivity (Spearman $R^2 = 0.900$; $P = .04$) and HR function in a panel of ovarian cancer cells (OV2008, C13*, SKOV3, A2780, CaOV3) (Supplementary Figures 1 and 2, available online). Moreover, BRCA1 downregulation by small interfering RNA (siRNA) sensitized C13* cells to cDDP by impairing HR function (Supplementary Figure 3, available online). However, methylation-specific polymerase chain reaction (PCR) revealed that none of these cells have *BRCA1* promoter methylation (Supplementary Figure 4). Thus, we believe there are mechanisms other than methylation involved in BRCA1 dysregulation in ovarian cancer.

Genome-wide miR library screening has been successfully used to identify miRs that regulate the expression of proteins involved in drug sensitivity (14,15); however, this strategy is both time- and labor-intensive. Therefore, we designed a bioinformatics-driven screening approach in which we used comprehensive bioinformatics analysis as a filter to generate a selective miR library to perform subsequent screening. A total of 28 miRs were successfully identified as candidate miRs (Supplementary Table 1, available online).

Reverse screening with the candidate miR library was carried out by luciferase assay. miR-9 remarkably reduced the normalized luciferase activity to 60.3% (95% CI = 52.0% to 68.5%) compared with miR NC (100%; $P < .001$) (Figure 1A). The RNA22 algorithm showed that the bases from 928 to 933 in the *BRCA1* 3′-UTR have perfect complementarity to the "seed" sequence of miR-9 (Figure 1B). Quantitative reverse-transcription PCR (qRT-PCR) in the five aforementioned cell lines indicated an inverse expression trend between miR-9 and BRCA1 (Figure 1C), which was further validated in six ovarian cancer cells of NCI60 ($r = -0.733$; $P = .04$) (Supplementary Figure 5, available online). Transient transfection of miR-9 in C13* cells reduced BRCA1 expression (Figure 1D), whereas it had no statistically significant effect on cell cycle distribution (Supplementary Figure 6, available online), indicating that reduction of BRCA1 expression by miR-9 is not due to blocking of cell cycle progression. Furthermore, qRT-PCR showed that BRCA1 mRNA was reduced to 33.9% in miR-9–overexpressing C13* cells (95% CI = 5.3% to 62.4%; $P < .001$). BRCA1 mRNA was 1.61-fold higher in miR-9 inhibitor transfected OV2008 cells (95% CI = 1.03- to 2.18-fold; $P = .02$), 4.15-fold higher in miR-9 inhibitor transfected A2780 cells (95% CI = 3.61- to 4.68-fold; $P < .001$) (Figure 1E), and 2.04-fold higher in miR-9 inhibitor transfected SKOV3 cells (95% CI = 1.37- to 2.71-fold; $P < .001$) (Supplementary Figure 7) than in control cells. These results suggest that miR-9 regulates BRCA1 expression in ovarian cancer cells.

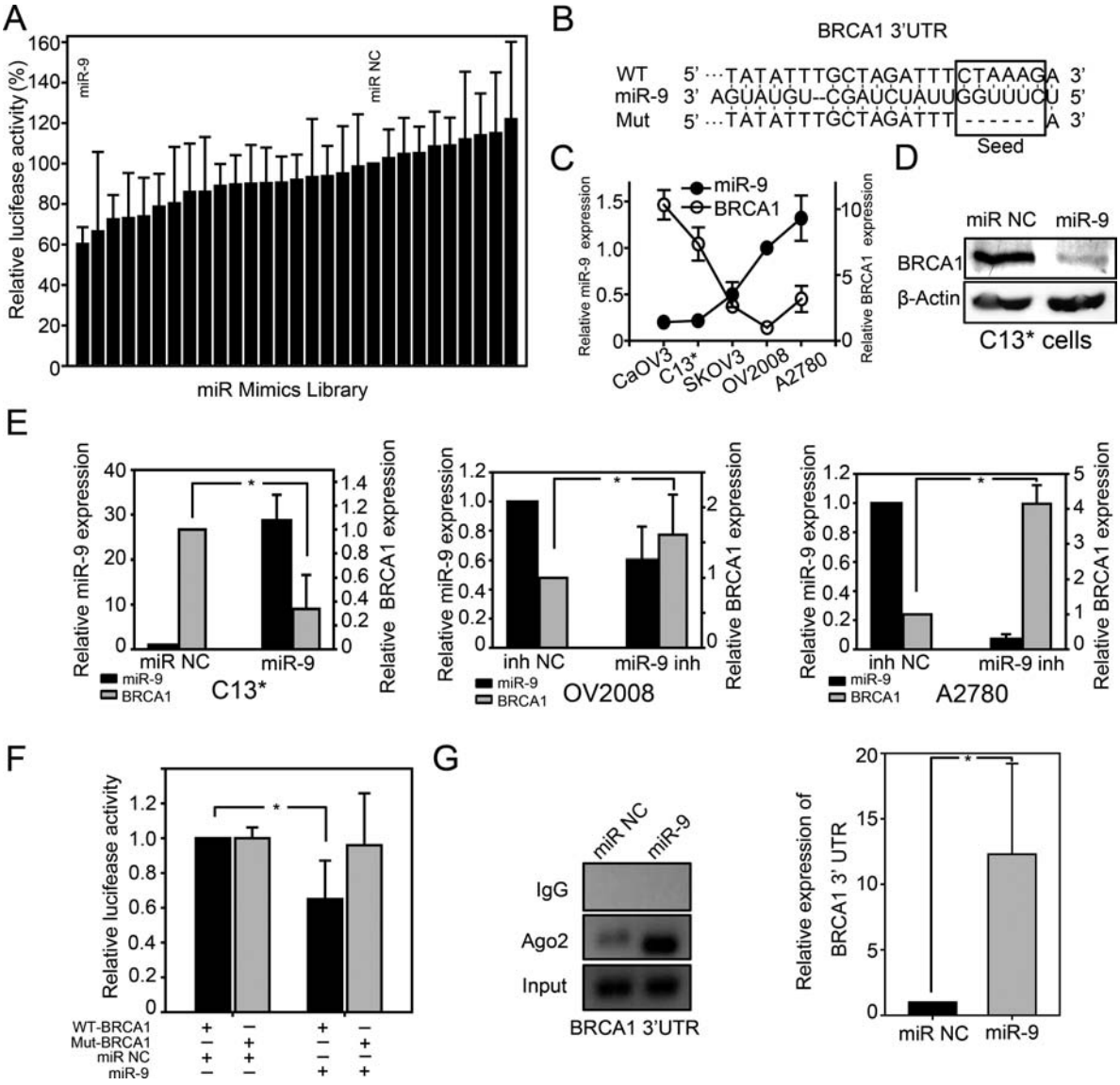

Figure 1. MicroRNA (miR)–9 targeting of *BRCA1* in ovarian cancer cells. **A**) Reverse miR library screening using the luciferase reporter system. Two hundred ninety-three cells were cotransfected with the wild-type (wt) *BRCA1* 3′-UTR reporter plasmid and the candidate miR-mimic, which was predicted by at least four bioinformatics algorithms, or the miR mimics negative control (miR NC) for 36 hours. Relative Renilla luciferase activity changes were normalized to control that was cotransfected with the miR NC and shown as the mean ± 95% confidence interval from four independent experiments. **B**)The predicted duplex formation between human wt *BRCA1* 3′-UTR and miR-9 (**upper and middle sequence**), and the ideograph of the mutated *BRCA1* 3′-UTR with six seed nucleotide deletions are shown (**bottom sequence**). **C**) Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis in these five ovarian cancer cell lines showed an inverse expression trend between miR-9 and BRCA1 expression. The expression levels of miR-9 and BRCA1 were normalized to OV2008 (set at 1). **D**) Western blot analysis of miR-9 dependent down-regulation of BRCA1 protein in C13* cells. **E**) C13* cells that contain low endogenous levels of miR-9 were transiently transfected with miR-9 mimics or miR NC for 48 hours. qRT-PCR showed miR-9 overexpression reduced BRCA1 mRNA levels (*P < .001, two-sided Student t test). Data represent the mean ± 95% confidence interval from three independent experiments (**left panel**). OV2008 and A2780 cells that contain high endogenous levels of miR-9 were transfected with the miR-9 inhibitor or miR inhibitor NC for 48 hours. qRT-PCR showed miR-9 inhibitors increased *BRCA1* mRNA expression (*P = .02 and *P < .001, respectively; two-sided Student t test) (**middle and right panel**). Data represent the mean ± 95% confidence interval from three independent experiments. **F**) Relative Renilla luciferase activity of the wt or mutant (mut) *BRCA1* 3′-UTR reporter gene in C13* cells transfected with the miR-9 mimics or miR NC for 36 hours (P = .003, two-sided Student t test). Data represent the mean ± 95% confidence interval from three independent experiments. **G**) C13* cells were transfected with miR-9 or the miR NC for 48 hours. Ribonucleoprotein immunoprecipitation showed that the miR-9/Ago2 complex was enriched in *BRCA1* 3′-UTR more than the control group (**left panel**). qRT-PCR showed that miR-9 overexpression enriched binding to the *BRCA1* 3′-UTR by approximately 12-fold. (P = .002, two-sided Student t test) Data represent the mean ± 95% CI from four independent experiments (**right panel**). IgG = immunoglobulin G.

To substantiate the site-specific repression of miR-9 on *BRCA1*, we constructed a mutated *BRCA1* 3′-UTR luciferase reporter (Figure 1B), which completely restored luciferase activity (Figure 1F). To further prove that miR-9 binds to *BRCA1* 3′-UTR in cells, the ribonucleoprotein immunoprecipitation (RNP-IP) technique was used. In cells that overexpress a specific miRNA, RNP-IP of Ago2 selectively enriched for the overexpressed miRNA and its corresponding target mRNAs. We found that the miR-9/Ago2 complex associated selectively with the *BRCA1* 3′-UTR at levels remarkably higher than the control group with obvious

differences shown by RT-PCR (Figure 1G, left panel). qRT-PCR showed that miR-9 overexpression enriched *BRCA1* 3′-UTR to almost 12.30-fold (95% CI = 5.38- to 19.22-fold; P = .002) (Figure 1G, right panel).

Effect of miR-9 on cDDP Sensitivity and the HR Pathway

BRCA1 is an integral component of the cellular DNA damage response. Thus, miR-9–mediated BRCA1 downregulation is hypothesized to increase the sensitivity of cells to cDDP. miR-9 overexpression decreased BRCA1 protein levels of C13* cells and sensitized C13* cell to cDDP (control: mean IC_{50} [the half maximal inhibitory concentration] = 87.1 μM, 95% CI = 82.0 to 92.6 μM; miR-9: mean IC_{50} = 31.4 μM, 95% CI = 26.1 to 37.7 μM; BRCA1 siRNA: mean IC_{50} = 37.7 μM, 95% CI = 33.1 to 42.9 μM) (Figure 2A). Next, cells transfected with miR-9 demonstrated reduced colony formation rates by approximately 60% compared with control after exposure to cDDP (50 μM) (Figure 2B) (mean of 38.8% compared with control as 100%; 95% CI = 26.1% to 51.5%; P < .001). miR-9 overexpression caused a reduction of BRCA1 and Rad51 foci, but not γ-H2AX foci, when treated with cDDP (50 μM) for 24 hours (Figure 2, C and D). Comet array revealed that C13* cells with ectopic overexpression of miR-9 had higher residual DNA damage (miR-9: mean = 27.3%, 95% CI = 17.9% to 36.7%; control: 2.9%, 95% CI = 1.6% to 4.2%; P = .004) (Figure 2E). In a reciprocal experiment, the cell-surviving fraction after cDDP treatment was statistically significantly increased in high miR-9 background OV2008 and A2780 cells with miR-9 inhibition compared with control (Figure 2, F and G). However, we found that miR-9 levels had no statistically significant impact on paclitaxel sensitivity (Supplementary Figure 8, available online).

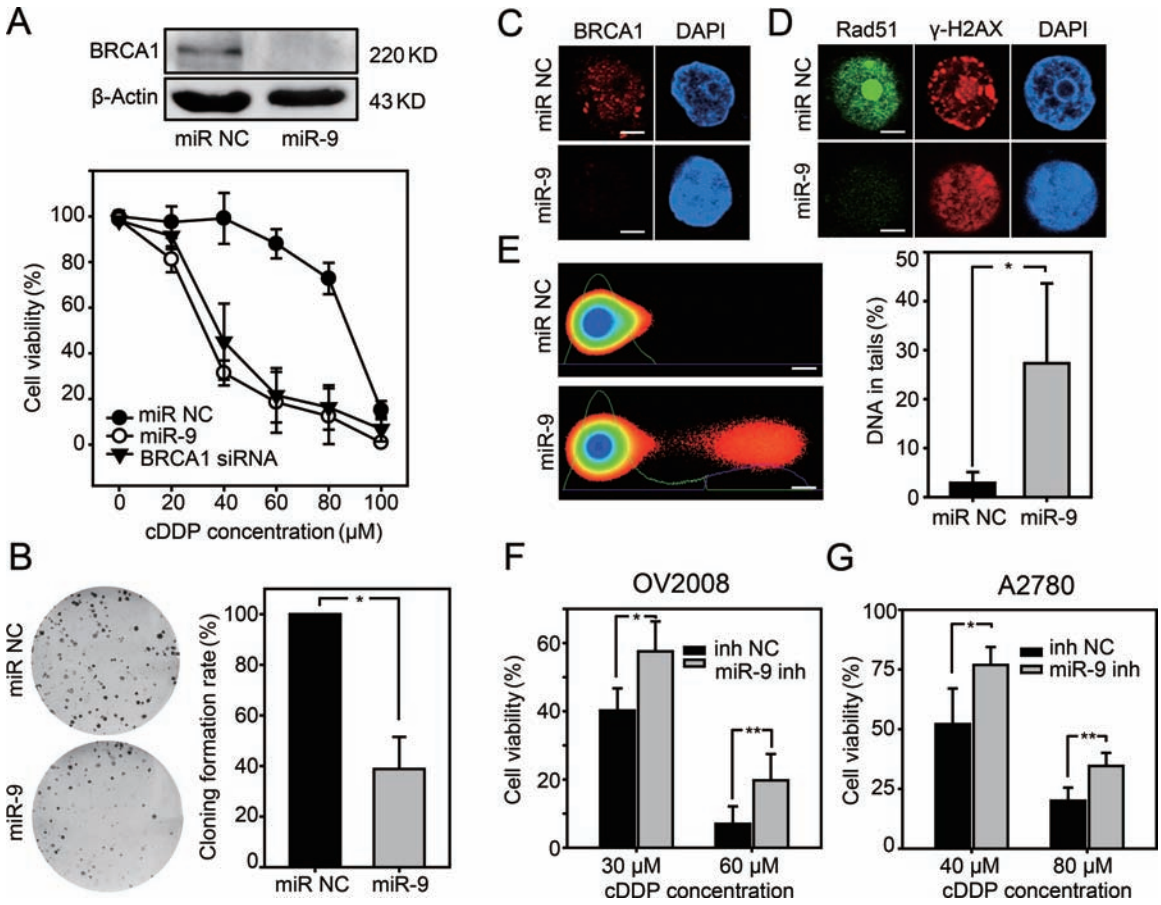

Figure 2. Effect of microRNA (miR)–9 on cisplatin (cDDP) sensitivity and the homologous-recombination (HR) pathway through BRCA1 inhibition. **A**) C13* cells were transfected with miR-9 mimics or the miR mimic negative control (NC) for 48 hours. Cell viability was assayed after treatment with increasing concentrations of cDDP for 48 hours by CCK-8. Western blot analysis validated the knockdown of BRCA1 by miR-9 overexpression (**upper panel**). BRCA1 small interfering RNA was used as a positive control. Data are shown as the mean ± 95% confidence interval from three independent experiments. **B**) Colony formation of C13* cells transfected with miR-9 mimics or miR NC after exposure to cDDP for 12 hours (50 μmol/L) (*P < .001 vs miR NC, two-sided Student t test). **C** and **D**) C13* cells were transfected with miR-9 mimics or the miR NC and treated with cDDP (50 μmol/L) for 24 hours and then fixed for immunofluorescence staining of BRCA1 (**C**) and γH2AX/Rad51 (**D**). Nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI). Representative images of immunofluorescence are shown. Scale bars = 5 μm. **E**) C13* cells were transfected with miR-9 mimics or miR NC for 48 hours. Transfected cells were incubated with cDDP (50 μmol/L) for 24 hours and analyzed by single-cell gel electrophoresis. Representative images are shown. Scale bars = 5 μm. Residual DNA damage after cDDP treatment is increased in miR-9 mimic transfected cells (*right panel*). (*P = .004, two-sided Student t test). **F** and **G**) OV2008 and A2780 cells were transfected with the miR-9 inhibitor (inh) or miR inhibitor NC and then treated with an increasing concentration of cDDP for 48 hours. The miR-9 inhibitor statistically significantly changed cell sensitivity to cDDP in OV2008 (*P = .003, **P = .001, two-sided Student t test) (**F**) and A2780 (*P = .003, **P = .005, two-sided Student t test) (**G**). Data in panels (**B**) and (**E**–**G**) are the mean ± 95% confidence interval from three experiments.

Prognostic Role of miR-9 in Ovarian Cancer Patients

The physiological relevance of the BRCA1/miR-9 interaction was further established by evaluating the endogenous expression pattern of miR-9 and BRCA1 in ovarian cancers patients. miR-9 and BRCA1 mRNA levels were quantified using qRT-PCR. Among these 58 samples, BRCA1 was higher (approximately twofold; low miR-9: mean relative expression level = 0.0049, 95% CI = 0.0032 to 0.0066; high miR-9: mean relative expression level = 0.0024, 95% CI = 0.0013 to 0.0036; P = .009) in the miR-9 low group than in the miR-9 high group (median as the cutoff value) (Figure 3A). Using Spearman correlation analysis, an inverse correlation, with R^2 = 0.379, was observed between miR-9 and BRCA1 (P = .003), suggesting the existence of miR-9– dependent regulation of BRCA1 (Figure 3B). We also analyzed *BRCA1* promoter methylation status in these patients. Only 5 patients (8.62%; n = 5 of 58) presented with *BRCA1* methylation, and there was no statistically significant difference of BRCA1 mRNA expression between methylated and nonmethylated patients (P = .77) (Supplementary Figure 9, available online). We next determined the levels of miR-9 expression by in-situ hybridization and the expression of BRCA1 by immunohistochemistry in another series of 113 ovarian tumors for which clinical and prognosis data were available. Basic clinical characteristics are summarized in Table 1. There were no statistically significant associations between BRCA1/miR-9 expression and clinical variables, such as age, stage, lymph node involvement, and grade. However, high miR-9 expression was associated with better therapy response (P = .002) and platinum sensitivity (P = .030). We found a similar inverse correlation between BRCA1 and miR-9 expression (Spearman R^2 = 0.198; P = .04). Representative images are showed in Figure 3C. Moreover, platinum-resistant patients showed higher BRCA1 expression (P < .001) and lower miR-9 expression (P = .03) (Figure 3D). The associations of platinum resistance and PFS with clinical variables including BRCA1 and miR-9 expression are presented. Similar to low BRCA1 expression (low vs high BRCA1 expression: median PFS = 37.3 months, 95% CI = 22.6 to 50.1 months vs median PFS = 15.2 months, 95% CI = 10.3 to 20.1 months, P = .03), high expression of miR-9 is associated with platinum sensitivity (P = .03) (Supplementary Table 2, available online) and longer PFS (high vs low miR-9 expression, median PFS = 26.4 months, 95% CI = 13.8 to 39.0 months vs median PFS = 15.4 months, 95% CI = 6.8 to 23.9 months, P = .02) (Figure 3E; Supplementary Table 3, available online). These data support the hypothesis that miR-9–dependent

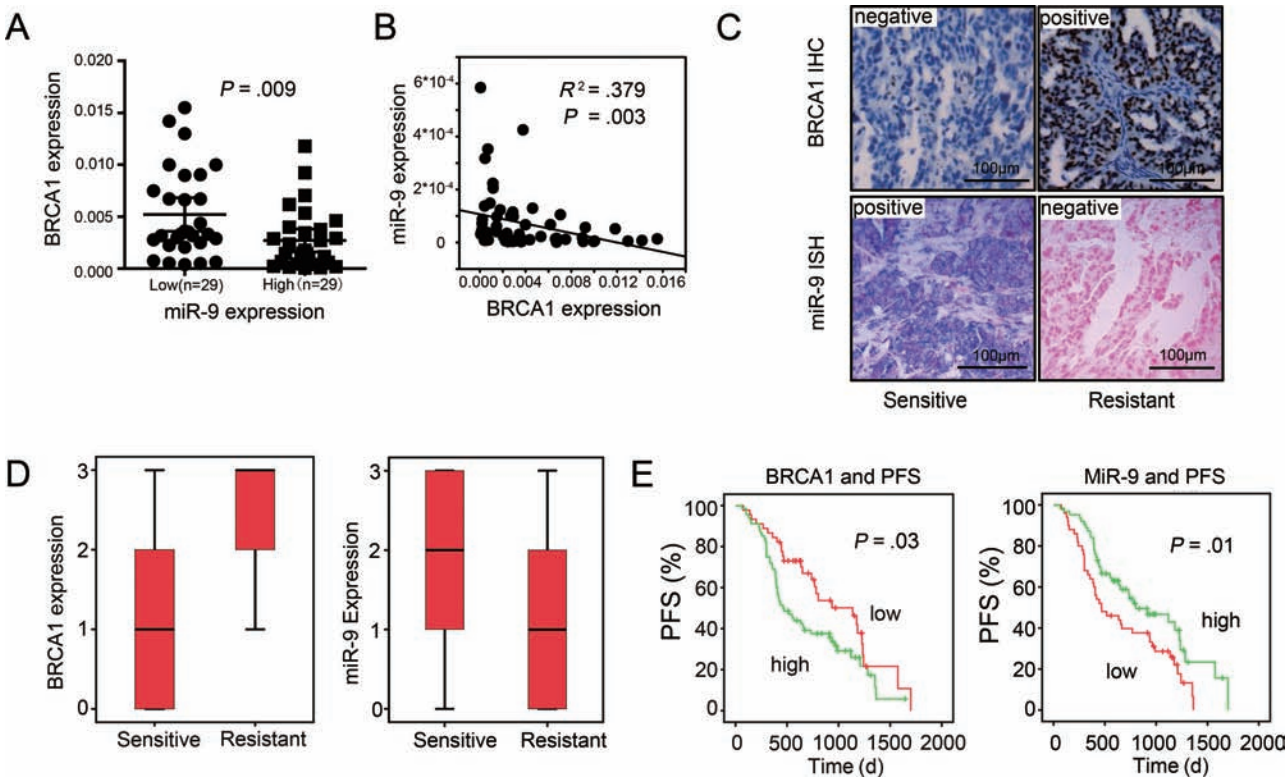

Figure 3. Prognostic role of microRNA (miR)–9 in ovarian cancer patients. **A** and **B**) BRCA1 protein and miR-9 expression are inversely correlated in ovarian cancer samples. miR-9 expression was quantified by quantitative reverse-transcription polymerase chain reaction (normalized to U6) in 58 clinical ovarian cancer tissue samples. BRCA1 expression was quantified with β-actin as a control. Patients were divided equally into two groups according to miR-9 expression level, and BRCA1 expression showed considerable differences between miR-9 low and miR-9 high groups. (P = .009, Mann–Whitney U test) (**A**). A plot of the relative expression of miR-9 vs BRCA1 showed an inverse correlation between them. Correlation index R^2 and P values were calculated using Spearman rank test (R^2 = 0.379; P = .003) (**B**). **C**) Paraffin-embedded, formalin-fixed ovarian cancer tissues were incubated with a locked nucleic acid anti-miR-9 probe for in-situ hybridization (ISH) and anti-BRCA1 antibody for immunohistochemical (IHC) analysis with scrambled probe and phosphate-buffered saline as a negative control, respectively. Representative photographs are shown. Scale bars = 100 μm. **D**) The differences of BRCA1 (**left panel**) and miR-9 expression (**right panel**) between platinum-based chemotherapy–sensitive and –resistant patients in ovarian cancer specimens is shown. Platinum-based chemotherapy–resistant patients showed higher BRCA1 expression (P < .001, Mann–Whitney U test) (**right panel**) and lower miR-9 expression (P = .03, Mann–Whitney U test), when compared with chemotherapy-sensitive ovarian patients (**left panel**). **E**) A Kaplan–Meier analysis of progression-free survival (PFS) for ovarian cancer patients with the corresponding expression profiles of BRCA1 (**left**) and miR-9 (**right**) is shown (P = .03 and P = .01, respectively, log-rank test). All statistical tests were two-sided.

Table 1. Clinicopathological characteristics of ovarian cancer patients and association with BRCA1 and miR-9 expression*

Variables	All (n = 113) No.	BRCA1 Low (n = 45) No. (%)	BRCA1 High (n = 68) No. (%)	BRCA1 P	miR-9 Low (n = 50) No. (%)	miR-9 High (n = 63) No. (%)	miR-9 P
Age, mean (SD)	50.0 (9.1)	50.6 (9.1)	49.7 (9.1)	.60	47.8 (9.3)	51.8 (8.6)	.02¶
Stage†							
IIIC	97	40 (88.9)	57 (83.8)	.45	44 (88.0)	53 (84.1)	.56
IV	16	5 (11.1)	11 (16.2)		6 (12.0)	10 (15.9)	
Grade‡							
High	19	9 (20.0)	10 (14.7)	.66	7 (14.0)	12 (19.0)	.74
Moderate	41	17 (37.8)	24 (35.3)		18 (36.0)	23 (36.5)	
Low	53	19 (42.2)	34 (50.0)		25 (50.0)	28 (44.4)	
Lymph nodes							
Negative	48	22 (48.9)	26 (38.2)	.67	23 (46.0)	25 (39.7)	.50
Positive	65	23 (51.1)	42 (61.8)		27 (54.0)	38 (60.3)	
Residual tumor							
≤1cm	88	32 (71.1)	56 (82.4)	.16	38 (48.0)	50 (68.3)	.67
>1cm	25	13 (28.9)	12 (17.6)		12 (52.0)	13 (31.7)	
Response to primary therapy§							
CR	88	39 (76.0)	49 (55.7)	.07	32 (64.0)	56 (88.9)	.002¶
Non-CR	25	6 (24.0)	19 (44.3)		18 (36.0)	7 (11.1)	
Platinum status‖							
Sensitive	67	34 (75.6)	33 (48.5)	.004¶	24 (35.8)	43 (64.2)	.03¶
Resistant	46	11 (24.4)	35 (51.5)		26 (56.5)	20 (43.5)	

* CR = complete response; SD = standard deviation.

† Stage based on International Federation of Gynecology and Obstetrics.

‡ Grade based on histological features.

§ Non-CR includes partial response, stable disease, and progressive disease assessed per Response Evaluation Criteria In Solid Tumors (RECIST) criteria.

‖ Platinum resistance or sensitivity was defined as relapse or progression within 6 months or after 6 months from the last platinum-based chemotherapy, respectively.

¶ Statistically significant by two-sided χ^2 test with *P* less than .05.

regulation of BRCA1 is associated with platinum sensitivity and prognosis of ovarian cancer patients.

Effect of miR-9 on Ovarian Cancer Cells Response to the PARP Inhibitor In Vitro

BRCA1 mutation–associated ovarian tumors have been shown to be selectively sensitive to PARP1 inhibitors (16), and use of PARP1 inhibitors is a promising personal therapeutic strategy for the elimination of these tumors (17,18). Thus, we confirmed that miR-9–induced low BRCA1 expression leads to an increased sensitivity to PARP-1 inhibitor (AG014699) in C13* cells (control: mean IC_{50} = 81.6 μM, 95% CI = 73.6 to 90.5 μM; miR-9: mean IC50 = 52.3 μM, 95% CI = 42.2 to 64.7 μM) (Supplementary Figure 10, available online).

Effect of miR-9 on Tumor Growth In Vivo Combined With cDDP or PARP Inhibitor

To further demonstrate the role of miR-9 in chemosensitization, we used miR-9 agomiR, a cholesterol-conjugated 2′-O-methyl-modified miR-9 that has suitable pharmacokinetic properties for in vivo studies (19). Mice were treated with vehicle alone, cDDP plus miR-9 agomiR, or cDDP plus scramble agomiR. cDDP plus scramble agomiR had no statistically significant effects on tumor growth. However, in mice treated with cDDP plus miR-9 agomiR, tumor growth was statistically significantly delayed, with a 60% (*P* = .003) and 65% (*P* < .001) reduction in tumor volume at day 28 compared with vehicle or cDDP plus scramble agomiR, respectively (cDDP plus miR-9 agomiR: mean tumor volume = 68.5 mm^3, 95% CI = 29.7 to 107.3 mm^3; vehicle alone: mean tumor volume = 173.5 mm^3, 95% CI = 114.4 to 232.6 mm^3; cDDP plus scramble agomiR: mean tumor volume = 197.8 mm^3, 95% CI = 143.9 to 251.6 mm^3) (Figure 4A). Moreover, tumor weights on day 35 after tumor cell injection with cDDP plus miR-9 agomiR were statistically significantly lower than vehicle or cDDP plus scramble agomiR, with a 60% (*P* < .001) and 55% (*P* < .001) reduction, respectively (cDDP plus miR-9 agomiR: mean tumor weight = 77.5 mg, 95% CI = 55.0 to 100.0 mg; vehicle alone: mean tumor weight = 184.2 mg, 95% CI = 153.9 to 214.4 mg; cDDP plus scramble agomiR: mean tumor weight = 179.2 mg, 95% CI = 146.2 to 212.2 mg) (Figure 4B). These data support the notion that miR-9 is a potent cDDP sensitizer in vivo.

Next, we tested whether AG014699 can abrogate the outgrowth of miR-9–overexpressing tumors. As anticipated, treatment with AG014699 retarded tumor outgrowth in miR-9–overexpressing mice. However, in mice without miR-9 treatment, the tumors grew at a comparable rate in the presence or absence of AG014699 (AG014699 plus miR-9 agomiR: mean tumor volume = 14.3 mm^3, 95% CI = 2.1 to 26.4 mm^3; vehicle alone: mean tumor volume = 178.8 mm^3, 95% CI = 113.4 to 244.1 mm^3; AG014699 plus scramble agomiR: mean tumor volume = 158.5 mm^3, 95% CI = 88.9 to 228.1 mm^3) (Figure 4C). Tumor weights showed similar results (AG014699 plus miR-9 agomiR: mean tumor weight = 42.4 mg, 95% CI = 33.6 to 51.2 mg; vehicle alone: mean tumor weight = 176.7 mg, 95% CI = 133.5 to 219.8 mg; AG014699 plus scramble agomiR:

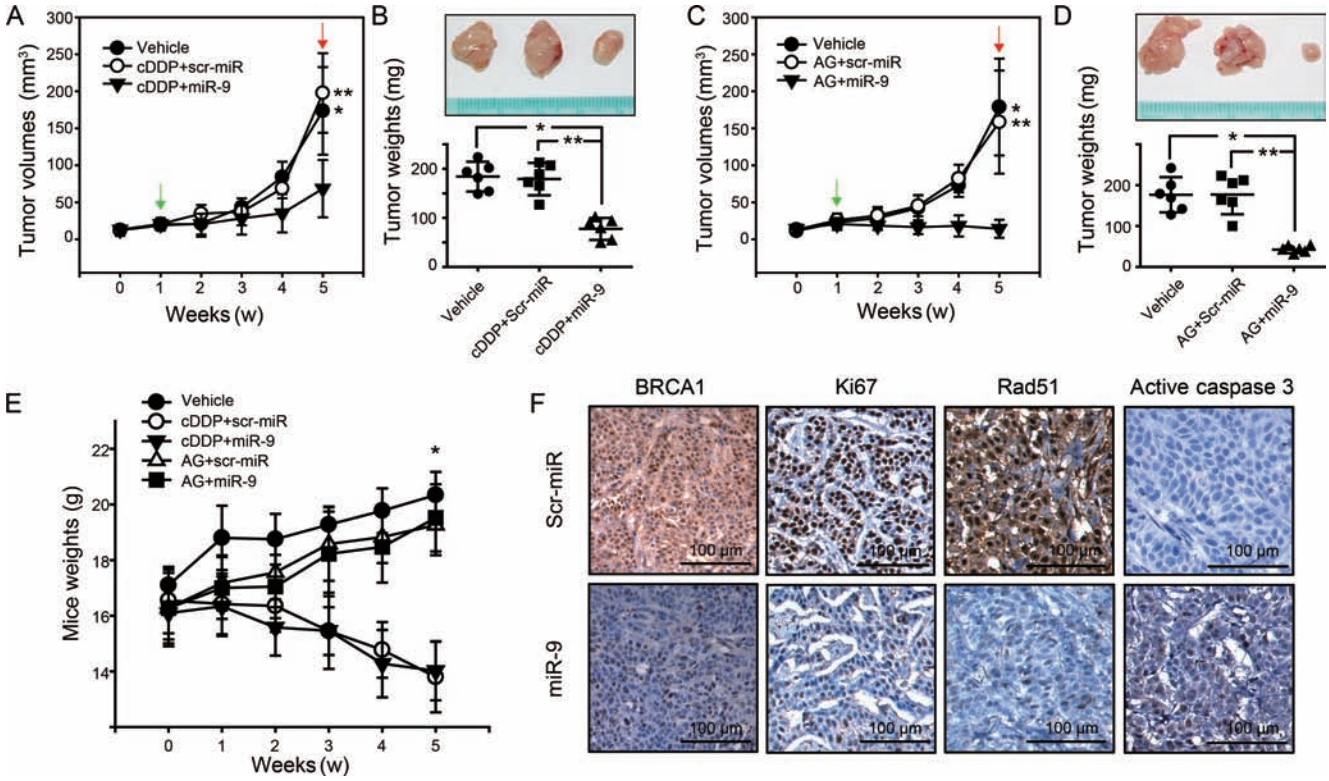

Figure 4. Effect of microRNA (miR)–9 on tumor growth in vivo combined with cisplatin (cDDP) or PARP inhibitor. **A**) Growth curves of C13* subcutaneous xenograft tumors treated with vehicle, cDDP (5 mg/kg, intraperitoneally every 4 days) plus agomiR-9 (1 nmol, intratumoral injection, every 4 days), or cDDP (5 mg/kg, intraperitoneally every 4 days) plus agomiR- scramble-NC (1 nmol, intratumoral injection, every 4 days) are shown. Tumor volumes were calculated as length × (square of width)/2. **Green** and **red arrows** indicate the start and end of treatment, respectively. n = 6 per group. (*P = .003, **P < .001, two-sided Student t test). **B**) The gross morphology of tumors (**upper panel**) and the final xenograft tumor weights (**lower panel**) measured on day 35 after tumor cell injection. (*P < .001, **P < .001, two-sided Student t test). **C**) Growth curves of C13* subcutaneous xenografts treated with vehicle, AG014699 (20mg/kg, intraperitoneally daily) plus agomir-9 (1 nmol, intratumoral injection, every 4 days) or AG014699 (20mg/kg, intraperitoneally daily) plus agomiR-scramble NC (1 nmol, intratumoral injection, every 4 days) are shown. **Green** and **red arrows** indicate the start and end of treatment, respectively. n = 6 per group. (*P < .001, **P < .001, two-sided Student t test). **D**) The gross morphology of tumors (**upper panel**) and primary tumor weights (**lower panel**) in nude mice were measured on day 35 after tumor cell injection. (*P < .001, **P < .001, two-sided Student t test). **E**) The changes in weights of mice treated as indicated above were plotted. (*P < .001, two-sided analysis of variance). **F**) The immunohistochemistry analyses for BRCA1, Rad51, and Ki67, and active caspase 3 staining were carried out on C13* xenograft tumor sections collected from mice treated with the indicated treatments. Representative staining are shown. Scale bars = 100 μm. Data in (**A–E**) are the mean values ± 95% confidence intervals. scr-miR: agomiR-scramble NC; AG: AG014699.

mean tumor weight = 177.2 mg, 95% CI = 129.0 to 225.4 mg) (Figure 4D). Moreover, treatment with AG014699 did not result in any obvious signs of toxicity such as weight loss over the course of the treatment; however, cDDP treatment caused severe toxicity, with a mean weight loss of 30% when compared with the vehicle group (cDDP: mean mice weight = 13.8 g, 95% CI = 12.9 to 15.1 g; AG014699: mean mice weight = 19.3 g, 95% CI = 18.2 to 20.3 g; vehicle alone: mean mouse weight = 20.3 g, 95% CI = 19.5 to 21.2 g; P < .001) (Figure 4E).

Mice were killed after 28 days of treatment. miR-9 overexpression in xenografts was confirmed by qRT-PCR. Tumors overexpressing miR-9 had a lower level of BRCA1 protein than control tumors by IHC (Supplementary Figure 11). Furthermore, miR-9–overexpressing tumors had a higher apoptosis index according to active caspase 3 stain and showed lower Ki67 and Rad51 staining, which indicates that miR-9–overexpressing tumors have lower proliferative potential and lower HR function (Figure 4F). Therefore, these data suggest that miR-9–dependent downregulation of BRCA1 plays a positive role in the treatment of ovarian cancer. Furthermore, miR-9 is a mediator of the cellular response to PARP inhibitor.

Discussion

In previous studies, low BRCA1 expression was common in EOC (range = 34%–87.80%; mean 55.2%) (20–27) (Supplementary Table 4, available online); moreover, aggregated hazard ratio indicated that low BRCA1 expression was a better prognostic factor for survival (HR = 0.63; 95% CI = 0.51 to 0.78) (Supplementary Figure 12, available online). The mechanism of BRCA1 suppression in sporadic tumors, however, is unclear. Although promoter methylation may result in low levels of BRCA1 (10,11), aberrant methylation of the *BRCA1* promoter is only found in a moderate percentage of sporadic ovarian cancers, and there is no statistically significant association with prognosis (4,28). miRs are crucial for the expression of many proteins that are involved in complex functions (29,30). In this study, we found that miR-9 may target the 3′-UTR of *BRCA1* through a reversal library screening. We verified that miR-9 expression was negatively correlated with BRCA1 levels in ovarian cancer cell lines and tissues. miR-9–transfected C13* cells exhibited less Rad51 foci formation, which represented a state of impaired HR, and showed more sensitivity to cisplatin

and PARP inhibitors in vitro and in vivo. Clinical analyses showed that elevated levels of miR-9 or decreased expression of BRCA1 was associated with platinum sensitivity and prognosis. These data suggest that miR-9 could expand cisplatin efficacy by inhibiting BRCA1 expression in ovarian cancers.

Another interesting issue is the role of BRCA1 in paclitaxel sensitivity, the most commonly used combination drug with platinum. Currently, the evidence is conflicting in ovarian cancer. Although in vitro experiments using breast cancer cells showed that BRCA1-negative cells were less sensitive to paclitaxel (31), some studies of ovarian cancer showed that loss of BRCA1 does not affect the sensitivity to paclitaxel (32) or may even increase sensitivity (33,34). This study concludes that BRCA1 affects chemosensitivity mainly through the regulation of sensitivity to platinum both experimentally and clinically, which is consistent with the fact that platinum is the most important drug to determine outcomes of EOC patients, and recurrent cases are often described as platinum resistant according to National Comprehensive Cancer Network guidelines (ovarian cancer), version 1.2013 (http://www.nccn.org/professionals/physician_gls/pdf/ovarian.pdf).

miR-9 has been reported to be highly expressed in many types of malignant tumors, and it can promote cancer cell proliferation and metastasis (35–38). Our study showed that high miR-9 expression statistically significantly sensitized ovarian cancer cells to cisplatin in vivo and in vitro. Moreover, miR-9 expression levels may serve as a prognostic marker to predict the chemosensitivity of ovarian cancers, which is consistent with previous reports that miR-9 levels were low in recurrent ovarian cancer patients (39).

PARP-1 is a key enzyme in base excision repair, a complex process that repairs DNA single-strand breaks caused by chemotherapy (40). Inhibiting PARP1 is a recently developed strategy for cancer therapy that exploits HR defects (such as BRCA1 mutation) in a subset of cancers (17,18,41,42). Moreover, for BRCA-proficient cancer populations, creating a state of "BRCAness" is also a rational approach for expanding the efficacy of PARP inhibitors. By targeting Cdk1, an inhibitory kinase of BRCA1, Johnson et al. selectively sensitized the transformed BRCA-positive cells to PARP inhibition (43). This strategy led us to hypothesize that elevated miR-9 expression may also make BRCA-positive ovarian cancer cells more sensitive to PARP-1 inhibitors. In vitro analysis showed that BRCA-rich C13* exhibited profound resistance to the PARP inhibitors. When C13* cells were transfected with miR-9, this resistance decreased dramatically. Furthermore, miR-9 overexpression reduced tumor growth in xenograft mice when treated with the PARP-1 inhibitor AG014699. Compared with the group treated with cDDP plus miR-9, the AG014699 plus miR-9 group had more statistically significant tumor growth control and less weight loss during the period of treatment (4 weeks), which suggests an important advantage of AG014699 (with its excellent therapeutic index and low toxicity) over cDDP. Thus, our results suggest that ovarian cancer cells overexpressing miR-9 are susceptible to PARP inhibition in vivo and in vitro.

This study had several limitations. Although we observed that miR-9 could effectively improve chemosensitivity by being injected into subcutaneous tumors, it might be better to use an intraperitoneal ovarian cancer model, in view of the character of ovarian cancer invasion, to evaluate miR-9 effects in vivo. In addition, problems such as in vivo stability of miR, specificity of targeting, and safety must be resolved before miR-9 can be used a therapeutic target.

In conclusion, we found, for the first time to our knowledge, that miR-9 directly regulates BRCA1 expression in ovarian cancer. PARP-1 inhibitors may exert more therapeutic potential in the treatment of malignancies bearing positive BRCA1 when treated together with miR-9. This approach avoids the use of toxic DNA-damaging chemotherapeutic drugs and provides the potential to extend well-tolerated PARP inhibition for the treatment for BRCA-proficient cancers when an miR delivery system becomes feasible.

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Note

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