Enhanced expression of DNA polymerase eta contributes to cisplatin resistance of ovarian cancer stem cells

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Cancer stem cells (CSCs) with enhanced tumorigenicity and chemoresistance are believed to be responsible for treatment failure and tumor relapse in ovarian cancer patients. However, it is still unclear how CSCs survive DNA-damaging agent treatment. Here, we report an elevated expression of DNA polymerase η (Pol η) in ovarian CSCs isolated from both ovarian cancer cell lines and primary tumors, indicating that CSCs may have intrinsically enhanced translesion DNA synthesis (TLS). Down-regulation of Pol η blocked cisplatin-induced CSC enrichment both in vitro and in vivo through the enhancement of cisplatin-induced apoptosis in CSCs, indicating that Pol η-mediated TLS contributes to the survival of CSCs upon cisplatin treatment. Furthermore, our data demonstrated a depletion of miR-93 in ovarian CSCs. Enforced expression of miR-93 in ovarian CSCs reduced Pol η expression and increased their sensitivity to cisplatin. Taken together, our data suggest that ovarian CSCs have intrinsically enhanced Pol η-mediated TLS, allowing CSCs to survive cisplatin treatment, leading to tumor relapse. Targeting Pol η, probably through enhancement of miR-93 expression, might be exploited as a strategy to increase the efficacy of cisplatin treatment.

cancer stem cell | cisplatin | translesion synthesis | DNA polymerase eta | miR-93

Ovarian cancer is the most lethal malignancy of the female reproductive tract with a 5-y survival rate of only 27% in advanced stages (1). The American Cancer Society estimates that in 2014, about 21,980 new cases of ovarian cancer will be diagnosed and 14,270 women will die of ovarian cancer in the United States (1). The mainline treatment of ovarian cancer is cytoreductive surgery followed by platinum (Pt)-based chemotherapy (2). Chemotherapy with Pt is initially effective for most patients. However, the majority eventually becomes refractory to Pt treatment, and around 70% of patients have tumor relapses (3). Poor understanding of the underlying mechanisms of this acquired drug resistance and tumor relapse poses a critical cancer research challenge.

cis-diamminedichloroplatinum(II) (cisplatin), the first member of Pt-based chemotherapeutic agents, has been widely used to treat various malignant tumors, including ovarian cancer (4). Mechanistically, cisplatin reacts with DNA bases to cross-link adjacent purines. These cross-links block DNA replication and invoke apoptosis in rapidly dividing cells (5). Thus, the preferential activation of the DNA damage responses, especially the efficient removal of these DNA lesions, or prompt rescue of the replication, will prevent replication fork collapse and promote survival of the cells upon cisplatin treatment, eventually leading to cisplatin resistance. The cisplatin-induced DNA cross-links are primarily removed by the nucleotide excision repair (NER) pathway (6) or bypassed during replication through translesion DNA synthesis (TLS) (7–10). TLS is mediated by specialized DNA polymerases (Pols), which are characterized by low fidelity and an ability to replicate across certain types of damaged sites in template DNA with the assistance of monoubiquitylated proliferating cell nuclear antigen (ub-PCNA) (11). TLS rescues cells from the collapse of the replication fork and thus is believed to contribute to the development of cisplatin resistance (8, 12–17).

It has been increasingly evident that heterogeneous ovarian cancers contain a subpopulation of cancer stem cells (CSCs) with enhanced tumorigenicity and chemoresistance. These CSCs are believed to be responsible for treatment failure and tumor relapse. Ovarian CSCs have been successfully isolated, based on the expression of distinctive cell surface markers CD44 and CD117 (18, 19), their ability to efflux the Hoechst 33342 fluorescent dye (Side population, SP) (20), the activity of ALDH (21), and their ability to grow as floating spheres in serum-free medium (19). The $CD44^+CD117^+$ cells, SP cells, $ALDH^+$ cells, and spheroid cells isolated from both ovarian cancer cell lines and primary human ovarian tumors fulfill all currently accepted criteria for the existence of a subpopulation of tumor-initiating cells (19, 22, 23). Most importantly, these CSCs also demonstrate increased cisplatin resistance. However, it is still unclear how CSCs survive cisplatin treatment. In this study, we demonstrated that the expression level of TLS Pol η is higher in ovarian CSCs isolated from both cancer cell lines and primary tumors than the bulk cancer cells. Down-regulation of Pol η expression blocked cisplatin-induced enrichment of the CSC population, through facilitating the killing of CSCs by cisplatin. Mechanistic investigation demonstrated that decreased expression of miR-93 in ovarian CSCs contributes, at least partially, to the enhanced expression of Pol η. Taken together, our study suggests that Pol

Significance

Cancer stem cells (CSCs) exhibit enhanced chemo/radiotherapy resistance, and their survival following cancer treatment is believed to be responsible for tumor recurrence and metastasis. Thus, understanding the mechanisms through which CSCs survive conventional chemotherapy is essential for identification of new therapeutic strategies to prevent tumor relapse. Our findings that ovarian CSCs survive cisplatin treatment through elevated expression of polymerase η represent an opportunity to eradicate CSCs and improve the survival of ovarian cancer patients. In addition, identification of miR-93 as the regulator of polymerase η expression provides a target to increase the efficacy of cisplatin treatment.

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η-mediated TLS could be a target to facilitate the eradication of ovarian CSCs by cisplatin.

Results

Reduced DNA Damage Formation and Enhanced DNA Repair Capacity Were Not Consistently Found in Ovarian CSCs upon Cisplatin Treatment. Inefficient formation of DNA lesions and enhanced DNA repair have been implicated in cancer therapy resistance in CSCs (24). We sought to determine whether ovarian CSCs are also resistant to the formation of cisplatin-induced DNA lesions and exhibit enhanced DNA repair capacity. $CD44^+CD117^+$ cells were isolated from ovarian cancer cell lines 2008 and C13 (25, 26) by using fluorescence-activated cell sorting (FACS) and have been considered CSCs based on their characteristics (19, 27). The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cell viability assay confirmed the cisplatin resistance of these $CD44^+$ $CD117^+$ cells compared with their corresponding unsorted cells (*SI Appendix*, Fig. S1 *A* and *B*). However, we were unable to demonstrate a consistently reduced formation of 1,2-intrastrand cross-links (Pt-GG) in ovarian CSCs compared with the unsorted bulk cancer cells following the same amount of cisplatin treatment (*SI Appendix*, Fig. S2 *A* and *C*). No significant reduction of Pt-GG formation was found in another CSC population isolated from ovarian cancer cell line SKOV3 based on the spheroid formation (27, 28) (*SI Appendix*, Fig. S2 *B* and *D*). The removal rates of Pt-GG in these cells were further analyzed after equivalent amounts of Pt-GG were generated. Again, we failed to find an enhanced capacity of 2008-CSCs, C13-CSCs, and SKOV3-CSCs to remove cisplatin-induced DNA lesions (*SI Appendix*, Fig. S2 *E–G*). Given that cisplatin can induce other DNA lesions besides Pt-GG (29), we performed inductively coupled plasma mass spectrometry to determine the Pt content in DNA to account for all DNA-Pt adducts. Similarly, neither significant reduction of DNA-Pt adducts nor significant increase of the removal rate of DNA-Pt adducts was found in these CSCs compared with their corresponding bulk cancer cells (*SI Appendix*, Fig. S3 *A–F*). Taken together, these data suggest that inefficient DNA lesion formation and enhanced DNA repair capacity are not likely to be the cause of increased cisplatin resistance in ovarian CSCs. It is worth noting that even more DNA-Pt adducts were formed in C13-CSCs than their corresponding bulk cancer cells (*SI Appendix*, Figs. S2 *A* and *C* and S3*B*). The differences in the formation of DNA lesion and in DNA repair capacity among these ovarian CSCs suggest the complexity of cisplatin resistance, and there may likely be a distinct major mechanism for cisplatin resistance in ovarian CSCs with different backgrounds.

Expression of TLS Pol η Is Elevated in Ovarian CSCs. One of the important cell survival mechanisms following cisplatin treatment is TLS, which is mediated by specialized DNA Pols (11). The analyses of expression levels of various TLS Pols in ovarian CSCs demonstrated that POLH mRNA (encoding Pol η) is highly expressed in 2008-CSCs, C13-CSCs, and SKOV3-CSCs (Fig. 1 *A–C* and *SI Appendix*, Fig. S4). Interestingly, the POLH mRNA levels in 2008, 2008-CSCs, C13, and C13-CSCs correlate well with cisplatin sensitivity (*SI Appendix*, Figs. S1 vs. S5). We wished to extend these observations to the CSCs isolated from primary human ovarian tumors. Ovarian serous adenocarcinomas were disaggregated and subjected to different growth conditions, either for regular monolayer adherent growth of tumor cells or for the selection of self-renewing, nonadherent spheroids, which have been demonstrated to be CSCs (19). Disaggregated tumor cells from five patients were able to form spheroids under CSC-selective culture conditions (*SI Appendix*, Fig. S6*A*). The spheroids displayed significantly enhanced expression of *Nanog*, a marker of CSCs, compared with the bulk tumor cells (*SI Appendix*, Fig. S6*B*). Most importantly, the spheroid cells isolated from four out of five primary ovarian tumors also exhibited significantly increased expression level

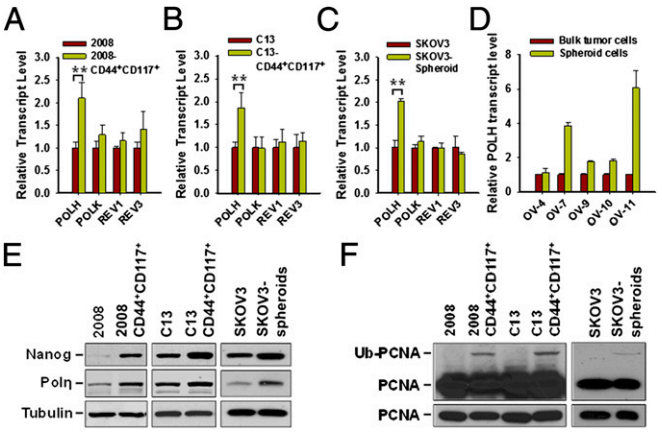

Fig. 1. Enhanced TLS in ovarian CSCs. (*A-C*) Expression of various TLS Pols in ovarian cancer cell lines 2008 (*A*), C13 (*B*), and SKOV3 (*C*), as well as their corresponding CSC populations were determined using quantitative RT-PCR (qRT-PCR). $n = 3$; Bar, SD; **, $P < 0.01$. (*D*) The mRNA expression level of POLH was determined in primary tumor cells isolated from five freshly removed ovarian tumors and their corresponding spheroid cells, using qRT-PCR. $n = 3$; Bar, SD. Analysis by the linear mixed model indicates that POLH expression increased significantly in spheroid cells compared with bulk cancer cells ($P < 0.0001$). (*E*) Protein levels of Nanog and Pol η in ovarian cancer cell lines and their corresponding CSC populations were determined using immunoblotting. (*F*) Monoubiquitylated PCNA in ovarian cancer cell lines and their corresponding CSC populations were determined using immunoblotting. Results shown here are from one of three experiments with identical results.

of POLH mRNA (Fig. 1*D*). In support of this finding, we also demonstrated an enhanced protein level of Pol η in 2008-CSCs, C13-CSCs, and SKOV3-CSCs compared with their corresponding bulk cancer cells (Fig. 1*E*). In addition, an elevated basal level of ub-PCNA was also revealed in these CSCs (Fig. 1*F*). These observations suggest an enhanced TLS activity in ovarian CSCs mainly mediated by elevated expression of Pol η.

Pol η Is Required for Cisplatin-Induced Enrichment of the CSC Population. Cisplatin treatment efficacy is inversely correlated to the expression level of Pol η in various cancers (30–32). To determine whether Pol η down-regulation affects the efficacy of cisplatin treatment in ovarian cancers, we established a 2008 cell line with Pol η stable knockdown and generated xenografts by injecting cells s.c. into Athymic nude mice. Upon tumor presentation, mice were chronically treated with cisplatin six times during a period of 74 d. As shown in *SI Appendix*, Fig. S7 *A* and *B*, POLH-deficient transplants exhibited an enhanced response to cisplatin relative to POLH-proficient controls, indicating that down-regulation of Pol η is also able to sensitize ovarian cancer to cisplatin.

Cisplatin treatment is capable of inducing enrichment in a population of cells with CSC properties, probably due to killing of cisplatin-sensitive bulk cancer cells and survival of cisplatin-resistant CSCs (33, 34). We sought to assess the contribution of Pol η to cisplatin-induced enrichment of CSCs. In 2008, C13, and SKOV3 cells, treatment with cisplatin did increase the percentage of the CSC population defined as $CD44^+CD117^+$ cells. Down-regulation of Pol η expression by transient transfection of POLH siRNA into these cells reduced the cisplatin-enriched CSC population (Fig. 2 *A–F* and *SI Appendix*, Fig. S8 *A–C*). Consistently, treatment with cisplatin failed to enrich the CSC population in a 2008 cell line with POLH stable knockdown (*SI Appendix*, Fig. S9 *A* and *B*). We further validated this finding in a xenograft model with in vivo cisplatin treatment. Mice bearing POLH-proficient or POLH-deficient xenografts (Fig. 3*A*) were treated with cisplatin twice. Similar to the chronic treatment, this

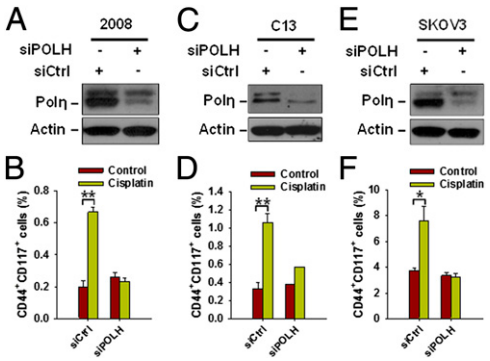

Fig. 2. Down-regulation of Pol η in ovarian cancer cells blocked cisplatin-induced enrichment of CSCs in vitro. (*A*, *C*, and *E*) Ovarian cancer cell lines 2008 (*A*), C13 (*C*), and SKOV3 (*E*) were transiently transfected with either POLH siRNA or control siRNA for 24 h, and the expression of Pol η was determined using immunoblotting. (*B*, *D*, and *F*) The siRNA transfected 2008 (*B*), C13 (*D*), and SKOV3 (*F*) cells were treated with cisplatin for 3 d and stained with anti–CD44-FITC and anti–CD117-PE antibodies. The percentage of $CD44^+CD117^+$ cells was analyzed using FACS. $n = 3$; Bar, SD; *, $P < 0.05$; **, $P < 0.01$.

short-term cisplatin treatment also inhibited the growth of all xenografts, with POLH-deficient tumors exhibiting a less progressive growth dynamics and more significant tumor regression following treatments (Fig. 3*B*), in comparison with POLH-proficient transplants. The tumor cells were isolated 2 d after the second treatment and subjected to FACS to determine the percentage of $CD44^+CD117^+$ cells. As expected, in vivo cisplatin treatment enriched CSCs in the POLH-proficient, but not POLH-deficient xenografts (Fig. 3 *C* and *D*). Taken together, these data suggest Pol η plays a critical role in reducing the efficacy of cisplatin to shrink ovarian tumors and in the enrichment of CSC population upon cisplatin treatment.

Down-Regulation of TLS Pol η Sensitizes Ovarian CSCs to Cisplatin Treatment. To assess the contribution of Pol η to cisplatin resistance in CSCs, the expression of Pol η in 2008-$CD44^+$ $CD117^+$, C13-$CD44^+$ $CD117^+$, and SKOV3-spheroid cells was knocked down with either POLH siRNA or shRNA, and the cell sensitivity to cisplatin was determined. Down-regulation of Pol η promoted cisplatin-induced cell killing in all these ovarian CSC populations, with 2.1–8.3-fold reduction of IC50 (Fig. 4 *A*–*C* and *SI Appendix*, Fig. S10 *A* and *B*). Annexin V staining and FACS analysis of SKOV3-spheroid cells further revealed that the number of apoptotic cells increased in POLH–down-regulated cells upon cisplatin treatment (Fig. 4*D*). All these data suggest that Pol η-mediated TLS facilitates CSCs to survive cisplatin treatment, leading to an enrichment of the CSC population.

Decreased Expression of miR-93 Is Responsible for the Increased Pol η Expression in Ovarian CSCs. Micro RNAs (miRNAs) can be differentially expressed in CSCs and regulate their characteristics (35). By using the web-based algorithms miRDB and miRanda, we identified two miRNAs that have high potential to bind to 3′-UTR of POLH (miR-93 and miR-20b) (Fig. 5*A* and *SI Appendix*, Fig. S11*A*). qRT-PCR analyses demonstrated that miR-93 level was significantly lower in all CSC populations derived from three ovarian cancer cell lines compared with their corresponding bulk cancer cells (Fig. 5 *B* and *C* and *SI Appendix*, Fig. S12*A*). The CSC populations derived from primary tumors exhibited significantly lower miR-93 level compared with their corresponding bulk tumor cells as well (Fig. 5*D*). However, a lower level of miR-20b was only found in C13 and SKOV3 CSCs (*SI Appendix*, Figs. S11 *B* and *C* and S12*B*), and no significant difference for miR-20b expression was found in the CSC population and bulk cancer cells derived from primary tumors (*SI Appendix*, Fig. S11*D*). Interestingly, those CSC populations (OV-7, OV-9, OV-10, and OV-11) showing lower levels of miR-93 also exhibited higher levels of POLH (Figs. 1*D* vs. 5*D*). These data suggest an inverse correlation between miR-93 and POLH expression levels in ovarian CSCs and indicate that miR-93 might regulate the expression of POLH.

To establish the regulatory role of miR-93 in POLH expression, 2008, C13, and SKOV3 cells were transfected with miR-93 inhibitors, whereas 2008-$CD44^+CD117^+$, C13-$CD44^+CD117^+$, and SKOV3-spheroid cells were transfected with miR-93 mimics. qRT-PCR analyses demonstrated that down-regulation of miR-93 in 2008 and C13 cells enhanced the POLH mRNA levels (*SI Appendix*, Fig. S13 *A* and *B*), whereas overexpression of miR-93 in 2008-CSCs and C13-CSCs reduced the POLH mRNA levels (*SI Appendix*, Fig. S13 *C* and *D*). Furthermore, the inhibitory effect of miR-93 on the protein level of Pol η was also revealed by immunoblotting analyses (Fig. 6 *A* and *B*). In contrast, transfection of miR-20b mimics did not have influence on the expression of Pol η in these CSCs (*SI Appendix*, Fig. S11*E*). Collectively, these data suggest that reduced expression of miR-93 contributes to the elevated expression of POLH in ovarian CSCs.

Given that miR-93 down-regulates Pol η expression, overexpression of miR-93 should be able to enhance the sensitivity of ovarian CSCs to cisplatin treatment by decreasing the Pol η level. Indeed, transfection of miR-93 mimics into 2008-$CD44^+CD117^+$, C13-$CD44^+CD117^+$, and SKOV3-spheroid cells increased cisplatin-induced cell death and cellular apoptosis (Fig. 6 *C*–*H* and *SI Appendix*, Fig. S14 *A*–*C*), indicating that enforced miR-93 expression sensitizes ovarian CSCs to cisplatin treatment.

Discussion

TLS is believed to contribute to the development of cisplatin resistance because TLS is able to rescue the cells from the collapse of the replication fork induced by DNA intrastrand cross-links following cisplatin treatment (8, 12–17). However, it is still unclear

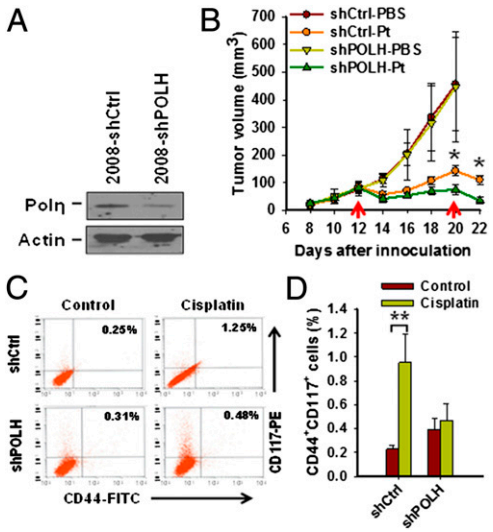

Fig. 3. Down-regulation of Pol η in ovarian cancer cells blocked cisplatin-induced enrichment of CSCs in vivo and sensitized xenografts to cisplatin treatment. (*A*) The 2008 cell lines with stable transfection of either shCtrl or shPOLH were established. (*B*) The 2008-shCtrl and 2008-shPOLH cells were injected into the flank of athymic nude mice *s.c.* ($n = 8$). Mice were treated with cisplatin (7 mg/kg) once every week for 2 wk after tumors were about 0.5 cm in diameter. Tumor sizes were recorded, and tumor growth curves were plotted. Red arrows indicate the cisplatin treatment. (*C* and *D*) Tumors were harvested after 2 d of the second treatment. Tumor cells were isolated, and the percentage of $CD44^+CD117^+$ cells was analyzed using FACS (*C*) and plotted (*D*). $n = 7$; Bar, SD; *, $P < 0.05$; **, $P < 0.01$.

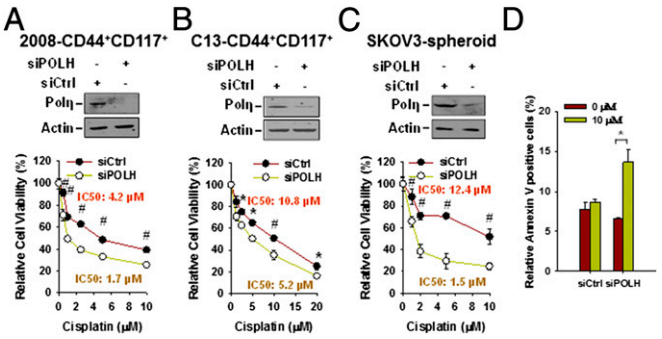

Fig. 4. Down-regulation of Pol η-sensitized CSCs to cisplatin treatment. (*A–C*) The 2008-CD44$^+$CD117$^+$ (*A*), C13-CD44$^+$CD117$^+$ (*B*), and SKOV3-spheroid (*C*) cells were transfected with either POLH siRNA or control siRNA, followed by treatment with cisplatin for 3 d. Cell survival was determined using the MTT assay. $n = 4$; Bar, SD; *, $P < 0.05$; $^{\#}$, $P < 0.01$ compared between control and POLH knockdown cells at each time point. (*D*) SKOV3-spheroid cells were transfected with either control or POLH siRNA for 48 h and then treated with cisplatin for 24 h. Apoptotic cells were stained with Annexin V and detected by FACS. $n = 3$; Bar, SD; *, $P < 0.05$.

whether the cisplatin-resistant property of CSCs is also due to enhanced TLS activity. TLS allows the DNA replication machinery to bypass an unrepaired DNA damage site using special polymerases (11, 36). Among many polymerases tested in vitro, the Y-family DNA Pol η is the most efficient and accurate at bypassing Pt-GG lesions (10, 37, 38). Pol η down-regulation results in increased sensitivity to cisplatin (13), whereas the increased Pol η level reduces the effectiveness of chemotherapy and the survival time of patients with non–small-cell lung cancer or metastatic gastric adenocarcinoma (30). Our mice xenografts study also demonstrated that down-regulation of Pol η significantly enhanced the response of ovarian tumor xenografts to cisplatin treatment. To further support this notion, we analyzed the public database of gene expression arrays (free online software Kaplan-Meier Plotter: www.kmplot.com) and found that the higher POLH mRNA expression in ovarian tumors is negatively correlated with the outcome of patients (*SI Appendix*, Fig. S15 *A* and *B*). In contrast, we did not find such a correlation between *REV1* or *REV3L* mRNA expression level and the overall survival of patients (*SI Appendix*, Fig. S15 *C* and *D*), although it has been reported that REV1 or REV3L depletion sensitized lymphoma to cisplatin (14), and REV3L depletion sensitized lung adenocarcinoma to cisplatin (17).

From the in vivo xenografts study, we noticed that the regrowth rate of xenografts after each cisplatin treatment is lower in Pol η–down-regulated ovarian tumor xenografts than controls. Given that CSCs are believed to be responsible for the initiation and regrowth of tumors, our data indicate that down-regulation of Pol η may facilitate the eradication of ovarian CSCs by cisplatin. Indeed, we did demonstrate an elevated expression of Pol η at both mRNA and protein levels in ovarian CSC populations and a protective role of Pol η in ovarian CSCs upon cisplatin treatment. Thus, Pol η must be a critical contributor to the chemoresistant property of CSCs, and inhibition of the Pol η-mediated TLS pathway in CSCs would be a promising therapeutic strategy to promote the eradication of CSCs by cisplatin.

Our data also demonstrated that the elevated Pol η expression in ovarian CSCs can be attributed to the reduced expression of miR-93. miR-93 belongs to the miR106b-25 cluster that has been reported to be overexpressed in different types of cancer, such as gastric, prostate, and pancreatic neural endocrine tumors, as well as neuroblastoma and multiple myeloma (39). However, highly depleted miR-93 was found in mouse mammary stem cells characterized with ALDH+ (40) and breast CSCs characterized by their expression of ALDH$^+$ or CD44$^+$CD24$^-$ (41). The role of miR-93 in tumor growth is still controversial. miR-93 is able to enhance cell survival, promote sphere formation, and augment tumor growth by targeting integrin-β8 in the glioblastoma U87 cell line (42). In contrast, miR-93 was reported to inhibit tumor growth and metastasis by decreasing the CSC population in SUM159 breast cancer cells (41). Our data also showed a decreased level of miR-93 in CSCs isolated from both ovarian cancer cell lines and primary ovarian tumors and demonstrated that low miR-93 level is critical to the cisplatin resistance property of ovarian CSCs. We for the first time to our knowledge showed an inverse correlation between miR-93 and POLH expression in various ovarian CSCs. The use of miR-93 mimics led to attenuated Pol η expression in ovarian CSCs, whereas the use of miR-93 inhibitors caused significant increase in Pol η expression in bulk ovarian cancer cells. Thus, miR-93 is able to target POLH 3′-UTR and inhibit the expression of Pol η. As a result, the low level of miR-93 causes enhanced expression of Pol η, which facilitates TLS to promote CSCs survival after cisplatin treatment. Collectively, the findings implicate a miR-93–Pol η axis affecting the survival of ovarian CSCs upon cisplatin treatment. However, it is also worth noting that miR-93 can target several important pathways, such as STAT3 and AKT pathways (41). Given that elevated expression of STAT3 and AKT3 is associated with cisplatin resistance in various cancers (43, 44), it is possible that the low level of miR-93 also contributes to cisplatin resistance through affecting other critical pathways.

miR-93 is located at chromosome 7q22, in the intron 13 of the host gene minichromosome maintenance 7 (*MCM7*), where they are cotranscribed in the context of *MCM7* primary transcript (39). Although MCM7 overexpression has been identified in various tumors and considered a bad prognostic indicator in prostate cancer (45, 46), MCM7 expression is lower in various CSCs, including SP of human lung cancer cells (47) and prostate cancer cells (48), as well as ALDH+ breast cancer cells (41). In addition, an analysis of 12 publically available microarray datasets revealed a down-regulation of the *MCM7* gene in various cancer stem-like cells in all datasets, although only three of them showed significant change (*SI Appendix*, Table S1). Thus, this reduced expression of *MCM7*, probably due to relative quiescence of CSCs (49, 50), could be a contributor to the decreased expression of miR-93 in CSCs.

In summary, our data demonstrated a previously unidentified mechanism of cisplatin resistance in ovarian CSCs. A low level of miR-93 in ovarian CSCs enhances the expression of Pol η, which promotes the bypass of cisplatin-induced, unrepaired DNA intrastrand cross-links, leading to the elevated survival of CSCs. Therefore, Pol η inhibitors could be exploited as chemotherapy-enhancing agents. A combination of cisplatin and Pol η inhibitors

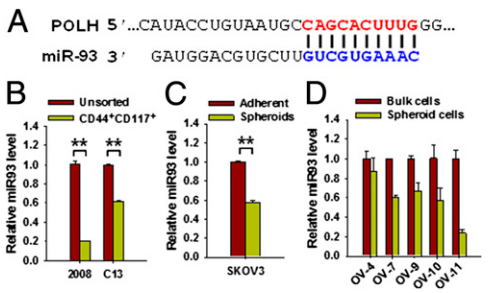

Fig. 5. Reduced miR-93 expression in ovarian CSCs. (*A*) Predicted binding of miR-93 to the 3′UTR of POLH. (*B* and *C*) Expression of miR-93 was determined in various ovarian cancer cell lines and their corresponding CSCs by qRT-PCR. $n = 3$; Bar, SD; **, $P < 0.01$. (*D*) Expression of miR-93 in bulk primary tumor cells and their corresponding spheroid cells was analyzed using qRT-PCR. $n = 3$, Bar, SD. Analysis indicates that there was significantly decreased miR-93 expression in the spheroid cells compared with bulk cells ($P = 0.047$).

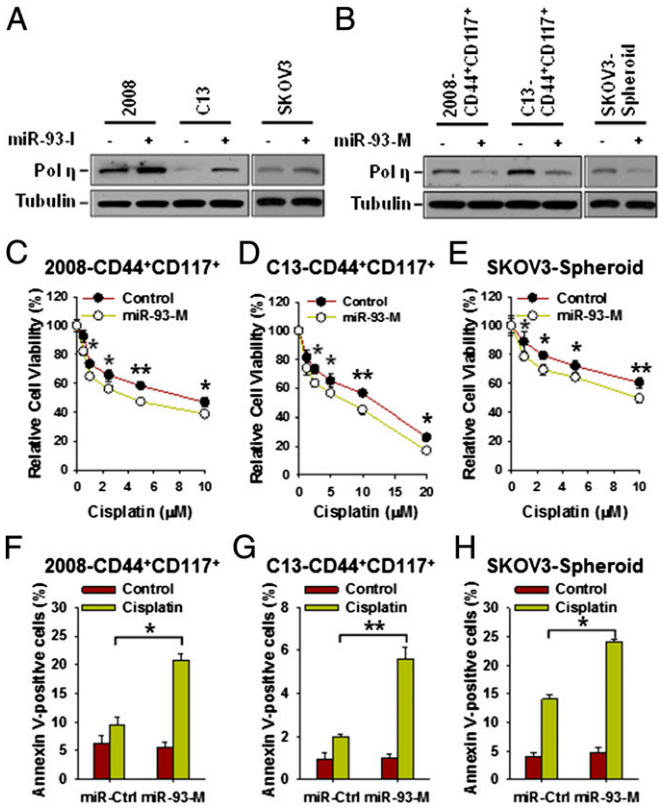

Fig. 6. Reduced expression of miR-93 contributes to the elevated POLH expression and enhanced cisplatin resistance in ovarian CSCs. (*A* and *B*) Western blot analyses of the protein level of Pol η in ovarian cancer cell lines with miR-93 inhibition (*A*) or their corresponding CSCs with miR-93 overexpression (*B*). The results are representative of three experiments with similar results. (*C–E*) MTT assay was used to determine the effect of enhanced miR-93 expression on the sensitivity of 2008 (*C*), C13 (*D*), and SKOV3 (*E*) CSCs to cisplatin. $n = 4$; Bar, SD; *, $P < 0.05$; **, $P < 0.01$ compared between control and miR-93 overexpressed cells at each concentration. (*F–H*) Effect of enhanced miR-93 expression on cisplatin-induced apoptosis in 2008 (*F*), C13 (*G*), and SKOV3 (*H*) CSCs was determined with Annexin V staining and FACS. $n = 3$; Bar, SD; *, $P < 0.05$, **, $P < 0.01$.

may allow reduced cisplatin dosage and increase the efficacy of anticancer treatment.

Materials and Methods

Cell Culture and Cisplatin Treatment. Human ovarian cancer cell line 2008 and its resistant cell line C13 (25) were kindly provided by Dr. Francois X. Claret (University of Texas, M. D. Anderson Cancer Center). The SKOV3 ovarian cancer cell line was provided by Dr. Thomas C. Hamilton (Fox Chase Cancer Center). The 2008 cells with stable knockdown of Pol η (2008-shPOLH-1) were successfully established in our laboratory. All cell lines were authenticated by DNA (short tandem repeat) profiling and maintained in Roswell Park Memorial Institute medium 1640 supplemented with 10% (vol/vol) fetal bovine serum. The 2008-CD44$^+$CD117$^+$, C13-CD44$^+$CD117$^+$, and SKOV3-spheroid cells were maintained in ultralow attachment plates in knockout Dulbecco's modified Eagle medium/F12 medium supplemented with 20% knockout Serum Replacement (Life Technologies), 20 ng/mL epidermal growth factor, and 10 ng/mL basic fibroblast growth factor. All cells were grown at 37 °C in humidified atmosphere of 5% (vol/vol) CO_2 in air. Cisplatin (Sigma) stocking solution was prepared freshly with PBS and further diluted to the desired concentration with culture medium for cell treatment.

Flow Cytometry Analysis and Cell Sorting. Anti–CD117-PE and anti–CD44-FITC (BD Pharmingen) were used for flow cytometric analysis and cell sorting. Briefly, cells were incubated with antibodies on ice for 40 min in the dark. After washing with cold PBS, cells were resuspended in 200 μL PBS and subjected to FACS analyses on a BD FACS Aria III at The Ohio State University Analytical Cytometry Shared Resource.

Isolation of Primary Tumor Cells from Freshly Removed Human Ovarian Tumors. Freshly removed ovarian tumors were obtained from Department of Pathology at the Ohio State University within 4 h after surgery in accordance with a protocol approved by the Ohio State University's IRB. The single tumor cells were obtained by enzyme digestion and cultured enrichment of epithelial tumor cells and CSCs under different culture conditions. See *SI Appendix, Materials and Methods* for detailed procedure.

qRT-PCR Analysis. Total RNA was extracted using TRIzol reagent (Invitrogen), and the first-strand cDNA was generated by the High-Capacity cDNA Reverse Transcription kit (ABI) in a 20-μL reaction containing 1 μg of total RNA. A 2.5-μL aliquot of cDNA was amplified by Fast SYBR Green PCR Master Mix (Life Technologies) in each 20 μL reaction. PCR reactions were run on the ABI 7900 Fast Real-Time PCR system in the Ohio State University Comprehensive Cancer Center (OSUCCC) Nucleic Acid Core Facility. See *SI Appendix, Materials and Methods* for primer sequences.

Immunoblotting. Whole-cell lysates were prepared by boiling cell pellets for 10 min in SDS lysis buffer [2% (wt/vol) SDS, 10% (vol/vol) Glycerol, 62 mmol/L Tris·HCl, pH 6.8, and a complete miniprotease inhibitor mixture (Roche Applied Science)]. After protein quantification, equal amounts of proteins were loaded, separated on a polyacrylamide gel, and transferred to a nitrocellulose membrane. Protein bands were immunodetected with appropriate antibodies, e.g., goat anti-Pol η (Abcam), rabbit anti-Nanog (Cell Signaling Technology), mouse anti-Tubulin (Millipore), and mouse anti-Actin (Santa Cruz Technology).

miRNA Detection. For miRNA detection, a TaqMan MicroRNA Assay Kit (Applied Biosystems), including the following assays, was used: miR-20b (Assay ID: 00104) and miR-93 (Assay ID: 001090). All quantitative real-time PCR runs were carried out according to manufacturer's instructions. RNU6B (Assay ID: 001093) and 18S rRNA (Applied Biosystems) were used for normalization. All PCR reactions were performed in triplicate.

Xenograft Tumor Growth. Nonobese diabetic/severe combined immunodeficiency and Athymic nude (NCr-nu/nu) mice (6–8 wk, female, 20–25 g body weight) were obtained from National Cancer Institute (Frederick, MD). Animals were maintained in accordance with institutional policies, and all studies were performed with approval of the Institutional Animal Care and Use Committee at the Ohio State University. To generate xenografts, 5×10^6 cells were mixed (1:1) with Matrigel (BD Biosciences) and injected s.c. into the flank of each mouse. Animals were treated with cisplatin i.p. twice (7 mg/kg; weekly) after xenografts reached 0.5 cm in diameter. Tumor growth was measured using calipers, and volumes were calculated based on the formula $V = (a \times b^2)/2$, in which a is the longest and b is the shortest diameter of the tumor. Xenograft cells were isolated after 2 d of the second treatment with the help of collagenase digestion and RBC lysis (eBioscience).

Detection of Cell Viability. After 24 h of transfection with siRNA or shRNA, cells were reseeded and cultured for another 24 h in a 96-well plate at a density of 1,000 cells per well, then treated with cisplatin for 3 d. Cell viability was assessed by the MTT cell proliferation assay kit according to the manufacturer's instruction (ATCC).

Detection of Apoptotic Cells. The 2008-CSCs, C13-CSCs, and SKOV3-CSCs growing in ultralow attachment plates with CSC selective medium were transfected with either 100 nM siCtrl or siPOLH or miR-93 mimics with lipofectamine for 24 h and treated with cisplatin for 48 h. Cells were harvested and stained with Annexin V FITC assay kit (Cayman Chemical) and analyzed by FACS.

Statistical Analysis. Linear mixed effect models were used to take into account the observations from the same subject for the tumor growth studies. Tumor growth over time was compared among groups from those models. For the RT-PCR studies, the data were first normalized to the internal controls, and then ANOVA or linear mixed effects models were used for analysis for cell line studies and primary cell studies, respectively. All results were presented as mean ± SD, with a P value < 0.05 considered as statistically significant.

Additional laboratory reagents used are described in *SI Appendix, Materials and Methods*.

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