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THERAPY

# Circular RNA Cdr1as Upregulates SCAI to Suppress Cisplatin Resistance in Ovarian Cancer via miR-1270 Suppression

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**The aim of this study is to explore the roles of circular RNA (circRNA) Cdr1as on cisplatin resistance in ovarian cancer and explore the underlying mechanisms. We investigated the expression of circRNAs in five paired cisplatin-sensitive and cisplatin-resistant tissues of ovarian cancer by microarray analysis. The quantitative real-time PCR analysis was to investigate the expression pattern of Cdr1as in cisplatin-resistant ovarian cancer patient tissues and cell lines. Then, the effects of Cdr1as on cisplatin resistance, cell proliferation, and apoptosis were assessed in ovarian cancer cells. In this study, Cdr1as was observed to be downregulated in cisplatin-resistant patient tissues and cell lines. Overexpression of Cdr1as inhibited cell proliferation and promoted the cisplatin-induced cell apoptosis in ovarian cancer cells. Then we demonstrated that repressed Cdr1as promoted the miR-1270 expression, and miR-1270 could bind to the predicted binding site of Cdr1as. Furthermore, we found that miR-1270 displayed its role via modulating the Suppressor of Cancer Cell Invasion (SCAI) expression. Importantly, we demonstrated that Cdr1as was downregulated in serum exosomes from cisplatin-resistant patients. In summary, our study demonstrated that Cdr1as sensitizes ovarian cancer to cisplatin by regulating the miR-1270/ SCAI signaling pathway.**

## INTRODUCTION

Ovarian cancer is the most frequent cause of mortality among gynecological malignancies, which usually are diagnosed at an advanced metastatic stage.[1] Ovarian cancer is conventionally treated with surgery and cisplatin-based chemotherapy. Although cisplatin is effective in many patients, it is associated with development of resistance.[2] The 5-year overall survival (OS) rate of ovarian cancer is between 35% and 40%, and cisplatin chemoresistance is the main factor restricting the long-term survival.[3,4] However, the molecular mechanisms contributing to the chemotherapy resistance of ovarian cancer are obscure. It is necessary to elucidate the mechanisms of chemotherapy resistance and develop new target drugs.

Circular RNAs (circRNAs) recently have been identified as members of the non-coding RNA (ncRNA) family that play a significant role in many cancers' progression and numerous kinds of cellular biological

and pathological processes.[5] circRNA can function as a competitive endogenous RNA (ceRNA) to regulate the biological activity of microRNA (miRNA) and completely or partially restore the inhibitory function of miRNA in the target gene.[6] A growing body of research suggests that circRNAs participate in many biological and pathological processes.[7] Cdr1as, also known as a circRNA sponge for miR-7 (ciRS-7) or CDR1NAT, is formed by reverse splicing of the antisense strand of the cerebellar degeneration-associated antigen 1 (CDR1) gene.[8] The genomic length of Cdr1as is 1,485 bp, and the spliced mature sequence length is 1,485 bp. It is located at chrX:139865339–139866824. Cdr1as was confirmed to have approximately 70 conserved miR-7 binding sites and to act as an miR-7 sponge to perform biological functions.[9] Moreover, CDR1as promotes the proliferation and invasion of several types of carcinoma cells.[10,11] However, the role of Cdr1as in the development of cisplatin chemoresistance in ovarian cancer still remains unknown. In the present study, we investigated the relationship between Cdr1as and the sensitivity of ovarian cancer to cisplatin, and explored the potential miRNA and downstream target gene Suppressor of Cancer Cell Invasion (SCAI) mechanistically. Our findings will provide new insights into the regulatory mechanisms of Cdr1as in tumorigenesis and cisplatin resistance of ovarian cancer.

## RESULTS

### Profile of circRNAs in Cisplatin-Resistant Ovarian Cancer Tissues

To analyze the expression pattern of circRNAs in cisplatin-sensitive and cisplatin-resistant tissues of ovarian cancer, we identified the expression profiles of dysregulated circRNAs using high-throughput microarray assay. We found 339 circRNAs were aberrantly expressed with fold change $\geq$ 2.0 and p < 0.05. Among them, 148 circRNAs were upregulated and 191 circRNAs were downregulated. Hierarchical clustering showed the 10 most upregulated and downregulated circRNAs between cisplatin-sensitive and cisplatin-resistant tissues
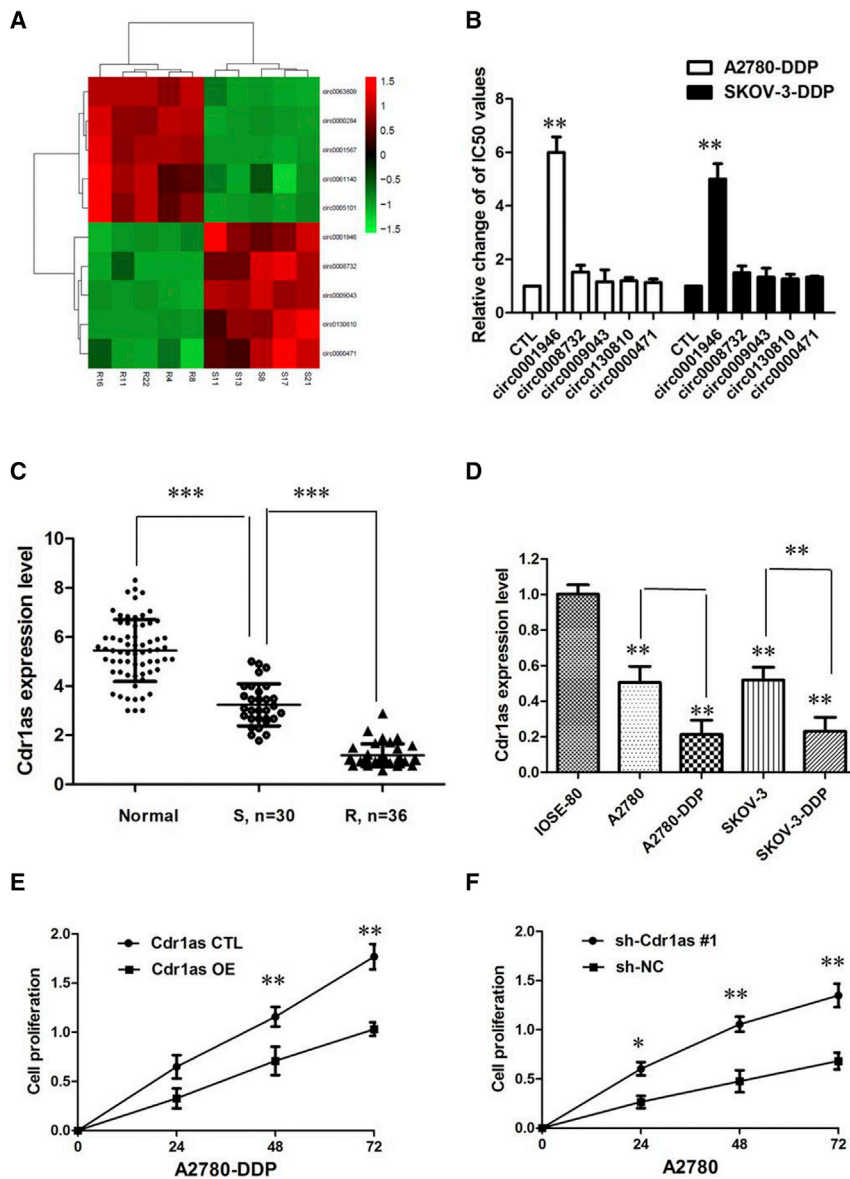
**Figure 1. Cdr1as Is Preferentially Downregulated in Cisplatin-Resistant Ovarian Cancer Patient Tissues**

(A) circRNA microarray data of 5 paired patients, 5 with cisplatin-resistant ovarian cancer and 5 with cisplatin-sensitive ovarian cancer, are presented in a heatmap. (B) Determination of $IC_{50}$ values of cisplatin for both resistant cell lines after overexpression of various circRNA. (C) Relative expression of Cdr1as in cisplatin-resistant ovarian cancer and cisplatin-sensitive ovarian cancer. (D) Relative expression of Cdr1as in a panel of ovarian cancer cell lines. (E) CCK-8 assay showed that Cdr1as overexpression could dramatically inhibit the proliferation of A2780-DDP in the presence of cisplatin (20 μM). (F) CCK-8 assay showing inhibition of Cdr1as promoted the proliferation of A2780 cells in the presence of cisplatin (20 μM). All tests were performed at least three times. Data were expressed as mean ± SD. ***p < 0.001; **p < 0.01.

expression of Cdr1as in normal ovarian tissues and ovarian cancer tissues from cisplatin-resistant or cisplatin-sensitive patients. As shown in Figure 1C, there was a decreasing trend in Cdr1as levels from normal ovarian tissues to cisplatin-sensitive ovarian cancer tissues and then to cisplatin-resistant ovarian cancer tissues, and the differences among the three groups were significant (p < 0.001). Then, experiments were performed at the cellular level. Cdr1as expression was distinctively lower in resistant ones and obviously higher in normal ovarian surface epithelial cells IOSE-80 (p < 0.01; Figure 1D).

**Cdr1as Enhances the Cisplatin Chemosensitivity of Ovarian Cancer *In Vitro***

To further validate the expression level of Cdr1as on cisplatin resistance, we performed loss- and gain-of-function studies by knocking down or overexpressing in ovarian cancer cells. A2780 and SKOV-3 cells were transfected with three kinds of Cdr1as knockdown lentivirus (respectively, sh-Cdr1as #1, sh-Cdr1as #2, or sh-Cdr1as #3) or GFP lentivirus (sh-control [sh-CTL]). The quantitative real-time PCR analysis confirmed that Cdr1as expression level was significantly downregulated in A2780 and SKOV-3 cells by sh-Cdr1as #1 instead of sh-Cdr1as #2 and sh-Cdr1as #3 (p < 0.01; Figures S1A and S1B), so we chose sh-Cdr1as #1 subsequently for the following experiments. Meanwhile, we infected A2780-DDP and SKOV-3-DDP cells with the Cdr1as-overexpressing adenovirus (Cdr1as OE) or control GFP adenovirus (Cdr1as CTL). The quantitative real-time PCR assay indicated the relative abundance of Cdr1as in A2780-DDP and SKOV-3-DDP cells infected with adenovirus (p < 0.01; Figures S1C and S2D).

of ovarian cancer (Figure 1A). Then, we further confirmed the expression of these five most downregulated circRNAs in five matched cisplatin-sensitive and cisplatin-resistant tissues of ovarian cancer by quantitative real-time PCR. Among them, we found that overexpression of Cdr1as reversed cisplatin resistance in both A2780-DDP and SKOV-3-DDP cell lines, whereas the other four circRNAs showed little effect (Figure 1B). Hence we focused on the functional role of circRNA Cdr1, also named ciRS-7 or Cdr1NAT, which is 1,500 nt long and is transcribed in the antisense orientation with respect to the cerebellum degeneration-related antigen 1 (CDR1) gene.

To investigate the clinical significance of Cdr1as expression in cisplatin sensitivity of ovarian cancer patients, we analyzed the

CCK-8 assay showed that Cdr1as overexpression could dramatically inhibit the proliferation of A2780-DDP and SKOV-3-DDP in the
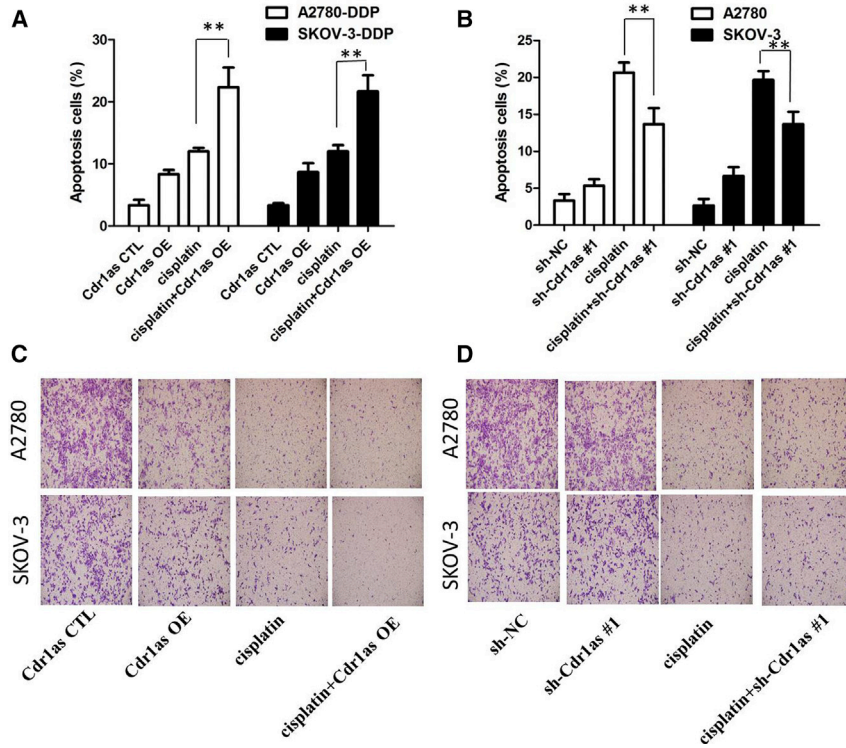
**Figure 2. UCA1 Affects Apoptosis and Migration of Ovarian Cancer *In Vitro***

(A) Flow cytometry analysis showed that overexpression of Cdr1as promoted the cisplatin-induced cell apoptosis of resistant ovarian cancer in the presence of cisplatin (20 μM). (B) Flow cytometry analysis showed that inhibition of Cdr1as significantly decreased the cisplatin-induced cell apoptosis of resistant ovarian cancer in the presence of cisplatin (20 μM). (C) Transwell assay showed that deregulation of Cdr1as significantly promoted the migratory capacities of A2780 and SKOV-3 cells in the presence of cisplatin (20 μM). (D) Transwell assay showed that overexpression of Cdr1as dramatically decreased the migration capacity of A2780-DDP and SKOV-3-DDP cell lines in the presence of cisplatin (20 μM). All tests were performed at least three times. Data were expressed as mean ± SD. **p < 0.01.

## Cdr1as Enhances the Cisplatin Chemosensitivity of Ovarian Cancer *In Vivo*

To determine the effect of Cdr1as *in vivo*, SKOV-3 cells stably transfected stable Cdr1as knockdown (sh-Cdr1as) or negative control (sh-NC) were injected into the flanks of nude mice. According to the treatment, the tumors on the mice were actually assigned to the following groups: group 1, sh-Cdr1as-transfected cells + cisplatin; group 2, sh-Cdr1as-transfected cells + NS; group 3, sh-NC-transfected cells + cisplatin; and group 4, sh-NC-transfected cells + NS. The results showed that cisplatin treatment significantly inhibited the growth of tumor cells when compared with control groups (group 1 versus group 2 and group 3 versus group 4, respectively). More importantly, with cisplatin treatment, tumor cells infected with sh-Cdr1as grew faster than controls (group 1 versus group 3), suggesting that Cdr1as enhances the cisplatin chemosensitivity *in vivo* (p < 0.01; Figure 3A). Tendencies in tumor weight were consistent with those in tumor volume (p < 0.01; Figure 3B). Moreover, immunohistochemistry assay showed that the tumors treated with sh-Cdr1as plus cisplatin displayed an increased proliferation percentage of Ki-67-positive tumor cells compared with the control group (group 1 versus group 3) (Figure 3C; p < 0.01). Taken together, these results demonstrated the reversion of cisplatin resistance by inhibition of Cdr1as expression *in vivo*.

## Cdr1as Functioned as a Molecular Sponge of miR-1270 in Ovarian Cancer Cells

Up to now, accumulating evidence indicated that Cdr1as exerted the function by interacting with miRNAs. Therefore, to investigate the effect of Cdr1as on the expression of miRNAs, we performed the bioinformatics prediction analysis by TargetScan and miRanda database. As shown in Figure 4A, miR-1270 harbor a complementary binding sequence of Cdr1as. In order to further validate the interaction, Cdr1as sequence containing the putative or mutated miR-1270 binding site was cloned into the downstream of luciferase reporter gene,

presence of cisplatin (20 μM) (p < 0.01; Figure 1E; Figure S2A), implying that Cdr1as inhibits proliferation of resistant ovarian cancer cells. However, the opposite phenomenon was observed after inhibition of Cdr1as, and the growth rate of A2780 and SKOV-3 cells was significantly increased compared with the control group (p < 0.01; Figure 1F; Figure S2B).

To further confirm the effect of Cdr1as on cisplatin resistance, we analyzed rates of apoptosis using annexin V-allophycocyanin (APC)/DAPI double staining and flow cytometry. Overexpression of Cdr1as promoted the cisplatin-induced cell apoptosis of resistant ovarian cancer in the presence of cisplatin (20 μM) (p < 0.01; Figure 2A). However, cell apoptosis assays revealed that following inhibition of Cdr1as, the apoptosis of A2780 and SKOV-3 cells was significantly decreased compared with the control group in the presence of cisplatin (20 μM) (p < 0.01; Figure 2B). These results demonstrate that Cdr1as enhances the cisplatin chemosensitivity of ovarian cancer cells.

To further confirm the effect of Cdr1as on ovarian cancer cell migration, we investigated cell migration by transwell assay. The results showed that deregulation of Cdr1as significantly promoted the migratory capacities of A2780 and SKOV-3 cells in the presence of cisplatin (20 μM) (p < 0.01; Figure 2C). Furthermore, the transwell assay showed that overexpression of Cdr1as dramatically decreased the migration capacity of A2780-DDP and SKOV-3-DDP cell lines in the presence of cisplatin (20 μM) (p < 0.01; Figure 2D).
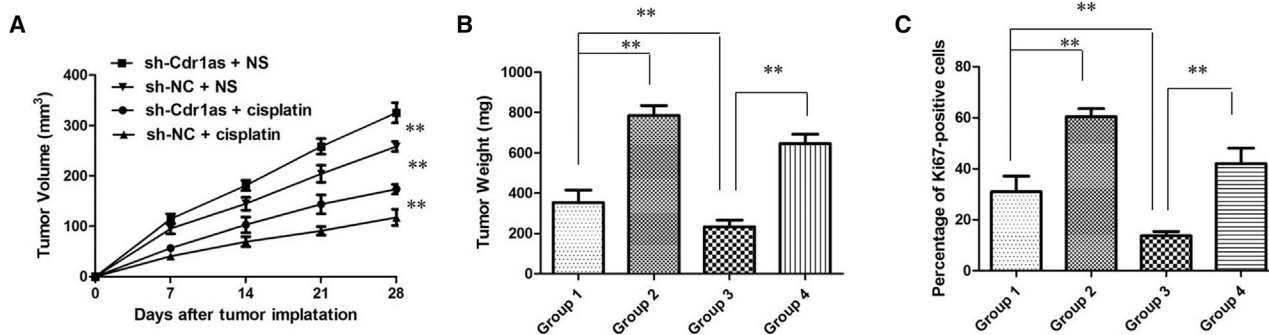
**Figure 3. Cdr1as Enhances the Cisplatin Chemosensitivity of Ovarian Cancer *In Vivo***

(A) Volume of tumors that developed in xenografts from different groups. (B) Weights of tumors that developed in xenografts from different groups. The group settings are as follows: group 1, sh-Cdr1as-transfected cells + cisplatin; group 2, sh-Cdr1as-transfected cells + NS; group 3, sh-NC-transfected cells + cisplatin; and group 4, sh-NC-transfected cells + NS. All tests were performed at least three times. **$p < 0.01$ compared with respective groups. (C) The percentage of KI67-positive cells in xenografts from different groups. All tests were performed at least three times. Data were expressed as mean ± SD. **$p < 0.01$.

generating wild-type (WT)-Cdr1as or mutant (MUT)-Cdr1as luciferase reporter plasmids. Then the effect of miR-1270 on WT-Cdr1as or MUT-Cdr1as luciferase reporter systems was determined. The results showed that miR-1270 mimic considerably reduced the luciferase activity of the WT-Cdr1as luciferase reporter vector compared with negative control, whereas miR-1270 mimic did not pose any impact on the luciferase activity of MUT-Cdr1as-transfected cells ($p < 0.01$; Figure 4B). In a further RNA immunoprecipitation (RIP) experiment, Cdr1as and miR-1270 simultaneously existed in the production precipitated by anti-AGO2 ($p < 0.01$; Figure 4C), suggesting that miR-1270 is Cdr1as-targeting miRNA. Subsequently, the effect of Cdr1as on miR-1270 expression was also observed in ovarian cancer cells. The results manifested that knockdown or overexpression of Cdr1as significantly affected miR-1270 expression ($p < 0.01$; Figures S2C and S2D). These outcomes indicated that the interaction of Cdr1as and miR-1270 was realized by the putative binding site.

### miR-1270 Plays an Oncogenic Role in Ovarian Cancer Cells and Reduces Their Cisplatin Chemosensitivity

To investigate whether miR-1270 expression is altered in ovarian cancer, we examined the miR-1270 expression in 66 pairs of ovarian cancer tissues and matched adjacent normal tissues via quantitative real-time PCR. As shown in Figure 5A, there was an increasing trend in miR-1270 levels from normal ovarian tissues to cisplatin-sensitive ovarian cancer tissues and then to cisplatin-resistant ovarian cancer tissues, and the differences among the three groups were significant ($p < 0.01$). We also confirmed that the expression of miR-1270 was obviously increased in cisplatin-resistant cells compared with that in cisplatin-sensitive cells, indicating the opposite result to Cdr1as expression ($p < 0.01$; Figure 5B).

We further performed rescue assays to confirm how miR-1270 modulated cisplatin resistance in ovarian cancer cells. We transfected miR-1270 mimics or inhibitor into ovarian cancer cell lines, and the proliferation curves were performed. Our results showed that miR-1270 inhibitor markedly inhibits the cell growth in cisplatin-

resistant cells when compared with cells transfected with miRNA control (miR-NC) ($p < 0.01$; Figures 5C and 5D), whereas cisplatin-sensitive cells transfected with miR-1270 mimics grew at a dramatically higher rate as compared with controls ($p < 0.01$; Figures 5E and 5F). Collectively, these data indicate that miR-1270 contributes to cisplatin resistance.

### SCAI Was a Direct Target of miR-1270, which Could Enhance the Cisplatin Chemosensitivity of Ovarian Cancer

According to several miRNA target prediction services (miRDB: http://www.mirdb.org/; TargetScan: http://www.targetscan.org/vert_72/), it was noticed that tumor suppressor gene SCAI had three DNA sequences on its 3′ UTR that could be putative binding sites for miR-1270 (Figure 6A). In order to verify whether miR-1270 may directly bind the SCAI gene, we conducted a WT or MUT SCAI 3′ UTR luciferase reporter vector. SCAI-WT or SCAI-MUT was co-transfected with miR-1270 mimics or negative control into cells. The relative luciferase activity was remarkably reduced in cells co-transfected with the SCAI-WT luciferase reporter and miR-1270 mimic compared with in the negative control cells. However, inhibitory effects were abolished when 3′ UTRs that contained both MUT binding sites were co-transfected with miR-1270, confirming that SCAI is a target of miR-1270 ($p < 0.01$; Figure 6B).

We examined SCAI expression in ovarian cancer patient tissues and cell lines. The real-time PCR analysis was performed to determine the expression levels of SCAI in normal ovarian tissues and ovarian cancer tissues from cisplatin-resistant or cisplatin-sensitive patients. The results demonstrated that the SCAI mRNA were lower in cisplatin-resistant ovarian cancer tissues compared with those in cisplatin-sensitive ovarian cancer tissues ($p < 0.01$; Figure 6C). The expression of SCAI was obviously decreased in cisplatin-resistant cells compared with that in cisplatin-sensitive sensitive cells ($p < 0.01$; Figure 6D). Our results showed that inhibitor of miR-1270 significantly upregulated the protein expression of SCAI in SKOV-3-DDP cells (Figure 6E).
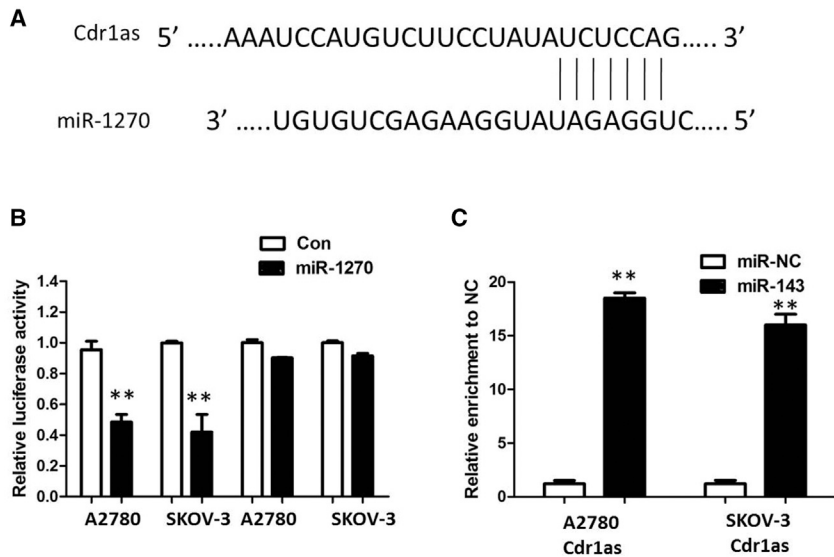
**A**

Cdr1as    5′ .....AAAUCCAUGUCUUCCUAUAUCUCCAG..... 3′

                          | | | | | | | |

miR-1270    3′ .....UGUGUCGAGAAGGUAUAGAGGUC..... 5′



**Figure 4. Cdr1as Functioned as a Molecular Sponge of miR-1270 in Ovarian Cancer Cells**

(A) StarBase v2.0 results showing the sequence of Cdr1as with highly conserved putative miR-1270 binding sites. (B) miR-1270 mimic considerably reduced the luciferase activity of the WT-Cdr1as luciferase reporter vector compared with negative control, whereas miR-1270 mimic did not pose any impact on the luciferase activity of MUT-Cdr1as-transfected cells. (C) Cdr1as and miR-1270 simultaneously existed in the production precipitated by anti-AGO2. All tests were performed at least three times. Data were expressed as mean ± SD. **$p < 0.01$.

### Serum Exosomal Cdr1a Level Is Downregulated in Cisplatin-Resistant Ovarian Patients

Finally, in our current study, we extracted exosomes from 66 serum samples from ovarian cancer patients who received cisplatin treatment. First, we characterized these vesicles by several methods such as electron microscopy (Figure 7A) and western blot (Figure 7B). The high presence of the exosomal markers TSG101 and HSP70 confirmed the purity of isolated ovarian cancer-secreted exosomes in the serum. Our results showed that Cdr1a expression is detectable in extracted serum exosomes and is less expressed in the cisplatin-resistant group than in the cisplatin-sensitive group ($p < 0.01$; Figure 7C). Furthermore, there was a significant inverse correlation between the expression levels of Cdr1a and miR-1270 in serum exosomes derived from ovarian cancer patients ($r = 0.679$; $p < 0.001$; Figure 7D). Altogether, these results indicate that exosomal Cdr1a in serum is stable and can serve as a promising biomarker for cisplatin-resistant ovarian cancer patients.

## DISCUSSION

In this study, we explored the effect of circRNA Cdr1as on the cisplatin chemosensitivity of ovarian cancer and demonstrated the regulatory mechanism of the miR-1270/SCAI signaling pathway. Our results indicate that upregulated Cdr1as could increase the cisplatin sensitivity of ovarian cancer cells. Cdr1as can function as a molecular sponge of miR-1270, which weakens the inhibitory effect of miRNA on the downstream target gene SCAI. Moreover, the dual-luciferase reporter system and RIP assay validated the direct interaction of Cdr1as, miR-1270, and SCAI. These results suggested that Cdr1as may have the potential to regulate the sensitivity of ovarian cancer cells to cisplatin, in turn promoting the progression of ovarian cancer.

circRNAs recently have been identified as members of the ncRNA family that play a significant role in many cancers' progression.[12,13]

Emerging evidence shows that dysregulation of circRNAs plays important roles in chemoresistance.[14] Gao et al.[14] identified 18 differentially expressed circRNAs in breast cancer by screening microarrays of drug-resistant cell lines and sensitive cell lines, and then explored the potential chemoresistance mechanism of hsa_circ_0006528. Therefore, targeting circRNAs and elucidating the underlying mechanisms of circRNAs may improve diagnostic and therapeutic strategies for ovarian cancer. Cdr1as, also named ciRS-7 or Cdr1NAT, is 1,500 nt long and is transcribed in the antisense orientation with respect to the CDR1 gene.[15] Recent studies have shown that Cdr1as can bind to miR-7 and function as an miRNA sponge in different diseases. In islet cells, Cdr1as regulates insulin transcription and secretion via miR-7 and its targets.[16] Moreover, it serves as an oncogene in hepatocellular carcinoma and non-small-cell lung cancer through targeting miR-7 expression.[17,18]

In recent years, the growing popularity of high-throughput sequencing technology has enabled researchers to further investigate the expression and action mechanism of circRNA. circRNA expression profiles for cisplatin-resistant ovarian cancer were screened using samples from five cases of cisplatin-resistant ovarian cancer patients and five cisplatin-sensitive individuals as controls. Results showed 148 circRNAs were upregulated and 191 circRNAs were downregulated between cisplatin-sensitive and cisplatin-resistant tissues of ovarian cancer. Cdr1as was downregulated in cisplatin-resistant cells and patient tissues. To further validate whether Cdr1as is functionally required for cisplatin resistance, we performed loss-of-function studies by overexpressed Cdr1as in two cisplatin-resistant ovarian cancer cell lines, A2780-DDP and SKOV-3-DDP. Meanwhile, we downregulated Cdr1as expression in cisplatin-sensitive ovarian cancer cells. Gain-of-function experiments revealed that ectopic expression of Cdr1as inhibited proliferation and promoted apoptosis of cisplatin-resistant cells in the presence of 4 μg/mL cisplatin, compared with negative control-transfected cells. Loss-of-function experiments revealed that knockdown of Cdr1as promoted the cisplatin-induced cell apoptosis and cell mobility of cisplatin-sensitive cells under cisplatin treatment. In addition, xenograft experiments showed that Cdr1as promoted the cisplatin chemosensitivity of ovarian cancer in vivo.
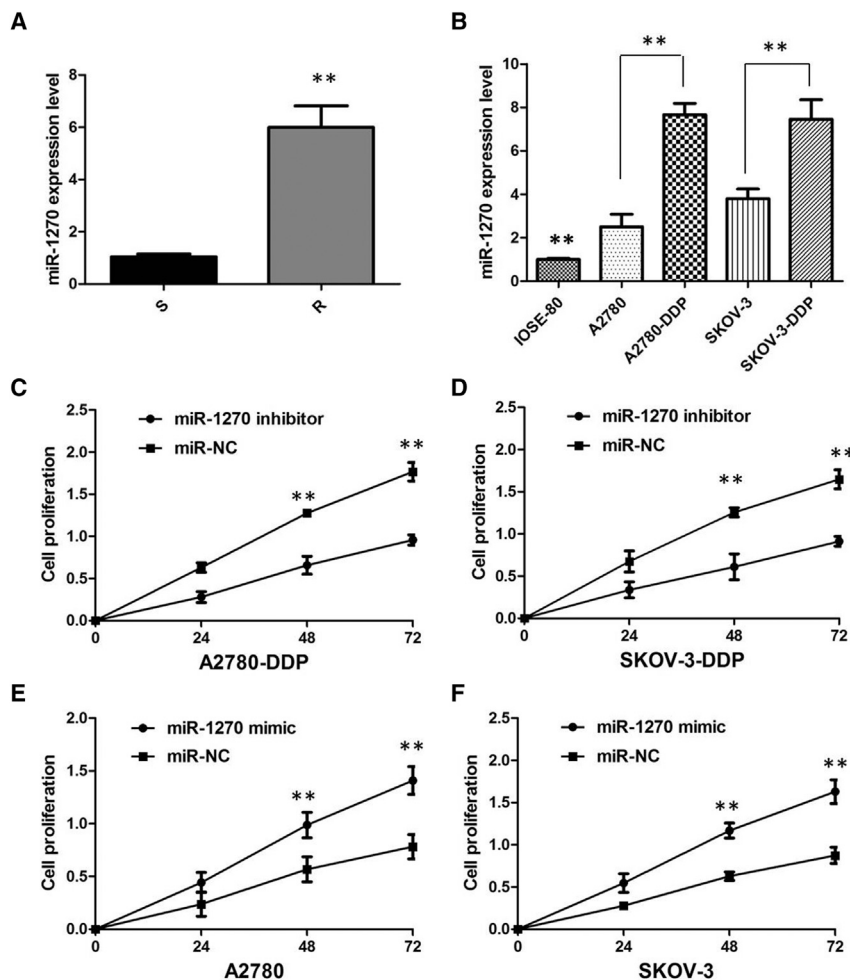
**Figure 5. miR-1270 Plays an Oncogenic Role in Ovarian Cancer Cells and Reduces Their Cisplatin Chemosensitivity**

(A) Relative expression of miR-1270 in the cisplatin-sensitive group and cisplatin-resistant group in ovarian cancer patients. (B) Relative expression of miR-1270 in a panel of ovarian cancer cell lines. (C) CCK-8 assay showed that the miR-1270 inhibitor markedly inhibited the cell growth of A2780-DDP cells when compared with cells transfected with miR-NC. (D) CCK-8 assay showed that miR-1270 inhibitor markedly inhibits the cell growth of SKOV-3-DDP cells when compared with cells transfected with miR-NC. (E) CCK-8 assay showed that miR-1270 overexpression markedly promoted the proliferation of A2780 cells when compared with cells transfected with miR-NC. (F) CCK-8 assay showed that miR-1270 overexpression markedly promoted the proliferation of SKOV-3 cells when compared with cells transfected with miR-NC. All tests were performed at least three times. Data were expressed as mean ± SD. **$p < 0.01$.

It is well-known that the ceRNA network is an important regulatory model and circRNAs could act as ceRNAs through regulating miRNAs.[19,20] Herein, using various assays, we found that Cdr1as enhances the cisplatin chemosensitivity, mainly through interaction with miR-1270. We found that the miR-1270 was significantly higher in cisplatin-resistant ovarian cancer tissue compared with cisplatin-sensitive ovarian cancer tissue. Next, we verified that Cdr1as had an endogenous sponge-like effect on miR-1270 in ovarian cancer. First, bioinformatics prediction and a luciferase reporter assay showed that Cdr1as and the SCAI 3′ UTR share identical miR-1270 response elements and might therefore bind competitively to miR-1270. Second, Cdr1as could bind directly to miR-1270 in an AGO2-dependent manner. Third, knockdown or overexpression of Cdr1as significantly affected miR-1270 expression. Finally, Cdr1as could control the SCAI level by provoking miR-1270. It has recently been reported that circRNAs can act as miRNA sponges to negatively control miRNA. Taken together, the study revealed that a Cdr1as/miR-1270/SCAI axis exists in ovarian cancer.

Exosomes have been reported to be involved in each process of cancer, such as angiogenesis, metastasis, epithelial mesenchymal transition (EMT), and immune escape.[21] Although several studies have shown that exosomal circRNAs are potential markers for cancer,[22] levels of exosomal circRNAs derived from ovarian cancer cells are still unknown. In this study, we performed transmission electron microscopy (TEM) to reveal the shapes and size of exosomes from plasma of ovarian cancer patients. Notably, we found that Cdr1as expression is detectable in extracted serum exosomes of ovarian cancer patients and is less expressed in the cisplatin-resistant group than in the cisplatin-sensitive group.

In summary, our results showed that Cdr1as was identified as a critical regulator gene in the formation of cisplatin resistance of ovarian cancer. Mechanistically, Cdr1as functions as a molecular sponge to downregulate miR-1270, thereby resulting in partial abolition of the translational repression of its target gene SCAI in ovarian cancer cells. This indicates that Cdr1as could be used to sensitize ovarian cancer cells to cisplatin.

## MATERIALS AND METHODS

### Clinical Specimens

Sixty-six paired ovarian cancer tissues and matched adjacent normal tissues were obtained from The First Affiliated Hospital of Zhengzhou University between 2012 and 2016. Tumor specimens and corresponding adjacent normal tissues were collected and stored in liquid nitrogen until use. For exosome purification, serum samples were collected from these patients. Patients with progressive disease during primary chemotherapy or those who suffered recurrent disease within 6 months of completing primary chemotherapy were termed
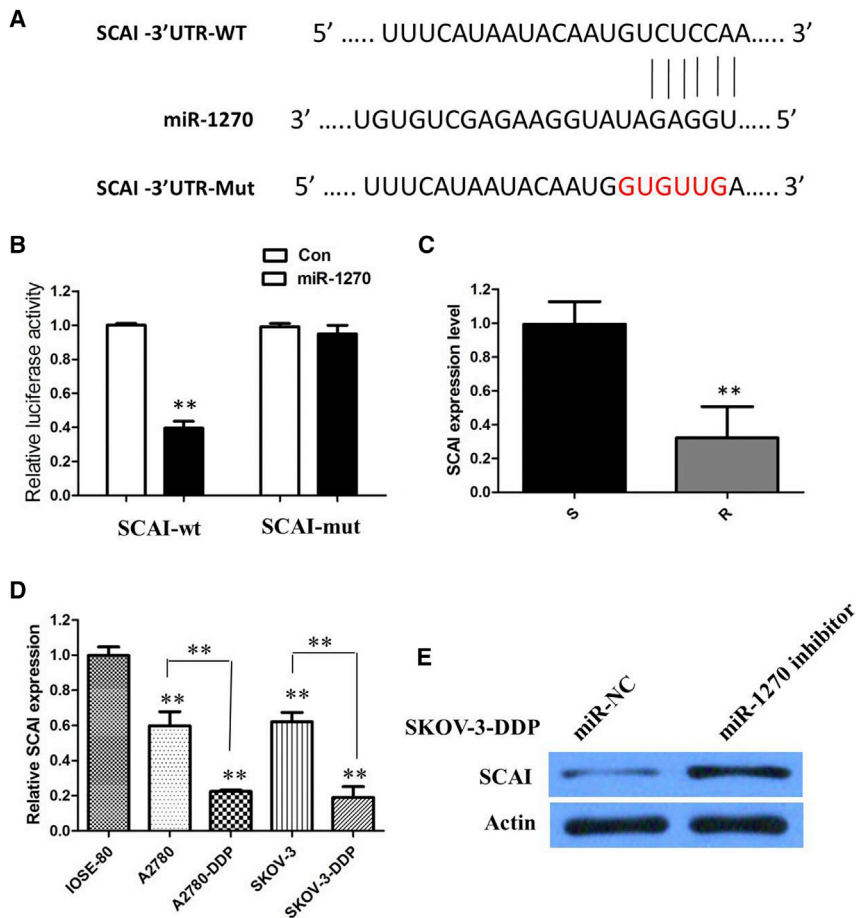
**Figure 6. SCAI Was a Direct Target of miR-1270, which Could Enhance the Cisplatin Chemosensitivity of Ovarian Cancer**

(A) Bioinformatics analysis revealed the predicted binding sites between SCAI and miR-1270. (B) Luciferase reporter assay demonstrated miR-1270 mimics significantly decreased the luciferase activity of SCAI-WT in ovarian cancer cells. (C) The real-time PCR analysis was performed to determine the expression levels of SCAI in normal ovarian tissues and ovarian cancer tissues from cisplatin-resistant or cisplatin-sensitive patients. (D) Relative expression of SCAI in a panel of ovarian cancer cell lines. (E) Inhibition of miR-1270 significantly increased the protein expression of SCAI in SKOV-3-DDP cells. All tests were performed at least three times. Data were expressed as mean ± SD. **$p < 0.01$.

on the circRNAs chips. Exogenous RNAs developed by the External RNA Controls Consortium (ERCC) were used as controls. circRNAs were enriched by digesting linear RNA with RNase R (Epicenter, Madison, WI, USA). Labeled RNAs were scanned using an Agilent Scanner G2505C (Agilent Technologies, Santa Clara, CA, USA). The circRNA microarray process was performed by KangChen Biotech (Shanghai, China).

### Cell Lines

Human ovarian cancer cell lines (A2780 and SKOV-3) and the normal ovarian epithelial cell line (IOSE-80) were purchased from ATCC (Manassas, VA, USA) and bena culture collection (BNCC; Beijing, China), respectively. Ovarian cancer cells were maintained in RPMI 1640 medium (Hyclone, South Logan, UT, USA) with 10% fetal bovine serum (FBS; Invitrogen, Gaithersburg, MD, USA), whereas normal ovarian cells were maintained in 90% DMEM (Invitrogen) with high glucose and 10% FBS. All cell lines were cultured in 5% $CO_2$ at 37°C. Resistant ovarian cancer cell lines A2780-DDP (Yuci Biotech, Jiangsu, China) and SKOV-3-DDP (Xinyu Biotech, Shanghai, China) were induced by cisplatin (Sigma, St. Louis, MO, USA). The definite operations were as follows: resistant cell lines were developed over a period of 6 months by stepwise increased concentrations of cisplatin. Cells were continuously maintained in cisplatin, with treatments beginning at the initial half maximal inhibitory concentration ($IC_{50}$) of the respective parent cell lines. Media containing cisplatin were changed every 2–3 days. Because cells displayed resistance to treatments of cisplatin, the concentration was subsequently increased with final treatment doses of 80 μm. Following each treatment they were allowed to fully recover before assessing their resistance to cisplatin and any experimental work. The acquired cell resistance to cisplatin was judged based on decreased cell death and increased proliferation of cells.

cisplatin-resistant (R, n = 36). Patients with recurrence beyond 6 months or without recurrence were termed cisplatin-sensitive (S, n = 30). All of the cancer samples analyzed were serous ovarian cancer, which were obtained from the primary untreated tumors. In all of the cases, the diagnoses were confirmed by two experienced pathologists, which were done in accordance with the principles laid down in the latest World Health Organization classification. Informed consent was also obtained from all of the patients, and the study was approved by the Medical Ethics Committee of The First Affiliated Hospital of Zhengzhou University. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki.

### Expression Profile Analysis of circRNAs

Five pairs of cisplatin-resistant ovarian cancer tissues and cisplatin-sensitive ovarian cancer tissues were used for circRNA microarray. Tissues specimens were obtained during operation and immediately frozen at −80°C until further use. The circRNAs chip (ArrayStar Human circRNAs chip; ArrayStar, Rockville, MD, USA) containing 5,639 probes specific for human circRNAs splicing sites was used. Following hybridization and washing of the samples, five pairs of paired cancerous and adjacent noncancerous tissues were analyzed
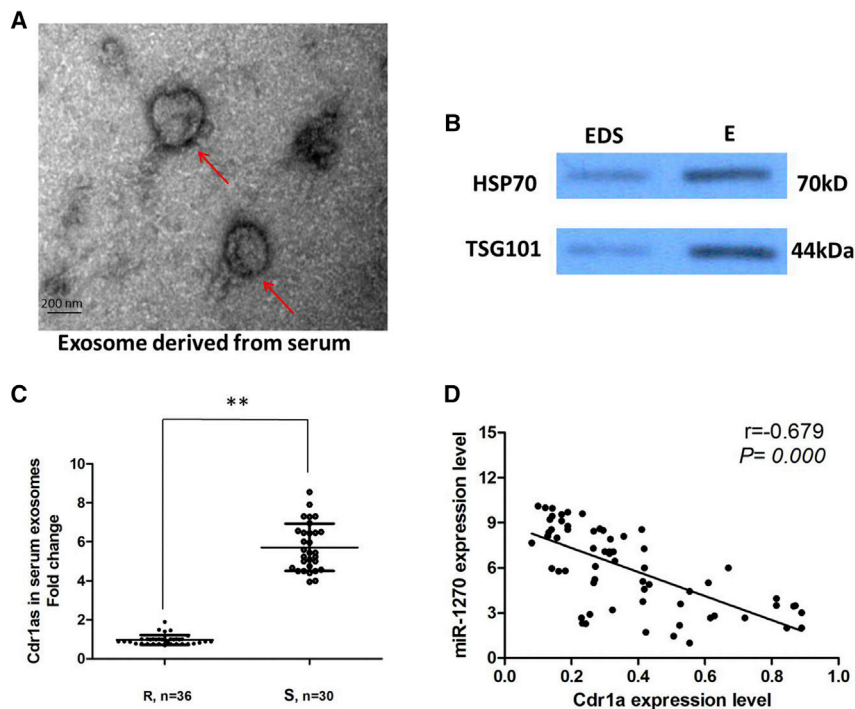
**Figure 7. Serum Exosomal Cdr1a Level Is Downregulated in Cisplatin-Resistant Ovarian Patients**

(A) Representative image of exosome (indicated by red arrows) derived from serum of ovarian cancer patients detected from electron microscope. (B) The markers of exosome from purified serum exosome were analyzed by western blotting in exosomes (E) and exosome-depleted supernatant (EDS). (C) Quantitative real-time PCR for the abundance of Cdr1a in serum exosomes. The levels of Cdr1a in serum exosomes from the cisplatin-resistant group were significantly lower than from the cisplatin-sensitive group. (D) In the exosomes extracted from serum of ovarian cancer patients, the expression levels of Cdr1a were negatively correlated with that of miR-1270.

480 (Roche, USA). Total RNAs, including circRNAs and miRNAs, were isolated from tissues and transfected cells by using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. cDNA was synthesized using HiScript II (Vazyme, China). Quantitative real-time PCR for circRNA and miRNA was performed on an AB7300 thermorecycler (Applied Biosystems, USA) or LightCycler 480 (Roche, USA). The $2^{-\Delta\Delta CT}$ method was used to analyze gene expression.

## RNA Oligoribonucleotides

The RNA oligoribonucleotides used in this study, including miR-1270 mimic, miR-1270 inhibitor, the small interfering RNAs (siRNAs) targeting CDR1as (si-CDR1as) or SCAI (si-SCAI), and the corresponding miRNA control (miR-NC) and siRNA control (si-NC), were purchased from GenePharma (Shanghai, China). The RNA oligoribonucleotides used in this study, including miR-1270 mimic, miR-1270 inhibitor, the siRNAs targeting CDR1as (si-CDR1as) or SCAI (si-SCAI), and the corresponding miRNA control (miR-NC) and siRNA control (si-NC), were purchased from GenePharma (Shanghai, China).

The lentivirus targeting human CDR1as was purchased from GeneChem (Shanghai, China). The targeting sequence was designed as follows: 1 sense 5′-TGCACCTGTGTCAAGGTCTTTT CAAGAGAAAGACCTTGACACAGGTGCTTTTTTC-3′ and 2 sense 5′-TGGTCTTCCAGCGACTTCAATTCAAGAGATTGAAG TCGCTGGAAGACCA-3′. miR-7 mimics and inhibitors were synthesized by RiboBio. The miRNA oligonucleotides were transfected using Lipofectamine RNAiMAX (50 nmol/L; Invitrogen, CA, USA).

## RNA Extraction and Quantitative Real-Time PCR

Total RNAs, including circRNAs and miRNAs, were isolated from tissues and transfected cells by using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. cDNA was synthesized using HiScript II (Vazyme, China). Quantitative real-time PCR for circRNA and miRNA was performed on an AB7300 thermorecycler (Applied Biosystems, USA) or LightCycler

## CCK-8 Assay

Cell proliferation was measured by the cell proliferation reagent CCK-8 (Roche, Basel, Switzerland). After the cells ($1 \times 10^3$/well) were plated in the 96-well microtiter plates (Corning, NY, USA), 10 μL CCK-8 regents was added to each well at the time of harvest. Two hours later, the absorbance was recorded at 450 nm to determine the cell viability.

## Cell Apoptosis Analysis

After detecting apoptosis by flow cytometry, we used an annexin V-allophycocyanin (APC)/DAPI double-staining kit (Thermo Fisher Scientific) to analyze cellular apoptosis. Cells were seeded in six-well plates ($5 \times 10^5$ cells/well) and then digested with trypsin (GIBCO trypsin-EDTA; Thermo Fisher Scientific), washed with PBS three times, suspended in 500 μL binding buffer, and then incubated with 5 μL fluorescein isothiocyanate (FITC)-conjugated annexin V and 3 μL propidium iodide (PI) for 15 min at room temperature in the dark. The stained cells were detected using the BD FACSAria II flow cytometer (BD Biosciences, Hercules, CA, USA).

## Cell Migration Assay

Cell migration was measured using the 24-well transwell chambers with 8-μm polycarbonate membrane (Corning, NY, USA). The filter was first pre-coated with 500 ng/mL Matrigel solution for cell migration assay (BD, Franklin Lakes, NY, USA) and incubated for 4 h at 37°C; then 500 μL 10% FBS medium was placed in the lower

chamber, and 100 μL serum-free medium was placed in the upper chamber. After cells were incubated at 37°C for 14–24 h, cells on the upper membrane surface were scraped off. Surface cells were fixed with a mixture of methanol and glacial acetic acid at the ratio of 3:1 for 30 min and then dried before they were stained with 15% Giemsa solution for 6–8 h. Then stained cells were observed and counted using an inverted microscope, and an average number within five randomly chosen fields was obtained.

### Tumor Xenograft Model

A total of 12 BALB/c female athymic mice at 4 weeks of age were housed and maintained in specific pathogen-free conditions. For injection, $1 \times 10^7$ SKOV-3 cells transfected with Lv-circRNA Cdr1as or Lv-NC were suspended in 100 μL PBS and injected subcutaneously in the flank. When tumors were palpable, the mice were randomized into treatment groups or control groups. Treatment lasted for 4 weeks until the xenograft tumor was stripped and the size was calculated. The experiments were performed in an observer-blinded and randomized manner. All experimental procedures took place at the animal center of The First Affiliated Hospital of Zhengzhou University and were approved by the Animal Ethics Committee of The First Affiliated Hospital of Zhengzhou University, and animal experiments were performed following the NIH *Guide for the Care and Use of Laboratory Animals*.

### Luciferase Reporter Assay

Sequences of potential binding sites of miR-1270 in circRNA Cdr1as full-length and SCAI 3′ UTR sequences and their MUT sequences were amplified by PCR and then cloned into a pmirGLO dual-luciferase vector (Promega, Madison, WI, USA) to construct luciferase reporter vector (Cdr1as-WT and SCAI-WT; Gene-Pharma). Cells were seeded into 24-well plates in triplicate. After 24 h, the cells were transfected with Cdr1as-WT (or Cdr1as-MUT) or SCAI-WT (or SCAI-MUT) and miR-1270 mimic or miR-NC using Lipofectamine 3000 (Invitrogen). Luciferase activity was measured in cell lysates 24 h after transfection using a Dual Luciferase Reporter System (Promega, Madison, WI, USA).

### RNA Immunoprecipitation (RIP)

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was used for RIP. Cells were lysed in complete RNA lysis buffer; then cell lysates were incubated with RIP buffer containing magnetic beads conjugated with human anti-Argonaute2 (AGO2) antibody (Millipore) or negative control mouse immunoglobulin G (IgG) (Millipore).

### Western Blotting

Total proteins from cells, tissues, and exosome samples were extracted using a radioimmunoprecipitation assay (RIPA) kit (Beyotime Biotechnology, Jiangsu, China). They were separated on polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-SCAI (ab124688; Abcam, Cambridge, UK) antibodies at 4°C overnight and were then

incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG at room temperature for 1 h. To identify exosome markers, we purchased primary antibodies against TSG101 from Abcam (ab125011; Cambridge, UK) and obtained primary antibodies against Hsp70 from Cell Signaling Technology (#4873; CST, Beverly, MA, USA). The secondary antibodies were F(ab)$_2$ fragments of donkey anti-mouse immunoglobulin or donkey anti-rabbit immunoglobulin linked to horseradish peroxidase (Jackson ImmunoResearch, USA). Immunoblotting reagents from an electrochemiluminescence kit were used (Amersham Biosciences, Uppsala, Sweden).

### Immunohistochemistry (IHC)

IHC analysis was performed under manufacturer's instructions. In brief, the slides were incubated with primary antibodies overnight at 4°C and then incubated with secondary antibodies at room temperature for 2 h. The expression was evaluated using a composite score obtained by multiplying the values of staining intensities (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) and the percentage of positive cells (0, 0%; 1, <10%; 2, 10%–50%; 3, >50%).

### Exosome Purification

Exosomes were extracted from serum samples using an ExoQuick precipitation kit (SBI; System Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions. In brief, serum was thawed on ice and centrifuged at 3,000 × g for 15 min to remove cells and cell debris. Next, 250 μL of the supernatant was mixed with 63 μL of the ExoQuick precipitation kit and incubated at 4°C for 30 min, followed by centrifugation at 1,500 × g for 30 min. Then, the supernatant was removed by careful aspiration, followed by another 5 min of centrifugation to remove the residual liquid. The exosome-containing pellet was subsequently re-suspended in 250 μL PBS. Exosomes were measured for their protein content using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, USA). Electron microscopy was applied to characterize the vesicles floated in PBS.

### TEM

Exosomes were suspended in 100 μL PBS and were fixed with 5% glutaraldehyde at incubation temperature and then maintained at 4°C until TEM analysis. According to the TEM sample preparation procedure, we placed a drop of exosome sample on a carbon-coated copper grid and immersed it in 2% phosphotungstic acid solution (pH 7.0) for 30 s. The preparations were observed with a transmission electron microscope (Tecnai G2 Spirit Bio TWIN; FEI, USA).

### Statistical Analysis

All data were expressed as mean ± SD, and the statistical analyses were practiced by GraphPad Prism (Version 7.0; GraphPad, La Jolla, CA, USA). Two-sided Student's t test, $\chi^2$ test, or Wilcoxon test were conducted to measure the differences between groups. $p < 0.05$ was considered to have statistical significance.

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.07.012.

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## AUTHOR CONTRIBUTIONS
Z.Z. and M.J. performed primers design and experiments. Q.W. contributed flow cytometry assay and animal experiments. N.H. collected and classified the human tissue samples. Y.L. contributed to RT-PCR and quantitative real-time PCR. M.J. analyzed the data. M.J. wrote the paper. All authors read and approved the final manuscript.

## CONFLICTS OF INTEREST
The authors declare no competing interests.

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**Supplemental Information**

**Circular RNA Cdr1as Upregulates SCAI**

**to Suppress Cisplatin Resistance in Ovarian**

**Cancer via miR-1270 Suppression**

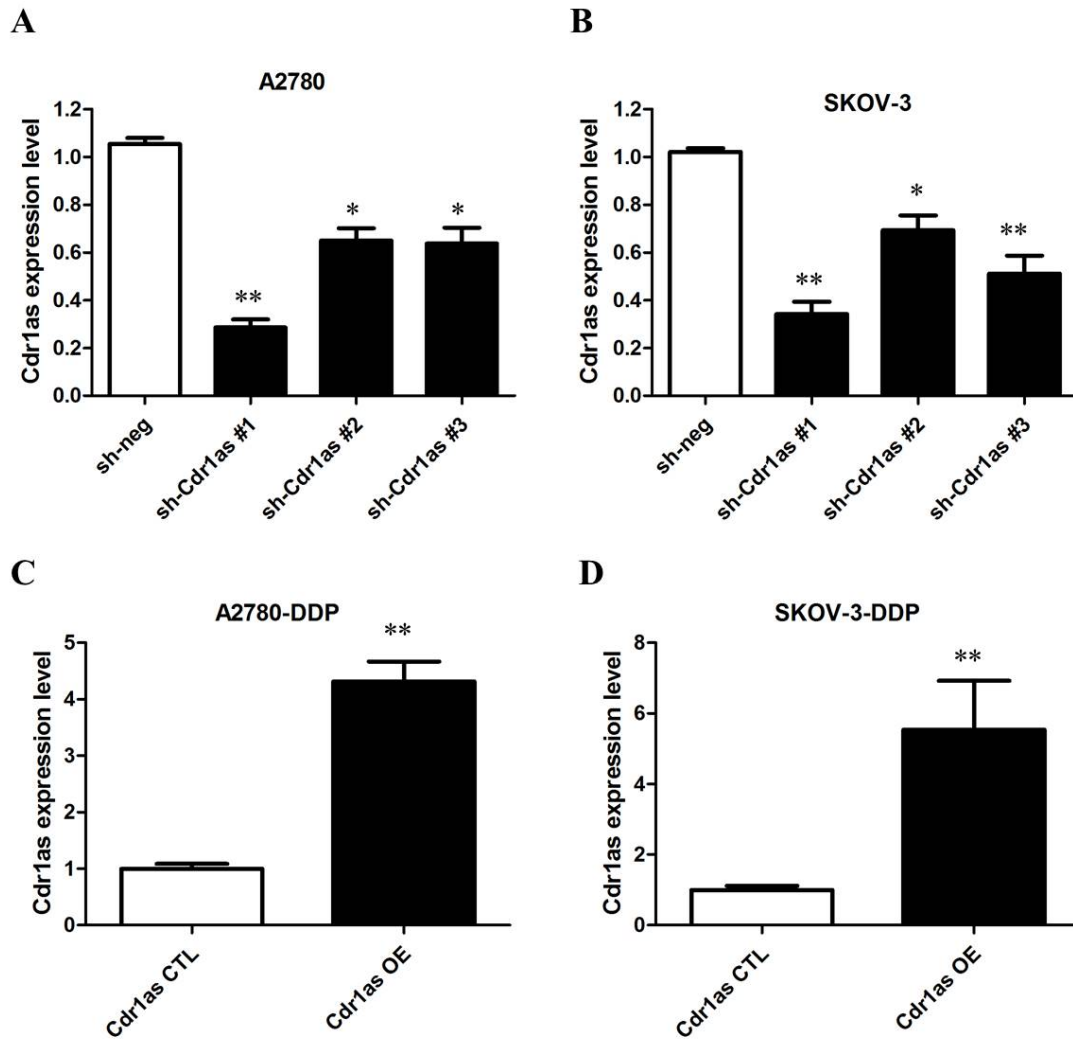Zhao Zhao, Mei Ji, Qianqing Wang, Nannan He, and Yue Li

**Figure S1** (A) The qRT-PCR analysis confirmed that Cdr1as expression level was significantly downregulated in A2780 cells by sh-Cdr1as #1 instead of sh-Cdr1as #2 and sh-Cdr1as #3; (B) The qRT-PCR analysis confirmed that Cdr1as expression level was significantly downregulated in SKOV-3 cells by sh-Cdr1as #1 instead of sh-Cdr1as #2 and sh-Cdr1as #3; (C) The qRT-PCR assay indicated the relative abundance of Cdr1as in A2780-DDP cells infected with Cdr1as overexpressing adenovirus (Cdr1as OE) or control GFP adenovirus (Cdr1as CTL); (D) The qRT-PCR assay indicated the relative abundance of Cdr1as in SKOV-3-DDP cells

infected with Cdr1as overexpressing adenovirus (Cdr1as OE) or control

GFP adenovirus (Cdr1as CTL); All tests were at least performed three

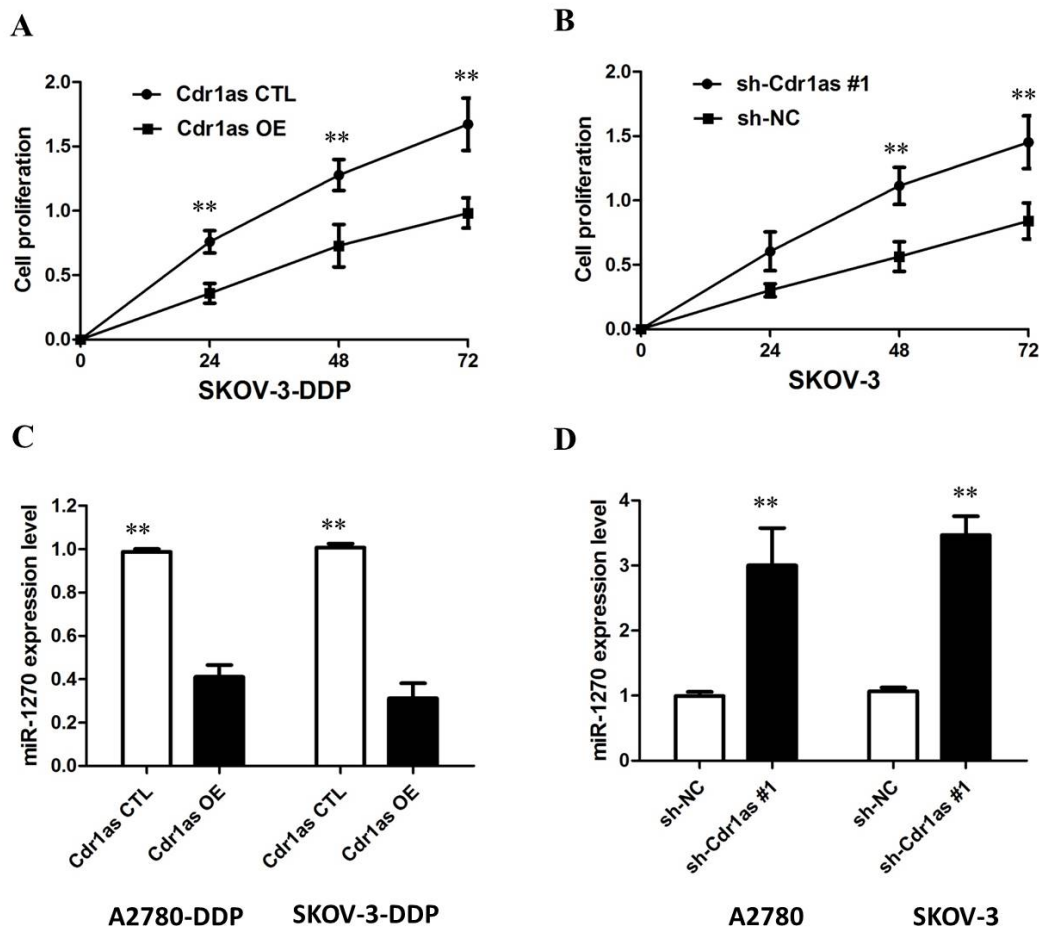times. Data were expressed as mean ± SD. ***P < 0.001;**P < 0.01;



**Figure S2** (A) CCK-8 assay showed that Cdr1as overexpression could

dramatically inhibit the proliferation of SKOV-3-DDP in the presence of

cisplatin (20 μM); (B) CCK-8 assay showed that inhibition of Cdr1as

promoted the proliferation of SKOV-3 cells in the presence of cisplatin

(20 μM); (C) Inhibition of Cdr1as increased the expression level of

miR-1270 in A2780 and SKOV-3 cells; (D) Overexpression of Cdr1as

decreased the expression level of miR-1270 in A2780-DDP and

SKOV-3-DDP cells; All tests were at least performed three times. Data were expressed as mean ± SD. ***P < 0.001;**P < 0.01;