Repression of Dicer is associated with invasive phenotype and chemoresistance in ovarian cancer

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sphere of 5% CO₂. The A2780/DDP cells used in the study were cultured in the absence of cisplatin for >1 month prior to use to exclude the stress reaction mediated by drug treatment.

The study was approved by the Ethics Committee of Huazhong University of Science and Technology, Wuhan, China.

**Transient Dicer small interfering RNA (siRNA) transfection.** Carboxyfluorescein (FAM)-labeled siRNA targeting Dicer and negative control siRNA were chemically synthesized (Invitrogen Life Technologies; Carlsbad, CA, USA). The siRNA sequences were as follows: Sense: 5'-UUUGUUGCCAGGCGAUUCCT-3' and anti-sense: 5'-GAUCAAGCCUCGCAACAAATT-3' for Dicer siRNA; sense: 5'-UCUCCGAGACGUGUACGTT-3' and anti-sense 5'-ACGUGACACGUUCCAGAATT-3' for negative control siRNA. Lipofectamine 2000 (Invitrogen Life Technologies) was used for transfection according to the manufacturer's instructions. The transfection efficiency was detected by fluorescent microscopy and the growth medium was replaced after 6 h. Forty-eight hours after transfection, the cells were harvested for analysis.

**RNA isolation and quantitative polymerase chain reaction (qPCR).** Total RNA was extracted from the cell lines using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Following confirmation of the quantity and quality of extracted total RNA by Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA), cDNA was synthesized using a reverse transcription kit (Toyobo; Osaka, Japan) according to the manufacturer's instructions. The primer sequences for Dicer mRNA detection were as follows: Upstream: 5'-GGTGTCCCTTGTTGATTTGCCC-3' and downstream: 5'-ATGTGGAGACATCGTGGA-3' (NM_001195573.1). The sequences of the β-actin primers were as follows: Upstream: 5'-GTCCTGCACCAGCAATGTCCTA-3' and downstream: 5'-TGACTGTCACCTTACCAGGTT-3'.

All reactions were performed in duplicate in an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems; Foster City, CA, USA). Each reaction system contained 1 µl cDNA sample, 12.5 µl SYBR-Green Real-time PCR Master Mix (Toyobo) and 1 µl of 10 µmol/l each primer, in a final volume of 25 µl. Reactions were performed under the following cycling conditions: Initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 15 sec and extension at 72°C for 45 sec. PCR products were identified by a melting curve analysis. The relative mRNA level of the Dicer gene was calculated using the comparative threshold cycle (Ct) method (2^ΔΔCt) normalized by β-actin expression (24).

**Protein extraction and western blot analysis.** Protein for western blot analysis was isolated from cells by a radioimmunoprecipitation assay buffer (RIPA; Beyotime, China) according to the manufacturer's instructions. The protein concentration was measured by biocinchonic acid (BCA) assay. Fifty micrograms of total protein was denatured by boiling for 5 min, then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes and blotted with mouse anti-Dicer (1:500 dilution; Abcam, Cambridge, MA, USA; Product No. ab14601) or mouse anti-β-actin (1:500 dilution; Santa Cruz Biotechnology Inc.; Santa Cruz, CA, USA). Primary antibodies were detected using horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5000; Santa Cruz Biotechnology, Inc.) and visualized by an enhanced chemiluminescence kit (Pierce; Rockford, IL, USA). Protein bands were quantified following scanning by Quantity One software (Bio-Rad; Hercules, CA, USA).

**Cell proliferation assay.** The cell proliferation assay was performed using a BrdU enzyme-linked immunosorbant assay (ELISA) kit (Calbiochem; San Diego, CA, USA) according to the manufacturer's instructions. The absorbance at 450-595 nm was measured with an iMark microplate reader (Bio-Rad; Serial No. 10601).

**Cell viability assays.** Cells were seeded in triplicate into 96-well plates (2000 cells/well) and then incubated with 3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) for 4 h, followed by dissolution in dimethylsulfoxide (DMSO) for 10 min every 24 h for 5 days. Growth curves were generated by calculating the mean value of the optical density measurements at 570 nm using the iMark microplate reader (Bio-Rad).

**Cell cycle analysis.** Cells were harvested, trypsinized, washed with ice-cold phosphate-buffered saline (PBS) and fixed in 70% cold ethanol at 4°C overnight. Following centrifugation (1200 × g for 5 min), fixed cells were washed with PBS, and then resuspended in PBS containing 1 mg/ml RNase A for 30 min at 37°C. Subsequently, cells were incubated with 50 µg/ml propidium iodide for 30 min at 4°C. Cell cycle analysis was performed using a LSR flow cytometer (Becton Dickinson; San Jose, CA, USA) with ModFit LT software (Verity Software House; Topsham, ME, USA).

**Cell migration assay.** The cell migration assay was performed using a Boyden chamber. Cells (1×10⁵/well) were trypsinized, resuspended in serum-free RPMI-1640 medium and then added to the transwell inserts (6.5 mm diameter, 8 µm pore size, polycarbonate membrane; Corning Costar; Cambridge, MA, USA). RPMI-1640 medium (600 µl) with 10% FBS was added to the lower chamber beneath the insert membrane. The transwell chambers were then incubated for 24 h under culture conditions. Migrated cells on the lower surface of the membrane were fixed with 70% ethanol and stained with crystal violet, and were then counted in 10 randomly selected high-power fields (x400) under a microscope. The average value was used as parameter to evaluate the migration ability of the cells. All assays were performed in triplicate.

**Drug cytotoxicity assays.** Assessment of chemoresistance to cisplatin was determined by the MTT assays. Cells were seeded in triplicate at a density of 5000 cells/well in 96-well plates. Cells were treated with cisplatin at various concentrations ranging from 2.5-40 µg/ml for an additional 24 h. Subsequently, 20 µl of 5 mg/ml MTT was added to each well and, after 4 h, cells were dissolved in 150 µl of DMSO.
for 10 min. The absorbance at 570 nm was measured using wells without cells as blanks on an iMark microplate reader (Bio-Rad; Serial No. 10601). The percentage of cell survival at each dose was calculated as the absorbance ratio of treated to untreated cells. The 50% inhibitory concentration (IC$_{50}$) values were calculated by linear interpolation. Data shown are representative of three independent experiments.

Statistical analysis. Data were expressed as mean ± standard deviation. The statistical significance of differences were estimated by a two-tailed Student’s t-test or a one-way analysis of variance (ANOVA), as appropriate, using the Statistical Package for the Social Sciences (SPSS) software, version 13 (SPSS Inc.; Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Knockdown of Dicer by siRNA. To study the function of Dicer in ovarian cancer, transient Dicer-knockdown A2780 cells were generated using Dicer siRNA (siDicer). Untransfected A2780 cells and negative control siRNA (siNC)-transfected cells were used as controls. An 84.33% decrease in the level of Dicer mRNA was observed in siDicer-A2780 cells compared with untransfected A2780 cells by qPCR (P<0.001, Fig. 1A); whereas no significant difference was identified between siNC-A2780 and untransfected cells. The knockdown of Dicer was further confirmed by western blot analysis (Fig. 1B).

Downregulation of Dicer promotes cell proliferation in ovarian cancer cells. To investigate the effect of Dicer knockdown on cell growth and proliferation in cancer cells, MTT and BrdU assays were performed for siDicer-A2780 and SKOV3 cells. The MTT assay revealed that siDicer transfection significantly increased the cell viability of A2780 and SKOV3 cells 96 h post-transfection compared with siNC-transfected cells 96 h post-transfection (Fig. 1D).

To study whether the growth elevation upon Dicer depletion in ovarian cancer cells is associated with cell cycle regulation,
Cell cycle analysis was conducted by propidium iodide (PI) staining and flow cytometry. Dicer knockdown significantly increased the percentage of S and G2/M phase cells (P=0.002 and P=0.022, respectively), which was accompanied by a fall in the percentage of G0/G1 phase cells (P<0.001; Fig. 1E), suggesting that Dicer is a regulator of the cell cycle, impacting cell proliferation in ovarian cancer.

Knockdown of Dicer promotes ovarian cancer cell migration. Transwell migration assays for siDicer-transfected cells were subsequently performed. siDicer-A2780 and siDicer-SKOV3 cells exhibited an increased ability to migrate through an 8-µm pore size polycarbonate membrane compared with siNC-transfected cells (P<0.001 for both cells; Fig. 2). The results thus far suggest that downregulation of Dicer in ovarian cancer may be required for disease progression.

Downregulation of Dicer contributes to cisplatin resistance in ovarian cancer cells. To delineate the role of Dicer in drug resistance, we first compared the expression of Dicer in A2780 and cisplatin-resistant cells derived from these (A2780/DDP) by qPCR and western blot analysis. A marked downregulation of Dicer expression was observed at both the mRNA (57.3% decrease; P<0.01; Fig. 3A) and protein (52.6% decrease; P<0.001; Fig. 3B) level in A2780/DDP cells compared with parental A2780 cells.

To verify the effect of Dicer knockdown on cisplatin sensitivity, the cell viability was assessed by MTT assays following treatment with various concentrations of cisplatin. Cell survival following cisplatin treatment was significantly increased in Dicer small interfering RNA (siDicer)-A2780 cells compared with negative control siRNA (siNC)-A2780 cells.

Loss of EZH2 increased Dicer expression in ovarian cancer cell lines. To investigate whether EZH2 is involved in the regulation of Dicer expression in ovarian cancer, we analyzed the alteration in Dicer expression following EZH2 depletion mediated by shRNA (Fig. 4A) using qPCR and western blot
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analysis. qPCR revealed that the Dicer mRNA level increased by 55.7% in shEZH2-A2780 cells compared with NC (P<0.001; Fig. 4B) and a corresponding increase (56.0%) at the protein level was revealed by western blot analysis (P<0.001; Fig. 4C). This result suggests that EZH2 is involved in the regulation of Dicer expression.

Discussion

In the present study, reduced expression of Dicer in ovarian cancer was demonstrated to be associated with activated tumor cell proliferation, enhanced migration ability and increased cisplatin resistance. A number of studies have demonstrated similar effects of Dicer silencing in other types of cells. Dicer knockdown substantially increased the invasion ability of breast cancer cells (14) and the migratory capacity of human embryonic kidney (HEK) 293T cells in vitro (15). Additionally, for the first time, the role of Dicer in cisplatin resistance in ovarian cancer cells was investigated. Knockdown of Dicer in A2780 cells by siRNA was observed to promote cell cycle progression and to decrease sensitivity to cisplatin. A previous study had demonstrated that ablation of Dicer in the MCF-7 breast cancer cell line led to significant G1 arrest and increased sensitivity to cisplatin (20), suggesting that the role of Dicer in the regulation of the cell cycle and drug response is tumor type-specific. However, the effect on the invasion of Dicer silencing compared with that on cell survival and cisplatin resistance was observed to be more significant, suggesting that there are other factors besides Dicer affecting these pathways.

Although there are numerous studies concerned with Dicer, the regulation of its expression is poorly understood. Merritt et al measured the Dicer mRNA level in specimens of invasive epithelial ovarian cancer from 111 patients. Decreased Dicer expression was observed in 60% of cases. Mutational analysis in a subgroup of ovarian cancer specimens revealed rare missense mutations (2/37) in the Dicer gene, but its presence or absence was not correlated with the level of Dicer mRNA expression (9). Tokumaru et al demonstrated that let-7 miRNA inhibits the expression of Dicer, representing a negative feedback loop on overall miRNA production (21). Furthermore, Wiesen and Tomasi revealed that Dicer is post-transcriptionally regulated by cellular stresses and interferons (22). Previous studies demonstrated that EZH2 is upregulated in ovarian cancer and contributes to tumor progression and the development of cisplatin resistance in vitro and in vivo (13,23). To validate the regulation pathway and to explore the mechanism whereby EZH2 regulates Dicer expression requires further investigation.

In summary, we have demonstrated that loss of Dicer is capable of promoting cell proliferation, increasing cell migratory capacity and decreasing ovarian cancer sensi-

Figure 4. Loss of EZH2 increases Dicer expression in A2780 cells. (A) Western blot analysis shows downregulation of EZH2 protein in shEZH2-A2780 cells. (B) Quantitative real time-polymerase chain reaction (qRT-PCR). Dicer mRNA expression is elevated in short hairpin (sh) EZH2-A2780 cells compared with untransfected A2780 cells and shVector-A2780 cells. All data are representative of three independent experiments and normalized to β-actin. (C) Dicer protein is overexpressed in shEZH2-A2780 cells compared with untransfected A2780 and shVector-A2780 cells. β-actin, internal control. Bottom: representative western blot analysis of Dicer; top: histogram of quantified results of western blot analysis. All data are representative of three independent experiments.
tivity to cisplatin. Furthermore, for the first time, we provide evidence that implicates EZH2 in the regulation of Dicer expression. Further investigation into the function of Dicer in carcinogenesis and its regulation pathways in human ovarian cancer tissue, additional cell lines and animal models will promote our exploitation of novel anti-cancer targets.

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