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Epigenetic editing of *BRCA1* promoter increases cisplatin and olaparib sensitivity of ovarian cancer cells

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ABSTRACT

Drug resistance is the primary contributor to the high mortality rate of ovarian cancer (OC). The loss of *BRCA1*/2 function is linked to drug sensitivity in OC cells. The aim of this study is to enhance the drug sensitivity of OC cells by inducing *BRCA1* dysfunction through promoter epigenetic editing. Epigenetic regulatory regions within the *BRCA1* promoter, affecting gene expression, were initially discerned through analysis of clinical samples. Subsequently, we designed and rigorously validated epigenetic editing tools. Ultimately, we evaluated the cisplatin and olaparib sensitivity of the OC cells after editing. The *BRCA1* promoter contains two CpG-rich regions, with methylation of the region covering the transcription start site (TSS) strongly correlating with transcription and influencing OC development, prognosis, and homologous recombination (HR) defects. Targeting this region in OC cells using our designed epigenetic editing tools led to substantial and persistent DNA methylation changes, accompanied by significant reductions in H3K27ac histone modifications. This resulted in a notable suppression of *BRCA1* expression and a decrease in HR repair capacity. Consequently, edited OC cells exhibited heightened sensitivity to cisplatin and olaparib, leading to increased apoptosis rates. Epigenetic inactivation of the *BRCA1* promoter can enhance cisplatin and olaparib sensitivity of OC cells through a reduction in HR repair capacity, indicating the potential utility of epigenetic editing technology in sensitization therapy for OC.

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Introduction

The annual increase in ovarian cancer-related fatalities presents significant challenges in prevention and treatment. In 2022, ovarian cancer (OC) ranked as the fifth leading cause of cancer-related death among women in the United States [1]. In China, in 2015, there were approximately 52,100 new cases of OC along with 22,500 fatalities, making it the seventh leading cause of cancer-related death among Chinese women [2]. The most common histological subtype is high-grade serous ovarian cancer (HGSOC), initially responsive to platinum-based therapy [3]. However, up to 75% of patients experience disease recurrence after remission, leading to platinum resistance. The survival rates for HGSOC have exhibited minimal alteration over the span of several decades [3]. The 5-year relative survival rates for patients diagnosed between 2007 and 2013 were 42% and 26%, respectively [4]. Progress in OC treatment has been limited, emphasizing the urgency of addressing drug resistance through innovative sensitization and pharmaceutical approaches.

Platinum-based chemotherapy is the gold standard for first-line treatment of OC [5]. Drug resistance that occurs during chemotherapy may be due to genetic and epigenetic changes, assisting cancer cells in adapting to chemotherapy through various pathways such as stress, DNA damage, and apoptosis [6]. Platinum-based chemotherapeutics trigger DNA interstrand cross-linking (ICL), inhibit transcription and replication, and promote cell cycle arrest and cell death through apoptosis. Activation of the DNA damage response (DDR)

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is crucial for tumour cell survival and the eventual development of platinum resistance [7]. Platinum-induced ICLs can further progress to DNA double-strand breaks (DSBs), necessitating coordinated engagement of pathways like nucleotide excision repair (NER) and homologous recombination (HR) repair. While NER is essential for the removal of ICLs in quiescent cells, repair of ICLs in replicating cells relies on the HR pathway [8]. Therefore, HR repair in tumours is closely associated with treatment outcomes. Currently, it is known that genetic mutations in 22 genes can lead to HR deficiency (HRD), including *BRCA1/2*, *ATM*, and *CDK12* [9]. The most common causes of HRD are germline *BRCA* mutations or somatic *BRCA* mutations [10]. Moreover, abnormal epigenetic processes, such as DNA methylation and histone modifications, also play an important role in the initiation and progression of platinum resistance in OC [11,12]. DNA methyltransferases (DNMTs) are reported to be highly expressed in OC and are speculated to be associated with platinum resistance. Hypomethylating agents (HMAs) can resensitize ovarian tumours to platinum-based drugs and enhance clinical outcomes [13–16]. Changes in DNMTs expression can lead to widespread alterations in the epigenome, including transcriptional inactivation of tumour suppressor genes (TSGs) [14]. Numerous TSGs in OC exhibit high methylation and epigenetic silencing, including the hypermethylation of the *BRCA1* promoter in 10–15% of OC tissues, which is associated with drug sensitivity [16,17].

Site-specific gene editing technology presents a promising avenue for ovarian cancer (OC) treatment, offering the potential to combat drug resistance and enhance patient outcomes [18]. These innovative approaches encompass gene-specific knockouts and the regulation of gene expression [18]. Notably, several studies have shown the potential of these approaches: CRISPR-Cas9-mediated knockdown of *ABCB1* reversed doxorubicin resistance in OC cells [19], BRCA1 knockout synergistically suppressed *poly(ADP-ribose) polymerase (PARP)-1* expression in a mouse OC cell line (ID8), increasing sensitivity to cisplatin and PARP inhibitors [20,21]. Moreover, the stabilization of Snail, knockout of *USP1*, or the reduction of *USP1* expression enhanced the sensitivity of tumour cells to chemotherapeutic agents [22]. However, the lack of safe and effective delivery systems and off-target effects limit the further application of gene editing technologies [23]. CRISPR-dCas9-based epigenetic editing may serve as an alternative approach to apply to sensitize chemotherapy for OC [18]. Epigenetic editing offers a safer method for OC treatment, as it possesses three advantages not typically found in traditional gene editing: 1) independence from DNA repair pathways, high efficiency, and minimal side effects; 2) reversibility, in contrast to the essentially irreversible nature of gene editing; and 3) risk of off-target effects compared to traditional gene editing [24]. Presently, epigenetic editing for chemotherapy sensitization encounters several challenges, encompassing a lack of adequate epigenetic editing tools, difficulties of *in vivo* targeted delivery, and the need to explore chemo-sensitivity epigenetic regulatory sites [24].

In this investigation, we identified promoter regions responsible for epigenetically regulating *BRCA1* expression and devised corresponding editing methods using the epigenetic editor CRISPRoff [24]. After validating the editing effects, we found that epigenetic inactivation of *BRCA1* in OC cells significantly enhanced their sensitivity to platinum and olaparib drugs

Materials and methods

DNA methylation analysis of OC samples in the dataset

The analysis of DNA methylation within the *BRCA1* promoter and gene expression data in OC tumour samples utilized publicly available datasets from The Cancer Genome Atlas (TCGA). Correlation analysis between *BRCA1* promoter methylation and gene expression in 398 TCGA Ovarian Serous Cystadenocarcinoma samples was conducted via cBioPortal (https://www.cbioportal.org/). The promoter region was defined as the region spanning 2000 bp upstream to 1000 bp downstream of the transcription start site (TSS). Data encompassing DNA methylation, SNP Array information, and survival data from 602 OC samples were

obtained from TCGA (https://portal.gdc.cancer.gov). Survival rates associated with DNA methylation were estimated using the Kaplan-Meier (K-M) method, employing the survminer v0.4.9 R package. This package utilized the log-rank test for comparing survival times, with significance set at $p < 0.05$.

Plasmids construction

The Plasmid CRISPRoffv2.1 was acquired from Addgene (Addgene #167981). The sgRNA plasmids were generated by employing restriction cloning of protospacers downstream of a U6 promoter using the BsmBI cut site within the pLentiGuide-puro plasmid. It is noteworthy that the CRISPRoff plasmid also expresses a blue fluorescent protein (BFP) marker to facilitate the measurement of transfection efficiency. The selection of sgRNA sequences was guided by our previously established algorithm designed to predict active CRISPRi sgRNAs [25]. The sgRNA sequences targeted the *BRCA1* promoter were presented as follows: 5'-TGAAGGCCTCCTGAGCGCAG-3' targeting chromosome 17 coordinates at position 41,277,324 and 5'-TTACCCAGAGCAGAGGGTGA-3' targeting chromosome 17 coordinates at position 41,277,307.

Cell culture and transfections

Four human cell lines, 293T, A2780, OVCAR3, and SKOV3, were cultivated. The 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM), while the OC cell lines A2780, OVCAR3, and SKOV3 were cultured in RPMI-1640 medium. These cell cultures were supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) antibiotics. The incubation conditions involved maintaining the cells at 37°C in an environment with 5% CO_2 to ensure proper humidification. Transfections experiments in 293T, A2780, OVCAR3, and SKOV3 were carried out when the cells reached 70%–80% confluence, using 300 ng of CRISPRoff plasmid and 150 ng of sgRNA-encoding plasmids combined with Lipofectamine 3000 (Thermo Fisher Scientific, USA) in Opti-MEM medium in 24-well plates.

Clinical sample collection

For validation analysis, a collection of 81 OC formalin-fixed paraffin-embedded (FFPE) samples were obtained from the Xiangya Hospital, School of Basic Medical Sciences, Central South University (Changsha, China) between January 2017 and December 2020. The patients had an average age at diagnosis of 50.6 ± 11.0 years. Among these, we obtained 46 high-grade serous ovarian carcinoma (HGSOC) samples, along with matched peripheral blood samples for HRD analysis. Ethical approval for our study was granted by the local committee, and all participants provided written informed consent.

Calculation of HRD score and BRCA1/2 mutation analysis

Genomic scar signatures were employed to calculate the HRD score using an R program, as previously described [26]. This calculation encompassed determining the Telomeric Allelic Imbalance (TAI), Large-Scale Transition (LST), and Loss of Heterozygosity (LOH) scores. TAI represented regions of allelic imbalance extending to the telomeres, LOH denoted chromosomal LOH regions longer than 15 Mb, and LST referred to breakpoints between regions longer than 10 Mb, after filtering out regions shorter than 3 Mb. The HRD score was computed as the sum of the TAI, LST, and LOH scores. In the TCGA cohort, HRD scores were calculated from SNP microarray data (Affymetrix GenomeWideSNP6 array). Both tumour and corresponding normal tissue was analysed. The same analytical process used for next-generation sequencing (NGS) was applied to analyse clinical tumour samples. The 3DMed Onco GPS Tissue NGS kit (3DMed, China) targeting 733 tumour-associated genes, including *BRCA1/2*, was used. Target genes were captured using probes and then sequenced on the HiSeq platform (Illumina, Inc., USA). *BRCA1/2* germline mutations were also assessed using this gene panel. Briefly, raw sequencing data were aligned to the reference human genome (Feb. 2009, GRCh37/hg19) using BWA software (version 0.5.9) and SAMtools (version 0.1.18). Genome Analysis Toolkit (version 3.5) was used for variant calling, employing parameters such as UnifiedGenotyper, HaplotypeCaller, and VariantFiltration. Subsequently, ANNOVAR was applied for variant annotation. The interpretation

of variants adhered to the American College of Medical Genetics and Genomics (ACMG) guidelines. Pathogenic or likely pathogenic variants encompassed frameshift insertions or deletions (indels), nonsense mutations, uncorrected splice-site variants, large-scale deletions, and missense mutations with impaired protein function, as determined by functional assays. Only pathogenic or likely pathogenic variants were classified as *BRCA1/2* mutations.

Bisulfite amplicon sequencing (BSAS)

DNA from cell line samples and FFPE samples was extracted using the High Pure PCR Template Preparation Kit (Roche, USA) and GeneRead DNA FFPE Kit (QIAGEN, Germany), respectively. DNA methylation levels of candidate genes were assessed via bisulphite amplification and next-generation sequencing (NGS). Specifically, 10 ng of genomic DNA underwent bisulphite conversion with the EZ DNA Methylation-Lightning Kit (Zymo Research, USA). Subsequently, we performed PCR amplification of the target sequences separately and constructed the second-generation sequencing libraries with the TaKaRa EX Taq Hot Start Version Kit (TaKaRa, China). The primers employed for amplification were as follows: *BRCA1* forward: 5'-GGAAAGGGATAGGGGGTTTAAGTGATGTT-3,'*BRCA1* reverse: 5'-AATTCACAARGCCTTA-RGCCTCTCAAATTC-3.' The library was 2 × 150 bp paired-end sequenced using the NGS HiSeq platform (Illumina, Inc., USA). The quality of the sequencing data was verified using the FastQC package. Adapter removal and quality trimming were performed using Cutadapt v1.9.1, the reads were aligned to a reference genome (UCSC hg19) using Bismark. Methylation levels of individual cytosines were determined through the utilization of the R package MethylKit v1.26.0.

Quantitative real time polymerase chain reactions

Total RNA was extracted from cultured cells and tissue samples using TRIzol Reagent (SIGMA, USA) according to the manufacturer's instructions. Reverse transcription was performed with a mix of oligo dT primer and random primer for mRNA and with stem-loop primer for miRNA using the PrimeScript^{RT} Reagent Kit (TaKaRa, China). qPCR was performed with a standard three-step amplification protocol of SYBR Premix Ex Taq^{TM} (TaKaRa, China). qPCR reactions were performed by using CFX96 Real-Time PCR detector (Bio-Rad, USA) and SYBR Green PCR Mix (TaKaRa, China). And the comparative Cq method was used to calculate the fold change. Amplification conditions were 60 min at 60°C, then 5 min at 95°C, 15 s at 95°C, and 1 min at 60°C, 45 PCR cycles. *BRCA1* primer (forward, 5'-ACAGCTGTGTGGTGCTTCTGTG-3," reverse 5"-CATTGTCCTCTGTCCA-GGCATC-3"), *GAPDH* primer (forward 5"-TCATTGACCTCAACTACATGGTTT-3," reverse 5"- GAAGATGGTGATGGGGATTTC-3').

Chromatin immunoprecipitation (ChIP)-qPCR

A2780 cells were briefly chilled in 125 mM glycine at room temperature for 5 minutes following fixation in 1% formaldehyde for 10 minutes. After fixation, the cells were subjected to centrifugation at 1,000 g for 10 minutes at 4°C and washed thrice with PBS. Subsequently, cell pellets were resuspended in 10 mL of ChIP lysis buffer (comprising 10 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Igepal, and protease inhibitors) and were incubated at 4°C for 60 minutes on a rotary shaker. The nuclei were then isolated by centrifugation (10 minutes, 4°C, 1,000 g), reconstituted in SDS lysis buffer (containing 1% SDS, 10 mM EDTA, and 50 mM Tris-HCl at pH 8.0), and incubated on ice for 30 minutes. SDS was diluted by adding 1× TE, and sonication was performed using a Covaris M220 (Covaris, Inc., USA). The efficiency of sonication was assessed via agarose gel electrophoresis. Subsequently, ChIP assays were conducted at 4°C for 16 hours using 2 μg of H3K27ac antibody (A7253, ABclonal, China). Immune complexes were captured with 20 μl of magnetic protein A/G beads (Thermo Fisher Scientific, USA) for 2 hours at 4°C. The beads underwent two washes with RIPA, three washes with ChIP wash buffer (containing 100 mM Tris pH 8, 500 mM LiCl, and 1% deoxycholic acid), and one wash with ChIP wash buffer supplemented with 150 mM NaCl. ChIP complexes were eluted in 100 μl of ChIP elution buffer (comprising 50 mM $NaHCO_3$ and 1% SDS) for 30 minutes, and cross-links were reversed in the presence of 0.5 M NaCl overnight at 65°C. ChIP DNA was purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). Quantitative

PCR was performed on ChIP and input control samples using the SYBR Premix Ex TaqTM (TaKaRa, China) and the CFX96 Real-Time PCR detector (Bio-Rad, USA) with SYBR Green PCR Mix (TaKaRa, China). The ChIP amplification primers targeting the *BRCA1* promoter are as follows: 5'-CGTATTCTGAGAGGCTGCTG-3' and 5'-GGAAGTCTCAGCGAGCTCAC-3.' ChIP enrichment was determined relative to input samples using the dCq method (dCq = Cq[ChIP] - Cq[input]).

Cell proliferation assay

Twenty-four hours prior to transfection, A2780 cells were seeded in 6-well plates and allowed to reach a confluence of 70%-90%. Transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific, USA) with a co-transfection of methylation editing plasmid pLentiGuide-puro-BRCA1 and CRISPRoff-v2.1 (500 ng each per well). Plasmids without editing effects were used as controls. Twenty-four hours after transfection, cells were collected and seeded at a density of 2×10^4 cells per well in a 96-well plate. After 24 hours of plating, different concentrations of cisplatin (DDP) (0.75 μg/ml and 1 μg/ml)(SIGMA, USA) and olaparib (10 μM and 20 μM) (MedChemExpress, USA) were added for drug sensitivity experiments. Cell Counting Kit-8 (DOJINDO, China) was added at days 0, 1, 3, 4, and 7 post-drug administration. Optical density at 450 nm (OD450) was measured after a 2-hour incubation period to analyse cell proliferation.

Detection of cell apoptosis

After trypsin digestion of adherent cells without EDTA, cells were collected by centrifugation at 300 × g for 5 minutes at 4°C. Apoptosis rates were measured by Cell Cycle and Apoptosis Analysis Kit (Yeasen, China). Specifically, cells were washed twice with pre-cooled PBS. Approximately $1–5 \times 10^5$ cells were collected. PBS was aspirated, and cells were resuspended in 100 μL of 1× Binding Buffer. Five microlitres of Annexin V-FITC and 10 μL of PI Staining Solution were added and gently mixed. The mixture was incubated in the dark at room temperature for 10–15 minutes. After adding 400 μL of 1× Binding Buffer and thorough mixing, the sample was placed on ice and analysed using a flow cytometer within 1 hour.

Homologous recombination (HR) assay

To assess HR capacity in A2780 and SKOV3 cells, a dual-immunofluorescence staining of γH2AX and RAD51 was conducted. Cells were transfected with pLentiGuide-puro-BRCA1 and CRISPRoffv2.1 or control plasmids. Following a 5-day incubation, cells were exposed to 10 μM DDP (SIGMA, USA) for 24 hours. Subsequently, the cells were subjected to nuclear extraction with buffer containing 20 mM HEPES (pH 7.4), 20 mM NaCl, 5 mM MgCl2, 0.5% NP-40, 1 mM DTT, and protease inhibitors for 10 minutes on ice. Afterwards, they were rinsed with ice-cold PBS and fixed with 4% PFA for 10 minutes at room temperature. The cells were then blocked in an immunofluorescence (IF) blocking buffer composed of 10% goat serum, 0.5% NP-40, and 0.5% saponin in PBS for 30 minutes. Subsequently, they were incubated with primary antibodies: mouse anti-RAD51 (GeneTex, USA) and rabbit anti-γ-H2AX (Cell Signaling, USA) antibodies diluted in blocking buffer for 2 hours at room temperature. Following primary antibody incubation, the cells were washed with PBS (3× 5 minutes) and stained with fluorescent secondary antibodies: Goat anti-Rabbit IgG (H+L) Secondary Antibody, DyLight 488 (Thermo Fisher Scientific, USA) and Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (Thermo Fisher Scientific, USA) for 1 hour at room temperature. After the secondary antibody staining, cells were washed as previously described, mounted in ProLong Gold mounting media (Thermo Fisher Scientific, USA), and imaged using a Ti-E+A1R+STORM10–1 (Nikon, Japan). Data were collected from 100 cells in five to ten fields in two independent experiments.

Statistical analysis

Graphs and statistical tests were analysed using GraphPad Prism version 9.0 (GraphPad Software, USA). Non-parametric Mann-Whitney test or

unpaired t-test was utilized to compare the mean values of the control and tested groups, and $p < 0.05$ was considered significant.

Results

Analysis of DNA methylation characteristics in the BRCA1 gene promoter in OC

BRCA1 is situated at 17q21.31, with a TSS positioned at 41,277,500 (Hg19) in the reverse orientation. Adhering to the promoter's definition, we selected a DNA segment ranging from 2,000 bp upstream to 1,000 bp downstream of the TSS (41,276,500–41,279,500) for scrutiny. Within this promoter region, there are three CpG islands, located at 41,279,075–41,277,986, 41,277,694–41,277,244, and 41277,206–41,276,698. The latter two CpG islands are contiguous, forming a region encompassing the TSS, exon 1, and part of intron 1. This region, along with the first CpG island, constitutes two CG-dense regions, in which there are eight CG dinucleotides with methylation data in the TCGA OC database (Figure 1a). Analysis of methylation levels of these CGs and their correlation with gene expression in the TCGA OC datasets unveiled that six CGs proximal to the TSS were predominantly hypomethylated, with a minority exhibiting hypermethylation in tumour tissues. In contrast, DNA methylation levels of the two CGs within the CpG island, 1000 base pairs upstream of the TSS, were generally high, with a few samples displaying hypomethylation. Moreover, DNA methylation levels of six CGs in the region near the TSS exhibited a highly significant negative correlation with gene expression, while the correlation between methylation and expression of two CGs upstream of the TSS was negligible or weak (Figure 1b).

We collected OC tissue samples and conducted a comprehensive analysis of DNA methylation levels in the two regions of the *BRCA1* promoter. Employing the BSAS assay, we amplified and sequenced two segments within these regions located on chromosome 17: 41277,787–41,277,627 and 41,275,000–41,277,199, respectively (Figure 1a). The analysis revealed that DNA methylation levels of all eight CGs proximate to the TSS were less than 1% in normal ovarian tissues. Conversely, within OC tissues, certain samples exhibited elevated methylation levels, with an average methylation level ranging from 10% to 15%, thereby signifying a noteworthy disparity between these two groups (Figure 2a). Conversely, the eight CGs within the CpG island upstream of the TSS were hypermethylated both in normal ovarian tissues and OC tissues, with no significant difference between the two groups (Figure 2b). These results are consistent with TCGA sample results. It can be inferred that the methylation levels of CGs near the TSS are intricately linked to the expression level of the tumour suppressor gene *BRCA1*, and escalated methylation levels may be associated with the inactivation of the *BRCA1* promoter in OC. Furthermore, we examined *BRCA1* promoter methylation in clinical cases, dividing them into HRD-positive (HRD score ≥ 42) and HRD-negative (HRD score < 42) groups. HRD-positive samples showed significantly higher DNA methylation levels at eight CG sites near the TSS compared to HRD-negative samples (Figure 2c). In contrast, methylation levels of eight CG sites within the CpG islands upstream of the TSS were similar across different HRD score groups (Figure 2d). Notably, both in our OC samples and the TCGA OC samples, samples with hypermethylation (>20%) at promoter CG sites displayed higher HRD scores, with mean values approaching or exceeding those of samples harbouring mutations in the *BRCA1/2* genes (Figure 2e, g). Furthermore, a significant positive correlation between promoter CG site methylation levels and HRD scores was observed (Figure 2f, h). Survival data analysis of TCGA samples found that hypermethylation in the region near the TSS was associated with higher survival rate (Figure 2i). These results further suggest that DNA methylation levels in the region near the TSS are associated with homologous recombination defects and may affect survival time through differential response to treatment.

Design and validation of the epigenetic editor for the BRCA1 promoter

The epigenetic editing plasmid CRISPRoff (DNMT3A-DNMT3L-XTEN80-dCas9-HA-2×NLS-BFP-KRAB) serves to augment DNA methylation

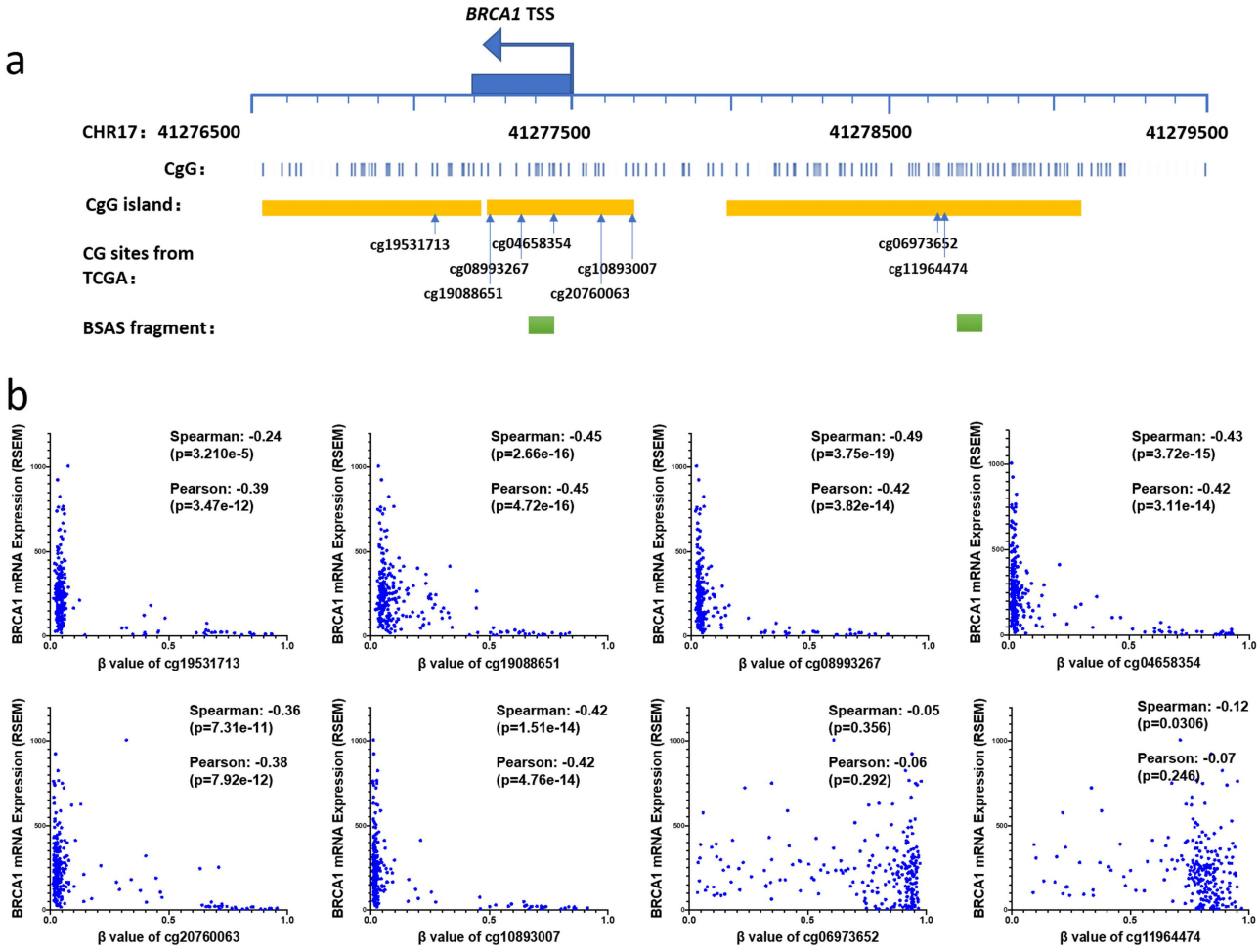

Figure 1. The relationship between methylation at CG sites within the *BRCA1* promoter and gene expression levels.

a. Schematic representation of the *BRCA1* promoter region. The first exon is depicted as a blue rectangle, while the direction of transcription is indicated by an arrow. The thin blue lines denote the CpG sites, and the orange rectangles represent the CpG islands. The green rectangles denote the regions subjected to bisulphite amplicon sequencing (BSAS). b. Correlation between methylation levels at 8 CG sites within *BRCA1* promoter and the expression level of *BRCA1*, utilizing data from The Cancer Genome Atlas (TCGA) ovarian cancer (OC) database. The x-axis displays the methylation levels of individual CG sites in the sample, and the y-axis represents the RNA-Seq by Expectation-Maximization (RSEM) value of the gene.

while diminishing histone modifications like H3K27ac, with the ultimate objective of repressing gene expression. Our approach involved the design of two sgRNAs (targeting chromosome 17 coordinates at positions 41,277,324 and 41,277,307, respectively) with a specific focus on the *BRCA1* TSS region, followed by the construction of the pLentiGuide-puro plasmids for precise site-specific epigenetic modification (Figure 3a). Co-transfection of CRISPRoff plasmid and pLentiGuide-puro plasmids was carried out in 293T, A2780, and OVCAR3 cells. Subsequently, we conducted a five-day observation of the fluorescent protein expression on the plasmids using fluorescence microscopy, aimed at assessing expression efficiency and stability. The results distinctly indicated the complete disappearance of fluorescent protein expression on the fifth day, signifying that the epigenetic editing plasmid exhibited transient expression within the cells.

We further examined changes in DNA methylation levels after editing. A significant escalation in DNA methylation levels was observed within a 400bp range upstream and downstream of the sgRNA binding site at 48 hours post transfection in OC cells A2780 and OVCAR3 cells as well as in 293T cells (Figure 3b-d). The elevation in DNA methylation is higher in A2780 and 293T cells, some CGs had increase of methylation more than 50%. However, most of the CGs in OVCAR3 cells had less increased methylation levels, between 5–

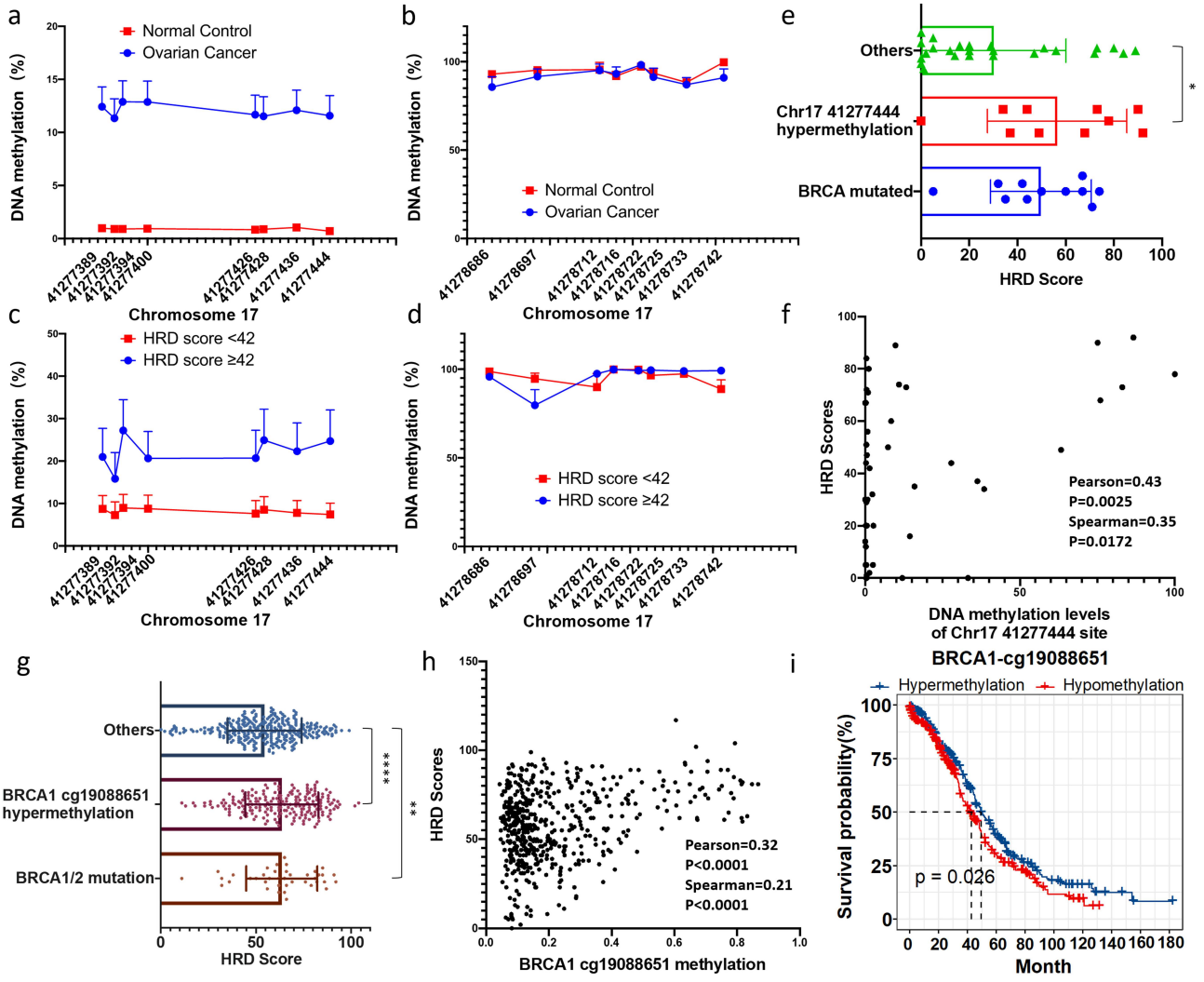

Figure 2. Methylation status of the *BRCA1* promoter and its implications for OC development and homologous recombination defects (HRD).

a, b, c, &d: *BRCA1* promoter methylation levels at CG sites near the transcription start site (TSS) in OC versus normal ovarian tissues (a) and in HRD-positive OC samples (HRD score ≥ 42) versus HRD-negative samples (HRD score < 42) (c); similarly, the methylation levels of CG sites upstream of the TSS in OC versus normal ovarian tissues (b) and in HRD-positive versus HRD-negative samples (d). e&f: Bar graph (e) and scatter plots (f) illustrating the relation between DNA methylation levels at the Chr17 41,277,444 site in *BRCA1* promoter, *BRCA1* mutations and HRD scores in OC samples. g&h: Bar graphs (g) and scatter plots (h) presenting the association between methylation levels of cg19088651, *BRCA1* mutations and HRD scores in TCGA OC samples. Statistical analysis utilized a two-tailed Student's t-test, with significance denoted as *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$. i: Kaplan-Meier survival analysis assessed the impact of hypermethylation (depicted in blue) and hypomethylation (depicted in red) of cg19088651 in TCGA OC samples, with survival rates reported in days post-surgery and significance set at $p < 0.05$.

20%. Notably, minimal changes in DNA methylation occurred at the sgRNA binding site itself. Continuous cultivation of edited cells unveiled a gradual reduction in DNA methylation; however, it is worth noting that in A2780 cells, the DNA methylation level within the TSS region remained significantly elevated compared to the control group even on the 28th day (Figure 3e), whereas 293T cells sustained heightened methylation for a duration of 14 days (Figure 3f). In addition, analysis of *BRCA1* gene expression in A2780 cells showed significantly lower mRNA levels than in unedited control cells from the second to the eighth day after editing (Figure 3g). Regarding the histone modification in A2780 cells, we found that the level of H3K27ac modification was

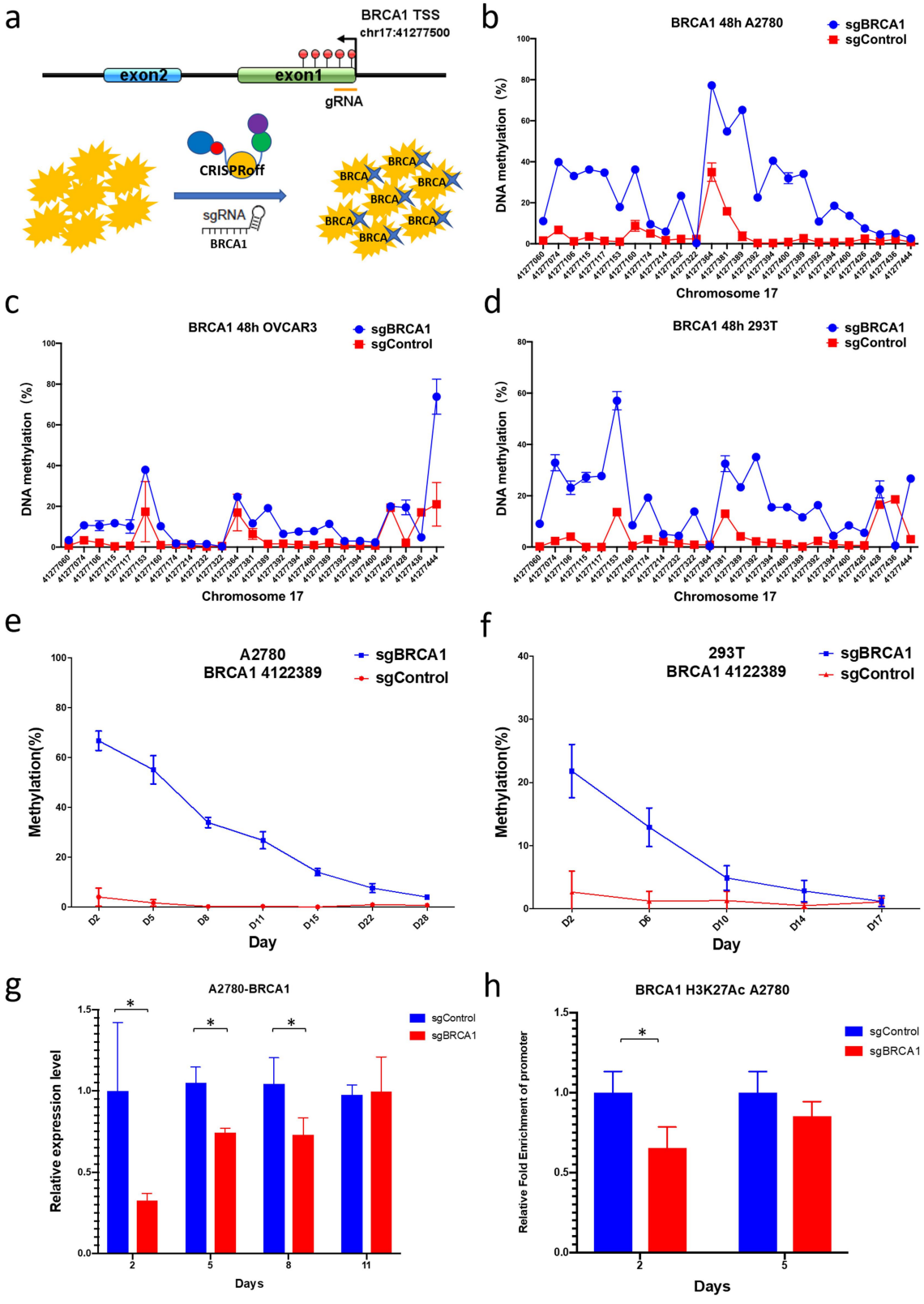

Figure 3. Design and validation of the epigenetic editor for the *BRCA1* promoter.

a: Schematic diagram depicting epigenetic editing strategy applied to the *BRCA1* promoter. b, c &d: DNA methylation alterations within a 400bp range around the sgRNA binding site 48 hours post-transfection in OC cells A2780 (b), OVCAR3 cells (c), and in 293T cells (d). The horizontal axis represents the chromosomal locations of the CG sites. e&f: Alterations in CG methylation levels at the *BRCA1* promoter region, chromosome 17, locus 4,122,389, tracked over increasing days of cell culture in A2780 (e) and 293 cell lines (f). g: *BRCA1* gene expression levels in A2780 cells post-epigenetic editing of promoter. The horizontal axis indicates the number of

significantly reduced compared to controls (Figure 3h). Collectively, these findings unequivocally substantiate our achievement of *BRCA1* expression suppression in ovarian cancer cells via epigenetic editing.

Epigenetic editing of the BRCA1 promoter affects HR repair capacity and increases drug sensitivity in OC cells

After confirming significant epigenetic modification changes and gene expression alterations in the *BRCA1* promoter region after editing, we examined the altered HR repair capacity of the cells and their altered sensitivity to platinum-based drugs and poly(ADP-ribose) polymerase inhibitors (PARPi). We first determined the appropriate concentrations of cisplatin and olaparib in A2780 cells and found that 1 μg/ml of cisplatin and 10 μM olaparib inhibited approximately about half of the cell growth after five days by cell proliferation assay, which was selected as the subsequent experimental drug concentration (data not shown). Cells were transfected with the *BRCA1* epigenetic editing plasmid for 5 days, 1 μg/ml of cisplatin was added, and HR capacity was determined by Dual-immunofluorescence staining of γH2AX/RAD51 after 1 day of treatment. Foci of γH2AX represent the location on the chromosome where the double-strand breaks have occurred, and the RAD51 foci represent ongoing HR repair. The ratio of the two co-localizations represents the HR repair capacity. The results showed that the RAD51 foci of *BRCA1* promoter-edited cells treated with drugs were significantly reduced in both OC cells, A2780 (Figure 4a) and SKOV3 (Figure 4b). Counting results showed that in A2780 cells, the mean number of RAD51 foci in edited A2780 cells was 15.8 compared to the mean number of 18.7 in unedited controls (Figure 4c), whereas in SKOV3 the mean number of foci in unedited and edited cells was 34.5 and 29.3, respectively (Figure 4d). These results suggest that epigenetic inactivation of the *BRCA1* promoter would reduce HR repair capacity. For drug sensitivity test, A2780 cells were treated with 1 μg/ml of cisplatin and 10 μM olaparib for five consecutive days 48 hours after epigenetic editing, and cell counts showed a significant reduction in cell proliferation after epigenetic editing of the *BRCA1* promoter (Figure 4e). The number of cells with promoter epigenetic editing was reduced by 32% on day five after cisplatin treatment compared to unedited cells, while it was reduced by 19% in the olaparib-treated group (Figure 4f). The above results indicate that the increased epigenetic modifications in the *BRCA1* promoter region that inhibit expression lead to a significant increase in cellular sensitivity to platinum-based and PARPi drugs. Interestingly in the control group without drug treatment, the number of cells in the sg*BRCA1* group was reduced by 14% on day 5, which was also significantly lower than in the control group (Figure 4f). We analysed the apoptosis of cells after drug treatment (Figure 4g), and the percentage of apoptosis was significantly higher in A2780 cells epigenetically edited for the *BRCA1* promoter under DDP treatment than in unedited cells (Figure 4h). Even in the absence of drug treatment, epigenetically edited cells still exhibited a higher percentage of apoptosis (Figure 4h). This result suggested that epigenetic editing of the *BRCA1* promoter may increase drug sensitivity by promoting apoptosis.

Discussion

Treatment resistance in OC is a challenging issue that significantly impacts patient prognosis. While mechanisms behind this resistance have been widely studied, effective strategies to reverse or sensitize drug-resistant tumours remain elusive. This investigation seeked to leverage epigenetic editing techniques to induce hypermethylation in the *BRCA1* promoter, thereby enhancing cellular

days in cell culture post-editing. h: Levels of H3K27Ac modification of the *BRCA1* promoter post-editing. The horizontal axis represents the number of days in cell culture following epigenetic editing. Statistical analyses were conducted using a two-tailed Student's t-test (*$p < 0.05$).

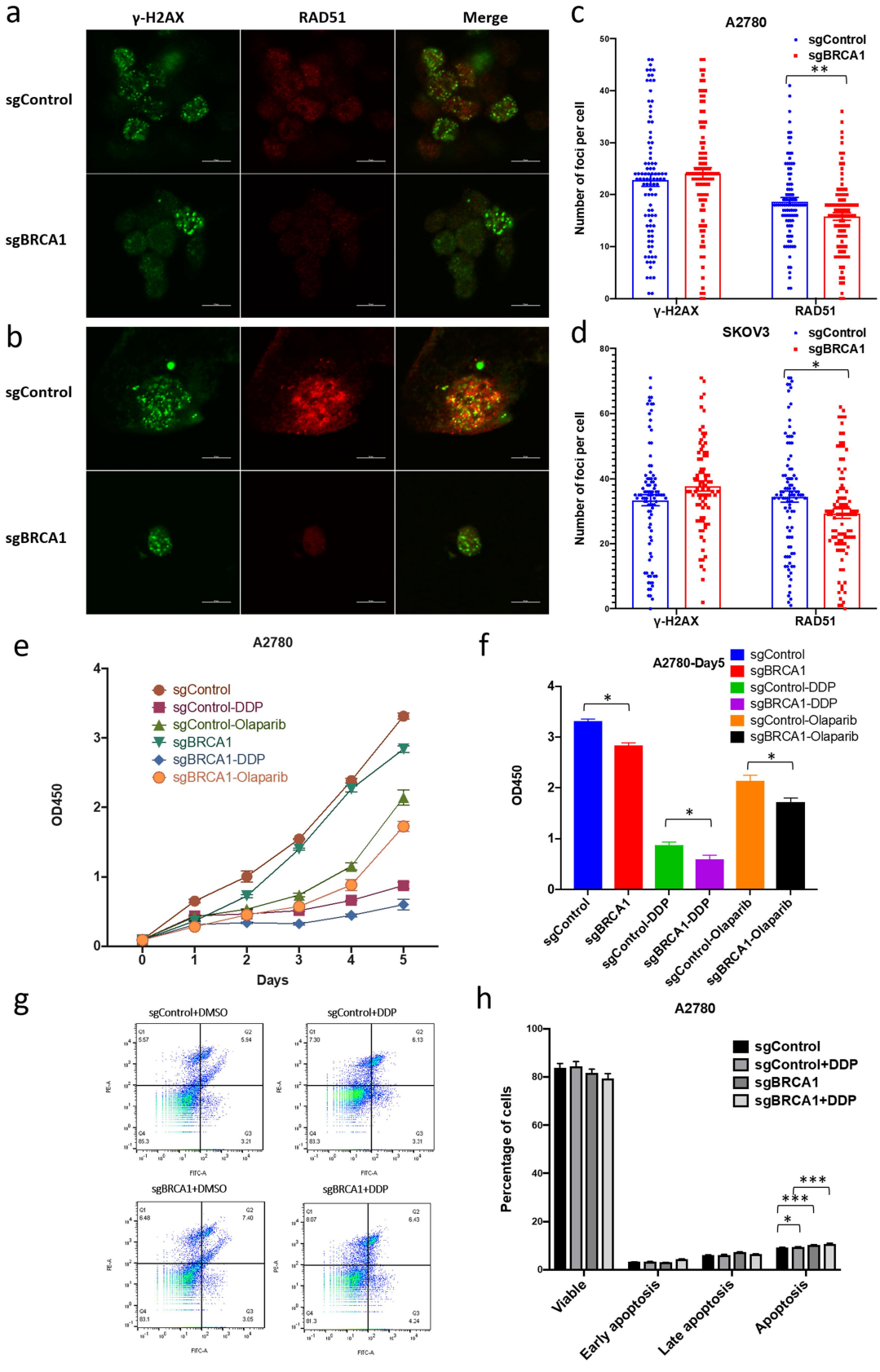

Figure 4. The impact of epigenetic editing of the *BRCA1* promoter on HR repair capacity and drug sensitivity in OC cells.

a&b: Dual immunofluorescence staining of RAD51/γH2AX in A2780 (a) or SKOV3 (b) cells exposed to 1 μg/ml DDP. Scale bars are indicated as 10 μm. c&d: Quantification of γH2AX and RAD51 foci in up to 100 cells of each groups in A2780 (c) or SKOV3 (d) cells. e: The proliferation curve of A2780 cells treated with 1 μg/ml of cisplatin or 10 μM olaparib over 5 days in culture, 48 hours post-

drug sensitivity. Through the analysis of databases and our clinical samples, we identified critical promoter regions influenced by DNA methylation that profoundly affect *BRCA1* transcription. We designed editing tools to increase DNA methylation in this region and remove H3K27ac modifications, resulting in sustained suppression of *BRCA1* expression. Consequently, edited cells reduced HR repair capacity, promoted apoptosis, and had significantly increased sensitivity to platinum and PARPi drugs. These findings validate the relationship between epigenetic modifications of the *BRCA1* promoter and platinum or PARPi resistance in OC, suggesting that targeted epigenetic regulation of *BRCA1* represents a viable strategy for sensitizing OC to treatment.

In addition to mutations, the inactivation of the *BRCA1* promoter leads to reduced *BRCA1* expression, correlating with increased risks of ovarian and breast cancers. Numerous studies have indicated that promoter methylation of *BRCA1* is a common occurrence in OC, particularly in HGSOC [27,28]. *BRCA1* functional loss, including promoter methylation, leads to HRD [29,30]. Approximately 50% of HGSOC exhibit HRD, representing a distinct subtype with significant clinical implications, particularly in terms of treatment. HRD OCs show increased sensitivity to platinum-based chemotherapy, making this therapeutic approach preferable. Additionally, novel targeted drugs, such as PARPi, have demonstrated marked cytotoxic effects on HRD tumour cells [31–34]. HR operates during the S and G2 phases of the cell cycle, relying on numerous proteins, including BRCA1 and BRCA2 [30,35]. When cells lack functional HR, often due to BRCA1 or BRCA2 deficiency, they rely on alternative repair pathways like non-homologous end-joining (NHEJ), which are less precise and prone to errors [36].

Clinical studies have found that OC with hypermethylation in the *BRCA1* promoter shares similar clinical and pathological features with OC harbouring *BRCA1*/2 gene mutations [27]. Although reported occurrence rates range from 5% to 89.9% [37,38], it is estimated that approximately 10%-15% of OC cases exhibit *BRCA1* promoter hypermethylation [10,39–41]. Additionally, *BRCA1*/2 germline mutations and somatic mutations can be observed in 15%-20% of OC cases [10,42]. *BRCA1*/2 mutations, along with *BRCA1* promoter hypermethylation, are associated with significantly higher response rates and extended progression-free survival in OC patients following platinum-based chemotherapy [10,43,44]. Analyses of *BRCA1* methylation in OC samples consistently demonstrate lower levels of BRCA1 protein and mRNA expression [40,45–47]. Cell line models of *BRCA1* methylation in OC exhibit specific sensitivity to platinum-based chemotherapy and PARP inhibitors [48]. Xenograft models of OC derived from cell lines and patients with *BRCA1* hypermethylation demonstrate significant sensitivity to platinum and/or PARP inhibitor therapy [48–50]. These reports align with our findings.

The contribution of hypermethylation of promoter and mutations in *BRCA1* to drug resistance differs. Several studies have shown that genotoxic drugs can lead to promoter demethylation, re-expression of *BRCA1*, and consequently, drug resistance. Exposure of *BRCA1*-highly methylated triple-negative breast cancer (TNBC) cells to platinum-based chemotherapy, whether as a clinical treatment or experimental *in vivo* exposure as patient-derived xenografts (PDX), results in promoter methylation loss, increased *BRCA1* expression, and platinum resistance [51]. Several studies have found that substances such as bisphenol A (BPA) and curcumin led to the re-expression of *BRCA1* in cancer cell lines with hypermethylation in the *BRCA1* promoter [52,53]. These results suggest that cancer cases with hypermethylation in the *BRCA1* promoter exhibit a high adaptability to environmental exposure. By reversing promoter

epigenetic editing, shown as OD450. f: Bar plot displaying OD450 after 5 days of drug treatment of A2780 cells, shown as means ± SEM. g&h: Apoptosis assay via flow cytometry, after staining with annexin V-FITC/propidium iodide (PI). A2780 cells were treated with 1ug/ml of cisplatin for 24 h post-*BRCA1* promoter epigenetic editing. Representative scatter plots of PI (y-axis) vs. annexin V (x-axis) are depicted (g), and the percentages of viable, early apoptotic, late apoptotic, and all apoptotic cells are presented (h). Data are shown as means ± SEM of triplicate experiments (*$p < 0.05$, **$p < 0.01$).

methylation level, *BRCA1* expression can be restored, leading to therapy resistance [51]. Therefore, in samples without *BRCA* mutations, the methylation status of this gene's promoter is a key factor in drug sensitivity. Novel approaches targeting epigenetic modifications of the *BRCA1* promoter may offer a new path to overcome resistance.

Epigenetic modifications are reversible and are carried out by various epigenetic modifying enzymes within cells. This makes it possible to regulate *BRCA1* expression through epigenetic editing. Developments in epigenetic editing techniques have revealed that simultaneous modification of DNA methylation and histone tail amino acids can produce sustained modification effects and effectively regulate gene expression. In OC, over half of the cases lack *BRCA* mutations and do not exhibit hypermethylation in the *BRCA* promoter. Epigenetic editing to induce promoter inactivation may benefit this subset of patients in chemotherapy. Additionally, some patients who have *BRCA1* reactivated during treatment may potentially benefit from re-closing *BRCA1* expression through epigenetic editing of the promoter. Our experimental results provide preliminary evidence that this strategy is feasible.

Certainly, our strategy requires validation in animal models. Furthermore, the method of *in vivo* drug administration presents another challenge, with potential approaches including viral vectors and non-viral lipid nanoparticles, among others.

Conclusion

In summary, we employed epigenetic editing to deactivate the *BRCA1* promoter, thereby enhancing the sensitivity of OC cells to cisplatin and olaparib. Initially, we identified crucial regions in the *BRCA1* promoter that are epigenetically controlled, both in TCGA ovarian cancer samples and our own clinical cases. Subsequently, we devised targeted epigenetic editing tools. Following the confirmation of the effectiveness of these edits in OC cells, we assessed HR repair capacity, apoptosis rates, and drug sensitivity. The results showed that inactivation of the *BRCA1* promoter through epigenetic editing can heighten cellular responsiveness to cisplatin and olaparib. It is imperative to undertake further *in vivo* experiments for validation.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author contributions

XN and QW designed the study. WH, HZ and QW collected the data from databases and performed analyses. WH, HZ and QW organized and arranged all the figures. WH, HZ and SZ performed the experiments and the formal analysis. GS, HL, MW and GY collected tissue samples. WH and QW prepared and wrote the original draft of the manuscript. XN reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

Ethics approval and consent to participate

The studies involving human participants were reviewed and approved by the Xiangya School of Medicine, Central South University (approval no. 2019030306). The patients/participants provided their written informed consent to participate in this study. The study was conducted in accordance with the Declaration of Helsinki.

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