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Clinical Cancer Research

Epigenetic Reprogramming Strategies to Reverse Global Loss of 5-Hydroxymethylcytosine, a Prognostic Factor for Poor Survival in High-grade Serous Ovarian Cancer

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Supplementary Materials and Methods

Cell Culture

Murine (4412, 4306) and human (A2780Sen, A2780Res, A2780Res+pTET2, OVSAHO, CaOV3, OVCAR4, OVCAR8, COV318, and SKOV3) cell lines were cultured in monolayer using either DMEM (4412, 4306, CaOV3, and OVCAR4), DMEM with 2mM L-Glutamine (COV318), or RPMI (A2780Sen, A2780Res, A2780Res+pTET2, OVSAHO, OVCAR8, and SKOV3), respectively, supplemented with 10% FBS (vol/vol) (Gemini Bio-Products) and 1% penicillin/streptomycin (vol/vol) (Invitrogen). RPMI medium for the A2780Res+pTET2 cell line was supplemented with puromycin for selection purposes. Cells being treated with drugs were grown in media without 1% penicillin/streptomycin.

Immunofluorescence Experiments

Tumor cell pellets collected from patient ascites samples were washed with PBS and tumor cells were grown on coverslips. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature with gentle rocking. Next, samples were permeabilized using 0.5% Triton-x in PBS for 30 min at room temperature followed by 3x PBS washes. Cells were then incubated in 1% BSA in 0.05% PBS-T for 1-2 hours with gentle rocking. Samples underwent primary antibody incubation diluted in 1% BSA – 0.05% PBS-T for 1-2 hours per manufacturer's instructions followed by 3x PBS wash. In a semi-dark room, samples were incubated in the corresponding secondary antibody (mouse or rabbit) dilution and rocked for 1 hour. Samples were then washed 3x with 0.05% PBS-T, mounted onto slides, and visualized.

Flow Cytometry

SP and NSP populations were isolated and analyzed as previously described[26]. ALDH+ cells were identified using Aldefluor reagent (Stem Cell Technologies) following manufacturer's instructions. LGR5 Clone OTI2A2 antibody (Origene) was incubated with cells at 1:100 for 40 min and attached to a fluorochrome secondary antibody (1:500) for 20 min. All percentages were determined based on 100,000 total events using the BD Aria Sorter.

RNA Extraction, cDNA Synthesis, and qRT-PCR Studies

Total RNA was extracted using the Qiagen RNeasy isolation kit following manufacturer's protocol, and up to 5µg was reverse-transcribed to cDNA using Superscript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR (qRT-PCR) was run using 100ng of template cDNA and the SYBR Green qPCR system (Qiagen) on the Applied Biosystems 7300 Real-Time PCR system. GAPDH was used as a loading control. Relative expression of TET for cells lines treated with 5-aza was measured using the $\Delta\Delta C_T$ method, with untreated cells acting as a control. qRT-PCR was run under the following conditions: 2 minutes at 50°C, denaturation for 10 minutes at 95°C, 40 cycles of amplification and elongation consisting of 15 seconds at 95 °C, 40 seconds at 60°C, and 15 seconds at 72°C. See table below for primer sequences:

Primer	Forward Sequence	Reverse Sequence
GAPDH	TGCACCACCACTGCTTAGC	GGCATGGACTGTGGTCATGAG
TET1	TCTGTTGTTGTGCCTCTGGA	GCCTTTAAAACTTTGGGCTTC
TET2	GAGACGCTGAGGAAATACGG	TGGTGCCATAAGAGTGGACA
TET3	CCCACAAGGACCAGCATAAC	CCATCTTGTACAGGGGGAGA

5-hmC Immunohistochemistry Tumor Staining

Deparaffinization occurred via three xylene washes for 10 minutes, which were followed by 6 washes in 100% alcohol for 2 minutes each. Sections were blocked with peroxide for 15 minutes then washed with running tap water for 10 minutes. For antigen retrieval DAKO Target retrieval solution (Citrate Buffer, pH 6) was used. The Biocare Decloaking Chamber, a pressure system for heat-induced epitope retrieval, was set up using the two programs SP1 at 123°C for 45 seconds and SP2 at 90°C for 10 seconds. Slides were loaded with Citrate buffer into the pressure cooker (Biocare Decloaking Chamber) and when the temperature reached 90°C, slides were removed, cooled, and washed with running water. Slides were rinsed with TBS/Tween20 buffer (TBST) followed by incubation with a 5-hmC primary antibody (Active Motif, 1:7500) for 40 minutes at room temperature. Samples were rinsed again with TBS/Tween20 for 10 minutes then incubated with Envision+ Mouse (Dako, Cat# 4007) for 30 minutes at room temperature followed by another TBST/Tween20 wash. Sections were incubated with DAB, washed with running water, and counterstained with hematoxylin. Finally, slides were dehydrated, cleared, and mounted.

Tumor Xenograft Studies

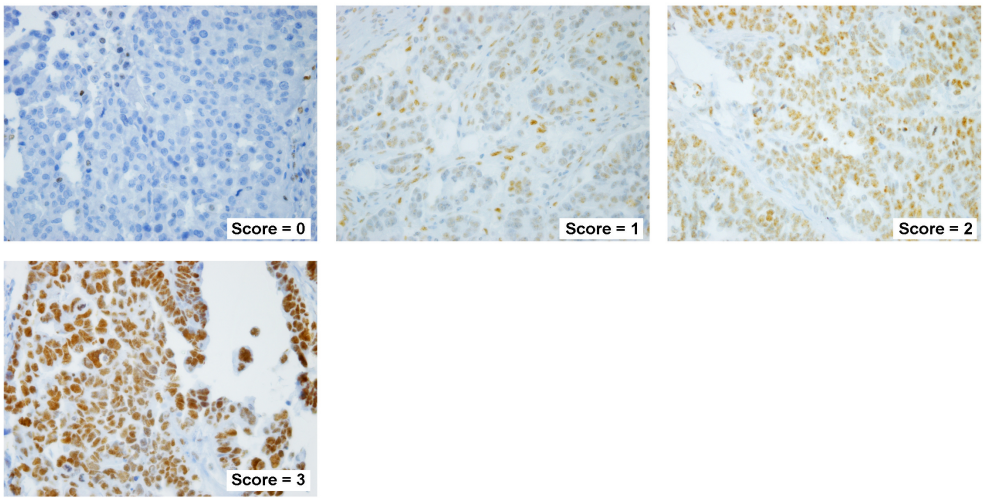
A2780Res tumor cells were transduced with a luciferin plasmid containing the mCherry fluorophore reporter. Cells were sorted using the BD FACSAria II cell sorter and grown in normal culture media. $1x10^6$ cells were resuspended in 100 µL PBS and injected into 6-8 week old female NCr nude mice (Taconic Biosciences). Treatments were initiated when substantial tumor growth was observed via luciferase-based imaging (IVIS Lumina II imaging system PerkinElmer, Inc.). For each treatment cycle, mice were pretreated twice with 5-aza (4 mg/kg) resuspended in PBS and injected intraperitoneally (i.p.) on subsequent days (days 1, 2) followed by CDDP therapy (3 mg/kg) on days 3 and 6 of each cycle. For each drug, the respective control groups were injected i.p. with 100 µl PBS. Murine tumor slides for 5-hmC IHC levels were scored using a composite (product) scoring method. Composite scores were the result of multiplying the cell number score (0-4) and staining intensity score (0-3). Cell number scores are as follows: 0 for <10% of tumor cells positively stained; 1 for 10-24% of tumor cells positively stained; 2 for 25-49% of tumor cells positively stained; 3 for 50-74% of tumor cells positively stained; 4 for >75% of tumor cells positively stained. A staining intensity score of 0 depicts no staining, 1 weak staining, 2 moderate staining, and 3 strong staining. Ki67 and cleaved Caspase 3 IHC slides were scored using the percentage of positive cells stained as shown above: 0 for <10% of tumor cells positively stained; 1 for 10-24% of tumor cells positively stained; 2 for 25-49% of tumor cells positively stained; 3 for 50-74% of tumor cells positively stained; 4 for >75% of tumor cells positively stained. All studies were carried out in agreement with the guidelines set by the Institutional Animal Care and Use Committees of the Brigham and Women's Hospital and Harvard Medical School.

Supplementary Figure 1

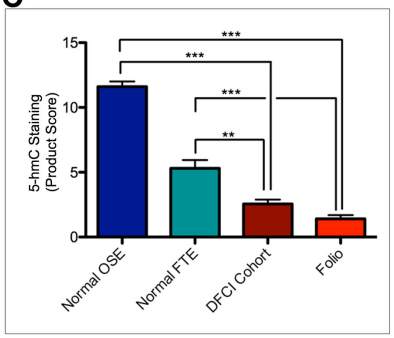
A

Cell Number Score	Number of Positive Cells
0	<1%
1	1-9%
2	10-24%
3	25-75%
4	>75%

B



C



Supplementary Fig. 1

(A, B) Scoring system used to assess composite scores in immunohistochemistry experiments. Composite (product) scores are the result of multiplying the cell number score (0-4) and staining intensity score (0-3). **(C)** Both DFCI HGSOC cases (n=136) and commercial Folio HGSOC (n=67) TMAs show statistically significant decreases in 5-hmC levels when compared to normal OSE (n=5) and FTE (n=5) (**p<0.01, ***p<0.001).

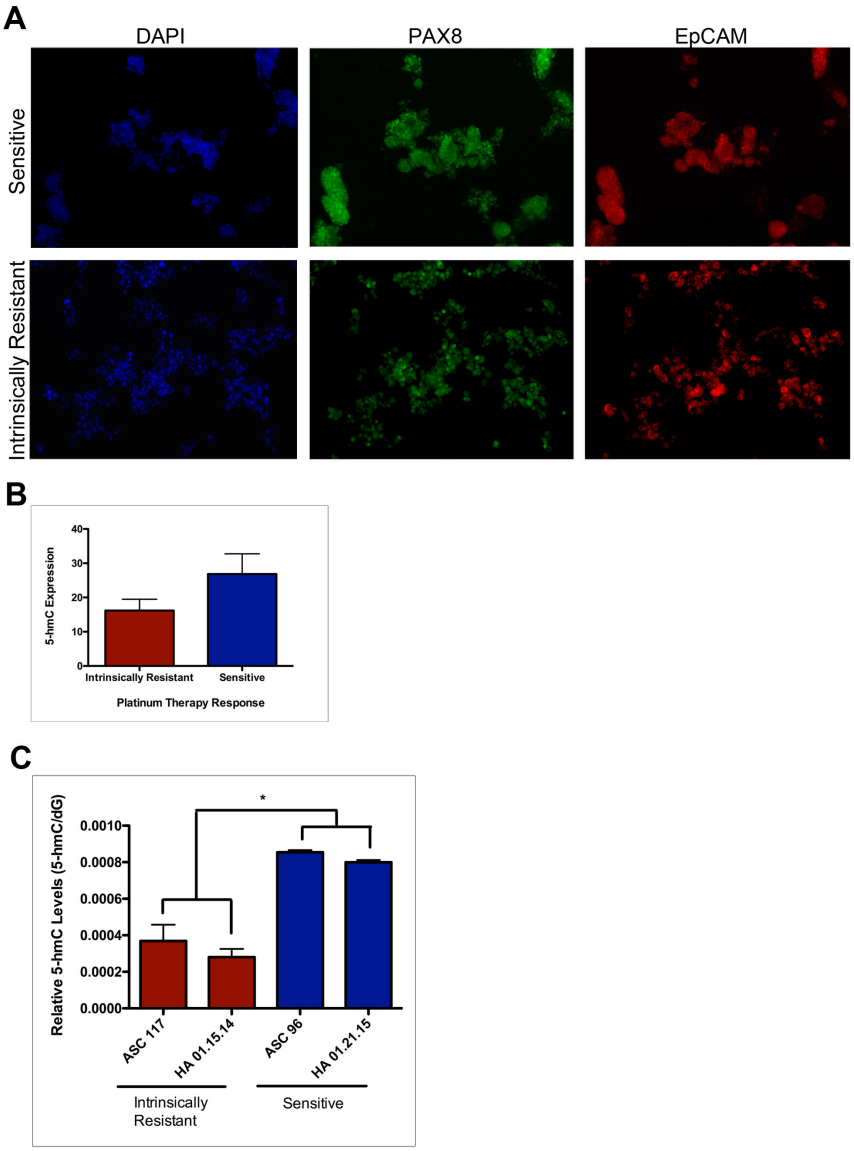
Supplementary Table 1

	Ovarian Cancer Cases (n=107)
Mean (SD)	
Age at Diagnosis (years)	60 (9.1)
N%	
Stage	
1	13 (12%)
2	8 (7%)
3	79 (74%)
4	7 (7%)
Grade	
1	2 (2%)
2	14 (13%)
3	91 (85%)
Debulking Status	
Optimal	72 (67%)
Suboptimal	9 (8%)
Unknown	26 (24%)

Supplementary Table 2

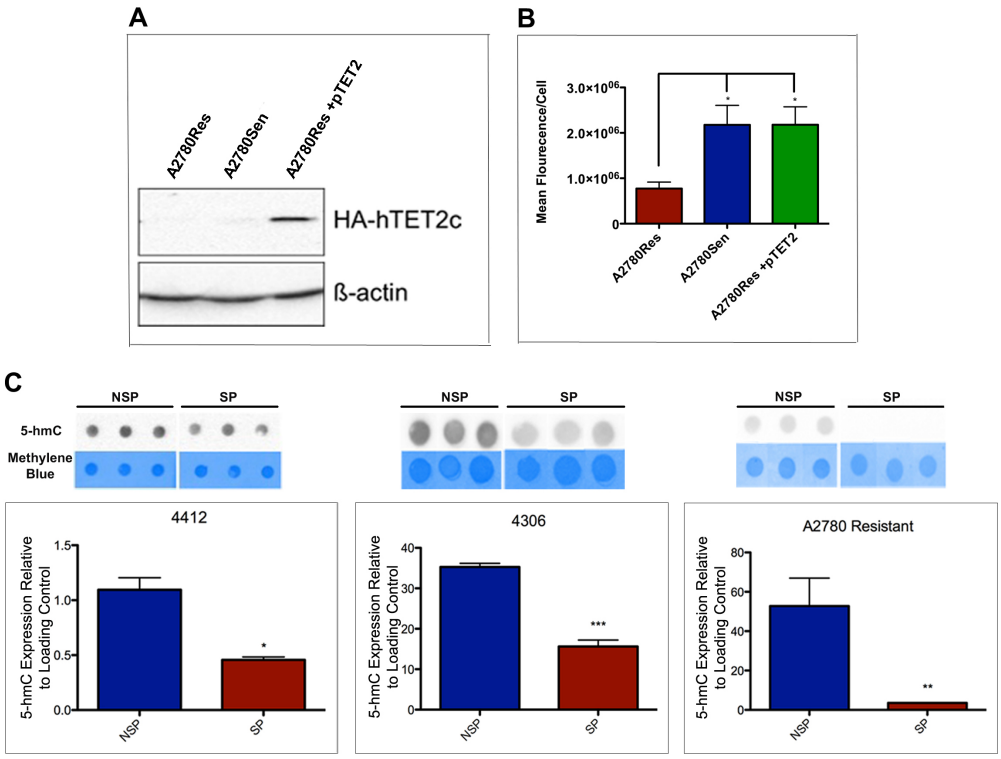
	Ovarian Cancer Cases (n=59)
Mean (SD)	
Age at Diagnosis (years)	60 (9.6)
N%	
Stage	
1	4 (7%)
2	6 (10%)
3	44 (75%)
4	5 (8%)
Grade	
1	0
2	8 (14%)
3	51 (86%)
Debulking Status	
Optimal	49 (83%)
Suboptimal	5 (8.5%)
Unknown	5 (8.5%)

Supplementary Figure 2



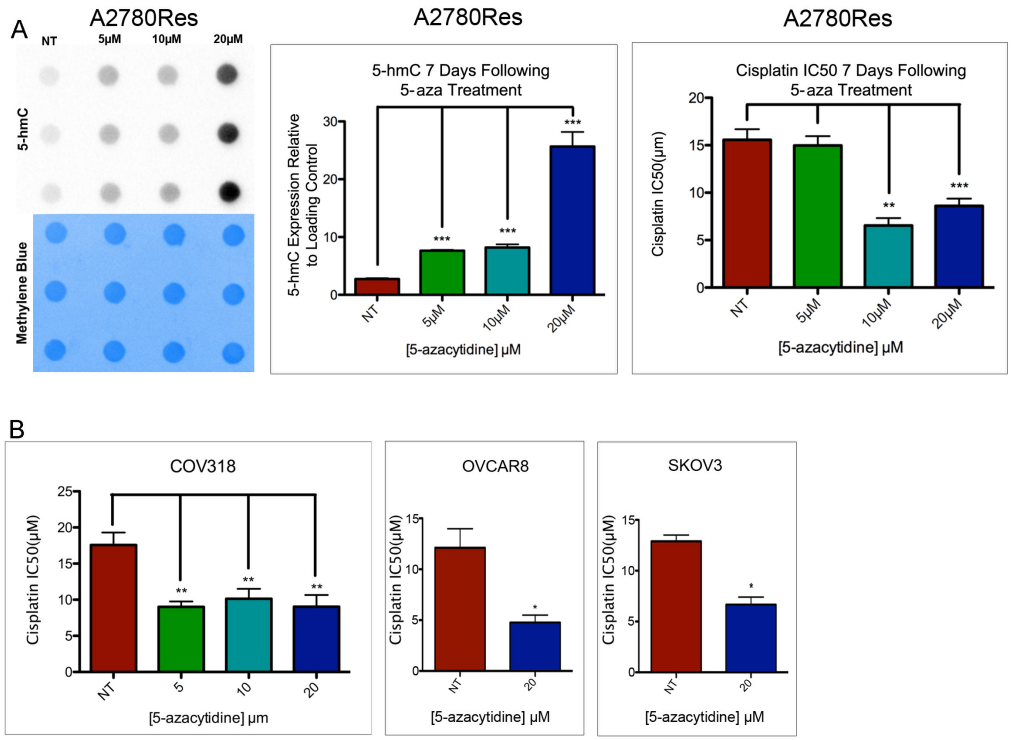
Supplementary Fig. 2
(A) Immunofluorescence (IF) studies in patient ovarian tumor ascites samples to validate the presence of tumor cells using PAX8 and EpCAM HGSOC markers. **(B)** Quantification of 5-hmC levels in patient ascites using a dot immunoblot assay. Tumor ascites were collected from newly diagnosed patients and deemed by IF to contain at least 50% tumor cells. Intrinsically platinum resistant patients (n=8) tended to have a lower mean 5-hmC score compared to platinum sensitive patients (n=4), although the difference did not reach statistical significance possibly due to the small number of samples analyzed. **(C)** Direct measurement of relative 5-hmC levels using mass spectrometry quantification for several platinum sensitive (ASC 96, HA 01.21.15) and intrinsic platinum resistant patient samples (ASC 117, HA 01.15.14), which were collected from ovarian tumor ascites and shown by IF to contain at least 50% tumor cells (*p<0.05).

Supplementary Figure 3



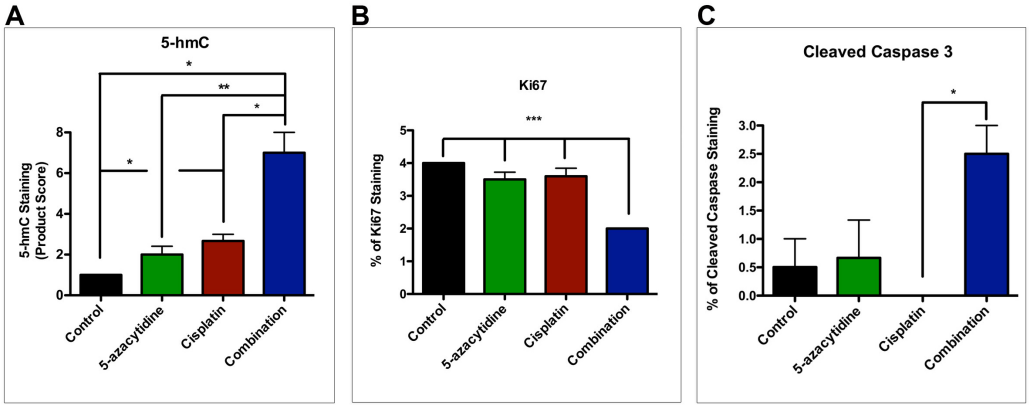
Supplementary Fig. 3
(A) Western blot analysis using an anti-HA-tag antibody validates TET2 overexpression in transfected A2780Res+pTET2 tumor cells. **(B)** Quantification of 5-hmC immunofluorescence. Both A27080 Sensitive and A2780 Restistant+pTET2 show a statistically significant increase (*p<0.05) in 5-hmC levels compared to A2780 Resistant as measured by mean fluorescence per cell (n=2). **(C)** 5-hmC immunoblot analysis of sorted SP compared to non-SP bulk tumor cells (NSP) in murine (4412, 4306) and human (A2780Res) ovarian cancer cell lines. Similar to somatic stem cells, cancer stem-like cells (SP) show statistically signifigant decreased 5-hmC levels when compared to NSP in murine lines (4412, *p<0.05; 4306, ***p<0.001), and human A2780Res tumor cells (**p<0.01).

Supplementary Figure 4



Supplementary Fig. 4
(A) Representative dot immunoblot analysis of 5-hmC levels in A2780Res tumor cells pretreated for 72 hours with various concentrations of 5-aza and then maintained in normal growth medium for 1 week. Methylene blue staining was used as a DNA loading control. Quantification of 5-hmC levels as assessed by immunoblot analysis indicates that all 5-aza pretreatments (5, 10, and 20 μM 5-aza, respectively) have a sustained effect. 5-hmC levels remain significantly higher (***p<0.001) than untreated (NT) A2780Res cells for at least 1 week following 5-aza pretreatment. Quantification of platinum IC50 values for A2780Res tumor cells, which were pretreated with 5-aza, maintained for 1 week in normal growth media, followed by exposure to CDDP. Tumor cells pretreated with 10 and 20 μM 5-aza maintained high 5-hmC levels and remained sensitive to platinum relative to untreated cells (**p<0.01, ***p<0.001) for at least 1 week, suggesting an optimal timing for platinum therapy following 5-aza pretreatment. **(B)** The platinum sensitivity of ovarian cancer cell line increases following pretreatment with 5-aza as shown by MTT analysis. COV318 (5, 10, and 20 μM 5-aza), OVCAR8 (20 μM 5-aza), and SKOV3 (20 μM 5-aza) tumor lines were pretreated for 72 hrs with 5-aza prior to CDDP therapy. The tested cell lines show increases in CDDP IC50 values (*p<0.05, **p<0.01).

Supplementary Figure 5



Supplementary Fig. 5
(A) Quantification of 5-hmC IHC levels in control, 5-aza, cisplatin (CDDP), and combination therapy tumors. Tumors treated with 5-aza and CDDP (combo) show a statistically significant increase in 5-hmC levels compared to the other groups (n=2-4/group, *p<0.05, **p<0.01). **(B)** Quantification of Ki-67 levels in control and treatment cohorts shows that tumors treated with 5-aza and CDDP (combo) have lower Ki-67 levels compared to the other groups (n=2-4/group, ***p<0.001). **(C)** Quantification of cleaved Caspase 3 expression in IHC studies. Tumors treated with combination therapy show a statistically significant increase in cleaved Caspase 3 levels compared to CDDP monotherapy (n=2-4/group, *p<0.05).