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Research Article

Toward understanding the roles of *matrix metallopeptidase 1* in ovarian cancer

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Abstract

Ovarian Cancer (OC) is the leading cause of gynecologic cancer-related deaths worldwide. The leading risk factors for OC-related death are OC recurrence and the development of chemotherapy resistance. Investigation into molecular differences that distinguish primary from recurrent disease and the role of the tumor microenvironment (TME) in OC progression may help identify therapeutic targets. Gene expression microarray data comparisons between 21 primary and 21 recurrent OC specimens (16 matched pairs) showed significantly increased expression of Matrix Metallopeptidase 1 (*MMP1*) in the recurrent specimens ($p = 0.03$). We, therefore, investigated *MMP1* expression regulation and how endogenous and exogenous *MMP1* expression influences OC cell proliferation, migration/invasion and chemosensitivity. Both endogenous *MMP1* knockdown and low levels of exogenous *MMP1* increased cell proliferation of the OC cell line, CAOV2 ($p < 0.01$ and $p < 0.001$, respectively). Furthermore, CAOV2 cells cultured with low exogenous *MMP1* exhibited increased invasion ($p = 0.04$ and $p = 0.002$, respectively, for two shRNA-conditioned mediums, sh*MMP1*-1 or sh*MMP1*-2) and faster migration by wound healing assay relative to controls without *MMP1* knockdown. CAOV2 *MMP1* knockdown cells were also more resistant than controls to carboplatin ($p = 0.04$) and paclitaxel ($p = 0.017$). To explore the functions of cancer environmental *MMP1* in different cancer cells, 3 OC cell lines (CAOV2, HEYA8 and SKOV3) were tested for their proliferation when cultured under a low *MMP1* conditioned medium. Interestingly, while the proliferation was increased in CAOV2 and HEYA8 cells, it was reduced when SKOV3 OC cells were cultured with low exogenous *MMP1* (CAOV2: * $p = 0.01$, HEYA8: **** $p = 0.0004$, SKOV3: ** $p = 0.002$). These results likely reflect inherent *MMP1* expression variability in OC tissues and cell lines that is at least partly dependent on other endogenous parameters of the TME, including pH, metabolic state, and oxygenation, all of which were found to alter levels of endogenous *MMP1*. Given the ability of *MMP1* to promote oncogenic or tumor-suppressive behaviors, further study will be necessary to better understand how *MMP1* contributes to promoting or restraining tumor progression in an individualized manner.

Abbreviations

MMP: Matrix Metallopeptidase; OC: Ovarian Cancer; TME: Tumor Microenvironment; ECM: Extracellular Matrix; ROC: Recurrent Ovarian Cancer; POC: Primary Ovarian Cancer; CM: Conditioned Medium; CTL: Control; FBS: Fetal Bovine Serum; P/S: Penicillin Streptomycin; DMEM: Dulbecco's Modified Eagle's Medium; RMA: Robust Multiarray Analysis; MAPK: Mitogen-Activated Protein Kinase; DAC: 5-aza-2′-Deoxycytidine; NFT: Normal Fallopian Tubes; CAF: Cancer-Associated Fibroblasts

Introduction

Ovarian Cancer (OC) is the second most common gynecologic malignancy and the fifth leading cause of cancer-related death in women in the United States [1]. Treatment strategies have not significantly improved in the past 30 years, particularly with respect to reducing disease recurrence. The main treatment for primary OC remains surgical removal of primary cancer and cytoreduction of metastases and chemotherapy using platinum-based antineoplastic drugs [2]. Neoadjuvant chemotherapy followed by interval debulking

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and then additional chemotherapy is also sometimes used [3]. Some patients receive maintenance treatment with poly ADP ribose polymerase (PARP) inhibitors [4]. Although most patients achieve clinical remission after surgery and chemotherapy, within a few months to several years after primary treatment, 70-90% of patients with advanced OC experience tumor recurrence, which is an incurable disease despite potentially positive initial responses to treatment [5-7]. Although the molecular alterations of primary OC have been extensively studied, little data is available on recurrent tumors, as it is difficult to obtain recurrent tumor samples, even more so matched primary-recurrent tumor pairs [7]. Investigation into the genomic differences that characterize recurrent ovarian cancer, and how these differences contribute to tumor progression and chemotherapeutic response, is of great importance in the effort to identify novel targets that will allow for more durable treatment of patients with OC.

Here we examined microarray gene expression profiles for 21 primary and 21 recurrent serous epithelial ovarian cancers, including 16 matched pairs where primary and recurrent tumor tissue samples came from the same patient. We found that the seven most differentially expressed genes between these two groups belong to the extracellular matrix (ECM) superfamily, including two Matrix Metallopeptidase (MMP) genes, *MMP1* and *MMP13*. The ECM is an important contributor to the Tumor Microenvironment (TME), which has a prominent role in the development of chemoresistance [8,9]. The TME includes the blood vessels, immune cells, fibroblasts, signaling molecules, and ECM that surround a tumor [10,11]. The MMP family of proteins are zinc-dependent enzymes that function to degrade the ECM. Recent studies on MMPs have focused on their function during tissue injury in the skin and cardiovascular system [12]. *MMP1* plays a role in response to DNA damage caused by radiation and DNA repair [13,14]. In response to DNA damage in the skin, soluble cytokines, including TNF-α and IL–1, stimulate dermal fibroblasts to upregulate *MMP1* transcription through the p38 mitogen-activated protein kinase (MAPK) pathway [15,16]. Enhancing DNA repair can reduce *MMP1* expression in human skin cells and tissues [14]. Surprisingly, given the modulation of *MMP1* expression levels in association with DNA damage, no studies have yet determined if *MMP1* plays a role in chemotherapeutic response [17,18]. The association between *MMP1* and DNA damage is of particular importance in OC since first-line platinum-based drugs work by causing DNA damage and efficient mechanisms for DNA repair after exposure to chemotherapy are vital to the proliferation and invasion of cancer cells [19].

While there are studies investigating the role of *MMP1* in ovarian cancer, no studies have reported on *MMP1* in recurrent OC [20-25]. Given the very strong relationship between recurrent OC and mortality, we focused our investigation on genomic differences between primary and recurrent OC, with a long-term goal of exploiting these differences for the development of novel targeted therapies. The objective of the present study was to determine the role of endogenous and exogenous *MMP1* in OC proliferation, migration/invasion and chemosensitivity and to examine how environmental characteristics of the TME regulate *MMP1* expression.

Materials and Methods

Tumor Samples

We used 21 primary (POC) and 21 Recurrent OC (ROC) tissues from patients with stage III/IV serous epithelial OC from the Duke Gynecologic Oncology Tissue Bank. Of the 21 tumor pairs, 16 were matched pairs, from patients who contributed both primary and recurrent tumor samples. The primary tumor specimens were collected at the time of initial debulking surgery. Recurrent tumor tissue was obtained from the same patients during "second look" surgeries. Samples were obtained, prior to the initial surgery, after patients provided written, informed consent, in accordance with protocols approved by the Duke University Institutional Review Board.

DNA and RNA extraction

DNA and RNA were simultaneously extracted from each of the fresh-frozen tissue samples using the AllPrep DNA/RNA Mini Kit, according to the manufacturer's protocol (Qiagen; Germantown, MD; Cat#80204). Nucleic acid concentration and purity were assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific; Waltham, MA).

DNA methylation by illumina infinium humanmethylation 450 beadchip

DNA (500 ng) was bisulfite converted using the Zymo EZ DNA Methylation Kit (ZymoResearch, Tustin, CA), according to the manufacturer's protocol. The Illumina Infinium HumanMethylation450 BeadChip was used to generate quantitative DNA methylation data using bisulfite-modified genomic DNA from the 21 POC and 21 ROC specimens. This data is publicly available through the Duke Digital Data Repository, https://doi.org/10.7924/r4765hq57 [26].

Gene expression by microarray

Affymetrix Human Genome U133A Plus 2 arrays were used by the Duke DNA Microarray Facility to quantify gene expression, using total RNA isolated from the 21 POC and 21 ROC frozen ovarian tumor samples. This data is publicly available through the Duke Digital Data Repository, https://doi.org/10.7924/r43f4sx2k [27]. Previously published Affymetrix U133A gene expression data were used from two normal fallopian tube fimbriae epithelia, 12 stages I/II serous OCs, and 55 stages III/IV serous OCs [28]. We also used our Affymetrix HT Human Genome U133A Array gene expression data (NCBI GSE25428) for 22 OC cell lines treated for 72 hours with vehicle or 5 µM 5'-aza-2'-deoxycytidine (Decitabine, DAC; Sigma-Aldrich; St. Louis, MO; #A3656).

Cell culture and treatment

Ovarian cancer cell lines CAOV2 (aka OVCAR2), HEYA8, or SKOV3 were provided by the Gynecologic Oncology Tumor Bank at Duke University Medical Center. CAOV2 was used as the main study model, as it was obtained from ascites of a patient with ovarian adenocarcinoma [29] whose tumor was inherently resistant to cisplatin [30], which is relevant to chemoresistant,

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recurrent ovarian cancer. Cells underwent genetic authenticity testing using the GenePrint 10 kit (Promega; Madison, WI) at the Duke University DNA Analysis Facility and testing for mycoplasma at the Duke Cell Culture Facility prior to each expansion of new frozen stocks. The cells were cultured in RPMI 1640 (Sigma Aldrich; Milwaukee, WI) supplemented with 10% fetal bovine serum (FBS) (Invitrogen; Carlsbad, CA) and 1% penicillin-streptomycin (P/S) solution (Invitrogen; Carlsbad, CA). The embryonic kidney cell line, HEK293T, was cultured with Dulbecco's Modified Eagle's Medium DMEM (Sigma Aldrich; Saint Louis, MO) supplemented with 10% FBS (Invitrogen; Carlsbad, CA) and 1% P/S solution (Invitrogen, Carlsbad, CA). WI38 (ATCC; Manassas, VA), a human lung fibroblast cell line, was from a normal embryo at about three months gestation. WI38 cells were cultured with EMEM (EBSS) (Sigma Aldrich, Saint Louis, MO), 2 mM glutamine (Sigma Aldrich; Saint Louis, MO), 1% non-essential amino acids (NEAA) (Invitrogen; Carlsbad, CA), 10% FBS (Invitrogen; Carlsbad, CA) and 1% P/S solution (Invitrogen; Carlsbad, CA). All cell lines were grown in a 37 °C humidified incubator with 5% atmospheric CO_2. Both HEK293T and WI38 cell lines were tested for authenticity at the Duke Cell Culture Facility and were confirmed to be mycoplasma free.

Variable pH: Thirty-seven percent HCl (Millipore Sigma; St. Louis, MO) and 10N NaOH (Millipore Sigma; St. Louis, MO) solutions were added to RPMI 1640 (Sigma Aldrich; Milwaukee, WI), supplemented with 10% FBS (Invitrogen; Carlsbad, CA) and 1% P/S solution, in a dropwise fashion until the desired pH was achieved. pH was measured using a Benchtop pH-mV Meter (Cole Parmer; Vernon Hills, IL). The pH-adjusted culture medium was filtered using a 0.22 µm pore, 250 mL vacuum filter (Corning Life Sciences, Corning, NY).

Hypoxic culture: Anhydrous cobalt (II) chloride ($CoCl_2$) (Millipore Sigma; St. Louis, MO) was used for chemical hypoxia induction. Dosing of $CoCl_2$ was in accordance with previously published literature for establishing chemical hypoxia in breast and ovarian cancer cell lines [31]. $CoCl_2$ was dissolved in distilled water. The resulting aqueous $CoCl_2$ solution was directly added to the cell culture media at 100, 200 and 300 µM concentrations.

High glucose culture: D- (+)-glucose (Sigma Aldrich; Milwaukee, WI) was used to supplement glucose concentrations in the culture medium. Glucose solution was added to RPMI 1640 (Sigma Aldrich; Milwaukee, WI), supplemented with 10% FBS (Invitrogen; Carlsbad, CA) and 1% P/S solution, and stored at 4 °C for later use. Because normal blood sugar levels in vivo are approximately 5.5 mM D-glucose, we tested glucose levels in a cell culture medium ranging from 1.1 mM to as high as 33 mM for high glucose culture.

D-methionine: D-methionine (D-2-Amino-4-(methylthio) butanoic acid, (R)-2-Amino-4-(methyl mercapto) butyric acid, $C_5H_{11}NO_2S$, Sigma Aldrich; Milwaukee, WI) was added at 0.05 mM into RPMI 1640 (Sigma Aldrich; Milwaukee, WI), supplemented with 10% FBS (Invitrogen; Carlsbad, CA) and 1% P/S solution.

MMP1 knockdown

shRNA specific to *MMP1* was introduced into CAOV2, WI38 and HEK293T cells using a lentiviral transduction system according to the Addgene protocol (Watertown, MA). Briefly, HEK293T cells were transfected with 1µg of a lentivirus plasmid containing either control shRNA (Sigma Aldrich; Milwaukee, Wis., USA) or two independent *MMP1*-specific shRNAs (sh*MMP1*-1: 3334, sh*MMP1*-2: 3335; Duke Functional Genomics Shared Resource) together with packaging plasmids. FuGENE HD transfection reagent (Promega; Madison, WI) was used according to the manufacturer's recommendations. After 48 and 72 hours of transfection, the supernatants containing the virus particles were collected and stored at -80 °C until needed. The OC cell line CAOV2, fibroblast cell line WI38 (used to create conditioned medium for *MMP1* low expression), and embryonic kidney cell line HEK293T (used to create conditioned medium for *MMP1* low expression), were seeded the day before infection with lentiviral shRNA into 6-well plates at a density of 1×10^5 cells/well. The cells were 60% - 70% confluent at the time of infection. The medium was replaced with medium containing 8 µg/mL of Polybrene (Sigma Aldrich; Milwaukee, WI**).** The virus-containing control shRNA or *MMP1* shRNAs (~1 x 10^6 for the viral titer) from the last step was added to each cell line. After 72 hours of incubation, the cells were selected for two weeks using puromycin at 2 µg/mL for CAOV2, 1 µg/mL for WI38, and 5 µg/mL for HEK293T cells. Cells were then harvested to determine *MMP1* expression using qRT-PCR analysis.

RNA extraction and qRT-PCR

Total RNA extraction from CAOV2 shCTL and CAOV2 sh*MMP1* knockdown cells and WI38 shCTL and WI38 sh*MMP1* knockdown cells was achieved using TRIzol Reagent (Invitrogen; Carlsbad, CA). RNA quantity was measured on a Nanodrop 1000 spectrophotometer (Thermo Scientific; Waltham, MA). *MMP1* expression was analyzed by qRT-PCR with the one-step PCR ToughMix® (QuantaBio; Beverly, MA). Relative mRNA expression levels of *MMP1* were analyzed using an *MMP1* probe (hs00899658; ThermoFisher; Waltham, MA). A *B2M* (a housekeeping gene) probe (hs00196842; ThermoFisher; Waltham, MA) was used as an internal loading control. All assessments of *MMP1* expression levels in real-time qRT-PCR experiments were performed in duplicate, normalized to housekeeping gene (B2M) levels and the data presented represent the average of those measurements.

Pyrosequencing

The pyrosequencing assay was designed to analyze the DNA methylation of the *MMP1* gene at its promotor region. Four CGs at the *MMP1* promotor region were included in the sequence to analyze, 5'-GAA TTT YGA AGA GTT ATY GTA AAG TGA GTG TTG GGG GAG TTG AAT TTT AGT TAG TAT AGG TGT YGA ATA GTT ATT AGG TGY GTA GTG TTA GTA ATT TTA TTT TTT GTT T-3'. PCR was carried out with bisulfite-modified genomic DNA with primers, F: 5'-AGG TAG TTT AAT AAA GGT AGA AGG G-3', and biotinylated R primer: 5'-btn-AAT TTC TCC ACA CAC CTT ACT C-3' (Sigma Aldrich; Milwaukee, WI). Defined mixtures

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of fully methylated and unmethylated human genomic DNAs (EpiTect Control DNA; Qiagen) were used to validate the performance of the pyrosequencing assay. Bisulfite conversion was performed using 800 ng genomic DNA from OC cells using Zymo EZ DNA Methylation Kit (ZymoResearch, Tustin, CA). Bisulfite-converted DNAs (20 ng) were used in a total PCR reaction volume of 20 µL. PCR was performed at 95 ºC for 15 minutes followed by 55 cycles of 95 ºC for 30 seconds/65 ºC for 30 seconds/72 ºC for 30 seconds, with a final extension at 72 ºC for 10 minutes. The pyrosequencing was performed with the sequencing primer, 5'-GGT AGA AGG GAA TTT TAG A-3', with a PyroMark Q96 MD Pyrosequencer (Qiagen, CA).

Generation of conditioned medium

HEK293T and WI38 cells transfected with *MMP1*-specific shRNAs or control shRNA were seeded in 100-mm cell culture dishes at a density of 2.0 x 10^6 cells/plate with DMEM (Sigma Aldrich; Saint Louis, MO) supplemented with 10% FBS (Invitrogen; Carlsbad, CA) and 1% P/S solution (Sigma Aldrich; Saint Louis, MO). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Twenty-four hours later, the medium was collected and centrifuged to remove cells and cell debris. The supernatant was collected and used as the *MMP1* knockdown conditioned medium, labeled sh*MMP1* CM for the conditioned medium from HEK293T or WI38 cells. The non-silencing control medium obtained from shCTL-transfected HEK293T or WI38 cells was labeled as shCTL CM for the control conditioned medium from HEK293T or WI38 cells.

ELISA assay

HEK293T cells transfected with *MMP1*-specific shRNAs (shMMP1-1) or control shRNA (shCTL) were seeded in 100-mm cell culture dishes at a density of 2.0 x 10^6 cells/plate with DMEM (Sigma Aldrich; Saint Louis, MO) supplemented with 10% FBS (Invitrogen; Carlsbad, CA) and 1% P/S solution (Sigma Aldrich; Saint Louis, MO). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Twenty-four hours later, the medium was collected and centrifuged to remove cells and cell debris. The medium was used for measuring *MMP-1* by ELISA assay, using the kit from Invitrogen (human MMP-1, Cat# EHMMP1) in accordance with the manufacturer's instructions. The protein concentration was determined using Bradford protein assay, according to the instructions from the manufacturer (Bio-Rad). The test was done in triplicate for each CM from sh*MMP1*-1 or shCTL.

Cell proliferation assay with *MMP1* knockdown cells and *MMP1* low-conditioned medium

CAOV2 cells transfected with either control shRNA (shCTL) or *MMP1*-specific shRNA were cultured in the clear bottom, black 96-well plates (Corning Life Science; Corning, NY) in RPMI-1640 medium, with 10% FBS and 1% P/S, at 5,000 cells/well. After 48 hours, 50 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation; Madison, WI) was added to each well. The luminescence signal was measured using a microplate reader. Experiments were performed twice with three replicates for each test.

CAOV2 cells were cultured in 96-well plates with conditioned medium (CM) from *MMP1* knockdown WI38 cells, sh*MMP1* CM, or an off-target control shRNA, shCTL CM. After 48 hours, 50 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation; Madison, WI) was added to each well. The luminescence signal was measured using a microplate reader. Experiments were performed twice with four replicates for each test.

Chemosensitivity test with *MMP1* low-conditioned medium

CAOV2 cells were cultured in the clear bottom, black 96-well plates (Corning Life Science; Corning, NY) in RPMI-1640 medium with 100 µL conditioned medium (CM) collected from WI38 cells transfected with shCTL or sh*MMP1*. After 24 hours, the medium was replaced with a fresh conditioned medium containing carboplatin or paclitaxel. The concentration range for paclitaxel was 1 nM to 10 µM in 10-fold increments. For carboplatin, the range was 1 µM to 1000 µM in 10-fold increments. Both drugs were diluted in RPMI-1640 medium with 10% FBS and 1% P/S in a total volume of 100 µL per well. After 48 hours, 50 µL of CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation; Madison, WI) was added to each well. The luminescence signal was measured using a microplate reader. Experiments were performed twice with three replicates for each test.

Wound healing assay

For the wound healing experiments with the conditioned medium, we seeded equal numbers of CAOV2 cells into two wells of 6-well plates at a density of 3 x 10^5 cells/well using the conditioned medium from WI38 shCTL or WI38 sh*MMP1* (shCTL CM or sh*MMP1* CM). After 24 hours, the cells reached 90% - 95% confluence. A uniform "wound" was then generated by scratching a sterile 1 mL pipet tip across each cell monolayer. Micrographs for wounds and wound healing were taken to capture the process of cell migration starting at the time the scratch was introduced (time 0) until closure. Micrographs for wounds and wound healing were taken to capture the process of cell migration at times 0, 24 and 48 hours after "wounding". These experiments were independently performed twice.

Cell invasion assay

Invasion potential for CAOV2 cells was assessed using a 96-well–Invasion Assay (Cell Biolabs; San Diego, CA). CAOV2 cells were seeded at 5 x 10^4 cells/well in the top chamber of the assay dish with WI38 shCTL CM, sh*MMP1*-1 CM, or sh*MMP1*-2 CM. The bottom chamber of the assay dish contained either conditioned medium collected from sh*MMP1*-1, sh*MMP1*-2, or control (shCTL) WI38 cells containing 20% FBS. Each condition was performed in triplicate. The plate was incubated at 37°C in a 5% CO_2 humidified incubator for 48 hours. The cells in the bottom chamber were stained using the reagents provided in the kit and the absorbance at 560 nm was measured using a microplate reader.

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Statistical analysis

The microarray data from primary and recurrent OC samples were adjusted for the p values for multiple gene expression comparisons. The microarray data from mock-treatment or treatment of OC cell lines with 5-aza-2′-deoxycytidine (DAC) was analyzed using Student's t-test with GraphPad Prism 8 to compare *MMP1* gene expression. The microarray gene expression data from early OC, advanced OC and normal fallopian tube (NFT) samples were compared for *MMP1* expression using a Student t-test. The microarray data, from primary and recurrent OC samples, were analyzed by the Wilcoxon test to compare the gene expression between 21 unpaired primary and recurrent OC samples and between 16 matched primary and recurrent OC pairs. Heatmapper (http://www.heatmapper.ca) was used for the generation of heatmaps [32]. Results from cell proliferation, invasion and qRT-PCR assays are expressed as means +/- standard deviation (SD). Student's t-test was used to assess two group differences for continuous variables. The cell viability assays for cells cultured with shCTL CM or sh*MMP1* CM and treated with chemotherapeutic drugs were analyzed and compared between groups using a One-Way ANOVA assay. For all data analyses, a p - value <0.05 was considered statistically significant.

Results

MMP1 expression in fallopian tube precursor cells versus primary serous OC

Previous studies have indicated that *MMP1* expression is upregulated in OC as compared to normal ovarian tissue specimens [20]. However, abundant data now support that the site of origin for serous epithelial OC is the fallopian tube epithelia rather than ovarian surface epithelial cells [33,34]. To better understand the level of *MMP1* expression in primary OC as compared to the non-diseased tissue of origin, we analyzed existing Affymetrix U133A microarray data from 67 serous OCs, including 55 stages III/IV cases (30 who lived <3 years and 25 who lived >7 years) and 12 stages I/II cases [28]. These results were compared to microarray data from the fallopian tube epithelia of two patients without the disease. Advanced OC had significantly higher expression of *MMP1* compared to the normal fallopian tube (Figure 1A, p = 0.026), which is consistent with findings from other researchers (19). *MMP1* also showed significantly higher expression in advanced OC compared to early-stage OC (Figure 1A, p = 0.033). We found that the minimal expression in early-stage tumors was Robust Multiarray Analysis (RMA) = 0.04 and maximal expression was RMA = 2.37, a range of 2.32, whereas the minimal *MMP1* expression in advanced tumors was RMA = 0.11 and maximal expression was RMA = 3.77, range of 3.66. The data suggested that the early-stage OCs have narrower *MMP1* expression ranges than do the advanced tumors.

Differentially expressed genes in primary versus recurrent ovarian cancer

Analysis of gene expression microarray data from 21 primary (POC) and 21 recurrent ovarian cancer (ROC) samples identified about 900 genes showing significant differences in expression between POC and ROC (unadjusted $p < 0.05$). Seven of these showed >two-fold higher expression in ROC and five showed >two-fold higher expression in POC (*data not shown*). Interestingly, all seven of the upregulated genes in ROC belong to the ECM superfamily, including Periostin (*POSTN*), Collagen 11A1 (*COL11A1*), Tenascin C (*TNC*), Asporin (*ASPN*), Epiphycan (*EPYC*), Matrix Metallopeptidase 13 (*MMP13*), and Matrix Metallopeptidase 1 (*MMP1*). These results support the importance of the ECM, a component of the TME, in ROC. The *MMP1* expression differences between the 21 POC and 21 ROC are shown in Figure 1B ($p < 0.0001$). Among the 21 POC and 21 ROC samples, there were 16 primary-recurrent paired sets each obtained from the same patient (Figure 1C). Although changes in *MMP1* expression were observed across the two distinct disease time points for each pair, the direction of change was not consistent for each pair of POC and ROC (Figure 1C), with 8 pairs showing decreased and 8 pairs showing increased

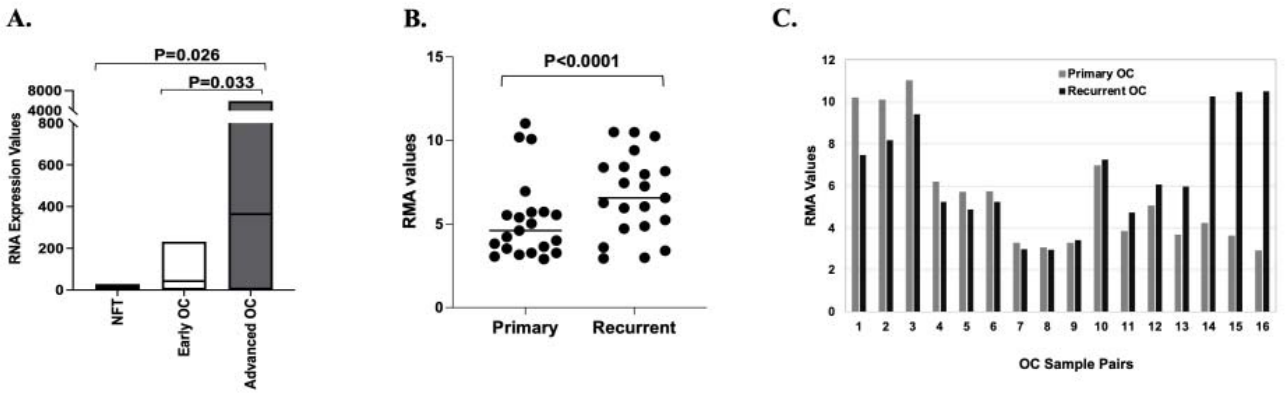

Figure 1: *MMP1* Expression is Increased in Ovarian Cancer vs Normal Tissues and Further Elevated in Recurrence.
A. Microarray data from two normal fallopian tube epithelial specimens (NFT), 12 early OC, and 55 advanced OC tissue samples from patients with stage III/IV serous epithelial OC (p = 0.026 NFT vs. advanced OC and p = 0.033 early OC vs. advanced OC, Student's t-test). **B.** RMA-normalized *MMP1* expression levels for the 21 primary and recurrent ovarian cancer samples (primary OC mean = 5.273, recurrent OC mean = 6.713, $p < 0.0001$, Wilcoxon test). **C.** RMA-normalized *MMP1* expression levels for the 16 paired primary and recurrent ovarian cancer samples (primary OC mean = 5.547, recurrent OC mean = 6.562, $p < 0.0001$, Wilcoxon test).

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expression of *MMP1* in the ROCs (primary OC mean = 5.547, recurrent OC mean = 6.562, $p < 0.0001$, Wilcoxon test). This seemingly conflicting data suggest that the importance of *MMP1* expression and thereby its functional role, depends on the individual tumor and that this logically would contribute to the large heterogeneity in phenotypes, tumor aggressiveness, and the therapeutic response observed in different individuals.

MMP1 expression is regulated by DNA methylation in ovarian cancer cells

Differences in the epigenetic profiles at *MMP1* could mechanistically contribute to the wide variability in *MMP1* expression observed in the OC tissues. Epigenetic changes are important contributors to cancer phenotype through altered regulation of cancer-related genes [35]. We, therefore, investigated whether *MMP1* gene expression is regulated by DNA methylation. Twenty-two OC cell lines were treated with 5 µM decitabine (DAC), a DNA methyltransferase inhibitor that has been used clinically for many types of cancer including OC [36]. RNA extraction was performed after 72 hours of mock treatment or treatment with DAC, followed by the generation of gene expression microarray data using the Affymetrix HT Human Genome U133A Array. Following DAC treatment, 15 of the 22 tested cell lines (68%) showed an increase in *MMP1* expression, indicating that *MMP1* is regulated directly or indirectly by DNA methylation in these cells (p=0.0016, Figure 2A). To validate the microarray data, we performed qRT-PCR (Figure 2B) with seven OC cell lines that were mock-treated or treated with 5 µM DAC for 72 hours. We were able to validate the gene expression microarray findings for 6 out of 7 tested cell lines, with upregulation of *MMP1* expression in the same seven showing upregulation by microarrays (Figure 2B).

To further confirm the relationship between DNA methylation and gene expression for *MMP1*, we designed a pyrosequencing assay to analyze four CpG sites at the *MMP1* promoter region, which includes one CG (cg18733315) which is also included on the Illumina Infinium HumanMethylation 450 BeadChip as shown in Figure 2C, the schematic graph of the *MMP1* promoter region. This region for pyrosequencing assay is about 400 bp upstream of the *MMP1* transcription start site. Pyrosequencing assay validation was performed using fully methylated and unmethylated genomic DNAs, including 0%, 50% and 100%. The average percent methylation of the four CGs were used for analysis. There was a high correlation between the input methylated DNA and measured levels of DNA methylation (R = 0.99) supporting the ability of the pyrosequencing assay to quantify methylation levels across the possible linear range (Figure 2C). Pyrosequencing of the *MMP1* promoter and qRT-PCR was performed in three cell lines: A2780, HEYA8 and OVCA429, which showed higher *MMP1* DNA methylation in mock-treated cells which was reduced with DAC treatment (Figure 2D) and a concomitant significant increase in *MMP1* expression (A2780 $p < 0.001$, HEYA8 $p < 0.01$ and OVCA429 $p < 0.05$, Figure 2E). Our data support that the expression of *MMP1* is at least partially regulated directly by DNA methylation.

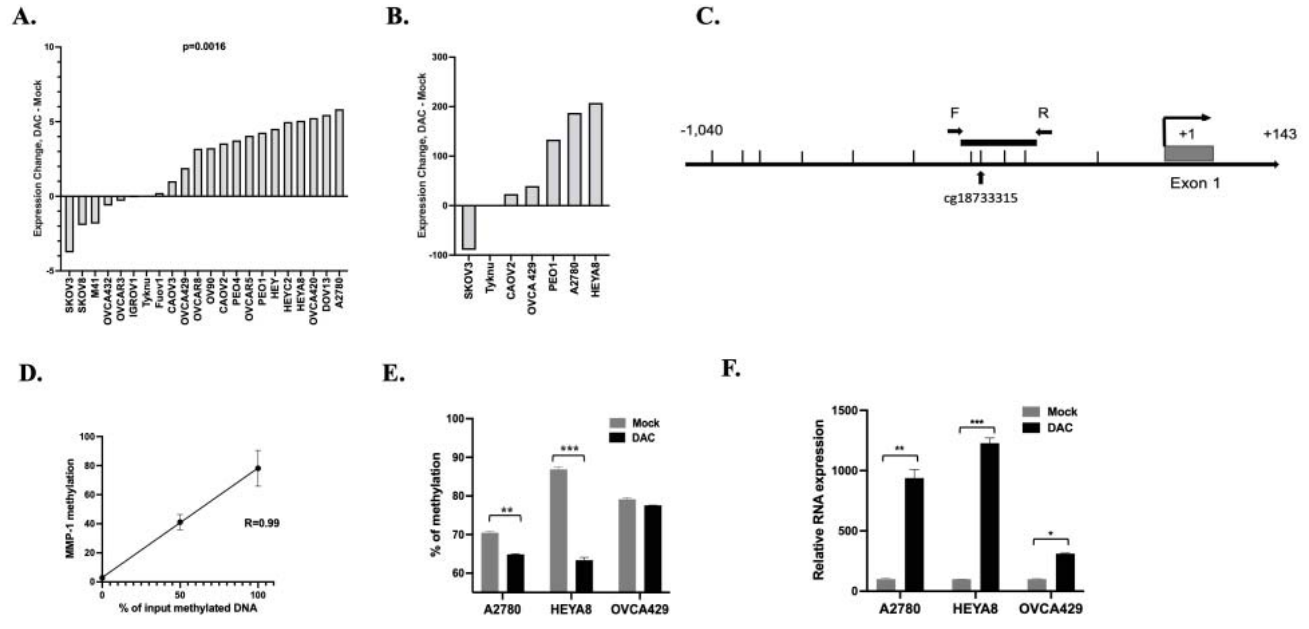

Figure 2: *MMP1* Expression in OC is Regulated by DNA Methylation.
A. Difference in *MMP1* expression (treated minus mock treated value) after OC cell lines were treated with DNMT inhibitor, decitabine (DAC). RMA-normalized *MMP1* gene expression levels were obtained for 22 OC cell lines following mock treatment or treatment with DAC ($p = 0.0016$, paired Student's t-test). **B.** Seven cell lines were analyzed using qRT-PCR to independently confirm the effect of DAC treatment on *MMP1* expression. The relative expression differences shown were obtained by subtracting the values for the mock-treated cells from those of the DAC-treated cells. **C.** Schematic graph of the *MMP1* promoter region showing the relative location of the pyrosequencing assay (F, forward PCR primer; R, reverse PCR primer). The promoter region for *MMP1* is 1,183 bp long containing 11 CGs, which are depicted as tick marks. The pyrosequencing assay was designed to cover four of these CG sites. One CG (cg18733315), which is also included on the Illumina Infinium HumanMethylation450 BeadChip, was shown with the arrow. **D.** *MMP1* pyrosequencing assay performance, using 0%, 50%, and 100% methylated DNAs. **E.** DNA methylation levels following DAC treatment. *** $p < 0.001$, ** $p < 0.01$ (paired Student's t-test). **F.** *MMP1* expression is increased in OC cells following treatment with DAC. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (paired Student's t-test).

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MMP1 expression in cancer cells is affected by the TME

Soluble factors secreted by the TME can contribute to abnormal proliferation, angiogenesis, metastasis and drug resistance [37,38]. Previous studies have demonstrated that cultured cancer cells upregulate the secretion of MMPs in the presence of conditioned media from tumor cells [39]. To determine how *MMP1* expression is influenced by the local microenvironment, we performed a series of experiments in which we altered pH, induced hypoxia, or provided additional glucose or methionine. We found that expression levels of *MMP1* indeed vary with the pH of the culture medium. Significant increased *MMP1* expression was observed under more acidic conditions (pH 6.2; $p = 0.003$) while *MMP1* repression was observed under more basic conditions (pH 8.2; $p = 0.04$) (Figure 3A). Hypoxic conditions were induced by increasing the $CoCl_2$ concentration in the cell culture medium, which tripled expression levels of *MMP1* (200 µM, $p = 0.002$; 300 µM, $p = 0.02$) (Figure 3B). We also observed a dose-dependent reduction in *MMP1* expression with increasing glucose concentration ($p < 0.05$; Figure 3C). Cells cultured in a methionine-depleted medium showed a nearly two-log increase in *MMP1* expression as compared with cells cultured with supplemental methionine ($p = 0.036$; Figure 3D). These data support that the level of *MMP1* expression in OC cells is responsive to, and greatly influenced by, specific and relevant TME cues.

MMP1 repression in cancer cells and the TME enhances proliferation and migration

To determine the effect of reducing the availability of endogenous *MMP1* in OC cancer cells, knockdown of *MMP1* expression in CAOV2 cells (Figure 4A) followed by proliferation analysis (Figure 4B) was performed. Contrary to existing data from other researchers using other cancer cell lines [21,22,40], CAOV2 showed significantly increased cell proliferation (Figure 4B, $p < 0.01$) when *MMP1* was downregulated in cancer cells using two shRNAs specific to *MMP1* (sh*MMP1*-1 and sh*MMP1*-2) or an off-target control shRNA (shCTL) (Figure 4A, $p < 0.01$ for sh*MMP1*-1 and $p < 0.001$ for sh*MMP1*-2). To further examine the functions of exogenous *MMP1*, like that produced in the ECM, we prepared conditioned media having low *MMP1* from WI38 cells in which *MMP1* was targeted for knockdown with shRNA (sh*MMP1* CM). QRT-PCR data confirmed the efficacy of shRNA-mediated knockdown of *MMP1* in WI38 cells using two shRNAs specific to *MMP1* (sh*MMP1*-1 and sh*MMP1*-2) or an off-target control shRNA (shCTL). Knockdown efficiency was ~80% *MMP1* as determined by qRT-PCR (*$p = 0.04$ and *$p = 0.03$ for sh*MMP1*-1 and sh*MMP1*-2, respectively; Figure 4C) when compared with the WI38 cells transfected with off-target control shRNA. CAOV2 cells cultured with the sh*MMP1* CM showed increased proliferation relative to cells in the shRNA control CM (shCTL CM), which had relatively higher *MMP1* expression ($p < 0.001$ for both sh*MMP1*-1 CM and sh*MMP1*-2

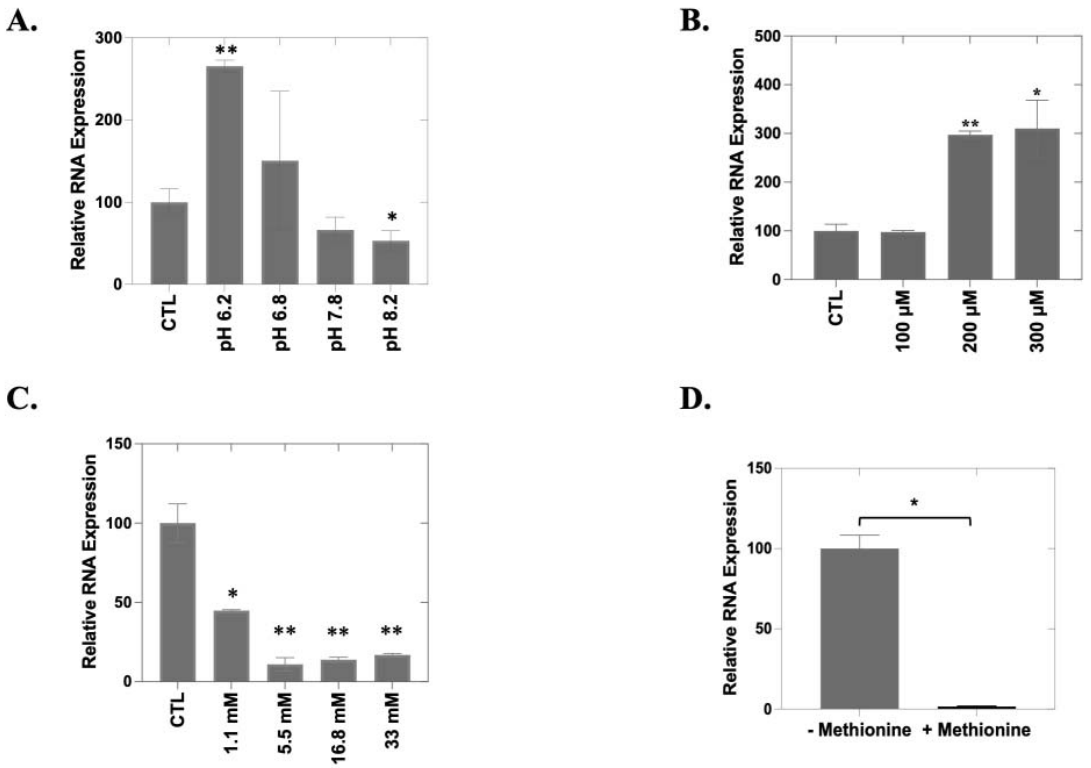

Figure 3: *MMP1* Expression in OC Cells is Regulated by the TME.
A. *MMP1* expression increased following culture in acidic medium (pH 6.2 or 6.8) and decreased in basic medium (pH 7.8 or pH 8.2) compared with cells cultured in neutral medium (pH 7.2; control). Results were confirmed by three independent experiments, each performed in duplicate. ** $p = 0.003$ and * $p = 0.04$ (paired Student's t-test). **B-D.** *MMP1* expression in CAOV2 cells cultured with the indicated concentrations of **(B)** $CoCl_2$ to induce hypoxia, **(C)** final glucose concentrations were at 1.1, 5.5, 16.8, and 33 mM, or **(D)** 0.05 mM methionine. ** $p < 0.01$, * $p < 0.05$ (paired Student's t-test).

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CM; Figure 4D). The *MMP1* protein expression in sh*MMP1* conditioned medium (CM) obtained from *MMP1* knockdown HEK293T cells was tested using ELISA assay (p = 0.0122) (Figure 4E).

Higher expression of MMPs is associated with increased invasion in many types of cancer [40,41]. Increased MMP secretion (such as *MMP2* in breast cancer) may assist in the breakdown of the basement membrane and lead to enhanced cancer cell invasion [42]. To determine the potential involvement of *MMP1* in OC cell invasion, we performed invasion assays using CAOV2 cells cultured with WI38 sh*MMP1* or shCTL CM. Knockdown of *MMP1* expression was confirmed in WI38 cells using qRT-PCR (Figure 4C). CAOV2 cells exhibited a more invasive phenotype when cultured with sh*MMP1* CM (p = 0.04 and p = 0.002, respectively for sh*MMP1*-1 or sh*MMP1*-2 conditioned medium; Figure 5A). Similar results were achieved with CM from HEK293T cell cells (*Data not shown*).

MMP1 is reported to promote cancer metastasis when secreted into the ECM by cancer cells [12,21,22]. To assess the effect of reducing exogenous *MMP1* on OC cell migration, we seeded 3 x 10^5 CAOV2 cells/well and added *MMP1* low CM (sh*MMP1* CM) or *MMP1* control (shCTL CM) from WI38 cells (refer to Figure 4C for knockdown efficiency). After 24 hours of culture, we introduced a gap in each cell monolayer using a sterile pipet tip and documented cell migration to close the gap with micrographs from time 0 to gap closure at 48 hours. Like the *MMP1* knockdown cells, CAOV2 cells cultured with sh*MMP1* CM were able to close the gap more rapidly as compared to cells cultured with shCTL CM (Figure 5B). Together and in agreement with the cell proliferation data, we found that low endogenous and exogenous *MMP1* levels are associated with increased invasion and migration.

MMP1 repression in OC cells enhances OC cell chemoresistance

TME factors have emerged as suggested key players in the development of chemoresistance and malignant progression [43]. We performed cell viability assays (MTS) after chemotherapeutic drug treatment for CAOV2 cells cultured under *MMP1* low-conditioned medium (sh*MMP1* CM) or shRNA control conditioned medium (shCTL CM) (Figure 6). The *MMP1* conditioned medium was generated from WI38 cells transduced by shCTL or sh*MMP1* (refer to Figure 4C for knockdown efficiency) and the cells were further treated with (Figure 6A) carboplatin (from 1 µM to 1000 µM in 10-fold increments) or (Figure 6B) paclitaxel (from 1 nM to 10 µM in 10-fold increments). The IC50 values for paclitaxel treatment were 768.2 nM for CAOV2 in shCTL CM and 1.587µM in shMMP1 CM. The IC50 values for carboplatin treatment were 57.49 µM for CAOV2 in shCTL CM and 190.8µM in shMMP1 CM. Consistent with the cell proliferation and invasion data, CAOV2 under sh*MMP1* CM showed significantly more resistance to chemotherapy treatment (p = 0.04 and p = 0.017 for carboplatin and paclitaxel treatment, respectively) when each was compared to cell viability for CAOV2 cultured under shCTL CM.

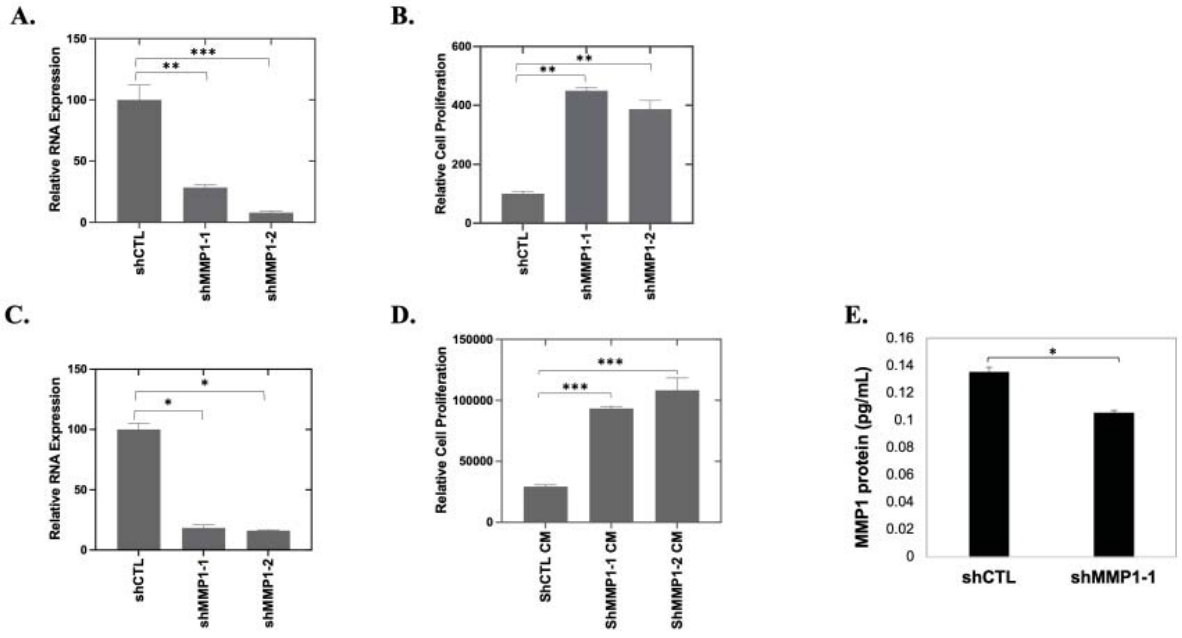

Figure 4: Reduced Endogenous or Exogenous *MMP1* Levels Increase OC Cell Viability.
A. QRT-PCR data showing the efficacy of shRNA-mediated knockdown of *MMP1* in CAOV2 cells using two shRNAs specific to *MMP1* (sh*MMP1*-1 and sh*MMP1*-2) or an off-target control shRNA (shCTL). ** p < 0.01, *** p < 0.001 (paired Student's t-test). **B.** *MMP1* knockdown in CAOV2 cells results in higher cell proliferation. ** p < 0.01 (paired Student's t-test). **C.** QRT-PCR data showing the efficacy of shRNA-mediated knockdown of *MMP1* in WI38 cells using two shRNAs specific to *MMP1* (sh*MMP1*-1 and sh*MMP1*-2) or an off-target control shRNA (shCTL). * p = 0.04 and * p = 0.03 for sh*MMP1*-1 and sh*MMP1*-2, respectively (paired Student's t-test). **D.** Culture of CAOV2 with conditioned medium (CM) from *MMP1* knockdown WI38 cells results in increased CAOV2 cell viability as compared to cells cultured in medium with WI38 control cells (shCTL). *** p < 0.001 for both sh*MMP1*-1 CM and sh*MMP1*-2 CM (paired Student's t-test). **E.** The protein expression of *MMP1* was determined by the *MMP1* ELISA kit for the *MMP1* knockdown conditioned medium (p = 0.0122). The test was done in triplicate for each CM from sh*MMP1*-1 or shCTL.

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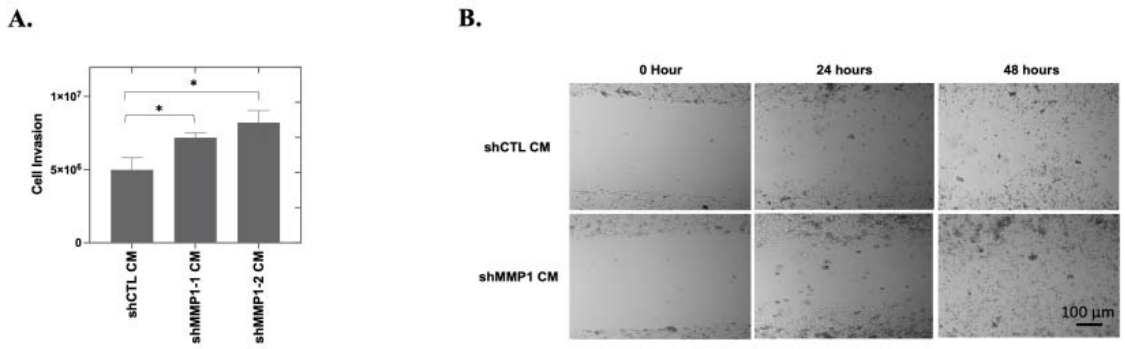

Figure 5: Reduced *MMP1* Expression in Tumor Microenvironment Enhances Migration and Invasion in OC Cells.
A. Invasion assay results from CAOV2 cells cultured with WI38 shCTL, sh*MMP1*-1, or sh*MMP1*-2 conditioned medium (CM). The assay was performed in triplicate for each shCTL and sh*MMP1* condition. * p = 0.04 and * p = 0.002, respectively for sh*MMP1*-1 or sh*MMP1*-2 conditioned medium (paired Student's t-test). **B.** Wound healing (migration) assay from CAOV2 cells cultured with WI38 shCTL or sh*MMP1* conditioned medium (shCTL CM and sh*MMP1* CM). The wound was created 24 hours after cell transfection. Micrographs were taken at times 0, 24, and 48 hours after "wounding". The scale bar in B is 100 mm.

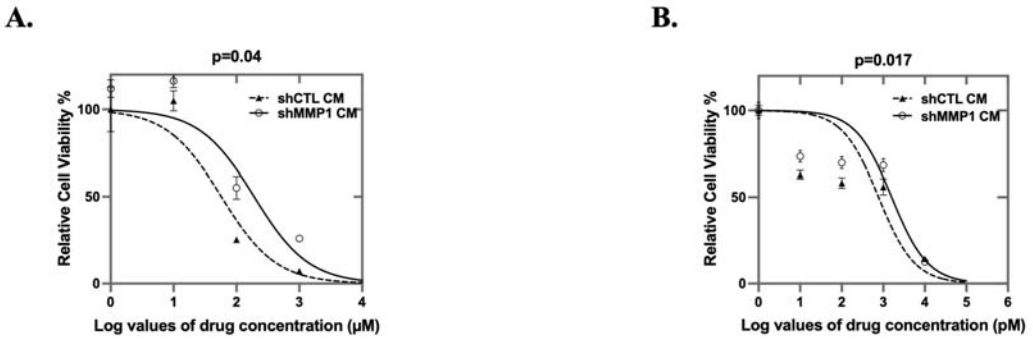

Figure 6: *MMP1* Knockdown Enhances OC Cell Chemoresistance.
Cell viability assays (MTS) for CAOV2 cells cultured under conditioned medium (CM) generated from WI38 cells transduced with shCTL or sh*MMP1* and treated with (**A**) carboplatin (from 1 µM to 1000 µM in 10-fold increments, p = 0.04) or (**B**) paclitaxel (from 1 nM to 10 µM in 10-fold increments, p = 0.017) (log(inhibitor) vs. normalized response and One-Way ANOVA).

Differential effects of *MMP1* on OC cell proliferation

The finding that low *MMP1* expression significantly increased cancer cell proliferation and migration was seemingly contrary to the findings from OC clinical samples. To determine if the *in vitro* results were cell line dependent, we tested the proliferation of CAOV2, HEYA8 and SKOV3 OC cells cultured under sh*MMP1* or shCTL CM from WI38 cells (Figure 7). The results showed cell proliferation was significantly increased in CAOV2 and HEYA8 cells (p = 0.01 and p = 0.0004 for CAOV2 and HEYA8, respectively), but reduced proliferation was seen in SKOV3 cells when they were cultured in low *MMP1* conditions (p = 0.002) (Figure 7).

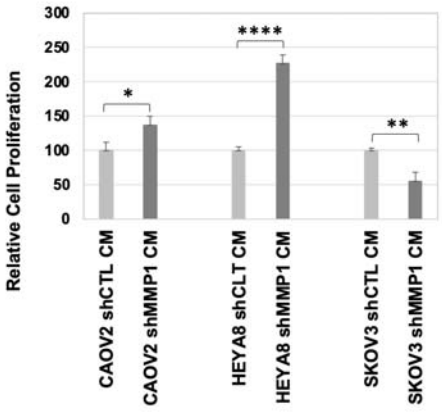

Figure 7: Differential Effects of *MMP1* on OC Cell Proliferation.
Cell proliferation assay (MTS) for CAOV2, HEYA8, and SKOV3 cells cultured with WI38 shCTL or sh*MMP1* conditioned medium. **** p = 0.0004, ** p = 0.002, * p = 0.01 (paired Student's t-test).

Discussion

This study examined the role of the TME in the regulation of *MMP1* expression in OC. *MMP1* expression was found to be higher in ROC and in tumors from OC patients who had short-term versus long-term survival post-diagnosis. We also showed that *MMP1* expression in OC cells is regulated epigenetically by DNA methylation at the promoter region. We showed that the expression of *MMP1* is significantly altered by changes relevant to the conditions in the TME, including pH, oxygen concentration and nutrient status in the culture media.

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Somewhat unexpectedly, we found that decreased availability of exogenous *MMP1* leads to a more aggressive cancer cell phenotype, characterized by increased cell proliferation, increased invasion/migration, and increased resistance to chemotherapeutic agents. Taken together, our data suggest that *MMP1* has different roles in ovarian cancer that are exquisitely sensitive to conditions within the TME and likely depend on the anatomical context of the cancer cells (e.g., in the tumor versus in the ascites) which warrant further studies.

Epigenetic modifications and their role in tumorigenesis are active areas of research. Using cancer-specific epigenetic changes as biomarkers for the detection and targeting of epigenetic readers and writers is a promising strategy used in cancer therapy [44,45]. In this study, we demonstrated increased *MMP1* expression in most OC cell lines in response to treatment with the DNA methyltransferase inhibitor, DAC, indicating that *MMP1* itself, and/or an upstream regulator(s) of *MMP1*, is repressed by DNA methylation. We then showed that DAC treatment is accompanied by a decrease in methylation at the *MMP1* promoter along with increased expression, indicating a direct functional role for methylation at the *MMP1* promoter. The DAC-mediated upregulation of *MMP1* expression in OC cells combined with our results shows that low levels of *MMP1* in OC are associated with a more aggressive phenotype, consistent with the idea that *MMP1* plays a tumor-suppressive role in serous epithelial ovarian cancer.

Matrix metalloproteinases (MMPs) can be secreted by both cancer cells and cancer-associated fibroblasts (CAFs), suggesting that the actions of cancer cells and the TME can be synergistic in regulating cancer progression [46]. Studies have suggested that MMP factors have dual roles in cancer progression, either promoting cancer growth and invasion by degrading the cancer matrix and enhancing angiogenesis [47] or, conversely, inhibiting cancer growth by limiting tumor neovascularization [48]. We found higher *MMP1* expression in ROC and in tumors from advanced OC patients, which is consistent with a more oncogenic role of *MMP1*. However, we also found that low exogenous *MMP1* was associated with increased CAOV2 cell proliferation, cell invasion and cell migration. Although this is consistent with findings examining *MMP1* knockdown in dermal fibroblasts [49], other studies have reported a more proliferative cancer phenotype when MMPs are overexpressed. This may be explained through a mechanism of releasing and activating growth factors, such as insulin-like growth factors (IGFs) and vascular endothelial growth factors (VEGF) [50,51]. MMPs are well-known key factors involved in ECM degradation that induce initiation in both physiological and pathological processes of angiogenesis [52]. However, the experimental evidence thus far demonstrates that MMPs also play a decisive role in the activation of pro-angiogenic and, in some cases, anti-angiogenic factors in cancer tissues [53]. Thus, MMPs can be considered angio-modulators, which could control new vessel formation necessary for cancer growth, progression, and spread. Therefore, MMPs have been speculated to participate in cancer angiogenesis in a cell context-dependent manner. Indeed, we found that low exogenous *MMP1* enhanced proliferation for CAOV2 and HEYA8 cells but reduced proliferation for SKOV3 cells (Figure 7). The CAOV2 cell line was derived from a metastatic site ascites fluid of a patient with an aggressive OC (NCIt: C4908)[54] and HEYA8 cells were derived from high-grade ovarian serous adenocarcinoma (NCIt: C105555) [54]. SKOV3 is a cell line with epithelial morphology that was isolated from the ovary of a 64-year-old, white female with ovarian adenocarcinoma (ATCC, HTB-77). This cell line is moderately well-differentiated adenocarcinoma consistent with ovarian primary cells. Differences in the cellular response to the low *MMP1* CM could also be a function of the overall available levels of endogenous plus exogenous *MMP1*. In this regard, it is noteworthy that *MMP1* transcription was enhanced in both CAOV2 and HEYA8 cells by treatment with decitabine but repressed in SKOV3 cells (Figure 2A and 2B). This supports the idea that CAOV2 and HEYA8 cells may have been inherently more responsive to differences in exogenous *MMP1* levels if endogenous levels were at least partially repressed by DNA methylation. In the context of low relative levels of endogenous *MMP1*, exogenous *MMP1* appears to play a role in suppressing aggressive cell behaviors. Higher endogenous levels of *MMP1* like that in SKOV3 cells may render reduction of exogenous *MMP1* irrelevant such that the normal repressive function is still evident. The seeming contradiction between our *in vitro* and *in vivo* findings might be explained by the enriched cellular content of the tumor tissues analyzed, which does not provide meaningful information about exogenous *MMP1*. Our results therefore altogether support the idea that exogenous *MMP1* normally helps suppress oncogenic behaviors of ovarian cancer cells but that higher endogenous *MMP1* levels may override this effect. It is known that tumor cells have extensive heterogeneity in their metabolism and phenotype relative to normal tissue across cancer types. Abnormalities coming from the tumor cells are tissue-specific, leading to adaptation to the microenvironment where they developed. The interesting observation of the MMP family is the large, robust specificity profile, which suggests that its role is controlled in a tissue-specific manner; that is, MMP types are expressed according to the regulatory proteins needed for the tissue.

A prior report showed that inhibition of *MMP2* and *MMP9* followed by cisplatin treatment resulted in a more cytotoxic effect in OC than cisplatin alone [55]; however, there are no published studies examining the impact of *MMP1* on chemotherapy resistance in OC. Our data, indicating that low levels of exogenous *MMP1* result in increased chemoresistance in OC is contrary to what has been suggested for other MMP factors, but is consistent with the summation of other phenotypes shown in this paper: reduced expression of exogenous *MMP1* was associated with more proliferative and invasive ovarian cancer cells. Additionally, we found *MMP1* expression was associated with cell environmental changes, such as local pH, hypoxia status, glucose level and methionine level alterations. Thus, regulating the local pH, hypoxia, or restricting methionine could be useful for anti-cancer treatment by exploiting their effects on *MMP1* expression and function(s).

The tumor immune microenvironment is distinct between primary and recurrent OC [56]. Immunologic factors in the

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TME evolve, such that factors associated with a poor prognosis in primary tumors can be associated with a positive prognosis in recurrent tumors [12]. For example, higher numbers of regulatory T cells (Tregs) in POC are associated with a decreased time to recurrence; yet higher levels of Tregs in recurrent tumors are correlated with longer overall survival [56]. Given these results, expanded studies are needed to explore potential functional differences in *MMP1* in primary and recurrent OCs and the relationship between endogenous and exogenous *MMP1* levels *in vivo*. Our results demonstrate that *MMP1* has important roles in OC and provide justification for deeper exploration into mechanisms that could lead to novel therapeutic approaches.

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Consent for publication

All authors consent to publication.

Author contributions

CH generated data, performed statistical analyses and drafted the manuscript. ZH designed and supervised the study and helped draft the manuscript. SKM, IC and AB reviewed and edited the manuscript. DY generated data and performed statistical analyses. ON, CG and PJ generated data. All authors had access to the study data, contributed to data interpretation, critically reviewed the manuscript and reviewed and approved the final manuscript.

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