Identification of candidate methylation-responsive genes in ovarian cancer
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Abstract

Background: Aberrant methylation of gene promoter regions has been linked to changes in gene expression in cancer development and progression. Genes associated with CpG islands (CGIs) are especially prone to methylation, but not all CGI-associated genes display changes in methylation patterns in cancers.

Results: In order to identify genes subject to regulation by methylation, we conducted gene expression profile analyses of an ovarian cancer cell line (OVCAR-3) before and after treatment with the demethylating agent 5-aza-deoxycytidine (5-aza-dC). An overlapping subset of these genes was found to display significant differences in gene expression between normal ovarian surface epithelial cells and malignant cells isolated from ovarian carcinomas. While 40% of all human genes are associated with CGIs, > 94% of the overlapping subset of genes is associated with CGIs. The predicted change in methylation status of genes randomly selected from the overlapping subset was experimentally verified.

Conclusion: We conclude that correlating genes that are upregulated in response to 5-aza-dC treatment of cancer cell lines with genes that are down-regulated in cancer cells may be a useful method to identify genes experiencing epigenetic-mediated changes in expression over cancer development.

Background

Gene expression profiling is now a common first approach toward the characterization of molecular changes occurring through cancer development and progression [1]. While recurrent changes in patterns of gene expression are beginning to emerge for a variety of cancers, the causal basis of the observed differences in expression remain to be delineated. One characteristic change associated with many cancers is the down regulation of genes involved in suppressing malignant transformation.
Down regulation of these "tumor-suppressor genes" may occur, not only by genetic (i.e., nucleotide substitution) changes but also by epigenetic modifications, such as DNA methylation [2,3]. DNA methylation largely occurs at cytosines associated with CpG dinucleotides. CpG rich regions are known as CpG Islands (CGIs). A CGI has been defined as a region of at least 200 bp with a GC content of 50% or more, and an observed/expected ratio of CpGs higher than 0.6 [4]. Methylation of CGIs in the promoter region of genes is known to transcriptionally repress those genes [3]. Numerous reports have shown that multiple genes are silenced during cancer progression through hypermethylation of the CGIs. Some examples of genes shown to be silenced in ovarian cancers due to hypermethylation include OPCML, RASSF1A, BRAC1 and p16 [5-7].

Treatment of cancer cell lines with the demethylating agent 5-aza-deoxycytidine (5-aza-dC) leads to changes in gene expression due to the loss of methylation in gene regulatory regions [8-11]. In this study, an ovarian cancer cell line (OVCAR-3) was treated with 5-aza-dC to identify genes regulated in cancer cells by methylation. We compared changes in gene expression patterns in the 5-aza-dC treated cancer cell line with observed differences in patterns of gene expression between normal and cancerous ovarian tissue to identify candidate genes undergoing epigenetic changes during the process of ovarian cancer development. The predicted change in methylation status of genes randomly selected from the list of candidate genes was experimentally verified. Our results indicate that correlating genes that are upregulated in response to 5-aza-dC would be expected to have a methylated CGI in or in proximity to promoter regions. Direct effects might include the altered expression of loci regulated by genes containing CGIs. To identify genes associated with CGIs, we utilized the CGI identification feature of the USCS Human Genome Browser Gateway. Of the 831 genes differentially expressed after 5-aza-dC treatment, 74% are known to be associated with CGIs.

The methylation pattern of the promoter region of five genes associated with CGIs and found to be upregulated after 5-aza-dC treatment (CAV1, CST6, MAGEA3, EMP3 and CXL6) was examined. With the exception of CXCL6, all have been previously shown to be hypermethylated in other cancers or cancer cell lines [12,15-17].

Figure 2 presents the results of sodium bisulfite sequencing analysis of CpG rich regulatory regions in each of the five genes. All genes showed extensive methylation of their promoter regions in the untreated ovarian cancer cells (OVCAR-3) and a variable decrease of methylation after 5-aza-dC treatment. CST6, CAV1, EMP3 and MAGEA3 displayed a decrease in methylation ranging from 13% to 61% while CXCL6 exhibited no methylation change in the promoter region examined. Variability in the response of different CpG regions to 5-aza-dC has been observed previously [9,18], therefore we can not exclude the possibility that expression of CXCL6 is controlled by the methylation levels of a different region than the one studied here.
Genes responsive to 5-aza-dC in the OVCAR-3 cell line display decreased expression in ovarian adenocarcinomas

We hypothesized that if epigenetic mediated changes in gene expression are occurring in ovarian cancer development, at least a subset of the genes upregulated in response to 5-aza-dC treatment in OVCAR-3 cells should display lower levels of expression in ovarian adenocarcinomas relative to controls. To test this hypothesis, we looked for overlap between genes responsive to 5-aza-dC treatment in the OVCAR-3 cells and genes displaying a significant change in expression between normal ovarian surface epithelium brushings (NOSE) and epithelial adenocarcinoma cells isolated from serous papillary epithelial ovarian tumors (EOC) by laser capture microdissection (LCM). Microarray analysis (Affymetrix U133 Plus 2.0) was performed on eight samples of NOSE brushings and eight LCM collections of epithelial cells from EOC. Differentially expressed genes between these samples were identified as described for the OVCAR-3 cell line experiment. Although a comprehensive analysis of the tissue microarray results will be published elsewhere, the average fold difference in expression levels between NOSE and EOC for the overlapping genes (see below) are presented in Additional File 1.

Of the 831 genes displaying a significant change in expression in response to 5-aza-dC treatment of OVCAR-3 cells, 123 displayed a significant decrease in expression between normal ovarian epithelia and adenocarcinomas [see Additional File 1]. Of these 123 genes, 102 were upregulated and 21 were down-regulated in response to the 5-aza-dC treatment in the OVCAR-3 cell line. Included in the list of 102 genes are genes previously identified as being hypermethylated and down-regulated in cancer (e.g., zinc finger protein 185 in prostate cancer [19], CAV1 in breast cancer [20], and DIRAS3 in ovarian cancer [21]). We thus predicted that the 102 genes upregulated in 5-aza-dC treated cells that overlapped with genes down-regulated in EOC were likely to have been so, at least in part, due to hypermethylation. To determine if these 102 candidate genes contained CGIs as our model predicts, we examined sequences in and immediately flanking each of the 102 genes. Ninety-six of the 102 candidate genes (94%) were found to have CGIs. In contrast, it has been estimated that only 40% of all human genes are associated with CGIs [22].

As an initial experimental test of the model, we randomly selected two genes from the list of candidate genes (CAV

### Table 1: Upregulated genes after 5-aza-dC treatment of OVCAR cells. Chromosomal location and fold change in gene expression are shown for 5-aza-dC treated/untreated OVCAR 3 cells.

| RefSeq Transcript ID | Symbol | Gene Name                | Chr. | Fold Change |
|---------------------|--------|--------------------------|------|-------------|
| NM_001351           | DAZL   | Deleted in azoospermia-like | 3p24 | 27.52       |
| NM_052957           | ACRC   | Acidic repeat containing  | Xq13 | 27.07       |
| NM_144594           | FLJ32942 | Hypothetical protein FLJ32942 | 12q13 | 22.73       |
| NM_032858           | MAEL   | Maelstrom homolog        | 1q24 | 18.75       |
| NM_002364           | MAGEB2 | Melanoma antigen family B, 2 | Xp21 | 18.73       |
| NM_005363           | MAGEA6 | Melanoma antigen family A, 6 | Xq28 | 16.31       |
| NM_012253           | TKT1   | Transketolase-like I     | Xq28 | 15.02       |
| NM_023930           | KCTD14 | Potassium channel tetramerisation domain | 11q14 | 11.34       |
| NM_013453           | SPANX  | Sperm protein associated with the nucleus, X-linked | Xq27 | 10.99       |
| NM_005362           | MAGEA3 | Melanoma antigen family A, 3 | Xq28 | 10.56       |
| NM_005335           | HCLS1  | Hematopoietic cell-specific Lyn substrate 1 | 3q13 | 10.41       |
| NM_006228           | Pnoc   | Prepronociceptin          | 8p21 | 9.89        |
| NM_152578           | FRG11B | Fragile X mental retardation 1 neighbor | Xq27 | 9.24        |
| NM_005367           | MAGEA12 | Melanoma antigen family A, 12 | Xq28 | 8.81        |
| NM_003289           | TPM2   | Tropomyosin 2 (beta)     | 9p13 | 7.00        |
| NM_080618           | CTCLF  | CCCTC-binding factor (zinc finger protein)-like | 20q13 | 6.39       |
| NM_001011544        | MAGEA11 | Melanoma antigen family A, 11 | Xq28 | 6.32        |
| NM_005213           | CSTA   | Cystatin A               | 3q21 | 5.80        |
| NR_002196           | H19    | H19, imprinted maternally expressed untranslated mRNA | 11p15 | 5.71        |
| NM_173571           | LOC255313 | Hypothetical protein LOC255313 | Xq24 | 5.28        |
| NM_003480           | MFAP5  | Microfibrillar associated protein 5 | 12p13 | 4.79        |
| NM_001323           | CTS6   | Cystatin E/M             | 11q13 | 4.79        |
| NM_001327           | CTAG1B&A | Cancer-testis antigen 1B/cancer-testis antigen 1A | Xq28 | 4.58        |
| NM_001011548        | MAGEA4 | Melanoma antigen family A, 4 | Xq28 | 4.48        |
| NM_00826            | SYT13  | Synaptotagmin XIII       | 11p11 | 4.37        |
| NM_013238           | DNAJC15 | DnaJ (Hsp40) homolog, subfamily C, member 15 | 13q14 | 4.33        |
| NM_005602           | CLDN11 | Claudin 11               | 3q26 | 4.23        |
| NM_001002915        | IGF2   | Insulin growth factor-like family member 2 | 1q21 | 4.09        |
| NM_004988           | MAGEA1 | Melanoma antigen family A, 1 (directs expression of antigen MZ2-E) | Xq28 | 3.73        |
and CXCL6) and one CGI-associated gene not on the list of candidate genes (EMP3) for sodium bisulfite sequencing analysis. Consistent with the prediction of the model, CAV-1 and CXCL6 were found to be hypermethylated in ovarian cancer relative to control tissues while essentially no difference in EMP3 methylation pattern was detected. The observed fold changes in gene expression between control (NOSE) and ovarian cancer (EOC) samples were significant (p < 0.01) for CAV 1 (-30.88) and CXCL6 (-4.22) [see Additional File 1] but not significant (p < 0.06) for EMP3 (+1.10). Consistent with these relative changes in levels of gene expression, the relative increase in levels of methylation was greater for Cav 1 than CXCL6 and minimal for EMP3 (Figure 3).

**Discussion**

The potential contribution of epigenetic changes to cancer development and progression is a topic of much recent interest [e.g., 23-25]. For example, several studies have implicated changes in DNA methylation with changes in the expression of a number of putative tumor suppressor and oncogenes in a variety of cancers [e.g., 2,12-21]. In an effort to establish a more global perspective of genes that may be prone to changes in DNA methylation during cancer development, a number of investigators have carried out gene profile analyses of a variety of cancer cells treated with demethylating agents (e.g., 5-aza-dC) [e.g., 8-11,26-28]]. The rationale is that genes that are transcriptionally repressed in cancer cells due to hypermethylation may be released from such constraint after treatment with demethylating agents and thereby identified by a significant increase in gene expression. If valid, such a strategy could be useful in identifying a candidate subset of methylation-regulated genes among the thousands of genes that are typically found to be down-regulated during cancer development. While intuitively appealing, such a strategy rests upon the questionable assumption that all or most genes displaying a significant increase in expression after treatment with demethylating agents are directly responding to an induced change in methylation. The purpose of our study was to conduct a preliminary evaluation of the validity of this assumption and of the overall utility of this approach within the context of ovarian cancer.

We selected for our study the ovarian cancer cell line OVCAR 3 because we had previously determined that it displays a gene expression profile most similar to that of ovarian cancer epithelial cells (data not shown). Comparing the gene expression profile of OVCAR 3 cells before and after treatment with 5-aza-dC revealed a significant (p < 0.01) change in expression for 831 genes. We deter-

| RefSeq Transcript ID | Symbol | Gene Name | Chr. | Fold Change |
|---------------------|--------|-----------|------|-------------|
| NM_001038360        | MADI1I | MAD1 mitotic arrest deficient-like 1 (yeast) | 7p22 | -4.27       |
| NM_004888           | NRG2   | Neuregulin 2 | 5q31 | -3.95       |
| NM_00100331         | ATP2B2 | ATPase, Ca++ transporting, plasma membrane 2 | 3p25 | -2.88       |
| NM_001719           | BMP7   | Bone morphogenetic protein 7 (osteogenic protein 1) | 20q13 | -2.56       |
| NM_032932           | RAB11FIP4 | RAB11 family interacting protein 4 (class II) | 17q11 | -2.49       |
| NM_014914           | CENP12 | Centaurin, gamma 2 | 2q37 | -2.30       |
| NM_013372           | GREM1  | Gremlin 1, cysteine knot superfamily, homolog | 15q13 | -2.28       |
| XM_373734           | ...    | Hypothetical LOC38388 | 3p13 | -2.28       |
| NM_020761           | Raptor | Raper | 17q25.3 | -2.26       |
| NM_00131693         | HHHL3  | HERV-H LTR-associating 3 | --- | -2.20       |
| NM_003598           | TEAD2  | TEA domain family member 2 | 19q13 | -2.18       |
| NM_022773           | FLJ12681 | Hypothetical protein FLJ12681 | 16p13.3 | -2.18       |
| NM_004389           | CTNNA2 | Catenin (cadherin-associated protein), alpha 2 | 2p12 | -2.09       |
| NM_32996            | MICAL3 | Microtubule associated monooxygenase, calponin and LIM domain containing 3 | 22q11 | -2.08       |
| NM_006200           | NOS1   | Nitric oxide synthase 1 (neuronal) | 12q24 | -2.06       |
| NM_006567           | FAR52 | Phenylalanine-tRNA synthetase 2 | 6p25 | -1.20       |
| NM_003466           | PAX8   | Paired box 8 | 2q13 | -1.99       |
| NM_006613           | GRAP   | GRB2-related adaptor protein-like | 17p11 | -1.97       |
| NM_022138           | SMOC2  | SPARC related modular calcium binding 2 | 6q27 | -1.96       |
| NM_017594           | DIRAS2 | DIRAS family, GTP-binding RAS-like 2 | 9p22 | -1.96       |
| NM_021018           | HIST1H3F | Histone 1, H3f | 6p22 | -1.92       |
| NM_004192           | ASMTL  | Acetylsorotonin O-methyltransferase-like | Xp22 | -1.92       |
| NM_002412           | MGNT   | O-6-methylguanine-DNA methyltransferase | 10q26 | -1.91       |
| NM_001466           | FZD2   | Frizzled homolog 2 (Drosophila) | 17q21 | -1.91       |
| XM_49871            | LOC147272 | Hypothetical protein LOC147272 | 19p13 | -1.90       |
| NM_003562           | SLC15A11 | Solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11 | 17p13 | -1.88       |
| NM_004565           | PEX14  | Peroxisomal biogenesis factor 14 | 1q36 | -1.87       |
| NM_003955           | SOCS5  | Suppressor of cytokine signaling 3 | 17q25 | -1.87       |
| NM_007037           | ADAMTS8 | ADAM metallopeptidase with thrombospondin type 1 motif, 8 | 11q24 | -1.85       |
| NM_022479           | WBSRN17 | Williams-Beuren syndrome chromosome region 17 | 7q11 | -1.84       |
mined that 615 of these 831 genes (74%) are associated with CpG islands (CGIs). This is a substantially higher fraction than what has been estimated for the human genome overall (40%) [22], and suggests that, as expected, CGI-associated genes are especially sensitive to 5-aza-dC treatment. Only slightly more than half (465 or 56%) of the 831 5-aza-dC-responsive genes were significantly upregulated after 5-aza-dC treatment as would be expected if they had been hypermethylated in the cancer cell. The explanation as to why the remaining 366 genes displayed a significant decrease in expression after 5-aza-dC treatment is unknown but could, in part, be due to hypomethylated CpG-associated genes being accessed by repressor proteins or to indirect regulatory effects (e.g., non-CpG-associated genes responding to an upregulated repressor protein) or to some unknown gene toxic effect of the treatment. Regardless, we can conclude that up to nearly half of the 5-aza-dC-responsive genes in the OVCAR 3 cells may not have been directly responding to 5-aza-dC-induced hypomethylation. On the other hand, support for the hypothesis that the majority of those genes upregulated in the cell line after treatment are directly
responding to 5-aza-dC-induced hypomethylation comes from the fact that 5 of the 6 upregulated genes that we directly tested were indeed hypomethylated in response to the 5-aza-dC treatment.

To evaluate the possible relevance of the results from the cell line experiment to actual changes in gene expression that occur during ovarian cancer development, we correlated the OVCAR 3 expression profiles with those from an ovarian cancer tissue analysis recently completed in our laboratory. We reasoned that if methylation dependent changes in gene expression are occurring in ovarian cancer development, at least a subset of the genes upregulated in response to 5-aza-dC treatment in OVCAR 3 cells should display lower levels of expression in ovarian cancer cells relative to controls. Of the 465 genes upregulated in the OVCAR 3 experiment, 102 (22%) were found to be downregulated in the ovarian cancer cells isolated from tumor samples. Since 102 genes is a small fraction (~0.02%) of the ~5000 genes that were down-regulated in ovarian cancer cells and that we estimate that nearly half of these are associated with CGIs (data not shown), it seems unlikely that monitoring the gene expression response of OVCAR 3 cells to 5-aza-dC is an effective way to identify all candidate genes that may experience functionally significant changes in methylation over cancer development. How-

**Figure 3**

Methylation analysis of the EMP3, CAV1 and CXCL6 genes in ovarian cancer (EOC) and control (NOSE) tissues. Summary of sodium bisulfite sequencing analysis of CGIs associated with the EMP3, CAV1 and CXCL6 genes in ovarian ovarian cancer and control (NOSE) tissues. For EMP3, 16 clones for 4 cancer samples and 23 clones for 5 control (NOSE) samples were sequenced. For CAV1, 17 clones for 4 cancer samples and 18 clones for 4 control (NOSE) samples were sequenced. For CXCL6, 19 clones for 4 cancer samples and 24 clones for 5 normal (NOSE) samples were sequenced. Each circle displays the percent methylation of all clones for a single CpG dinucleotide (open circle, 100% unmethylated; filled circle, 100% methylated). Average percent methylation for cancer (EOS) and control (NOSE) samples is shown next to each row of circles. (Vertical bars, CpG dinucleotides; arrow, position of the transcription start site; horizontal black rectangle, region amplified and sequenced after sodium bisulfite).
ever, we believe the approach may be useful in identifying a subset of genes that are most likely to experience functionally significant changes in methylation over cancer development. This approach can provide researchers with a useful method by which to identify a candidate set of genes for detailed epigenetic analysis. Our methylation analysis of randomly selected genes from the list of 102 overlapping genes, supports our conclusion.

Conclusion
We conclude that correlating genes that are upregulated in response to 5-aza-dC treatment of cancer cell lines with genes that are down-regulated in cancer cells may be a useful method to identify genes experiencing epigenetic-mediated changes in expression over cancer development.

Methods
Cell lines and cell culture
OVCAR-3 cells were grown in RPMI 1640 supplemented with 20% v/v heat-inactivated FBS (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Mediatech Inc., Herndon, VA), 10 mM HEPES buffer (Sigma, St. Louis, MO) penicillin (100 U/ml), streptomycin (100 μg/ml), 1 mM sodium pyruvate (Mediatech Inc.), and 0.01 mg/ml bovine insulin (Sigma).

5-aza-dC treatment
OVCAR-3 cells were plated at a concentration of 1 × 10^6 in 10 cm² cell culture dishes. Cells were allowed to adhere to the plates overnight, and the medium was replaced the next morning. Five μM 5-aza-deoxycytidine (Sigma) or PBS was added to the medium and the plates were incubated for 3 days at 37°C in a 5% CO² atmosphere. Each treatment was done in triplicate.

Microarray
OVCAR-3 cells were washed once in the plate with HBSS prior to the extraction of RNA and DNA by established techniques (Trizol). The integrity of the RNA was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Biotin labeled cRNA was synthesized, hybridized to Affymetrix U133 Plus 2.0 oligonucleotide arrays and analyzed with a GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA).

Microarray data analysis
CEL files generated by the Affymetrix Gene Chip Operating System (GCOS) were converted to expression level values using the GCRMA version1.1.0 (ref. below) package implemented from within the Spotfire DecisionSite for Microarray Analysis (DSMA). Probe set intensities

### Table 3: Primers used for RT-PCR and Sodium Bisulfite sequencing

| Gene   | Primer   | Sequence                        | Size  | Tm  | Ref. |
|--------|----------|---------------------------------|-------|-----|------|
| CAV1   | RT-PCR_F | 5'-CGACCCTAAAACACCTCAACGATG-3' | 278   | 55°C| [15] |
|        | RT-PCR-R | 5'-GCAGACGCAAGCGGATACGAC-3'    |       |     |      |
| MOD_F  | 5'-TGTGTATTTTGGAAATATGGGTATAATTCGG-3' | 537   | 58°C| [15] |
| MOD_R  | 5'-CATTTCCTCCTACTCTAAGACAC-3'     |       |     |      |
| SFN    | RT-PCR_F | 5'-GTGTGTCAGCCCAAGCCATGG-3'    | 161   | 60°C| [23] |
|        | RT-PCR-R | 5'-ACCTTCCTCCCTACTCCAG-3'      |       |     |      |
| CST6   | RT-PCR_F | 5'-AAAGACAGGCTCAGTGGAAGA-3'    | 163   | 60°C| [12] |
|        | RT-PCR-R | 5'-CCGGGACCTTTACACAGCTCC-3'    |       |     |      |
| MOD_F  | 5'-AGTTGGAATGTTGAGTGGTGT-3'      | 430   | 55°C| [12] |
| MOD_R  | 5'-CCGGACACACAACTACCA-3'         |       |     |      |
| MAGEA3 | RT-PCR_F | 5'-TGGAGGACGACGACCGCC-3'       | 725   | 68°C| [16] |
|        | RT-PCR-R | 5'-CGGAGGATTATACGAGGACCGTCG-3' |       |     |      |
| MOD_F  | 5'-AGATTGTGTGAGGAGGAGTGAAGGT-3'  | 405   | 60°C| [16] |
| MOD_R  | 5'-AACCCGAGACAAACTACACTAAAC-3'   |       |     |      |
| EMP3   | RT-PCR_F | 5'-TGCTCTCCCTCATTCTCCTGTCG-3'  | 274   | 60°C| [17] |
|        | RT-PCR-R | 5'-GCCGCTGATGGATGGATGGATG-3'   |       |     |      |
| MOD_F  | 5'-TAGTATATAGGAGGAGGAGAG-3'      | 340   | 62°C| [17] |
| MOD_R  | 5'-CCTCCCAAACACTACACTCCCA-3'     |       |     |      |
| CXCL6  | RT-PCR_F | 5'-CTCTGAGAGAGGGCAAGG-3'       | 135   | 60°C|      |
|        | RT-PCR-R | 5'-GACCTCAMGTCCTGTGATGT-3'     |       |     |      |
| MOD_F  | 5'-GAGGGATGATGAGGATGAGGAG-3'     | 616   | 60°C|      |
| MOD_R  | 5'-AACTCCCAAATCCCAACAACTACCTTT-3' |       |     |      |
| GAPDH  | RT-PCR_F | 5'-GAAATCCCATCACCATCTCC-3'     | 312   | 56°C|      |
|        | RT-PCR-R | 5'-ATGAGTCTCCCTCACAGTACAAAG-3' |       |     |      |

Size, product size in bp; Tm, annealing temperature
were filtered with DSMA using a modulation threshold of 5 to include only those probe sets with at least a log base 2 expression value of ≥ 5. Differentially expressed probe sets were identified using the t-test function of the Profile Anova Tool of DSMA. SAM (Significance Analysis of Microarrays v.2.21) was used to calculate fold change values for the differentially expressed probe sets [29]. Annotations for probe sets were obtained from the NetAffx website [30].

RT-PCR analysis
1 μg of total RNA was reverse transcribed using oligo (dT) primers from the Superscript III cDNA synthesis kit (Invitrogen). PCR was performed in an Opticon 2 (MJ Research) using 1 μl of synthesized cDNA. Primer sequences and expected length of the products are provided in Table 3. Gene expression was normalized to the expression of the housekeeping gene GAPDH.

DNA methylation analysis
Genomic DNA, from 5-aza-dC treated and control OVCAR 3 cells and NOSE brushings and ovarian cancer tissues, was treated with sodium bisulfite to convert unmethylated cytosines to thymines. The EZ DNA Methylation-Gold Kit (Zymo, Orange, CA) was used to modify unmethylated cytosines to thymines. The EZ DNA Methylation analysis
Genomic DNA, from 5-aza-dC treated and control OVCAR 3 cells and NOSE brushings and ovarian cancer tissues, was treated with sodium bisulfite to convert unmethylated cytosines to thymines. The EZ DNA Methylation-Gold Kit (Zymo, Orange, CA) was used to modify unmethylated cytosines to thymines. The EZ DNA Methylation (Invitrogen). PCR was performed in an Opticon 2 (MJ Research) using 1 μl of synthesized cDNA. Primer sequences and expected length of the products are provided in Table 3. PCR fragments were gel extracted using the Tissue DNA Extraction Kit (Qiagen) and cloned into pCR 2.1-TOPO TA cloning vectors (Invitrogen). For the OVCAR 3 treated and untreated cell lines, 5 clones each were extracted using the Qiaprep Spin Miniprep Kit (Qiagen) and sequenced (University of Georgia, DNA sequencing Facility). Eighteen to 24 clones from 4 × 5 NOSE (control) samples and 16–19 clones from four cancer samples were sequenced by High-Throughput Sequencing Solutions at the University of Washington.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
LM carried out the methylation analyses and RNA extractions of OVCAR-3 cells, participated in the 5-aza-dC treatments and the microarray data analyses and drafted the manuscript. DW carried out the methylation analyses on normal (NOSE) and ovarian cancer (EOC) samples and assisted in the draft of the manuscript. LVM conducted the microarray analyses, EBD participated in the cell culture and 5-aza-dC treatments. NJB contributed to the design of the study and participated in the microarray data analyses. NP contributed to the bioinformatics analysis of the data. BBB provided the ovarian tissue samples. IFM contributed to the design and coordination of the study and composed the final draft of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1
Overlap between genes displaying a significant (p ≤ 0.01) changes of expression in OVCAR-3 after 5-aza-dC treatment and genes down-regulated in ovarian cancer tissue samples. Fold change in gene expression is shown for 5-aza-dC treated relative to untreated OVCAR 3 cells and for ovarian cancer (EOC) relative to control (NOSE) patient samples. Also shown is the chromosomal location of each gene (Chr).

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Acknowledgements
We are grateful to Kim Totten and Nina Schubert for valuable assistance in the collection of tissue samples. This research was supported by grants from the Georgia Cancer Coalition.

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