Supporting Information

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SI Methods

Cell Culture and Reagents. A2780, MA148, and 1A9 cells were cultured in RPMI (Invitrogen) supplemented with 10% (vol/vol) FBS and 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen) and grown at 37 °C and 5% (vol/vol) CO_2. Control morpholino and miR-199a morpholino (sequence: CCTAACCAATGTGCAGACTACTGTA) and the transfection reagent Endoporter were purchased from Gene Tools. All transfections were performed according to the manufacturers' instructions.

Custom Micro-RNA Microarray. Custom miRNA microarray experiments and analyses were performed as previously described (1). Microarray data have been deposited to the Gene Expression Omnibus (GEO) database (National Center for Biotechnology Information GEO database accession no. GSE32313). Pearson correlation coefficients for array analysis of the same RNA samples were between 0.90 and 0.99, indicating good reproducibility. Changes in miR expression levels were confirmed by quantitative PCR (qPCR).

Cell Culture Immunohistochemistry/Immunofluorescence. Cells were grown in eight-well culture slides (BD Falcon) fixed in Phemo Buffer (Pipes (0.068 M), Hepes (0.025 M), EGTA (0.015 M), $MgCl_2$ (0.003 M), DMSO [10% (vol/vol)], pH 6.8), supplemented with 3.7% (wt/vol) formaldehyde, 0.05% glutaraldehyde, and 0.5% Triton X-100, for 10 min at room temperature. After three washes, cells were blocked overnight with 5% (wt/vol) BSA in TBS plus 1% Tween 20 (TBS-T). Antibody to hypoxia-inducible factor 1α (HIF-1α) (BD Transduction Laboratories) was diluted in 5% (wt/vol) BSA/TBS-T, and the cells were incubated overnight. Secondary antibody, goat anti-mouse Alexa Fluor 647 (Invitrogen), was diluted in 5% (wt/vol) BSA/TBS-T and applied for 2 h. Fluorescence images were captured using an Olympus FluoView 1000 BX2 Upright Confocal system.

The luciferase-HIF1α 3′-UTR construct was created by amplifying the 3′-UTR of HIF-1α and ligating to a luciferase reporter. Luciferase-HIF-1α mutant 3′-UTR was constructed using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) to delete the ACTGG (seed site of miR-199a) in the 3′-UTR of HIF-1α.

Luciferase Assays. Cells were grown to 70% confluence and transfected with the vectors described above along with pRL-SV40 *Renilla* luciferase construct (Promega) at a 10:1 ratio. Cell extracts were prepared 24 h after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) in a TD 20/20 Luminometer (Turner BioSystems).

Real-Time Cell Migration and Cell Attachment Assays. xCELLigence system from ACEA Biosciences was used. Experiments were performed according to published protocols from Roche. Briefly, for proliferation experiments, each well was seeded with 25,000 cells and hypoxia simulated with 500 μM desferrioxamine (DFX). Treatment with nondegradable HIF-1α was achieved by transfecting cells 24 h before seeding with vehicle or P-to-A HIF-1α. Measurements were taken every 10 min for 80 h. For the migration experiments, the bottom chamber of the CIM plate (Roche) was filled with serum-containing media and the top chamber was filled with vehicle or P-to-A HIF1α-transfected cells (75,000 per well) in serum-free media. For lysyloxidase (LOX) inhibition assays, 300 μM β-aminoproprionitrile (β-APN) were added to the top chamber. Hypoxia was simulated as above. Measurements were taken every minute for 24 h. For attachment assays, well surfaces were coated with laminin, fibronectin, or type 1 collagen (50 μg/mL) for 24 h before seeding with A2780-GFP or A2780-199 cells. Experiments proceeded as described for proliferation assays with measurements taken every 4 min. All experiments were run in quadruplicate.

Lentiviral Vectors and Cell Transduction. Vectors (pCDH-CMV-MCS-EF1-copGFP) encoding either green fluorescent protein (GFP) or mir-199a and GFP were packaged into VSV-G pseudotyped viral particles with the pPACKH1 Packaging Plasmid Mix in HEK-293TN cells (System Biosciences). Lipofectamine and PLUS Reagent (Invitrogen) were also used in the packaging process, for which we followed System Biosciences' published protocols. Supernatant containing the virus was collected, and A2780 cells were treated with 10 μg/mL polybrene (Chemicon) and the supernatant from either the viral GFP-treated HEK cells or the viral 199/GFP-treated cells. After 48 h, the cells were harvested and subjected to fluorescence-activated cell sorting to collect only those cells strongly expressing GFP.

In Vivo Tumor Models. All animal experiment protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota (Minneapolis, MN). For tumor-seeding visualization experiments, 3 million A2780-GFP or A2780-199 cells were injected intraperitoneally (i.p.) into 4-wk-old, female athymic mice. Imaging was conducted using the CRi Maestro in vivo imaging system. For tumor burden experiments, A2780-GFP or A2780-199 cells were injected into 20 mice per group as described above. After 1 wk, carboplatin (CalBioChem) was injected i.p. to one-half of the animals (10 A2780-GFP mice and 10 A2780-199 mice) at a dose of 20 mg/kg every 4 d for 4 wk. Animals were then killed and imaged as above, and tumors were completely removed for weighing and snap frozen for RNA work and formalin-fixed for immunohistochemistry.

Tissue Immunofluorescence/Immunohistochemistry. Tumors were fixed in formalin and serially sectioned and paraffin embedded by Josh Parker and colleagues in the Veterinary Population Medicine Department (University of Minnesota, Minneapolis), who also performed the hematoxylin and eosin staining procedures. Processed (deparaffinized/antigen-retrieved) slides were blocked in 5% (wt/vol) BSA/TBS-T overnight. Blocked sections were then incubated for 6 h with biotinylated tomato lectin (Vector Laboratories), washed, and incubated with streptavidin-Texas Red (Vector Laboratories). DAPI (Invitrogen) was applied overnight and the slides were mounted for confocal analysis. Images were acquired as described above. Vessel density and length were measured as previously described (2).

Second Harmonic Generation Confocal Microscopy. Frozen sections (20 μm) of tumors were analyzed under FlowView 300 second harmonic generation using Mai Tai 80-fs, 5-W, 780- to 920-nm pulsed IR laser (University Imaging Center) (3).

1. Zhang X, Xu W, Tan J, Zeng Y (2009) Stripping custom microRNA microarrays and the lessons learned about probe-slide interactions. *Anal Biochem* 386(2):222–227.
2. Wild R, Ramakrishnan S, Sedgewick J, Griffioen AW (2000) Quantitative assessment of angiogenesis and tumor vessel architecture by computer-assisted digital image analysis: Effects of VEGF-toxin conjugate on tumor microvessel density. *Microvasc Res* 59(3):368–376.
3. Provenzano PP, et al. (2006) Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med* 4(1):38.

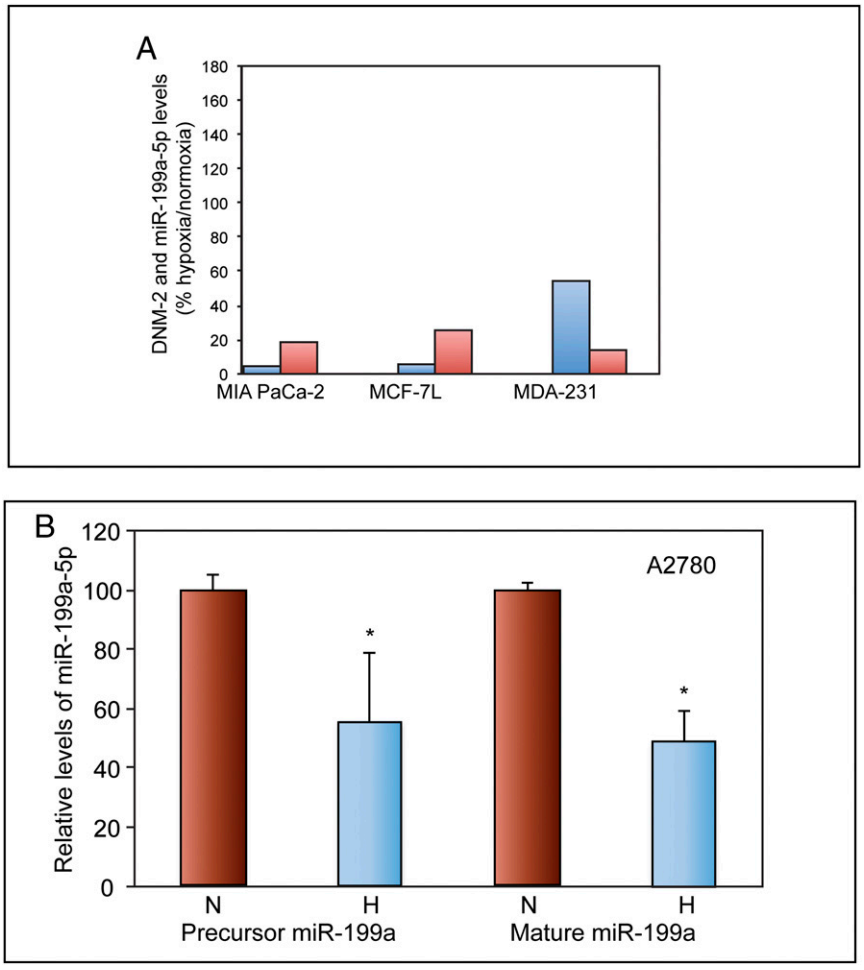

Fig. S1. (*A*) Hypoxia-induced changes in DNM2 and miR-199a-5p expression in breast and pancreatic cancer cell lines. Tumor cell lines were exposed to hypoxia for 24 h. Relative transcript levels and miR-199a-5p were determined by qPCR. β-Actin or GAPDH was used for normalizing the transcript levels of DNM2. SnoRNA, SNORD72.1, and U6 (Qiagen) were used for normalizing miR-199a expression. Each value is a mean of triplicates from three independent experiments. (*B*) Relative expression of precursor and mature miR-199a in A2780 cells cultured either in normoxia or hypoxia for 24 h. Specific primers (Qiagen) were used to determine precursor and mature microRNAs. U6 was used for normalization.

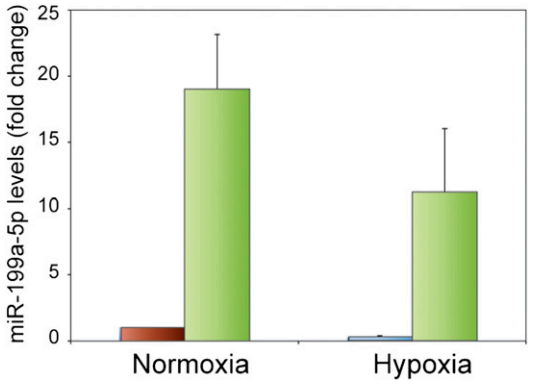

Fig. S2. miR-199a expression is increased in epithelial ovarian cancer cells (EOCCs) following treatment with miR-199a duplex. Levels of miR-199a-5p as determined by qPCR in A2780 cells transfected with scramble duplex (red and blue bars) or miR-199a duplex (green bars) and subjected to normoxia or hypoxia for 24 h. Normoxic, scramble duplex-treated levels are considered to be equal to 1. Values represent mean ± SD. $*P < 0.05$.

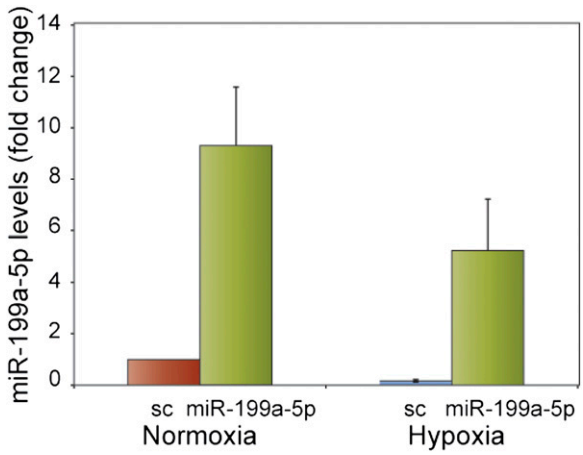

Fig. S3. miR-199a expression is increased in EOCCs following transduction with mir-199a–endcoding lentivirus. Levels of miR-199a-5p as determined by qPCR in A2780 cells transduced with GFP lentivirus (red and blue bars) or miR-199a/GFP lentivirus (green bars) and subjected to normoxia or hypoxia for 24 h. Normoxic, scramble duplex-treated levels are considered to be equal to 1. Values represent mean ± SD. $*P < 0.05$.

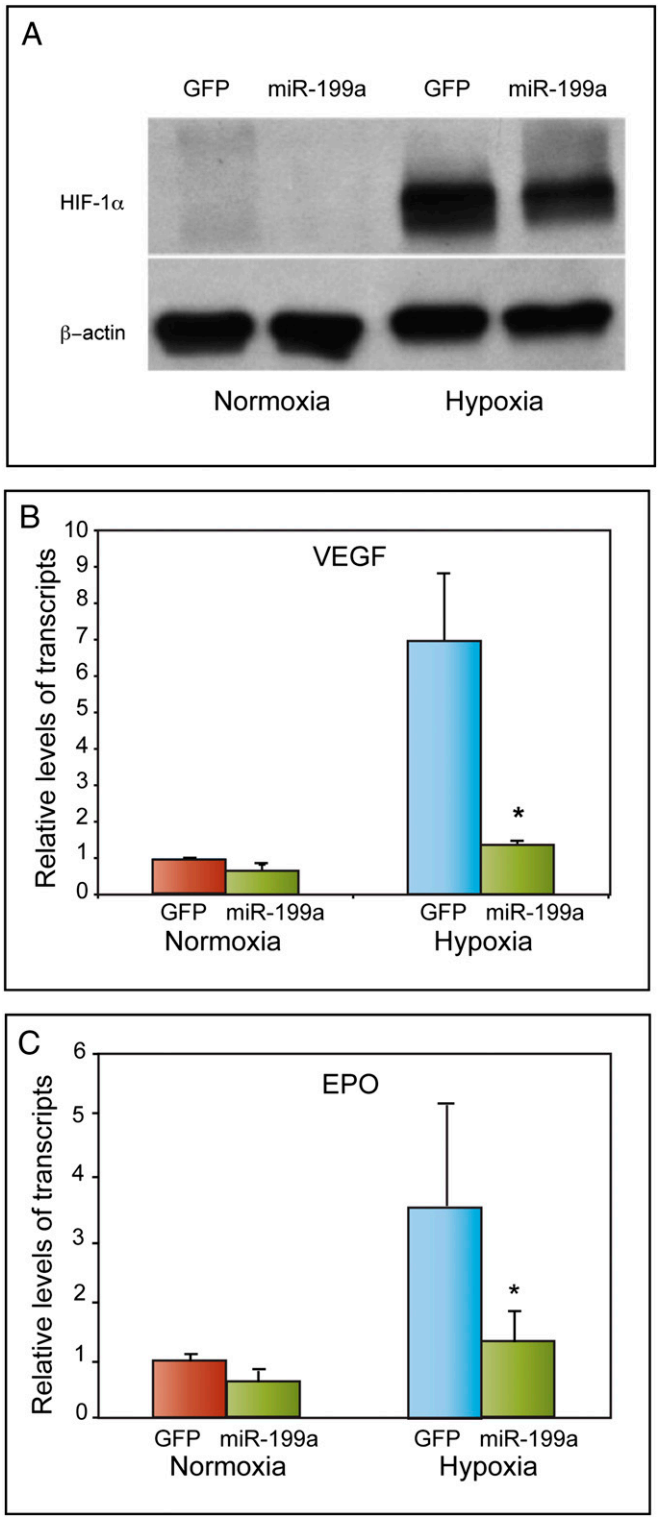

Fig. S4. miR-199a decreases hypoxia-induced stabilization of HIF-1α and HIF targets in EOCC. (*A*) A2780 cells transduced with either control lentivirus (GFP) or miR-199a-5p were exposed to normoxia or hypoxia for 24 h. Changes in HIF-1α were determined by Western blots. Actin was used for loading control. A representative blot is shown. In parallel experiments, total RNA was extracted and used in real-time PCR assays to determine changes in transcript levels of HIF targets. (*B*) miR-199a-5p expression attenuates hypoxia-induced increase in VEGF levels in A2780 cells both in normoxia and hypoxia. (*C*) In addition to VEGF, EPO transcript levels were also inhibited by miR-199a-5p.

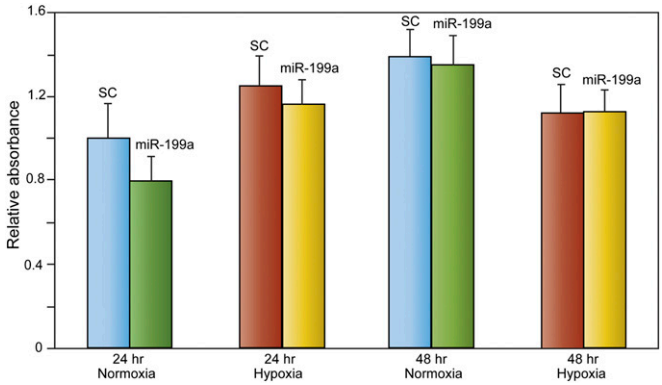

Fig. S5. miR-199a effect on EOCC proliferation. Bromodeoxyuridine incorporation assays comparing A2780 cells transduced with GFP lentivirus or miR-199a/GFP lentivirus subjected to 24 or 48 h of normoxia or hypoxia as indicated. Normoxic, scramble duplex-treated levels are considered to be equal to 1. Values represent mean ± SD.

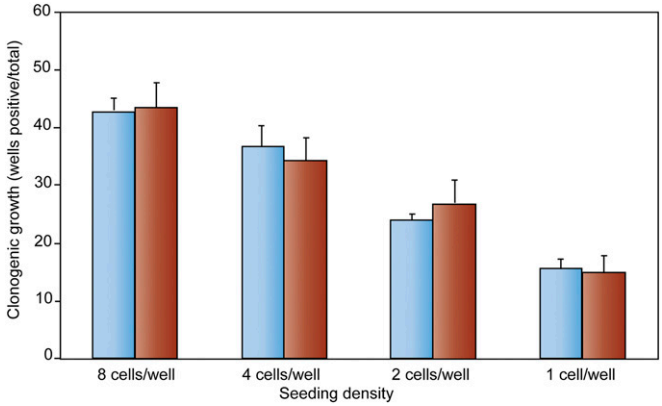

Fig. S6. Quantification of three separate clonogenic assays in which A2780 cells were transduced with GFP lentivirus (red bars) or miR-199a/GFP lentivirus (blue bars) and seeded at eight, four, two, or one cells per well as indicated. Values represent mean ± SD.

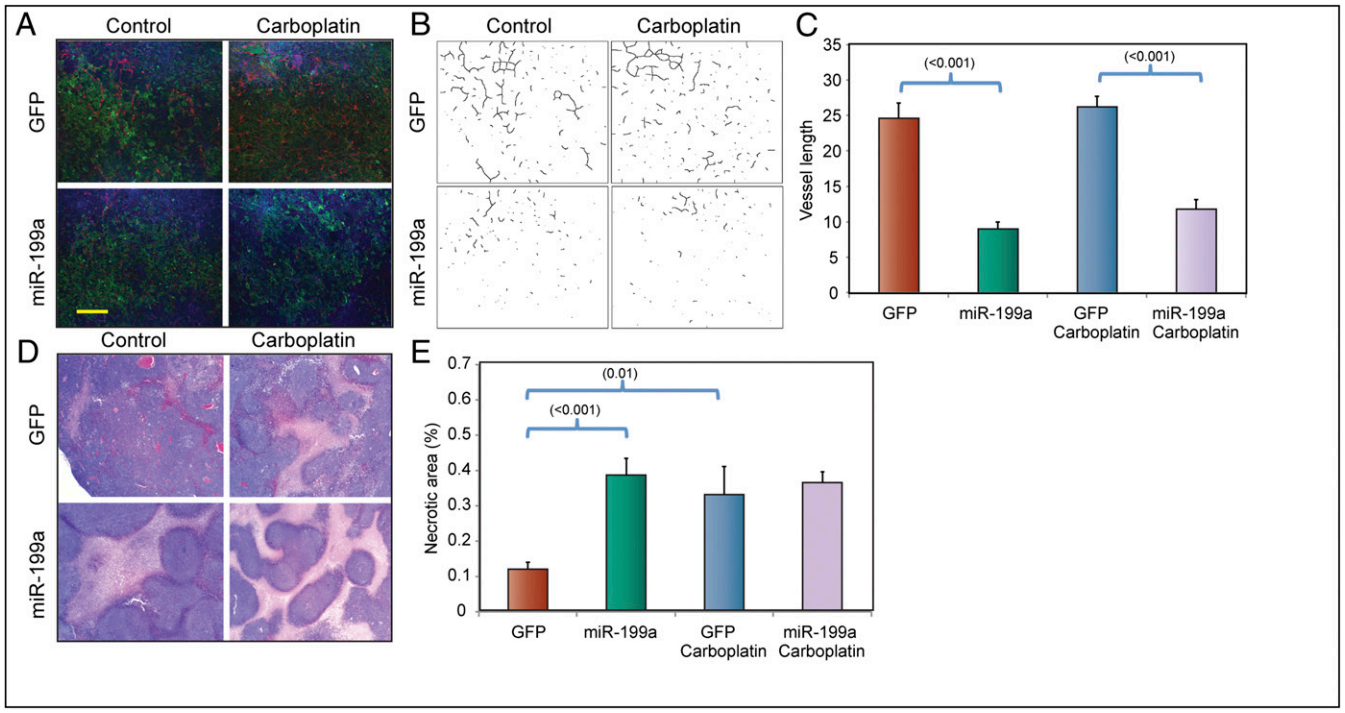

Fig. S7. (*A*) Representative images of tumor vasculature from mice injected intraperitoneally (i.p.) with GFP (*Upper*) or miR-199a/GFP (*Lower*) lentivirus-expressing A2780 cells without (*Left*) or with (*Right*) carboplatin treatment. Tumor sections were fixed in formalin and stained with lectin (red), an endothelial marker of vasculature, and DAPI (blue). (*B*) Skeletonization of lectin staining from *A*, showing method used to quantify total vessel length per field. (*C*) Quantification of vessel length based on skeletonization of lectin-stained tumor sections. (*D*) Representative images of hematoxylin and eosin-stained tumor sections are shown. The pink areas indicate areas of tumor necrosis, and the purple areas show viable tissue. (*E*) Quantification of necrotic areas compared with total tumor area from mice treated with carboplatin. Values represent mean ± SD. *P* values are as indicated in parentheses.

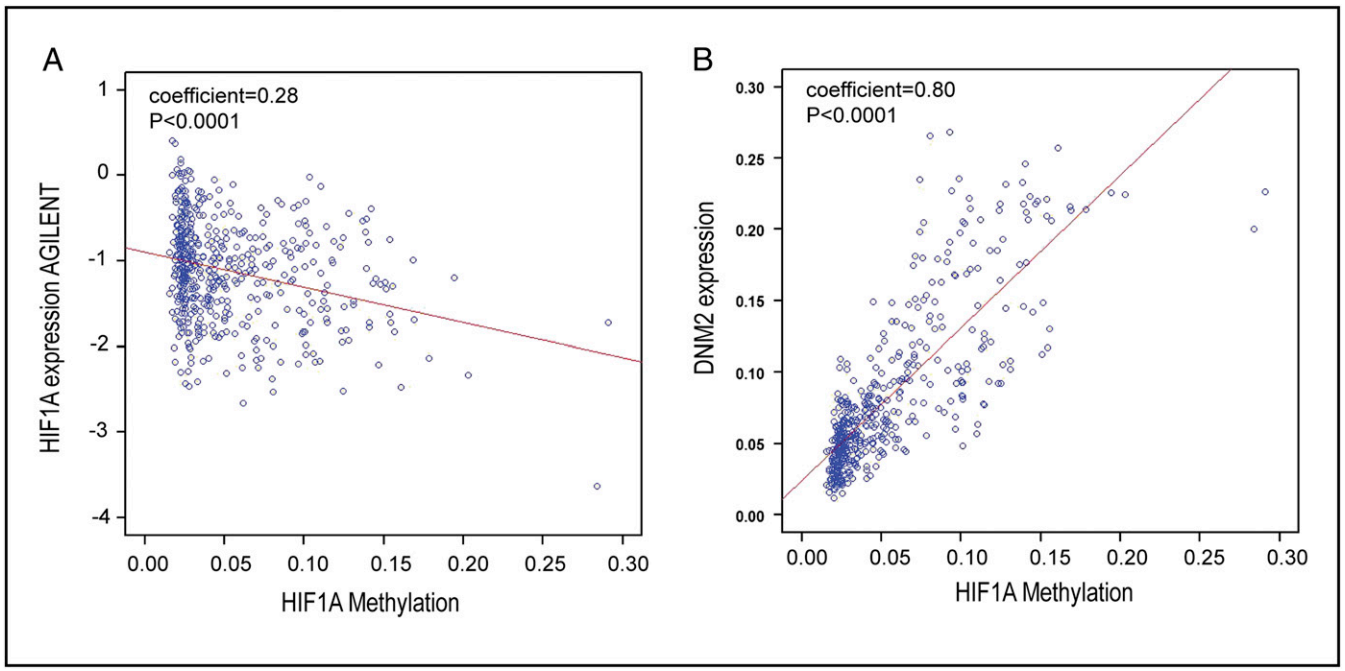

Fig. S8. Correlation between HIF1A promoter methylation and DNM2 expression. To validate reciprocal regulation in clinical settings, we analyzed the Cancer Genome Database for HIF-1 α and DNM2. (*A*) Data show that HIF-1α expression in tumor tissues negatively correlated with methylation status of its promoter ($P < 0.0001$). (*B*) Methylation of the HIF-1α promoter positively correlated (coefficient = 0.80, $P \leq 0.0001$) with DNM2 expression in ovarian cancer.