Angiostatic activity of DNA methyltransferase inhibitors

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Abstract

Inhibitors of DNA methyltransferases (DNMT) and histone deacetylases can reactivate epigenetically silenced tumor suppressor genes and thereby decrease tumor cell growth. Little, however, is known on the effects of these compounds in endothelial cell biology and tumor angiogenesis. Here, we show that the DNMT inhibitors 5-aza-2′-deoxycytidine and zebularine markedly decrease vessel formation in different tumor models. We show that DNMT inhibitors are antiproliferative for tumor-conditioned endothelial cells, without affecting endothelial cell apoptosis and migration. Furthermore, these compounds inhibit angiogenesis *in vitro* and *in vivo* as shown by inhibition of endothelial cells sprouting in a three-dimensional gel and inhibition of microvessel formation in the chorioallantoic membrane, respectively. 5-Aza-2′-deoxycytidine, as well as the histone deacetylase inhibitor trichostatin A, reactivates the growth-inhibiting genes *TSP1, JUNB*, and *IGFBP3*, which are suppressed in tumor-conditioned endothelial cells. Despite enhanced DNMT activity and increased overall genomic methylation levels in tumor-conditioned endothelial cells, silencing of these genes seemed not to be regulated by direct promoter hypermethylation. For *IGFBP3*, gene expression in endothelial cells correlated with histone H3 acetylation patterns. In conclusion, our data show that DNMT inhibitors have angiostatic activity in addition to their inhibitory effects on tumor cells. This dual action of these compounds makes them promising anticancer therapeutics. [Mol Cancer Ther 2006;5(2):467–75]

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Introduction

Epigenetic regulation of gene expression by DNA methylation and histone modifications involves the organization of chromatin in gene promoter regions, thereby affecting transcriptional activator complexes (1). These phenomena are essential in many biological processes, including genomic imprinting, X chromosome inactivation, and establishment of tissue-specific gene expression (2). Epigenetic modifications are also involved in pathology; aberrant epigenetic regulation has been observed in cancer cells and includes alterations in DNA methylation and histone modifications (3–5). DNA hypermethylation and histone deacetylation of CpG islands within the promoter regions of tumor suppressor genes result in undesirable gene silencing and are found in virtually every type of human cancer (6, 7). In contrast to genetic modifications, epigenetic changes are reversible, creating a target for therapeutic strategies in cancer. It has been shown that DNA methyltransferase (DNMT) as well as histone deacetylase (HDAC) inhibitors can reactivate epigenetically silenced tumor suppressor genes and decrease tumor cell growth *in vitro* and *in vivo* (8, 9). Because of these characteristics, these drugs are currently being tested in clinical trials (9, 10).

Tumor angiogenesis, a pivotal process in cancer, requires intricate regulation at the molecular level (11, 12). The rapid identification of novel genes involved in the generation of new vasculature is expected to contribute to the understanding of tumor angiogenesis (13–15). Little, however, is known about the role of epigenetics in tumor angiogenesis. Effects of DNMT inhibitors on endothelial cell biology and tumor angiogenesis have not been described thus far. Furthermore, there are no reports on epigenetic modifications of gene promoters in tumor endothelial cells during tumor angiogenesis. A link between HDAC inhibitors and angiogenesis has recently been suggested (16–18). In this study, we investigated the effects of DNMT inhibitors on endothelial cell biology and angiogenesis *in vitro* and *in vivo*. Furthermore, overall genomic methylation levels and DNMT activity, as well as epigenetic promoter modifications of growth-inhibitory genes, are studied in tumor-conditioned and quiescent endothelial cells.

Materials and Methods

Cell Cultures and Reagents

Human umbilical vein endothelial cells (HUVEC) were cultured in RPMI 1640 supplemented with 20% heat-inactivated human pooled serum, 2 mmol/L L-glutamine, 50 ng/mL streptomycin, and 50 units/mL penicillin in

0.2% gelatin-coated tissue culture flasks at 37°C, 5% CO_2. Tumor conditions were mimicked (19) by a 3-day exposure to 10 ng/mL basic fibroblast growth factor (bFGF; Peprotech, London, United Kingdom), 10 ng/mL vascular endothelial growth factor (VEGF; Peprotech), and, where indicated, 20% (v/v) of a 1:1 mixture of filtered culture supernatants of LS174T and CaCo-2 human colon carcinoma cell lines. Quiescent endothelial cells were prepared by culturing HUVEC for 3 days in the presence of 2% serum.

Mouse b.END5 brain endothelioma cells (European Collection of Animal Cell Cultures, Salisbury, United Kingdom) were cultured in DMEM containing 10% FCS, 2 mmol/L L-glutamine, and 5 μmol/l 2-mercaptoethanol (Sigma, St. Louis, MO). Bovine capillary endothelial cells were kindly provided by Dr. M. Furie (State University of New York, Stony Brook, NY) and cultured in gelatin-coated flasks in MEM-α supplemented with 10% FCS, 2 mmol/L L-glutamine, and antibiotics. Mouse B16F10 melanoma cells (kindly provided by Dr. J. Fidler, Houston, TX) were cultured using Hank's MEM containing 5% FCS, 1% nonessential amino acids, 1% sodium pyruvate, 1.5% MEM vitamins, and 2% sodium bicarbonate. Human LS174T colon tumor cells were grown in DMEM containing 10% FCS and 2 mmol/L L-glutamine. All culture media and standard cell culture materials were obtained from Life Technologies (Breda, the Netherlands).

DNMT inhibitor 5-aza-2′-deoxycytidine (DAC) was obtained from Sigma (Zwijndrecht, the Netherlands), zebularine was obtained from the National Cancer Institute (Bethesda, MD), and the HDAC inhibitor trichostatin A (TSA) from Wako (Neuss, Germany).

Mouse Tumor Models

The animal experiments were approved by the local ethical review committee. At day 0, 6-week-old C57BL/6 mice (obtained from Charles River, Maastricht, the Netherlands) were inoculated with 10^5 B16F10 mouse melanoma cells s.c. on the right flank. Between days 6 and 9, the tumors became visible in all mice and treatments were initiated. In the LS174T xenograft model, Swiss *nu/nu* mice (Charles River) were inoculated with 10^6 LS174T human colon carcinoma cells. Between days 10 and 14, the tumors became visible and treatment was initiated. DAC ($n = 5$; 10 mg/kg), zebularine ($n = 5$; 1,000 mg/kg; ref. 20), and TSA ($n = 5$; 1 mg/kg; ref. 16) were given daily by i.p. injection in a solution of 0.9% saline for 7 (B16F10) or 10 (LS174T) days. Tumor volumes were measured daily and calculated as follows: width^2 × length × 0.52. The microvessel density was analyzed as described previously (21).

Proliferation and Apoptosis Measurement

Endothelial cell proliferation was measured using a [^{3}H]thymidine incorporation assay as described previously (22). Tumor-conditioned HUVEC, cultured in a 96-well plate, were exposed for 3 days to a concentration range DAC, zebularine, or TSA, replacing drugs and culture medium every 24 hours. During the last 6 hours of the assay, the culture was pulsed with 0.3 μCi [methyl-^{3}H]-thymidine (Amersham Life Science, Roosendaal, the Netherlands) per well. Activity was measured using liquid scintillation. Four independent experiments were done, and in each experiment, measurements were done in triplicate.

Apoptosis was measured as described previously (22). Tumor-conditioned HUVEC were cultured for 72 hours with DAC, zebularine, or TSA, replacing drugs and culture medium every 24 hours. Serum deprivation of HUVEC (3 days) was used as a positive control for apoptosis.

Migration Measurement

HUVEC migration was measured using the wound assay (21). In brief, confluent monolayers of tumor-conditioned HUVEC cultured for 72 hours with DAC, zebularine, or TSA were wounded using the blunt end of a glass pipette. Cultures were washed, and medium and drugs were replaced. Wound width was measured in triplicate cultures at four predefined locations at start and at 2, 4, 6, 8, and 24 hours after wounding.

In vitro Angiogenesis

Sprouting and tube formation of bovine capillary endothelial cells was studied using Cytodex-3 beads overgrown with endothelial cells in a three-dimensional gel as described previously (22). Bovine capillary endothelial cells were mixed with gelatin-coated Cytodex-3 microcarrier beads (Sigma, Zwijndrecht, the Netherlands) and cultured for 48 hours in the presence of bFGF, VEGF, CaCo-2, and LS174T supernatants followed by a 3-day exposure to DAC, zebularine, or TSA, replacing drugs and culture medium every 24 hours. Next, the beads were placed in a three-dimensional gel and medium, containing 10 ng/mL bFGF, 10 ng/mL VEGF, and 20% of a 1:1 mixture of culture supernatants of LS174T and CaCo-2 human colon carcinoma cells, with or without DAC, zebularine, or TSA at concentrations as indicated, was applied on top of the gel. After 24 hours, photographs were taken and digitally analyzed.

Chorioallantoic Membrane Assay

The chorioallantoic membrane (CAM) assay was done in fertilized White Leghorn eggs as described previously (22). In brief, CAMs were treated by daily addition of sterile saline (0.9% NaCl), DAC (5 mmol/L), zebularine (100 mmol/L), or TSA (400 μmol/L) from days 10 to 13. The data from the *in vitro* assays, where extensive dose ranges were tested, as well as literature data have been used to extrapolate to testing in the CAM assay. For TSA, 400 μmol/L has been taken from literature (17). From this, we calculated a 10 times higher dose for DAC (as in the mice). For zebularine, a higher dose was used, which was found to be active already at 100 mmol/L. On day 14, the CAMs were photographed. Quantification of vascularization was done by enumeration of intersections with five concentric rings that were superimposed on the photographs.

High-Performance Capillary Electrophoresis

Tumor-conditioned HUVEC were treated for 72 hours with or without DAC, replacing drug and culture medium every 24 hours. Quantification of the degree of methylation was carried out as described previously (23). Quantification of the relative methylation of each DNA sample was

determined as the percentage of mC of total cytosines: mC peak area × 100 / (C peak area + mC peak area). Three analytic measurements were made per sample and experiments were done in duplicate.

Methyltransferase Assay and DNMT1 Western Blot

Tumor-conditioned HUVEC were treated for 72 hours with or without DAC, replacing drug and culture medium every 24 hours. DNMT assays were carried out as described previously (24). DNMT1 Western blot was done using rabbit polyclonal DNMT1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Quantitative Real-time Reverse Transcription-PCR

Tumor-conditioned HUVEC were treated for 72 hours with DAC or TSA, replacing drugs and culture medium every 24 hours. Total RNA isolation, cDNA synthesis, and quantitative real-time reverse transcription-PCR were done essentially as described previously (25) using SYBR Green PCR Master Mix (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands). Primer sequences are available on request.

Bisulfite Sequencing

Genomic DNA of quiescent HUVEC, tumor-conditioned HUVEC, or tumor-conditioned HUVEC treated with DAC for 72 hours (replacing DAC and medium every 24 hours) was isolated using the Wizard Genomic DNA Purification kit (Promega, Leiden, the Netherlands). Bisulfite modification of genomic DNA was carried out essentially as described previously (26). PCR products were cloned using the TA cloning kit (Invitrogen, Breda, the Netherlands) and single colonies were picked and sequenced. Primer sequences are available on request.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays on quiescent HUVEC, tumor-conditioned HUVEC, or tumor-conditioned HUVEC treated for 72 hours with DAC or TSA (replacing DAC, TSA, and medium every 24 hours) were done essentially as described previously (27) using anti-acetyl histone H3 antibody (Upstate Biotechnology, Lake Placid, NY). Primer sequences are available on request.

Statistical Analyses

All values are given as mean ± SE. Statistical analysis for the tumor volumes was done by the two-way ANOVA test. The Student's *t* test was used for statistical analyses of microvessel density levels in the mouse tumors and CAMs and for the migration assay. Statistical analyses of the proliferation, apoptosis and *in vitro* angiogenesis assays, DNMT activity assay, high-performance capillary electrophoresis, and quantitative real-time reverse transcription-PCR were done using the Wilcoxon-Mann-Whitney rank sum test, which was done in SPSS 10.0.5 software. All values are two sided and *P*s < 0.05 were considered statistically significant.

Results

DAC and Zebularine Inhibit Tumor Growth *In vivo*

To investigate the effects of DNMT inhibitors on tumor angiogenesis *in vivo*, B16F10 melanoma-bearing mice were treated with the DNMT inhibitors DAC or zebularine. Treatment of established tumors (~100 mm^3) with DAC (10 mg/kg i.p. daily) resulted in a significant abrogation of tumor growth ($P < 0.0001$), causing almost full stasis over the treatment period (Fig. 1A). The inhibitory activity of DNMT suppression on B16F10 tumor growth was confirmed by treatment with the DAC analogue zebularine (1,000 mg/kg i.p. daily; ref. 20), a compound recently found to have a similar functional activity but with a lower toxicity profile (Fig. 1A). Treatment of B16F10 tumors with the HDAC inhibitor TSA (1 mg/kg i.p. daily; ref. 16) also significantly inhibited tumor growth ($P < 0.0001$) by ~60%. The inhibitory effects of DAC and zebularine on growth of B16F10 tumors was associated with suppressed angiogenesis as suggested by significantly lower microvessel densities in tumors of treated mice (47% and 65% inhibition, respectively) compared with untreated control tumors (Fig. 1B and C; $P < 0.0001$). TSA treatment also significantly reduced microvessel density (52% inhibition; $P < 0.0001$) compared with untreated tumors (Fig. 1B and C),

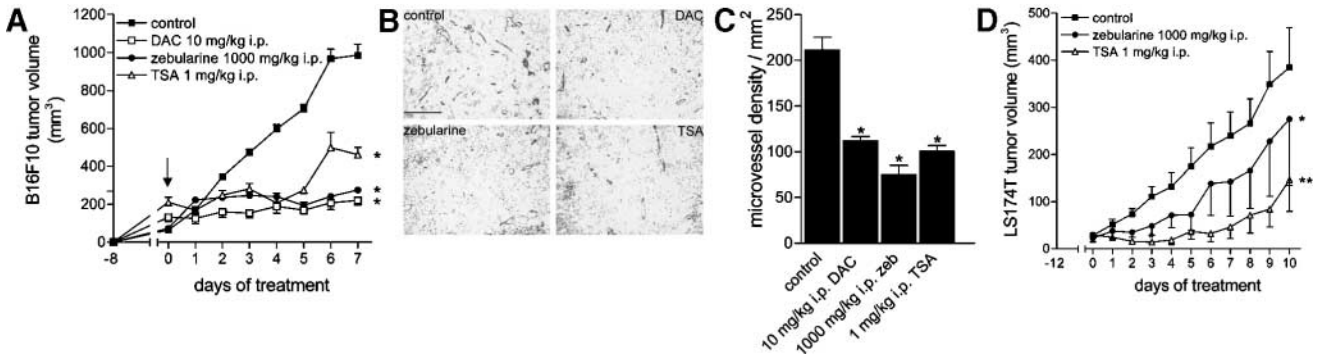

Figure 1. DAC, zebularine, and TSA inhibit tumor angiogenesis in mice. **A,** tumor growth inhibition of B16F10 mouse melanoma tumors in C57BL/6 mice by DAC, zebularine, and TSA treatment. *Points,* mean tumor volume (mm^3); *bars,* SE. *, $P < 0.0001$. *Arrow,* start of treatment. **B,** cryosections of tumors from control mice and treated mice stained with CD31 antibody for microvessel density assessment. *Bar*, 100 μm. **C,** quantification of microvessel density. *Columns,* mean number of vessels per mm^2; *bars,* SE. *, $P < 0.0001$. *zeb,* zebularine. **D,** tumor growth curves of human LS174T colon carcinoma in athymic mice either treated or not treated daily with zebularine (*, $P < 0.006$) or TSA (**, $P < 0.0001$).

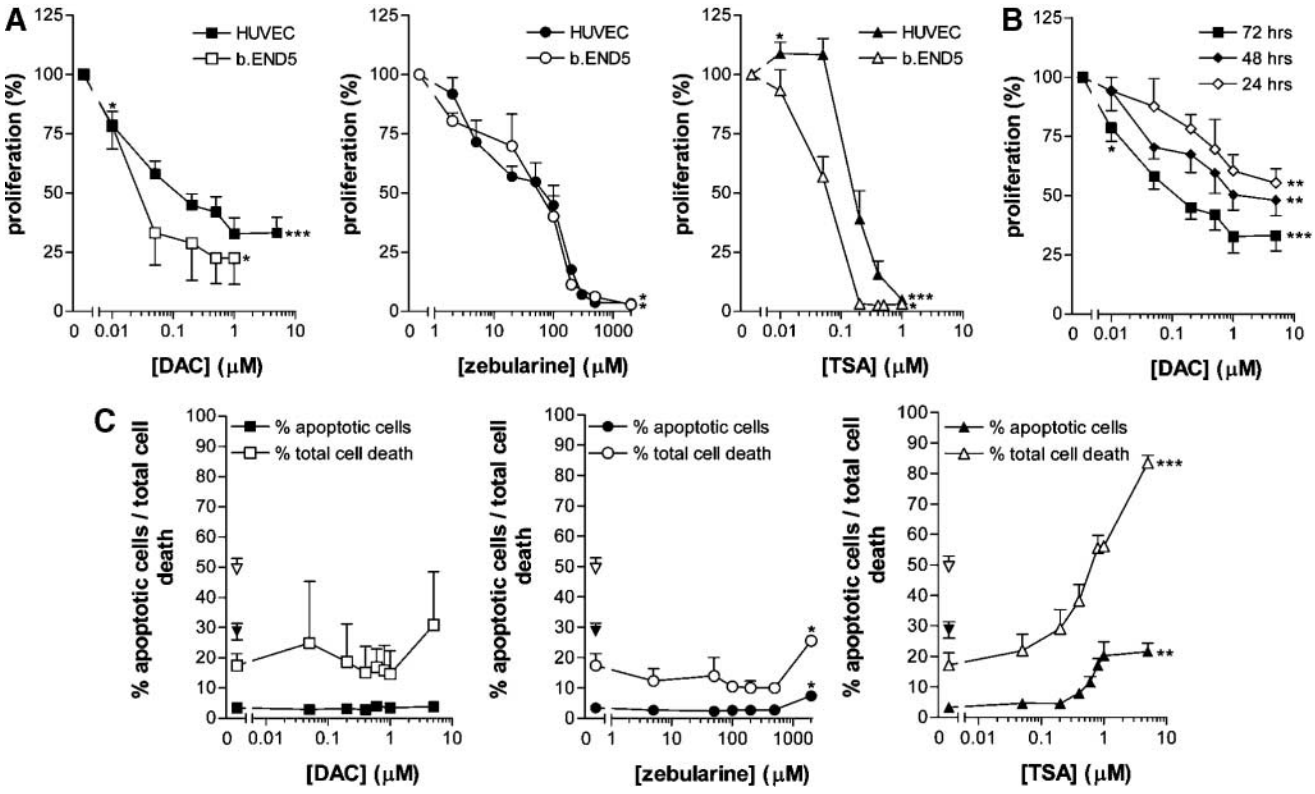

Figure 2. DAC, zebularine, and TSA inhibit endothelial cell growth characteristics. **A,** dose-response curves of DAC, zebularine, and TSA on growth factor – induced and spontaneous proliferation of HUVEC and b.END5 endothelioma cells, respectively, after 72 h of treatment. **B,** kinetic analysis of the response of tumor-conditioned HUVEC after 24, 48, and 72 h of treatment with DAC. *Points,* mean relative proliferation compared with untreated cultures of four independent triplicate experiments; *bars,* SE. *, $P < 0.037$; **, $P < 0.005$; ***, $P < 0.001$. **C,** dose-response curves of DAC, zebularine, and TSA on apoptosis (*solid symbols*) and total cell death (*open symbols*) of growth factor – stimulated HUVEC. HUVEC cultured in the presence of 1% serum was used as a positive control for apoptosis (▼, apoptosis; ▽, total cell death). *Points,* mean of three (DAC and zebularine) or six (TSA) independent triplicate experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.006$; ***, $P < 0.001$.

which confirms earlier data (16). Suppressive effects on angiogenesis and tumor growth by zebularine or TSA ($P < 0.006$ and $P < 0.0001$, respectively) were also observed in the human xenograft model of LS174T colon carcinoma in athymic mice (Fig. 1D). Although DAC in this model also inhibited tumor growth, the treatment was associated with toxicity, and the experiment was therefore halted.

DAC and Zebularine Inhibit Endothelial Cell Growth

Although an indirect effect of DNMT inhibitors on tumor angiogenesis *in vivo* can be expected due to inhibition of tumor cells, we explored whether these compounds have direct effects on endothelial cell growth. To that end, DAC and zebularine were tested for their ability to inhibit proliferation of activated cultured HUVEC using the [^{3}H]thymidine incorporation assay. Tumor conditions were mimicked by culturing cells in tumor-conditioned medium in the presence of bFGF and VEGF. DAC exhibited a concentration-dependent inhibition of HUVEC proliferation, with a half-maximal response (ED_{50}) at ~100 nmol/L (Fig. 2A). Similarly, zebularine also inhibited proliferation of activated HUVEC in a concentration-dependent way, although the effective concentrations of zebularine, which are standard concentrations (28), were ~100-fold higher than for DAC. Because inhibitory effects of HDAC inhibitors on endothelial cell growth have been described previously (16, 17), the HDAC inhibitor TSA was included as a positive control. As expected, TSA decreased endothelial cell growth, reaching an ED_{50} at ~200 nmol/L (Fig. 2A). Kinetic studies on the response of endothelial cells to DAC revealed that a 72-hour exposure resulted in stronger responses compared with treatment for 48 and 24 hours (Fig. 2B). This corresponds with the mechanism of action of this nucleoside analogue, which has to be incorporated into the DNA during replication before it can trap DNMTs during progression of the replication machinery (29). In contrast, TSA inhibited similarly at all time points (data not shown).

Antiproliferative effects of DAC, zebularine, and TSA were similar using HUVEC stimulated with bFGF or VEGF alone as well as in the human microvascular endothelial cell line (data not shown). In addition, DNMT and HDAC inhibitors had similar growth-inhibitory activity in b.END5 mouse endothelial cells (Fig. 2A). To assess the effect of DAC, zebularine, and TSA on proliferation of other non-neoplastic cell types, we analyzed the effects on proliferation of phytohemagglutinin-stimulated peripheral blood leukocytes. DAC had no significant effect on leukocyte

proliferation, whereas zebularine had a moderate inhibitory effect of 30% at 1 mmol/L ($P < 0.05$), which is minimal compared with the effect on endothelial cells (data not shown). In contrast, TSA significantly inhibited leukocyte proliferation with an ED_{50} at ~200 nmol/L ($P < 0.05$), which is comparable with effects on endothelial cells. Similar findings were observed for normal cultured fibroblasts (data not shown), suggesting that effects of DNMT and HDAC inhibitors are not specific for endothelial cells, as expected, although endothelial cells are more responsive to DAC and zebularine compared with blood leukocytes and normal fibroblasts.

To determine whether inhibition of endothelial cell growth was caused by inducing cell death, we quantified the percentage of dying cells in general as well as the percentage of cells undergoing apoptosis (21). At growth-inhibitory concentrations, DAC did not significantly affect endothelial cell apoptosis or total cell death as measured by the percentage of cells with subdiploid DNA content using flow cytometry (Fig. 2C). Similar results were observed for zebularine, although a small percentage of endothelial cells (7.5% compared with 3.4% of untreated cells) underwent apoptosis at the highest concentration tested ($P < 0.05$). In contrast to the cytostatic effect of the DNMT inhibitors, TSA caused a strong concentration-dependent cytotoxic effect, inducing apoptosis and total cell death (Fig. 2C), which might explain the stronger antiproliferative effect.

Effects of DNMT Inhibitors on Endothelial Cell Migration and Angiogenesis *In vitro* and *In vivo*

To assess the effects of DAC and zebularine on endothelial cell migration, the wound assay was used (21). Migration of endothelial cells was not significantly influenced by treatment with DAC at concentrations up to 1,000 nmol/L (Fig. 3A). Similar results were found for zebularine at concentrations up to 500 μmol/L. In contrast, TSA effectively inhibited migration of wounded confluent monolayers in a dose-dependent manner, which is in agreement with observations by Kim et al. (16). Significant effects ($P < 0.05$) were already observed 4 hours after wounding at 300 nmol/L concentration (Fig. 3A).

In a three-dimensional endothelial cell tube formation assay (22), DAC and zebularine dose-dependently inhibited growth factor–induced sprout formation of bovine capillary endothelial cells (Fig. 3B). TSA also showed a concentration-dependent inhibitory effect in this *in vitro* angiogenesis assay.

To study whether *in vivo* angiogenesis is perturbed by DAC and zebularine, we used the chick CAM assay, a model for developmental angiogenesis. In CAMs treated daily with DAC (5 mmol/L) from days 10 to 13, a profound inhibition (40%) of microvessel formation was observed, whereas larger preexisting vessels were apparently unaffected (Fig. 3C). These results were confirmed in zebularine-treated CAMs, in which maximal inhibition of microvessel formation was observed at 100 mmol/L concentration ($P < 0.023$). TSA also had angiostatic activity in the CAMs (32% inhibition of microvessel formation at 400 μmol/L; $P < 0.001$; Fig. 3C), as expected (17).

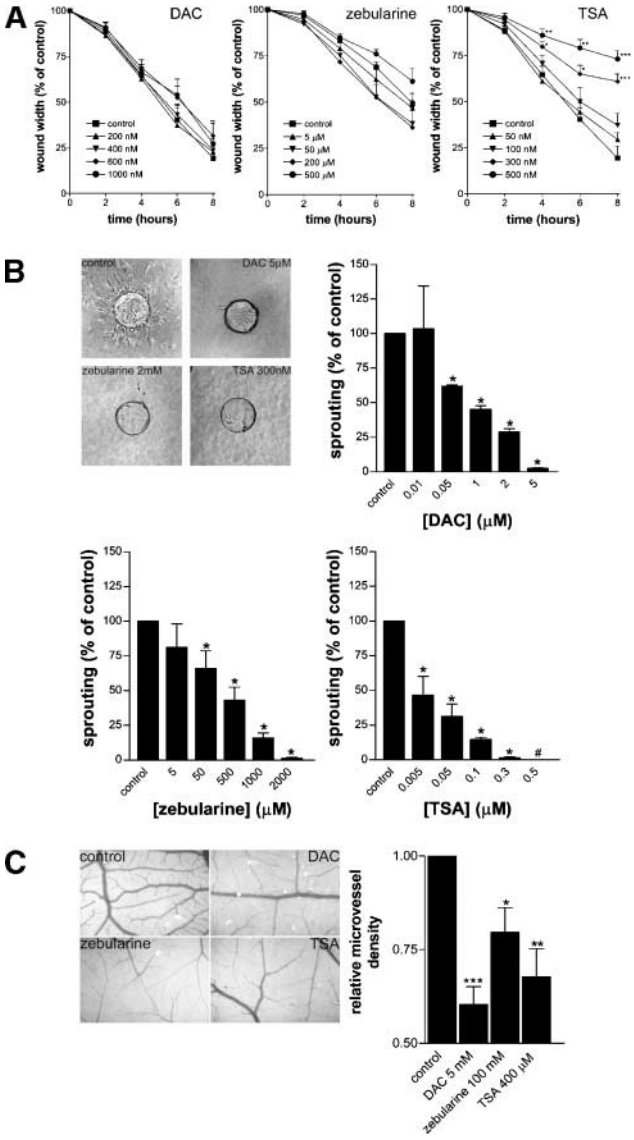

Figure 3. Effects of DAC, zebularine, and TSA on endothelial cell migration, angiogenesis *in vitro*, and chick CAM assay. **A,** relative wound width of dose ranges of DAC-, zebularine-, and TSA-treated cultures compared with untreated cultures. *Points,* mean of five independent experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **B,** sprouting of bovine capillary endothelial cells cultured on gelatin-coated Cytodex-3 beads into a collagen matrix. Sprout formation was induced by bFGF, VEGF, and tumor cell line conditioned medium (*control*). *Columns,* mean relative sprouting compared with untreated bovine capillary endothelial cells from three independent experiments; *bars,* SE. *, $P < 0.037$; #, $P < 0.046$. **C,** CAMs treated daily with saline (*control*), DAC, zebularine, or TSA from days 10 to 13. *Columns,* mean relative microvessel density of CAMs treated with DAC ($n = 7$), zebularine ($n = 4$), or TSA ($n = 5$); *bars,* SE. *, $P < 0.023$; **, $P < 0.001$; ***, $P < 0.0001$.

Increased 5-Methylcytosine Content and DNMT Activity in Tumor-Conditioned Endothelial Cells

Although altered DNA methylation levels have been studied in a variety of tumor cells, there are no reports on DNA methylation levels in tumor endothelial cells. Total

genomic 5-methylcytosine content in endothelial cells was quantified by high-performance capillary electrophoresis (23) in quiescent (HUVEC−) and tumor-conditioned (HUVEC+) endothelial cells. A significant hypermethylation was observed in activated tumor-conditioned HUVEC compared with quiescent HUVEC ($P < 0.004$; Fig. 4A). Furthermore, treatment of activated HUVEC with the DNMT inhibitor DAC at low dose (200 nmol/L) decreased genomic DNA methylation.

To examine whether the overall genomic hypermethylation in tumor-conditioned endothelial cells is caused by increased DNMT activity in these cells, we measured protein expression and activity of DNMT in activated and quiescent endothelial cells. HCT116 cells were used as a positive control (30). Overall, DNMT activity (Fig. 4B) and protein levels (data not shown) were lower in endothelial cells compared with the HCT116 tumor cell line. In activated endothelial cells, DNMT activity was significantly increased compared with quiescent endothelial cells (2.6-fold increase; $P < 0.05$), whereas DAC treatment almost completely eradicated DNMT activity in activated endothelial cells (Fig. 4B).

Reexpression of *IGFBP3, TSP1,* and *JUNB* in Activated Endothelial Cells by DNMTand HDAC Inhibitors through Methylation-Independent Effects

Although the inhibitory effects of DAC and zebularine on tumor angiogenesis *in vivo* can be indirect, via their effects on tumor cells, the inhibition of endothelial cell proliferation and angiogenesis *in vitro* by these compounds show that DNMT inhibitors directly affect endothelial cell growth and angiogenesis. We investigated whether these direct inhibitory effects could be explained by the reexpression of angiogenesis-inhibiting genes in activated endothelial cells by DNMT inhibitors. Screening the promoters of several well-known endogenous angiogenesis inhibitors (IFN-α/β, platelet factor-4, TSP1, transforming growth factor-β, IFN-γ-inducible protein-10, tumor necrosis factor-α, plasminogen activator inhibitor, bactericidal permeability-increasing protein, and pigment epithelium-derived factor) for the presence of 5′-CpG islands (GC content >60%, ratio of CpG-to-GpC >0.6, and minimum length 200 bp; ref. 31) revealed that only *TSP1* (32) contains a CpG island around the transcription start site. Furthermore, the expression levels of the angiogenesis-inhibiting tumor suppressor genes *p16INK4a, p73, maspin*, and *TIMP3*, which are prone to epigenetic silencing in tumor cells, were studied in endothelial cells. None of these genes met both criteria of significant down-regulation in activated versus quiescent HUVEC as well as up-regulation by DAC and TSA treatment (data not shown). Next to TSP1, we studied endothelial cell expression of some growth-inhibiting genes prone to epigenetic silencing in tumor cells, such as IGFBP3, a growth inhibitor that also decreases endothelial cell proliferation (33, 34), and JUNB, a negative growth regulator and potential tumor suppressor (35). Quantitative real-time reverse transcription-PCR revealed down-regulated transcript levels of these genes in activated compared with quiescent endothelial cells and reactivation by DAC, zebularine, or TSA treatment (Fig. 5A).

Reexpression of *IGFBP3, TSP1,* and *JUNB* by DAC, zebularine, and TSA in activated endothelial cells suggests that these genes might be silenced by epigenetic modifications in these cells. To study whether silencing of these growth-inhibiting genes in activated endothelial cells is caused by DNA methylation, promoter CpG island methylation was evaluated using genomic bisulfite sequencing. Interestingly, CpG islands in the promoters of *IGFBP3, TSP1,* and *JUNB* contained only a few methylated CpG sites (Fig. 5B). Furthermore, meaningful differences in promoter methylation patterns of these genes between silenced and activated endothelial cells were not present, indicating that silencing of these genes in activated endothelial cells and reexpression by DNMT and HDAC inhibitors occurs independently of direct promoter methylation. Therefore, chromatin immunoprecipitation of *IGFBP3, TSP1,* and *JUNB* was done to study whether gene silencing is associated with aberrant patterns of histone deacetylation. For each gene, the area with greatest CpG density in the promoter was analyzed, overlapping the region examined by genomic bisulfite sequencing. Interestingly, acetylated histone H3 was observed in the transcriptionally active *IGFBP3* promoter of quiescent HUVEC but was undetectable in activated HUVEC in the area from −2 to +323 (Fig. 5C). In cells treated with DAC or TSA, histone H3 acetylation reappeared in this promoter region. Thus, silencing of *IGFBP3* in activated HUVEC and reexpression by DAC and TSA occurred in conjunction with changes in histone H3 acetylation patterns. In contrast, for *TSP1* and *JUNB*, promoter histone H3 acetylation patterns do not correlate with silencing in activated endothelial cells and reexpression by DAC and TSA treatment (Fig. 5C), suggesting an indirect effect of DAC and TSA on *TSP1* and *JUNB* expression.

A

	% of total genomic 5-Methylcytosine content	
	Mean	SEM
HUVEC-	**3.65**	0.02
HUVEC+	**3.92***	0.03
HUVEC+ / DAC	**1.04#**	0.08

* p<0.004 versus HUVEC-, # p<0.004 versus HUVEC+

B

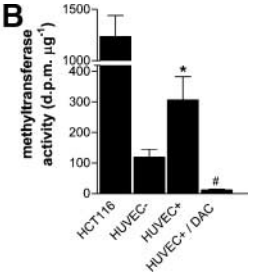

Figure 4. Global 5-methylcytosine content and DNMT1 activity in endothelial cells. **A,** measurement of 5-methylcytosine content as a percentage of the total cytosine pool in quiescent HUVEC (*HUVEC*−), tumor-conditioned HUVEC (*HUVEC*+), and tumor-conditioned HUVEC treated with 200 nmol/L DAC (*HUVEC*+*/DAC*). Three analytic measurements were made per sample and experiments were done in duplicate. **B,** DNMT1 activity in quiescent HUVEC, tumor-conditioned HUVEC, tumor-conditioned HUVEC treated with 200 nmol/L DAC, and HCT116. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$ versus quiescent HUVEC; #, $P < 0.05$ versus tumor-conditioned HUVEC.

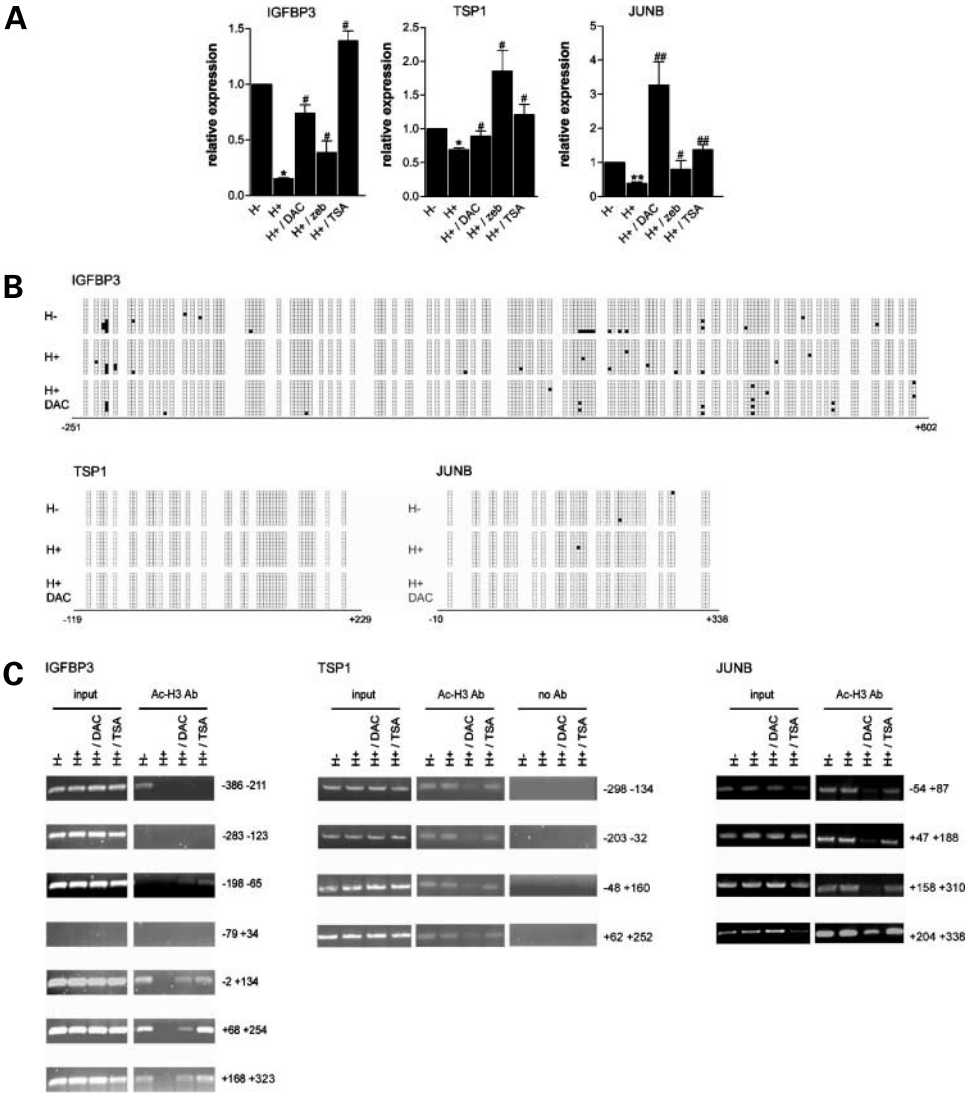

Figure 5. mRNA expression, promoter methylation, and histone deacetylation of *TSP1, JUNB*, and *IGFBP3* in endothelial cells. **A,** relative mRNA expression of IGFBP3, TSP1, and JUNB measured by quantitative real-time reverse transcription-PCR in quiescent HUVEC (*H−*), activated HUVEC (*H+*), and activated HUVEC treated with DAC (200 nmol/L; *H+/DAC*), zebularine (100 μmol/L; *H+/zeb*), or TSA (300 nmol/L; *H+/TSA*). *Columns,* mean relative mRNA expression compared with quiescent HUVEC from three independent experiments; *bars,* SE. *, $P < 0.04$ versus quiescent HUVEC; **, $P < 0.02$ versus quiescent HUVEC; #, $P < 0.05$ versus activated HUVEC; ##, $P < 0.03$ versus activated HUVEC. **B,** genomic bisulfite sequencing of 5′-CpG islands of IGFBP3, TSP1, and JUNB. In each clone, the methylation status of each CpG dinucleotide is represented. *Shaded box,* the position is methylated; *white box,* the position is not methylated. *Numbers,* position relative to transcriptional start site. **C,** chromatin immunoprecipitation of the 5′-CpG islands of IGFBP3, TSP1, and JUNB with anti-acetylated histone H3 antibody. *Numbers,* location of the DNA fragments amplified by PCR done on the DNA recovered from chromatin immunoprecipitation experiments and correspond with the *numbers* in **B**. For each primer set, PCR was done on nonimmunoprecipitated DNA (*input*), immunoprecipitated DNA (*Ac-H3 Ab*), and a no-antibody control DNA (*no Ab*). For IGFBP3 and JUNB, no bands were observed in the beads as shown for TSP1.

Discussion

We investigated whether DNMT inhibitors directly affect endothelial cell biology and tumor angiogenesis apart from potential indirect angiostatic activities *in vivo* via inhibition of tumor cells (8, 9). This report is the first to show that DNMT inhibitors act directly on activated endothelial cells and inhibit angiogenesis *in vitro* and *in vivo*, similar as described previously for HDAC inhibitors (16–18).

DAC and its analogue zebularine showed potent inhibition of tumor growth and angiostatic activity in two different mouse tumor models. Inhibition of tumor angiogenesis in B16F10 and LS174T tumor-bearing mice after treatment with DNMT inhibitors can be due to effects of these compounds on tumor cells (9, 10), which are known to influence tumor angiogenesis by release of proangiogenic and antiangiogenic factors. However, we show that the DNMT inhibitor DAC directly decreases proliferation of activated HUVEC and mouse b.END5 brain endothelioma cells, an observation that was confirmed using zebularine, a recently described DAC analogue with great potential in clinical use (28). Effective concentrations of zebularine were ~100-fold higher than DAC, which is in agreement with results in tumor cells and can be explained by differences in transport or metabolic activation as well as by the fact that zebularine is also incorporated into RNA (20). The significant inhibition of *in vitro* tube formation in the absence of tumor cells proves that these agents also directly inhibit endothelial cell sprouting. The potent inhibition of activated endothelial cells next to tumor cells makes DNMT inhibition a powerful anticancer therapy as reflected by the markedly decreased tumor volumes in mice treated with DAC and zebularine.

In tumor cells, global demethylation of the genome occurs despite regional promoter hypermethylation of tumor suppressor genes (5). This global hypomethylation in tumor cells has been proposed to cause chromosomal instability, harmful expression of endogenous viral sequences, and activation of oncogenes (36). We found an increase

in methylation upon activation of endothelial cells, which could explain why these cells are much less prone to genetic modifications. Despite the significant increase in total genomic 5-methylcytosine content in activated versus quiescent endothelial cells, silencing of the angiogenesis-inhibiting genes *TSP1, JUNB*, and *IGFBP3* in activated endothelial cells and reexpression by DAC, zebularine, and TSA occurs independently of direct promoter methylation of these genes. The angiogenesis inhibitor TSP1 blocks endothelial cell migration and induces endothelial cell apoptosis (32). JUNB negatively regulates cell growth by activating p16INK4A and decreasing cyclin D1 expression (35). IGFBP3, a key regulator of cell growth and apoptosis, potently inhibits VEGF-mediated HUVEC proliferation (33) and angiogenesis (34). Remarkably, previous studies have shown that silencing of the same genes in tumor cells is associated with promoter methylation [*TSP1* (37, 38), *JUNB* (39), and *IGFBP3* (40, 41)]. Thus, silencing of these angiogenesis-inhibiting genes in tumor cells and activated endothelial cells occurs through different mechanisms. Several methylation-independent effects of DNMT inhibitors have been described by others (42–44). Because endothelial cell death is not induced by DAC treatment, cytotoxicity can be excluded as a major cause of gene induction. A possibility is that DAC targets upstream regulators that are suppressed by promoter hypermethylation or that the minimal promoter comprises other regions than those analyzed by bisulfite sequencing. Furthermore, several studies have shown that DNA methylation serves to ''lock in'' rather than initiate gene silencing (45–47). Thus, despite the observed global hypermethylation, specific promoter hypermethylation might not have occurred within the timeframe of our experiments. This is supported by the absence of *IGFBP3* promoter hypermethylation in combination with histone H3 deacetylation after 3 days of endothelial cell activation. In addition, DNMTs have additional transcriptional repressor functions apart from their methylation ability (43, 44). By trapping DNMTs, DAC might inhibit these methylation-independent silencing functions of DNMTs and thus affect gene expression. Finally, gene silencing in activated endothelial cells and reactivation by DAC and TSA might be predominantly an HDAC-dependent mechanism, either HDACs directly or DNMT-mediated HDAC recruitment. Further studies are required to unravel whether (methylation-independent) epigenetic mechanisms are involved in silencing of *IGFBP3, TSP1,* and *JUNB* in activated endothelial cells and reexpression by DAC and TSA.

In conclusion, our data show for the first time that direct inhibitory effects of DNMT inhibitors regulate endothelial cell growth and angiogenesis. Although overall genomic methylation levels and DNMT activity are increased in tumor-conditioned endothelial cells, reexpression of growth-inhibiting genes in activated endothelial cells by DNMT and HDAC inhibitors seems to occur through methylation-independent effects. Current studies are focused on the exact role of DNMTs in regulation of endothelial cell growth and angiogenesis as well as in regulating expression of growth-inhibiting genes in tumor endothelial cells. The dual effects of DNMT and HDAC inhibitors on both tumor cell growth and tumor endothelial cells make them attractive anticancer therapeutics.

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References

1. Jenuwein T, Allis CD. Translating the histone code. Science 2001;293:1074–80.

2. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003;33 Suppl:245–54.

3. Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 2001;10:687–92.

4. Jones PA, Laird PW. Cancer epigenetics comes of age. Nat Genet 1999;21:163–7.

5. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 1983;301:89–92.

6. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042–54.

7. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001;61:3225–9.

8. Suzuki H, Gabrielson E, Chen W, et al. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet 2002;31:141–9.

9. Baylin SB. Reversal of gene silencing as a therapeutic target for cancer—roles for DNA methylation and its interdigitation with chromatin. Novartis Found Symp 2004;259:226–33.

10. Villar-Garea A, Esteller M. Histone deacetylase inhibitors: understanding a new wave of anticancer agents. Int J Cancer 2004;112:171–8.

11. Folkman J. Fundamental concepts of the angiogenic process. Curr Mol Med 2003;3:643–51.

12. Griffioen AW, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol Rev 2000;52:237–68.

13. St Croix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. Science 2000;289:1197–202.

14. Bicknell R, Harris AL. Novel angiogenic signaling pathways and vascular targets. Annu Rev Pharmacol Toxicol 2004;44:219–38.

15. van Beijnum JR, Griffioen AW. *In silico* analysis of angiogenesis associated gene expression identifies angiogenic stage related profiles. Biochim Biophys Acta 2005;1755:121–34.

16. Kim MS, Kwon HJ, Lee YM, et al. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. Nat Med 2001;7:437–43.

17. Deroanne CF, Bonjean K, Servotte S, et al. Histone deacetylases inhibitors as anti-angiogenic agents altering vascular endothelial growth factor signaling. Oncogene 2002;21:427–36.

18. Qian DZ, Wang X, Kachhap SK, et al. The histone deacetylase inhibitor NVP-LAQ824 inhibits angiogenesis and has a greater antitumor effect in combination with the vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584. Cancer Res 2004;64:6626–34.

19. Griffioen AW, Damen CA, Martinotti S, Blijham GH, Groenewegen G. Endothelial intercellular adhesion molecule-1 expression is suppressed in human malignancies: the role of angiogenic factors. Cancer Res 1996;56:1111–7.

20. Cheng JC, Matsen CB, Gonzales FA, et al. Inhibition of DNA methylation and reactivation of silenced genes by zebularine. J Natl Cancer Inst 2003;95:399–409.

21. van der Schaft DW, Dings RP, de Lussanet QG, et al. The

designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. FASEB J 2002;16: 1991 – 3.

22. van der Schaft DW, Toebes EA, Haseman JR, Mayo KH, Griffioen AW. Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. Blood 2000;96: 176 – 81.

23. Fraga MF, Uriol E, Borja Diego L, et al. High-performance capillary electrophoretic method for the quantification of 5-methyl 2′-deoxycytidine in genomic DNA: application to plant, animal and human cancer tissues. Electrophoresis 2002;23:1677 – 81.

24. Vertino PM, Yen RW, Gao J, Baylin SB. *De novo* methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5-)-methyltransferase. Mol Cell Biol 1996;16:4555 – 65.

25. Thijssen VL, Brandwijk RJ, Dings RP, Griffioen AW. Angiogenesis gene expression profiling in xenograft models to study cellular interactions. Exp Cell Res 2004;299:286 – 93.

26. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821 – 6.

27. Metivier R, Penot G, Hubner MR, et al. Estrogen receptor-α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 2003;115:751 – 63.

28. Cheng JC, Yoo CB, Weisenberger DJ, et al. Preferential response of cancer cells to zebularine. Cancer Cell 2004;6:151 – 8.

29. Vesely J. Mode of action and effects of 5-azacytidine and of its derivatives in eukaryotic cells. Pharmacol Ther 1985;28:227 – 35.

30. Rhee I, Jair KW, Yen RW, et al. CpG methylation is maintained in human cancer cells lacking DNMT1. Nature 2000;404:1003 – 7.

31. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. J Mol Biol 1987;196:261 – 82.

32. Bocci G, Francia G, Man S, Lawler J, Kerbel RS. Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. Proc Natl Acad Sci U S A 2003;100:12917 – 22.

33. Franklin SL, Ferry RJ, Jr., Cohen P. Rapid insulin-like growth factor (IGF)-independent effects of IGF binding protein-3 on endothelial cell survival. J Clin Endocrinol Metab 2003;88:900 – 7.

34. Iwatsuki K, Tanaka K, Kaneko T, et al. Runx1 promotes angiogenesis by downregulation of insulin-like growth factor-binding protein-3. Oncogene 2005;24:1129 – 37.

35. Passegue E, Wagner EF. JunB suppresses cell proliferation by transcriptional activation of p16(INK4a) expression. EMBO J 2000;19: 2969 – 79.

36. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. Nature 1998; 395:89 – 93.

37. Yang QW, Liu S, Tian Y, et al. Methylation-associated silencing of the thrombospondin-1 gene in human neuroblastoma. Cancer Res 2003;63: 6299 – 310.

38. Li Q, Ahuja N, Burger PC, Issa JP. Methylation and silencing of the thrombospondin-1 promoter in human cancer. Oncogene 1999;18: 3284 – 9.

39. Yang MY, Liu TC, Chang JG, Lin PM, Lin SF. JunB gene expression is inactivated by methylation in chronic myeloid leukemia. Blood 2003;101: 3205 – 11.

40. Chang YS, Wang L, Liu D, et al. Correlation between insulin-like growth factor-binding protein-3 promoter methylation and prognosis of patients with stage I non-small cell lung cancer. Clin Cancer Res 2002;8: 3669 – 75.

41. Fraga MF, Herranz M, Espada J, et al. A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. Cancer Res 2004;64:5527 – 34.

42. Schmelz K, Sattler N, Wagner M, Lubbert M, Dorken B, Tamm I. Induction of gene expression by 5-aza-2′-deoxycytidine in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) but not epithelial cells by DNA-methylation-dependent and -independent mechanisms. Leukemia 2005;19:103 – 11.

43. Fuks F, Burgers WA, Brehm A, Hughes-Davies L, Kouzarides T. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nat Genet 2000;24:88 – 91.

44. Fuks F, Burgers WA, Godin N, Kasai M, Kouzarides T. Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. EMBO J 2001;20:2536 – 44.

45. Bachman KE, Park BH, Rhee I, et al. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. Cancer Cell 2003;3:89 – 95.

46. Tamaru H, Selker EU. A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. Nature 2001;414:277 – 83.

47. Ting AH, Schuebel KE, Herman JG, Baylin SB. Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. Nat Genet 2005;37:906 – 10.

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