Abnormal Histone Methylation Is Responsible for Increased Vascular Endothelial Growth Factor 165a Secretion from Airway Smooth Muscle Cells in Asthma

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Abnormal Histone Methylation Is Responsible for Increased Vascular Endothelial Growth Factor 165a Secretion from Airway Smooth Muscle Cells in Asthma

Rachel L. Clifford,* Alison E. John,* Christopher E. Brightling,† and Alan J. Knox*

Vascular endothelial growth factor (VEGF), a key angiogenic molecule, is aberrantly expressed in several diseases including asthma where it contributes to bronchial vascular remodeling and chronic inflammation. Asthmatic human airway smooth muscle cells hypersecrete VEGF, but the mechanism is unclear. In this study, we defined the mechanism in human airway smooth muscle cells from nonasthmatic and asthmatic patients. We found that asthmatic cells lacked a repression complex at the VEGF promoter, which was present in nonasthmatic cells. Recruitment of G9A, trimethylation of histone H3 at lysine 9 (H3K9me3), and a resultant decrease in RNA polymerase II at the VEGF promoter was critical to repression of VEGF secretion in nonasthmatic cells. At the asthmatic promoter, H3K9me3 was absent because of failed recruitment of G9a; RNA polymerase II binding, in association with TATA-binding protein-associated factor 1, was increased; H3K4me3 was present; and Sp1 binding was exaggerated and sustained. In contrast, DNA methylation and histone acetylation were similar in asthmatic and nonasthmatic cells. This is the first study, to our knowledge, to show that airway cells in asthma have altered epigenetic regulation of remodeling gene(s). Histone methylation at genes such as VEGF may be an important new therapeutic target. The Journal of Immunology, 2012, 189: 000–000.
regulated transcriptionally (21), and wondered whether these transcriptional responses may be altered in cells from asthmatic donors. Interestingly, however, the only consistent difference we saw between the asthmatic and nonasthmatic airway smooth muscle cells was at the level of basal VEGF secretion, where asthmatic ASM cells secreted more VEGF than the nonasthmatic cells. This is in agreement with Simcock et al. (8). As the mechanisms of basal VEGF secretion are poorly characterized, in the current paper, we aimed to identify the mechanism of basal VEGF regulation and how this is altered in cells from asthmatic patients. We show that VEGF hypersecretion from asthmatic airway smooth muscle cells occurs via a transcriptional mechanism dependent on changes in chromatin structure. We report that differential histone H3 methylation and binding of Sp1 and basic transcriptional machinery at the asthmatic VEGF promoter is responsible for hypersecretion of VEGF from asthmatic airway smooth muscle cells. Interestingly, despite the presence of large CGIs within the VEGF gene, neither DNA methylation nor histone acetylation was involved. Our results shed light on an epigenetic mechanism controlling basal VEGF secretion and how this can become altered in inflammatory disease. Epigenetic changes are known to occur in cancer cells, but this is the first account, to our knowledge, of an aberrant epigenetic mechanism in an airway structural cell in asthma.

Materials and Methods

Cell culture

Primary cultures of HASM cells from seven asthmatic and six nonasthmatic individuals were isolated from bronchial biopsies and large airway tissue from subjects undergoing surgery at Glenfield Hospital (Leicester, U.K.) as described previously (22). Asthmatic subjects and nonasthmatic controls were recruited from Leicester, U.K. Subjects with asthma had a consistent history and objective evidence of asthma, as indicated by one or more of the following: 1) methacholine airway hyperresponsiveness (PC20, forced expiratory volume in 1 s [FEV1] < 8 mg/ml); 2) >15% improvement in FEV1 15 min after administration of 200 mg inhaled salbutamol; or 3) ≥20% of maximum within-day amplitude from twice daily peak expiratory flow measurements over 14 d. Severity of asthma was defined by Global Initiatives for Asthma treatment steps I–V (18). The study was approved by the Leicestershire Research Ethics Committees, and all patients gave their written informed consent (23). Four nonasthmatic individuals were non-smokers and two were ex-smokers. Four of the A individuals were non-smokers and three were ex-smokers. Cells were cultured to confluence in DMEM prior to experiment initiation. For determination of supernatant collected 24 h later and assayed by ELISA.

VEGF ELISA

The human VEGF-A and the human VEGF-A165 ELISA were purchased from R&D Systems. The assay was performed according to the manufacturer’s protocol as described previously (24). Concentrations were normalized to the number of cells per well using the Millipore Scepter 2.0 Handheld Automated Cell Counter.

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel) following the manufacturer’s protocol. Five microliters of RNA was reverse transcribed in a total volume of 25 μl, including 132 U Moloney murine leukemia virus reverse transcriptase, 26.4 U RNase inhibitor, 0.6 μg (dT)15 primer, 2 μM 2'-deoxycytidine 5'-triphosphates, and 1X Moloney murine leukemia virus reverse transcriptase buffer provided by Promega (10). The resulting reverse transcriptase products were used for real-time PCR amplification.

Quantitative real-time PCR

Total human VEGF expression was determined using the primer sequences shown in Table 1. GAPDH was used as the housekeeping gene. Splice variant-specific PCR was performed using primer sequences listed in Table 1 (slight modifications of previously published primers (25)).

One microliter of reverse-transcribed cDNA was subjected to real-time PCR using TaKaRa SYBR Premix Ex Taq (Lonza Group, Basel, Switzerland) and the MX3000P quantitative PCR system (Stratagene). Each reaction consisted of 1X SYBR Premix Ex Taq, 0.2 μM sense and anti-sense primers, 1 μl DNA, and H2O to a final volume of 25 μl. Thermal cycler conditions included incubation at 95˚C for 30 s, followed by 40 cycles of 95˚C for 5 s, 60˚C for 30 s, and 72˚C for 15 s. For splice variant PCR, the annealing temperature was 62˚C. Integration of the fluorescent SYBR green into the PCR product was monitored after each annealing step. Amplification of one specific product was confirmed by melting curve analysis.

Inhibitor studies

Actinomycin D. Cells were cultured to confluence in 6-well plates, followed by 24-h serum deprivation. Following serum withdrawal, cells were cultured in the presence or absence of 5 μM actinomycin D (DMSO was used as the vehicle control) for 4 h. Samples were taken for RNA isolation.

Mithramycin. Cells were cultured to confluence in 24-well plates and transferred to serum-free media for 24 h. Postserum starvation media were replaced with fresh serum-free media containing 0, 0.1, 1, or 10 μM mithramycin. DMSO was used as a vehicle control. Supernatants were collected 24 h later and assayed by ELISA.

NS398 and indomethacin. Cells were cultured to confluence in 24-well plates and transferred to serum-free media for 24 h. Subsequently, media were replaced with fresh media containing 1 μM indomethacin (Sigma-Aldrich), 1 μM NS398 (Sigma-Aldrich), or a DMSO control. Supernatants were collected 24 h later and assayed by ELISA.

Histone deacetylase inhibitors. Cells were cultured to confluence in 24-well plates and transferred to serum-free media for 24 h. Postserum starvation media were replaced with fresh media containing 0, 10−6, or 10−7 M MS-275 or suberoylanilide hydroxamic acid (SAHA) (Cayman Chemical, Ann Arbor, MI). DMSO was used as vehicle control. Supernatants were collected 24 h later and assayed by ELISA.

BIX-01294. Cells were cultured to ~75% confluence in 24 well plates in serum positive media. At ~75% confluence, media were changed for fresh serum-positive media containing 10−6, 10−7, 10−8, 10−9, 10−10, or 0 M 5-aza-2’d-deoxycytidine (5-aza). DMSO was used as the vehicle control. Forty-eight hours later, cells had reached confluence, and the media were replaced with serum-free media containing 5-aza at the above concentrations. Following 24-h serum starvation, the media were changed a final time, and cells were maintained in 5-aza for an additional 24 h. Supernatants were collected and assayed by ELISA.

Histone deacetylase inhibitors. Cells were cultured to confluence in 24-well plates and transferred to serum-free media for 24 h. Postserum starvation media were replaced with fresh media containing 0, 10−6, or 10−7 M MS-275 or suberoylanilide hydroxamic acid (SAHA) (Cayman Chemical, Ann Arbor, MI). DMSO was used as vehicle control. Supernatants were collected 24 h later and assayed by ELISA.

BIX-01294. Cells were cultured to ~75% confluence in 24 well plates in serum-positive media. At ~75% confluence, media were changed for fresh serum-positive media containing 10−6, 10−7, 10−8, 10−9, 10−10, or 0 M BIX-01294. DMSO was used as the vehicle control. Forty-eight hours later, cells had reached confluence, and the media were replaced with serum-free media containing BIX-01294 at the above concentrations. Following 24-h serum starvation, the media were changed a final time, and cells were maintained in BIX-01294 for an additional 24 h. Supernatants were collected and assayed by ELISA. Alternatively, samples were taken for ChIP at 2 h postserum starvation.

Cytoplasmic/nuclear extraction and separation

Cells were grown to confluence in 100-mm dishes, serum-starved for 24 h, and then collected by scraping. Cytoplasmic and nuclear fractions were separated using Cel-Lytic NuCLEAR extraction kit (Sigma-Aldrich).

Western blots

Cytoplasmic and nuclear protein samples were subject to electrophoresis in 10% SDS-polyacrylamide gel. Separated proteins were electroblotted to PVDF. Western blotting was performed according to standard conditions. Following 24-h serum starvation, the media were changed a final time, and cells were maintained in BIX-01294 for an additional 24 h. Supernatants were collected and assayed by ELISA.
bridge, U.K.) (1:2000 dilution with 5% fat-free dried milk in 0.1% TBST) for 1 h. The blot was washed again and then incubated with ECL Western blotting detection reagent (GE Healthcare). Details of the Abs used are given in Table II.

Transfection

The dominant-negative (D/N) Sp1 plasmid and pEGBV empty vector control were gifts from Prof. G. Thiel (University of Saarland Medical Centre, Saarbrücken, Germany) (26). Cells were transfected with 0.4 μg/well DNA using Fugene HD, according to the manufacturers’ guidelines. A 1:2 ratio of DNA/Fugene HD was used. Briefly, DNA/reagent mixes were made in serum and antibiotic-free media. Cells were transfected for 24 h in the presence of serum. Subsequently, cells were serum deprived for 24 h. The media were then changed a final time, and supernatants were collected 24 h later for ELISA.

The D/N G9A plasmid and pcDNA3.1 empty vector control were gifts from Prof. K.L. Wright (University of South Florida, Tampa, FL) (27). Cells were transfected with 0.1 μg/well DNA using Fugene HD and the above method.

The D/N SUV39H1 plasmid, Δ SET SUV39H1, and CMV empty vector control were gifts from Dr. T. Jenuwein (Max Planck Institute for Immunobiology, Freiburg, Germany) (28). Cells were transfected with 0.05 μg/well DNA using Fugene HD and the above method.

ChIP

ChIP was performed using the ChIP-IT Express kit (Active Motif) following the manufacturer’s protocol. Four micrograms of Ab was used, and input DNA underwent phenol/chloroform extraction before being used in PCR. The Abs used are listed in Table II. Products were amplified by quantitative real-time PCR using KAPA SYBR FAST Taq (supplemented with 0.8 M betaine) and the MX3000P quantitative PCR system (Stratagene). Primer sequences are shown in Table I.

Bisulfite sequencing

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer’s protocol. Two micrograms of genomic DNA was subject to bisulfite conversion using the Epitect Bisulfite kit (Qiagen). Eluted purified DNA was subject to seminested PCR using primers that amplified regions of CGIs throughout the VEGF gene (see Table III for sequences), as determined by Methyl Primer Express (Applied Biosystems) and HotStar Taq Plus (Qiagen). PCR products were run on a gel to confirm correct band size. Bands were excised and DNA extracted using the QIAEX II DNA extraction kit (Qiagen). For all products, except CpG1.3, the resulting DNA was sent for direct sequencing at the Bio-polymer Synthesis and Analysis Unit at the University of Nottingham on a 3130 ABI PRISM. Sequencing directly from PCR product was not successful for the CpG1.3 region, so a cloning step was necessary. Briefly, DNA from PCR cleanup was ligated into the pCR2.1 vector by overnight incubation at 14°C. The ligation product was transformed into competent Escherichia coli. Five positive colonies were picked per sample, grown overnight, and isolated using a Plasmid Mini kit (Qiagen). The resulting DNA was sequenced.

Statistical analysis

Data were expressed as means ± SEM of n determinants. N is stated in individual figure legends. Statistical analyses were performed using GraphPad Prism Software (versions 4 and 5). An unpaired two-tailed Student t test was used to determine the significant differences between the means for mRNA expression and protein secretion data; p < 0.05 was accepted as significantly significant.

Results

Increased asthmatic HASM cell VEGF secretion is transcription dependent and because of upregulation of a single isoform

To confirm VEGF hypersecretion from our asthmatic cells, non-asthmatic and asthmatic cells were serum deprived for 24 h, the media were replaced, and supernatants were collected 24 h later. The VEGF concentration in the supernatant was assessed by ELISA. VEGF concentrations were normalized to cell number. VEGF protein secretion was increased in asthmatic cells compared with NA cells (Fig. 1A). To begin elucidating the level at which VEGF hypersecretion was regulated, we determined the level of VEGF mRNA in the asthmatic and nonasthmatic cells by quantitative real-time PCR. Quantitative PCR showed increased VEGF mRNA levels in asthmatic HASM cells (Fig. 1B). Interestingly, although VEGF mRNA levels in the nonasthmatic HASM cells remained low and constant, the VEGF mRNA levels in the asthmatic HASM cells increased over time. For this reason, in later ChIP experiments, a 0- and a 2-h time point was used to identify any kinetic changes in basal promoter association. To confirm increased VEGF mRNA accumulation in asthmatic HASM cells was due to VEGF gene transcription, cells were cultured in the presence or absence of 5 μg/ml actinomycin D or DMSO vehicle control. Cells were lysed, and VEGF mRNA levels were assessed by quantitative real-time PCR. n = 3 nonasthmatic and 4 asthmatic HASM cell lines. *p < 0.05 compared with corresponding nonasthmatic result (B). Cells were lysed, and mRNA levels were assessed by quantitative real-time PCR. n = 3 nonasthmatic and 4 asthmatic HASM cell lines. *p < 0.05 compared with corresponding nonasthmatic result. (C) HASM cells were cultured for 4 h post serum deprivation in the presence of 5 μg/ml actinomycin D or DMSO vehicle control. Cells were lysed, and VEGF mRNA levels were assessed by quantitative real-time PCR. n = 3 nonasthmatic and 3 asthmatic HASM cell lines. PCR data are expressed as fold change relative to the mean control NA value. *p < 0.05 compared with DMSO control.

FIGURE 1. Increased asthmatic HASM cell VEGF secretion is transcription dependent. (A) Supernatants were collected 24 h after serum deprivation, and VEGF protein secretion was assessed by ELISA. n = 3 nonasthmatic and 3 asthmatic HASM cell lines. *p < 0.05 compared with corresponding nonasthmatic result. (B) Cells were lysed, and mRNA levels were assessed by quantitative real-time PCR. n = 3 nonasthmatic and 4 asthmatic HASM cell lines. *p < 0.05 compared with corresponding nonasthmatic result. (C) HASM cells were cultured for 4 h post serum deprivation in the presence of 5 μg/ml actinomycin D or DMSO vehicle control. Cells were lysed, and VEGF mRNA levels were assessed by quantitative real-time PCR. n = 3 nonasthmatic and 3 asthmatic HASM cell lines. PCR data are expressed as fold change relative to the mean control NA value. *p < 0.05 compared with DMSO control.
form has been identified for most conventional VEGF isoforms (Fig. 2A, 2B) (20). Many current assays for VEGF do not discriminate between these opposing isoforms. We used isoform-specific PCR (primers shown in Table I are taken/adapted from those published in Ref. 25) to establish which VEGF isoforms are present and regulated in asthmatic and nonasthmatic HASM cells. Both xxxa and xxxb isoforms were detectable by PCR in all HASM cells. As shown in Fig. 2C, the increase in asthmatic VEGF mRNA observed in Fig. 1B is composed entirely of the specific VEGF isoform VEGF165a. In agreement, an ELISA for VEGF165b was performed, but all samples were below minimum (data not shown).

**Basal VEGF expression is dependent on Sp1**

Sp1 is a ubiquitously expressed C2H2-type zinc finger-containing DNA binding protein that binds GC-rich regions of DNA to positively and negatively regulate gene transcription (31). Sp1 is a DNA binding protein that binds GC-rich regions of DNA to positively and negatively regulate gene transcription (31). Sp1 is a DNA binding protein that binds GC-rich regions of DNA to positively and negatively regulate gene transcription (31). Sp1 is a DNA binding protein that binds GC-rich regions of DNA to positively and negatively regulate gene transcription (31). Sp1 is a DNA binding protein that binds GC-rich regions of DNA to positively and negatively regulate gene transcription (31).

![FIGURE 2.](http://www.jimmunol.org/)

**FIGURE 2.** VEGF165a is specifically upregulated in asthmatic HASM cell. (A and B) Schematic of different VEGF isoforms. Conventional proangiogenic isoforms (A); more recently identified antiangiogenic isoforms (B). (C) Isoform-specific, quantitative real-time PCR was performed to assess splice variant expression, 4 h postserum starvation. n = 3 nonasthmatic and 3 asthmatic HASM cell lines. PCR data are expressed as fold change relative to the mean control NA value. *p < 0.05 compared with corresponding NA result.

To establish whether Sp1 is required for basal VEGF expression in nonasthmatic and/or asthmatic HASM cells, cells were incubated with mithramycin A. Mithramycin A selectively binds GC-rich regions of DNA to inhibit Sp1 binding (37). Mithramycin A inhibited VEGF secretion (Fig. 3A) from nonasthmatic and asthmatic HASM cells. To provide further evidence of a role for Sp1 in basal VEGF secretion we transfected A and NA cells with a D/N Sp1 construct. As shown in Fig. 3B, D/N Sp1 overexpression also inhibited basal asthmatic and nonasthmatic VEGF expression, when compared with the pEBGV empty vector control. This suggests Sp1 is required for nonasthmatic and asthmatic basal VEGF transcription.

Next, we analyzed basal Sp1 binding at the VEGF promoter by ChIP (Table II), in nonasthmatic and asthmatic HASM cells, at 0 and 2 h post 24-h serum deprivation. We analyzed two sections of the VEGF promoter, in this paper termed “distal” (−262 to −101 bp relative to the transcription start site) and “proximal” (−199 to +3 bp relative to the transcription start site), which both contain Sp1 binding sites. Both sections of the promoter, in asthmatic and nonasthmatic HASM cells, bind Sp1 at 0 h relative to the IgG control (Fig. 3C, 3D). Sp1 association was greater at the asthmatic promoter than at the nonasthmatic promoter. Furthermore, A Sp1 binding was further increased in the 2-h sample. These data suggest heightened and sustained recruitment of Sp1 to the asthmatic promoter contributes to increased VEGF secretion from asthmatic cells.

Sp1 can associate with members of the core promoter recognition complex TFIID, specifically TATA-binding protein-associated factor 1 (TAF1) to regulate transcription via recruitment of the core promoter complex or histone acetylation (38). Fig. 3E and 3F show increased binding of TAF1 (Table II) to the asthmatic VEGF promoter, suggesting TAF1 plays a role in asthmatic VEGF hypersecretion.

We have shown previously that PGE2 can induce VEGF secretion from HASM cells via increased Sp1 binding (21). To determine whether PGE2 is involved in basal VEGF production from nonasthmatic or asthmatic HASM cells, we incubated cells with the selective cyclooxygenase (COX)-2 inhibitor, NS398, and the nonselective inhibitor of COX-1 and COX-2, indomethacin. Incubation of nonasthmatic and asthmatic HASM cells with inhibitors of COX-1/2 had no effect on VEGF secretion (Fig. 3G), suggesting autocrine PGE2 production and signaling is not responsible for asthmatic VEGF hypersecretion.

Sp1-dependent regulation of VEGF can be dependent on levels of Sp1 expression (39). Western blot analysis showed that Sp1 (Table II) was present to the same extent in the nucleus of both asthmatic and nonasthmatic HASM cells under basal conditions (Fig. 3H).

Taken together, these data show that Sp1 is required for basal VEGF expression, that heightened Sp1 binding likely contributes to increased asthmatic VEGF secretion, and that increased Sp1 binding is not due to autocrine PGE2 signaling or increased Sp1 expression. We went on to investigate other modifications with the potential to affect Sp1 binding. Because IL-13 has also been shown to induce VEGF secretion from HASM cells (40), we also used an IL-13Rα2Fc chimera to establish whether basal VEGF secretion is IL-13 dependent but the blocking receptor had no effect (data not shown).

**DNA methylation of the VEGF gene is similar in asthmatic and nonasthmatic HASM cells**

Sp1-regulated promoters are often GC-rich DNA sequences. These GC-rich regions are known as CGIs, and methylation of cytosines...
within these regions can repress transcription. Sp1-dependent gene expression can be mediated by DNA methylation (41–43). Hypomethylation of the Abcc6 (44), Glutathione S-transferases M2 (GST-M2) (41), soluble epoxide hydrolase (sEH) (45), and KCTD11 (43) promoters results in increased gene expression and increased Sp1 binding. Furthermore, TAFs are stably recruited to CGI promoters (46). We found the VEGF gene had a multitude of CGI promoters (46). We found the VEGF gene had a multitude of promoters results in increased gene expression and increased Sp1 binding. Furthermore, TAFs are stably recruited to CGI promoters (46). We found the VEGF gene had a multitude of CGI promoters (46).

The first covers a large region (>3000 bp) including the VEGF promoter and exons 1 and 2, the second (~700 bp) is contained within exon 7, and the third (~700 bp) covers the 5’-end of exon 8. For the purpose of PCR amplification and sequencing CGI 1 was split into six sections (CpG1.1 to CpG 1.6), CGI 2 into two sections (CpG2.1 and 2.2), and CGI 3 into two sections (CpG 3.1 and 3.2). The positions are detailed along with the primer sequences in Table III. A single nonasthmatic basal DNA sample was used to optimize the bisulfite technique and to perform a preliminary determination of methylation status across the gene. Because VEGF transcription is lower in nonasthmatic cells, and DNA methylation is associated with repressed transcription, we were interested in methylated regions of the nonasthmatic VEGF gene. We found CpG1 of the nonasthmatic sample was unmethylated, whereas CpG2 and CpG3 were highly methylated (Fig. 4B). Because CpG2 and CpG3 were methylated in the NA sample, we investigated whether this methylation was lost in the asthmatic cells. Using basal samples from three asthmatic and three nonasthmatic HASM cells, we established that CGIs 2 and 3 are also fully methylated in the asthmatic HASM cells (Fig. 4C). We next considered whether DNA methylation was induced at the nonasthmatic VEGF CpG1. We took DNA samples 0, 2, and 4 h after 24-h serum starvation from three nonasthmatic HASM cell lines and analyzed them for CpG1 methylation. Fig. 4D shows that CpG1 maintained low methylation status across the time points. As final confirmation, we incubated A and NA cells with the DNA methyltransferase inhibitor 5-aza. 5-Aza had no effect on asthmatic or nonasthmatic VEGF secretion (Fig. 4E).

We concluded that DNA methylation was not regulating Sp1 association or VEGF expression and went on to investigate whether histone modifications were different between NA and A HASM cells.

**Asthmatic VEGF hypersecretion is not associated with hyperacetylated histone H3 or H4**

Epigenetic modification of DNA associated proteins regulates transcriptional activity. Histones are highly alkaline proteins around which DNA is tightly coiled to create nucleosomes. Histones can be modified in a number of ways to change their alkalinity resulting in an altered association with DNA. Acetylation of the N-terminal tail of histones decreases their basic charge and therefore their association with negatively charged DNA. As a result, the DNA is loosened, and access is granted to the basic transcriptional machinery and transcriptional regulators. Binding of Sp1 to gene promoters is often associated with hyperacetylation of histones (47), and Sp1 can directly associate with histone acetyltransferases (HATs), including TAF1 (38, 48). We therefore wanted to establish whether there was any difference in histone H3 or histone H4 acetylation (Table II) at the VEGF promoter between asthmatic and nonasthmatic HASM cells. We found no difference in histone H3 (Fig. 5A, 5B) or H4 (Fig. 5C, 5D) acetylation at the VEGF promoter by ChIP. Furthermore, when asthmatic and nonasthmatic cells were incubated in the presence of the histone deacetylase inhibitors, SAHA and MS275, they had no effect of VEGF expression (Fig. 5E, 5F).

**Table I. Primer sequences for quantitative PCR of cDNA samples and VEGF promoter ChIP**

<table>
<thead>
<tr>
<th>Target</th>
<th>Sense (5’-3’)</th>
<th>Antisense (5’-3’)</th>
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<tbody>
<tr>
<td>Total VEGF</td>
<td>AAAGCCAGGATGAGTCCCTGGTCGAG</td>
<td>GAGGTGATCCCTCGCCGTGTCG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCCAACAGCCGTTAAGACCTTCT</td>
<td>TCTTACGCGTACAGTCAG</td>
</tr>
<tr>
<td>VEGF 121a</td>
<td>GAAAGATATCTGCAGGCGTAGTTT</td>
<td>CTTGATTAAGATGATCTCTG</td>
</tr>
<tr>
<td>VEGF 121b</td>
<td>GAAATCTCCCTCAGCAGGAAA</td>
<td>CTTGATTAAGATGATCTTG</td>
</tr>
<tr>
<td>VEGF 165a</td>
<td>CAGGACAGAAGAAAATCTCCCTGG</td>
<td>AGAGGAGATCAGTCAGATCTTG</td>
</tr>
<tr>
<td>VEGF 165b</td>
<td>CAGGACAGAAGAAAATCTCCCTGG</td>
<td>AGAGGAGATCAGTCAGATCTTG</td>
</tr>
<tr>
<td>VEGF 189a</td>
<td>TGGGCTAGGCTTCCCCCTTGTG</td>
<td>CTTGACGCTTCCCCCTTGTG</td>
</tr>
<tr>
<td>VEGF 189b</td>
<td>TGGGCTAGGCTTCCCCCTTGTG</td>
<td>CTTGACGCTTCCCCCTTGTG</td>
</tr>
<tr>
<td>VEGFdistal</td>
<td>GCTGCTCTCTGAGACAGGATTT</td>
<td>AGCCTAGCGCTTCCCCCA</td>
</tr>
<tr>
<td>VEGFproximal</td>
<td>GCTGCTCTCTGAGACAGGATTT</td>
<td>AGCCTAGCGCTTCCCCCA</td>
</tr>
</tbody>
</table>

Asthmatic VEGF hypersecretion is associated with differential histone H3 methylation

Histone H3 can be mono, bi or tri methylated at a number of lysine residues (K4, K9, K27, K36, and K79), whereas only K20 on histone H4 can be methylated (49). The extent and position of the methylation correlate with gene activation or repression. Tri- and dimethylation of lysine 4 on histone H3 (H3K4me2/3) are found at actively transcribing genes, and H3K4me3 is highly enriched at transcription start sites. In agreement with this, we found by ChIP a small but consistent increase in H3K4me3 (Table II) at the asthmatic VEGF promoter, compared with the nonasthmatic promoter (Fig. 6A, 6B). The opposing histone methylation mark is histone H3 di, or trimethylated at lysine 9 (H3K9me2/3). H3K9me2/3 is associated with transcription repression and heterochromatin formation (49). We found a similarly consistent but larger difference in H3K9me3 (Table II) association between the asthmatic and nonasthmatic HASM cells than H3K4me3. Fig. 6C and 6D show that in nonasthmatic HASM cells the repressive H3K9me3 modification is induced, whereas in asthmatic cells, there is no H3K9me3 present at the VEGF promoter. The histone methylation pattern described by these data suggest that the asthmatic VEGF promoter is more active and is also devoid of a repression mechanism that is present at the nonasthmatic promoter. To our knowledge, this is the first time histone methylation at the VEGF promoter has been described.

**H3K9me3 regulates VEGF expression by regulating Sp1 and RNA polymerase II association with the VEGF promoter**

Having established that H3K9me3 was present at the nonasthmatic, but not the asthmatic, VEGF promoter, we wanted to determine a functional role for H3K9me3 in the regulation of VEGF transcription. Initially, we incubated cells with an inhibitor of the histone deacetylase inhibitors, SAHA and MS275, they had no effect of VEGF expression (Fig. 5E, 5F).

Histone H3K9me3 regulation of VEGF expression by regulating Sp1 and RNA polymerase II association with the VEGF promoter

We found a small but consistent increase in H3K4me3 (Table II) at the asthmatic VEGF promoter, compared with the nonasthmatic promoter (Fig. 6A, 6B). The opposing histone methylation mark is histone H3 di, or trimethylated at lysine 9 (H3K9me2/3). H3K9me2/3 is associated with transcription repression and heterochromatin formation (49). We found a similarly consistent but larger difference in H3K9me3 (Table II) association between the asthmatic and nonasthmatic HASM cells than H3K4me3. Fig. 6C and 6D show that in nonasthmatic HASM cells the repressive H3K9me3 modification is induced, whereas in asthmatic cells, there is no H3K9me3 present at the VEGF promoter. The histone methylation pattern described by these data suggest that the asthmatic VEGF promoter is more active and is also devoid of a repression mechanism that is present at the nonasthmatic promoter. To our knowledge, this is the first time histone methylation at the VEGF promoter has been described.
FIGURE 3. Basal VEGF secretion is Sp1 dependent. (A) VEGF protein levels following incubation with mithramycin A. \( n = 4 \) nonasthmatic and 3 asthmatic HASM cell lines. *\( p < 0.05 \) compared with asthmatic DMSO control. **\( p < 0.01 \) compared with nonasthmatic DMSO control. (B) VEGF protein levels after transfection with empty vector, pEBGV, or D/N Sp1. \( n = 3 \) nonasthmatic and 3 asthmatic HASM cell lines. *\( p < 0.05 \) compared with asthmatic DMSO control. **\( p < 0.005 \) compared with nonasthmatic DMSO control. Sp1 binding to the proximal(C) and distal(D) VEGF promoter was assessed by ChIP. TAF1 binding to the proximal(E) and distal(F) VEGF promoter was assessed by ChIP. Sp1/TAF1 binding were measured in confluent, serum-deprived nonasthmatic and asthmatic HASM cells 0 and 2 h after 24-h serum deprivation. IgG negative controls are shown. Data are expressed as fold change relative to the mean 0 h nonasthmatic value. \( n = 3 \) nonasthmatic and 2 asthmatic HASM cell lines. (G) VEGF protein levels after incubation with COX inhibitors, 1 \( \mu M \) indomethacin and 1 \( \mu M \) NS398. \( n = 4 \) nonasthmatic and 3 asthmatic HASM cell lines. (H) Nuclear Sp1 expression levels were assessed by Western blotting. Three nonasthmatic and three asthmatic cell nuclear lysates are shown.
effect on asthmatic VEGF levels but induced nonasthmatic VEGF levels to near asthmatic levels. To strengthen this observation, we transfected nonasthmatic and asthmatic cells with D/N G9A (Fig. 7B), and VEGF secretion was measured by ELISA. As with BIX-01294, D/N G9A had no effect on asthmatic cells but induced VEGF secretion from NA cells. SUV39H1 is the methyltransferase

Table II. Details of Abs used for Western blotting and ChIP

<table>
<thead>
<tr>
<th>Western blot</th>
<th>ChIP</th>
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<tbody>
<tr>
<td>Sp1 (sc-59X; Santa Cruz Biotechnology)</td>
<td>Lamin A/C (sc-7292; Santa Cruz Biotechnology)</td>
</tr>
<tr>
<td>Sp1 (sc-59X; Santa Cruz Biotechnology)</td>
<td>Total RNA Polymerase II (17-620; Millipore)</td>
</tr>
<tr>
<td>Acetyl histone H3 (39139; Active Motif)</td>
<td>ChIPAb+ Trimethyl-Histone H3 (Lys’) (17-625; Millipore)</td>
</tr>
<tr>
<td>G9A (sc-22877; Santa Cruz Biotechnology)</td>
<td>TAF1 (TAF II p250 (6B3)) (sc-735; Santa Cruz Biotechnology)</td>
</tr>
<tr>
<td>Acetyl histone H4 (06-598; Millipore)</td>
<td>Trimethyl-histone H3 (Lys4) (39159; Active Motif)</td>
</tr>
<tr>
<td>Normal goat IgG (sc-2028; Santa Cruz Biotechnology)</td>
<td>Normal rabbit serum (011-000-001; Jackson ImmunoResearch Laboratories)</td>
</tr>
<tr>
<td>Normal rabbit IgG (AB-105-C; R&amp;D Systems)</td>
<td></td>
</tr>
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</table>

FIGURE 4. Methylation status of the VEGF gene. (A) Schematic diagram of the VEGF gene showing exons 1–8 (top) and the position of CpG sites and CpG islands (bottom). Exon positions relative to CpG sites are approximated. (B) Preliminary screen of the methylation status of CpG sites within the three VEGF CpG islands. (C) Comparison of nonasthmatic (NA) and asthmatic (A) CGIs 2 and 3 methylation status. (D) Methylation status of nonasthmatic VEGF CpG island 1 over time. Black circle = methylated cytosine; white circle = unmethylated cytosine; × = nonsequenced site. For CpG 1.3, only half-filled circles = site methylated in at least one clone. (E) VEGF protein levels after incubation with 5-aza. n = 4 nonasthmatic and 3 asthmatic HASM cell lines.
responsible for trimethylation of H3K9. No inhibitor exists for SUV39H1; however, overexpression of D/N SUV39H1 mirrored the effect of D/N G9A transfection (Fig. 7C). These data suggest that H3K9me3 is able to regulate VEGF transcription from HASM cells and we were next interested in determining the molecular mechanism of this effect. Because we knew VEGF transcription was Sp1 dependent (Fig. 3), we investigated the effect of G9A inhibition on Sp1 association at the nonasthmatic VEGF promoter by ChIP. Fig. 7C and 7D show that in the presence of BIX-01294 (and therefore the absence of H3K9me3) Sp1 binding was increased at the nonasthmatic VEGF promoter. Thus, H3K9me3 is able to regulate Sp1 binding to the VEGF promoter.

Next, we investigated the presence of the central transcriptional enzyme RNA pol II (Table II) at the VEGF promoter, as a marker

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TABLE III. Primer sequences and annealing temperatures for bisulfite sequencing PCR

<table>
<thead>
<tr>
<th>CpG Site</th>
<th>First Round Primers</th>
<th>Annealing Temp (˚C)</th>
<th>Seminested Primer (F/R)</th>
<th>Annealing Temp (˚C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>F - TGTTTGAGTTAGTTGATAGGTTT</td>
<td>55</td>
<td>GGATATATTGTTTGTTTTTTTA (F)</td>
<td>55</td>
</tr>
<tr>
<td>1.2</td>
<td>R - ATTTACCACTTTTCCTTCATAATA</td>
<td>55</td>
<td>GGGAAGTTTAGGATTATTATTTT (F)</td>
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<tr>
<td>1.3</td>
<td>F - TAGTTGTTAGTTGATAGGTTT</td>
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<td>AAATACAAACAAAAATAATTTAAC (R)</td>
<td>48</td>
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<td>1.4</td>
<td>R - GTCCTCTCTCTACTTTTTCCTACATACT</td>
<td>55</td>
<td>TTGGAGTTTTGTTTTGTTTTT (F)</td>
<td>55</td>
</tr>
<tr>
<td>1.5</td>
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<td>55</td>
<td>AAACTAAAACAAAAACTGCTTTCCAATAA (R)</td>
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<tr>
<td>+1,069 to +1,517 bp</td>
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<td>55</td>
<td>ATGGGGGTTAATATGTTATGTTT (F)</td>
<td>55</td>
</tr>
<tr>
<td>2.1</td>
<td>F - AAGGTTGTTAGTTGATGATTG</td>
<td>55</td>
<td>CCTACTACACTAAAAACACAAACAACA (R)</td>
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<tr>
<td>+1,362 to +1,838 bp</td>
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<td>55</td>
<td>GCTAGGGTTGTTTTGTTTTT (F)</td>
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</tr>
<tr>
<td>2.2</td>
<td>F - TTGTGAAGTTTGTGGTTTTTGTTGTG</td>
<td>55</td>
<td>AAAACAAAACAAAAACCAACAAACAC (R)</td>
<td>55</td>
</tr>
<tr>
<td>+1,684 to +2,073 bp</td>
<td>R - AGTTTGGGTTGTTGATGATTG</td>
<td>55</td>
<td>GCTAGGGTTGTTTTGTTTTT (F)</td>
<td>55</td>
</tr>
<tr>
<td>3.1</td>
<td>F - ACCTCTCTCTTCTCCTTCTTCT</td>
<td>58</td>
<td>AACTCAAATCCAAAAACACAAACAACA</td>
<td>58</td>
</tr>
<tr>
<td>+1,408 to +1,432 bp</td>
<td>R - AGTTTGGGTTGTTGATGATTG</td>
<td>55</td>
<td>AACCAAGGTTTTGGTTTTT (F)</td>
<td>55</td>
</tr>
<tr>
<td>3.2</td>
<td>F - GTCCTCTCTCTACTTTTTCCTACATACT</td>
<td>55</td>
<td>GTGGGGTTAATATGTTATGTTT (F)</td>
<td>55</td>
</tr>
<tr>
<td>+1,430 to +1,479 bp</td>
<td>R - CTCCTCTCCTTACAAAAACCTACAACA</td>
<td>55</td>
<td>ATGGGGGTTAATATGTTATGTTT (F)</td>
<td>55</td>
</tr>
</tbody>
</table>

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FIGURE 5. Histone acetylation is not different between the asthmatic and nonasthmatic VEGF promoter. Acetylated histone H3 (AcH3) association with the proximal (A) and distal (B) VEGF promoter was assessed by ChIP. Acetylated histone H4 (AcH4) association with the proximal (C) and distal (D) VEGF promoter was assessed by ChIP. AcH3/4 association were measured in confluent, serum-deprived nonasthmatic and asthmatic HASM cells 0 and 2 h after 24-h serum deprivation. IgG negative controls are shown. n = 3 nonasthmatic and 3 asthmatic HASM cell lines. (E) VEGF protein levels after incubation with the HDAC inhibitor SAHA. n = 3 nonasthmatic and 3 asthmatic HASM cell lines. (F) VEGF protein levels after incubation with the HDAC inhibitor MS275. n = 6 nonasthmatic and 6 asthmatic HASM cell lines.
of active transcription. We saw that at times correlating to H3K9me3 in nonasthmatic HASMs, RNA pol II binding was reduced (Fig. 8A, 8B). Furthermore, when RNA pol II binding was measured in samples incubated with the H3K9me3 inhibitor BIX-01294, we found RNA pol II binding was recovered (Fig. 8C, 8D). Although the changes in RNA pol II association measured were small, they were consistent and in keeping with the poorly inducible nature of the VEGF promoter.

These data suggest that in nonasthmatic cells a transcription repression mechanism is present involving induction of H3K9 methylation and resultant reduction in RNA pol II binding and limitation of Sp1 binding. This repression mechanism is missing in asthmatic cells allowing unregulated binding of Sp1, maintenance of RNA pol II binding, and VEGF transcription. H3K9me3 is absent at the asthmatic promoter because of failed recruitment of G9A

Finally, we wanted to know why H3K9me3 in nonasthmatic HASMs, RNA pol II binding was reduced (Fig. 8A, 8B). Furthermore, when RNA pol II binding was measured in samples incubated with the H3K9me3 inhibitor BIX-01294, we found RNA pol II binding was recovered (Fig. 8C, 8D). Although the changes in RNA pol II association measured were small, they were consistent and in keeping with the poorly inducible nature of the VEGF promoter.

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H3K9me3 is absent at the asthmatic promoter because of failed recruitment of G9A

We show a dependence of VEGF hypersecretion on active transcription using actinomycin D. We have not ruled out that posttranscriptional mechanisms may also contribute to the differences we see in VEGF expression; however, low expression levels have prevented us from investigating this further, and the strong evidence we present for a transcriptional mechanism suggests a dominant role for transcriptional dysregulation. We also show selection of a specific VEGF isoform in the A cells. VEGF splice variants differ in functional activity. The presence of exons 6 and 7 determines bioavailability and biodistribution, whereas differential splicing of exon 8 can result in inhibitory isoforms (50). The molecular mechanisms of VEGF splice variant selection are unclear. It is thought that inducing VEGF transcription may also activate a milieu of splicing SR proteins, resulting in selection of specific VEGF isoforms.
of specific VEGF isoforms. Asthmatic HASM cells selectively produce VEGF165a, considered the most proangiogenic isoform.

Sp1 is a transcription factor belonging to the specificity protein/Krüppel-like factor family. Sp1 binds a consensus GC-rich region of DNA, which occurs at least 12,000 times in the human genome (31). We report that basal VEGF secretion from both asthmatic and nonasthmatic cells is dependent on Sp1 association with the VEGF promoter. Furthermore, Sp1 binding at the asthmatic promoter is increased and actively induced and concomitant with increased or retained binding of members of the basal transcriptional machinery, namely TAF1 and RNA pol II. As Sp1 binds GC-rich regions of DNA, it is not surprising that its association can be regulated by DNA methylation, a process in which cytosines within a CpG orientation are methylated, resulting in repression of transcription. The VEGF gene contains three large CpG islands (regions where the CpG orientation occurs at a greater than normal frequency). Data regarding the methylation status of these islands is scant. A recent paper (51) describing increased VEGF expression in lung cancer cells performed bisulfite sequencing of a portion of VEGF CpG1 (approximately CpG1.2 and 1.3) and showed no methylation. However, no normal control cells were sequenced. Considering our data, it seems likely the control cells would also have been unmethylated. Our study provides the most comprehensive whole VEGF gene methylation analysis to date. We did not see any differences between nonasthmatic and asthmatic HASM cell VEGF gene DNA methylation. Consistent with this, the DNMT inhibitor 5-aza was without effect.

We next considered histone modification as a potential regulatory mechanism of VEGF transcription. As Sp1 binding can occur in an environment of histone hyperacetylation (47) and Sp1 can associate with HATs, including TAF1 (38, 48), which we show to be increased at the asthmatic VEGF promoter, we first investigated histone H3 and H4 acetylation at the VEGF promoter. We found no difference in histone acetylation between nonasthmatic and asthmatic HASM cells. There is evidence for histone deacetylase (HDAC) inhibitor and HAT inhibitor/small interfering RNA effects on VEGF in the literature but without associated data on histone acetylation at the VEGF promoter (52, 53). Papers describing histone acetylation at the VEGF promoter detect regions of the DNA further upstream than those used in the current study and are in response to stimuli as opposed to basal levels (14).

Although it is possible that any differential histone acetylation occurs at a region of the promoter not covered by our primers, this would seem unlikely as we would have expected to observe an effect with the HDAC inhibitors if this was the case.

Having discounted histone acetylation we investigated histone methylation. We show that increased H3K4me3 is associated with the basal asthmatic VEGF promoter. To our knowledge, we believe this to be the first record of histone acetylation at the VEGF promoter. H3K4me3 is a well characterized marker of active transcription and frequently found at RNA pol II-associated promoters. When we looked at RNA pol II binding at the asthmatic and nonasthmatic promoters, to establish whether its association mirrored the differential levels of H3K4 trimethylation, rather than finding increased RNA pol II binding at the A promoter, we observed a loss of RNA pol II at the nonasthmatic VEGF promoter. Although this reduction in RNA pol II binding was small, it was consistent across the samples and correlated with
increased association of H3K9me3 at the nonasthmatic VEGF promoter. Using inhibitors and D/N constructs of the methylases responsible for H3K9me3, namely G9A and SUV39H1, the H3K9me3 mark at the nonasthmatic promoter was found to be functionally repressing VEGF transcription. To establish how H3K9me3 was repressing VEGF transcription, we used ChIP for Sp1 and RNA pol II in the presence of the G9A/H3K9me3 inhibitor BIX-01294. We show BIX-01294 could recover Sp1 and RNA pol II binding at the VEGF promoter, suggesting that H3K9me3 was preventing Sp1 binding and reducing RNA pol II association. Reduction in RNA pol II binding in the presence of FIGURE 8. H3K9me3 affects RNA polymerase II binding to the VEGF promoter. RNA pol II association with the proximal (A) and distal (B) VEGF promoter was assessed by ChIP. RNA Pol II association was measured in confluent, serum-deprived nonasthmatic and asthmatic HASM cells 0 and 2 h after 24-h serum deprivation. IgG negative controls are shown. Data are expressed as fold change relative to the mean 0 h nonasthmatic value. n = 3 nonasthmatic and 6 asthmatic HASM cell lines. RNA pol II association with the proximal (C) and distal (D) VEGF promoter, in the presence of BIX-01294 was assessed by ChIP as per Sp1. n = 3 nonasthmatic and 3 asthmatic HASM cell lines.

increased association of H3K9me3 at the nonasthmatic VEGF promoter. Using inhibitors and D/N constructs of the methylases responsible for H3K9me3, namely G9A and SUV39H1, the

FIGURE 9. G9A is not recruited to the asthmatic VEGF promoter. G9A association with the proximal (A) and distal (B) VEGF promoter was assessed by ChIP. G9A association was measured in confluent, serum-deprived nonasthmatic and asthmatic HASM cells 0 and 2 h after 24-h serum deprivation. IgG negative controls are shown. Data are expressed as fold change relative to the mean 0 h nonasthmatic value. n = 4 nonasthmatic and 4 asthmatic HASM cell lines.

H3K9me3 mark at the nonasthmatic promoter was found to be functionally repressing VEGF transcription. To establish how H3K9me3 was repressing VEGF transcription, we used ChIP for Sp1 and RNA pol II in the presence of the G9A/H3K9me3 inhibitor BIX-01294. We show BIX-01294 could recover Sp1 and RNA pol II binding at the VEGF promoter, suggesting that H3K9me3 was preventing Sp1 binding and reducing RNA pol II association. Reduction in RNA pol II binding in the presence of

FIGURE 10. Differences in complex formation at the nonasthmatic and asthmatic VEGF promoter regulate VEGF transcription. Both asthmatic and nonasthmatic cells have the same DNA methylation and histone acetylation pattern. A repressive complex is associated with the non-asthmatic promoter composed of histone H3 trimethylated in lysine 9, the histone methyltransferases G9A and SUV39H1 and low levels of Sp1. Repression of VEGF transcription to maintain “normal” levels is dependent on G9A recruitment and H3K9me3. The asthmatic promoter fails to recruit G9A and lacks the repressive complex. Instead the asthmatic VEGF promoter is occupied by a transcriptionally active complex containing histone H3 trimethylated at K4, components of the basal transcriptional machinery, namely RNA pol II and TAF1 and heightened levels of Sp1.
H3K9me3 agrees with published data investigating Kruppel-associated box-associated protein 1-mediated transcriptional repression via heterochromatin spreading (54). Furthermore, occlusion of Sp1 binding to the RASSF1A promoter is associated with increased H3K9me3 (55). However, in both these instances, other epigenetic modifications were present and contributed to binding, for example, decreased histone acetylation and increased DNA methylation contributed to loss of Sp1 binding, whereas decreased histone acetylation and increased HP1 binding contributed to loss of RNA pol II. We show that neither histone acetylation nor DNA methylation are different between asthmatic and nonasthmatic cells, and we were unable to detect HP1 by ChIP.

Finally, we show that reduced H3K9me3 at the asthmatic VEGF promoter is due failed recruitment of the methyltransferase G9A. Recently, evidence for a novel DNA conformation at the VEGF promoter has emerged. The VEGF promoter (within the regions that bind Sp1) contains a polyuridine tract consisting of five runs of three or more contiguous guanines separated by one or more bases, which is capable of forming a G-quadruplex DNA structure. These structures, when stabilized, can inhibit transcription of mammalian genes including c-myc, hypoxia-inducible factor 1α, and VEGF (56). G-quadruplex DNA structures can also form at telomere ends, and it is suggested that telomere repeat factor 2 can bind G-quadruplex DNA and affect H3K9me3 (57). It is possible therefore that H3K9me3 results in (or from) changes in the structure of the VEGF promoter DNA that affects G9A/Sp1/RNA pol II and transcription. Alternatively, evidence is also emerging that noncoding RNA (ncRNA) can epigenetically regulate gene transcription, for example, the Air ncRNA recruits G9A to the Slc22a3 promoter, resulting in H3K9me3 and transcription repression (58). Further work is required to establish whether either of these mechanisms contributes to VEGF regulation in HASM cells.

In summary, by comparing nonasthmatic and asthmatic HASM cell VEGF secretion, we have identified a mechanism of basal VEGF repression that is absent from asthmatic HASM cells. This mechanism is dependent on changes in histone methylation and associated changes in binding of transcription factors and constituents of the basal transcription machinery. The process requires induction of H3K9me3, but whereas previous studies show H3K9me3 to act in concert with reduced histone acetylation and induction of DNA methylation to repress transcription, in this instance, H3K9me3 alone appears functional. VEGF is critical to asthma pathogenesis. Bronchial biopsies from mild asthmatics are more vascular than those from controls and asthmatic bronchial vessels are larger than those of control subjects (59, 60). Increased vessel number extends into the medium and small airways and vascularity is increased in moderate asthmatics compared with mild asthmatics (61). In addition, in asthmatics, the number of vessels in the medium airways and FEV1 percentage of predicted are inversely correlated (61). VEGF levels in spu tum are higher in adult asthmatic patients compared with nonasthmatic patients (3, 4, 62-64) and levels correlate with vessel number (64), suggesting VEGF may contribute to the increased angiogenesis seen in the asthmatic lung. In agreement with this, the VEGF in asthmatic bronchoalveolar lavage fluid can induce angiogenesis in an in vitro endothelial cell and dermal fibroblast coculture system (65). Levels of induced sputum VEGF are also higher in asthmatic children during an acute attack compared with control children and the higher the asthma severity the greater the level of VEGF. In addition, there is a negative correlation between FEV1 percentage of predicted and VEGF levels in asthmatic children (66). VEGF staining of airway biopsies also shows higher levels of VEGF in asthmatic airways compared with control (64). Identification of mechanisms by which VEGF is regulated therefore provides targets for potential future asthma therapeutics. Histone methyltransferases have been described as “the most promising class of potential targets for the next generation of epigenetic therapies” (67) and therefore may represent a promising new asthma therapy.

Disclosures
The authors have no financial conflicts of interest.

References


