

Inhibition of lymphangiogenic factor VEGF-C expression and production by the histone deacetylase inhibitor suberoylanilide hydroxamic acid in breast cancer cells

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Abstract. Suberoylanilide hydroxamic acid (SAHA), a potent histone deacetylase (HDAC) inhibitor, has been shown to exert anticancer effects in various types of human cancer and is now used in the clinic for cancer treatment. In addition to cytostatic and cytotoxic activities, SAHA also represses angiogenesis to inhibit tumor growth. However, the effect of SAHA on tumor lymphangiogenesis, a step in which cancer cells produce pro-lymphangiogenic factors such as vascular endothelial growth factor-C (VEGF-C) to stimulate proliferation and migration of lymphatic endothelial cells, remains largely unclear. In this study, we investigated the expression of VEGF-C in breast cancer cell lines and found that VEGF-C was highly expressed in MDA-MB-231, MCF-7, MDA-MB-453 and BT-474 cells. SAHA inhibited VEGF-C expression in a dose-dependent manner in these cell lines. The secretion of VEGF-C into conditioned medium was also suppressed. We cloned human VEGF-C gene promoter and demonstrated that SAHA directly repressed promoter activity in MDA-MB-231 cells. Promoter deletion assay suggested that SAHA repressed VEGF-C via the -185/+38 region which contained several transcription factor binding sites. Notably, we found that SAHA reduced Sp1, but not Sp3 and NF- κ B protein levels. Treatment with Sp1 inhibitor mithramycin A also inhibited VEGF-C expression in breast cancer cells. In addition, enforced expression of Sp1 partially rescued the inhibition of VEGF-C by SAHA. Collectively, our results suggest that SAHA inhibits VEGF-C expression in breast cancer cells via transcriptional repression and this drug may exert anti-lymphangiogenic activity in cancer treatment.

Introduction

Epigenetic alteration is a general phenomenon during carcinogenesis (1). Both DNA methylation and histone modification play critical roles in the control of expression of oncogenes and tumor suppressor genes (2,3). One of the most important histone modifications is acetylation which is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). These enzymes are considered to be crucial targets for the development of anticancer drugs. Previous studies demonstrated that HDAC inhibitors show potent anticancer activities against different types of human cancer (4-6).

HDAC inhibitors are classified into different groups based on their chemical structures and their ability to inhibit individual HDACs (7,8). The first class is short chain fatty acids including butyrate and valproate. The second class is hydroxamic acid derivatives such as trichostatin A, suberoylanilide hydroxamic acid (SAHA, also known as vorinostat). The third class is benzamide and the fourth class is cyclic tetrapeptide. Among them, SAHA is one of the pioneer HDAC inhibitors that has entered clinical therapy and is now approved for the treatment of cutaneous T cell lymphoma (9,10). This drug is also undergoing clinical trials for the treatment of solid tumors, including non-small cell lung cancer and breast cancer. The anticancer effects of SAHA are mediated by different mechanisms. First, SAHA directly causes apoptosis in cancer cells (11,12). Second, SAHA upregulates anti-proliferative genes, such as p21, to inhibit proliferation (13,14). Third, SAHA acts as an immune modulating drug to improve anticancer immune surveillance (15,16). Fourth, SAHA suppresses angiogenesis to attenuate tumor growth *in vivo* (17).

Lymphangiogenesis is the process by which cancer cells promote the proliferation of lymphatic endothelial cells and enhance the migration of these cells toward tumor part (18). Previous studies demonstrated that induction of lymphangiogenesis is strongly associated with tumor metastasis and poor prognosis in cancer patients (19-21). The key step in inducing lymphangiogenesis is the production of lymphangiogenic factors by cancer cells. Among the lymphangiogenic factors studied, vascular endothelial growth factor-C (VEGF-C) has received considerable attention since lymphatic endothelial

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cells express high levels of its cognate receptor VEGFR3 and their proliferation is potently stimulated by VEGF-C.

Although SAHA was able to inhibit angiogenesis in different types of cancer, its effect on lymphangiogenesis has not been demonstrated. In this study, we investigated the expression of VEGF-C in breast cancer cell lines and its regulation by SAHA. In addition, we tried to elucidate the underlying mechanism by which SAHA modulated VEGF-C transcription.

Materials and methods

Cell culture. Human breast cancer cell lines MDA-MB-231, MCF-7, MDA-MB-453 and the normal breast cell line M10 were purchased from the cell bank of the National Health Research Institute (Maoli, Taiwan). MDA-MB-231 and MDA-MB-453 cells were grown in the L15 medium (Invitrogen, Carlsbad, CA, USA) containing 10% FCS, L-glutamate and antibiotics. MCF-7 and M10 cells were cultured in the MEM medium (Invitrogen) containing 10% FCS and antibiotics. BT-474 breast cancer cells were kindly provided by Dr Jin-Yuh Shew (National Taiwan University, Taiwan) and were maintained in the DMEM/F12 medium (Invitrogen) supplemented with 10% FCS, L-glutamate, nonessential amino acids, sodium pyruvate and antibiotics. All cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Reagents and antibodies. SAHA was purchased from LC Laboratories (Woburn, MA, USA). SAHA was dissolved in DMSO at a concentration of 20 mM and stored at -70°C. It was diluted in the culture medium to different working concentrations and used for cell treatment. Mithramycin A was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibody against VEGF-C was purchased from R&D Systems (Minneapolis, MN, USA). Antibody against NF-κB (p65) and Sp3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against actin and Sp1 were purchased from Millipore (Billerica, MA, USA). Antibody against acetyl lysine-histone 3 was purchased from Cell Signaling Technology (Beverly, MA, USA).

Construction of VEGF-C promoter plasmids and the luciferase assay. Genomic DNA was extracted from MCF-7 cells using an extraction kit (Qiagen, Hilden, Germany) and the -1046/+38 region from translational start site of VEGF-C gene (NM_005429) was amplified by PCR using two specific primers (VEGF-C-1046-forward 5'-ATACTCGAGCTTTTACAACCCCGAGGACA-3' and VEGF-C-1046-reverse 5'-AAAAAGCTTAGAGACACGCCACAGAGAAG-3'). A 1.1-kb DNA fragment was subcloned into the luciferase reporter gene vector pGL3 (Promega, Madison, WI, USA) by *Xho*I and *Hind*III to yield the luciferase reporter construct pGL3-1.1kb-VEGF-C (-1046/+38). Using this construct as a template, two 5'-deletion constructs were generated by the following primers: VEGF-C-439-forward 5'-ATACTCGAGCTCTCACTTCGGGGAAGG-3' and VEGF-C-185-forward 5'-ATACTCGAGGCCCTGCAAAGTTGGGAAC-3'. MDA-MB-231 cells were seeded into 6-well plates and transfected with 1 μg of serial VEGF-C promoter-luciferase plasmids. After 24 h, cells were incubated without or with SAHA (10 μM) for an additional 24 h. The luciferase activity was detected by reporter assay system (Promega),

Table I. PCR primers used in this study.

VEGF-C	F: 5'-CAGTTACGGTCTGTGTCCAGTGTAG-3'
VEGF-C	R: 5'-GGACACACATGGAGGTTTAAAGAAG-3'
Sp1	F: 5'-TATAGCAAATGCCCCAGGT-3'
Sp1	R: 5'-TTGCCATACACTTTCACACA-3'
Sp3	F: 5'-CCTGCAGATATTAGGATCAAGG-3'
Sp3	R: 5'-GCCTCTGTAATTCATCACTTCG-3'
RelA	F: 5'-TCAATGGCTACACAGGACCA-3'
RelA	R: 5'-ATCTTGAGCTCGGCAGTGTT-3'
Actin	F: 5'-TGTTACCAACTGGGACGACA-3'
Actin	R: 5'-GGGGTGTGAAGGTCTCAA-3'

F, forward; R, reverse.

according to the manufacturer's instructions, and normalized by protein concentration in cell lysates.

Transient cell transfection. GFP-Sp1 expression vector (kindly provided by Dr Jan-Jong Hung, National Cheng Kung University, Taiwan) and control vector were transfected into MDA-MB-231 cells using Lipofectamine 2000 (Invitrogen). After 48 h, cells were treated with SAHA for an additional 24 h. Conditioned medium and cell lysates were harvested for further analysis.

Reverse transcription-polymerase chain reaction (RT-PCR). MDA-MB-231, MCF-7, BT-474 and M10 breast cells were seeded into the 6-well plates. Following overnight incubation, cells were treated with different concentrations of SAHA for 24 h. Total RNA was isolated by RNeasy mini kit (Qiagen) and mRNAs were reverse-transcribed to cDNA by MMLV reverse transcriptase (Promega) using oligo-dT primers according to the manufacturer's instructions. PCR reaction was performed under the following conditions: initialization step for 5 min at 95°C, 30 cycles of amplification, with 40 sec at 95°C for denaturation and 40 sec at 60°C for annealing and 40 sec at 72°C for elongation, and 7 min at 72°C for further extension. The PCR primers used are shown in Table I.

Enzyme-linked immunosorbent assay (ELISA) for VEGF-C. MDA-MB-231 cells were incubated under various concentrations of SAHA in L15 medium for 24 h. The conditioned medium was collected and centrifuged at 1500 rpm for 5 min to remove cell debris. VEGF-C concentration was measured using Quantikines Human VEGF-C Immunoassay kit from R&D.

Western blotting. Cells were seeded into the 6-well plates. Following overnight incubation, cells were treated with different concentrations of SAHA for 6, 12 or 24 h. Cells were washed with cold PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.5 M sucrose, 0.25% sodium deoxycholate, 10% glycerol, 1% NP-40; protease inhibitors were added prior to use) on ice for 10 min. Cell debris was removed by centrifugation and cellular proteins were further fractionated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore).

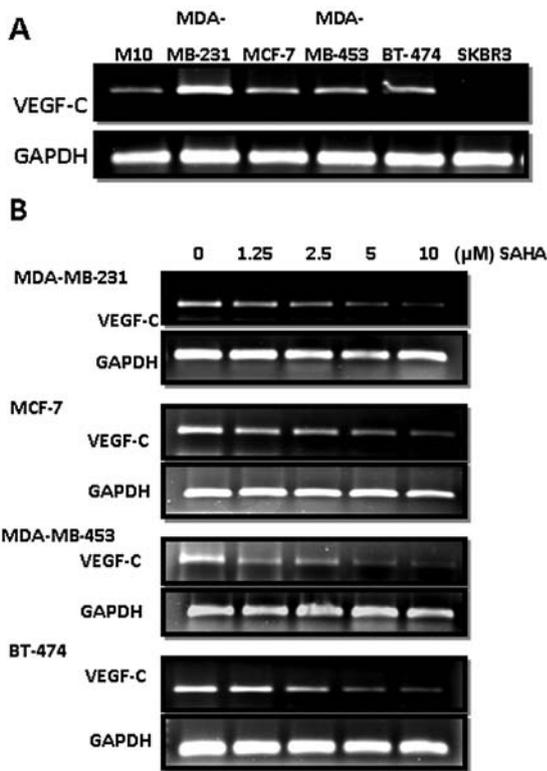


Figure 1. Expression of VEGF-C in breast cancer cell lines and its inhibition by SAHA. (A) VEGF-C expression of various breast cancer cell lines compared to the normal human mammary epithelial cell line M10. (B) Breast cancer cell lines were treated with different concentrations of SAHA for 24 h. VEGF-C mRNA was detected by RT-PCR.

After incubation with 5% non-fat milk in TBST, the membranes were probed with different primary antibodies followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies. Enhanced chemiluminescence (ECL) reagent (Millipore) was used to detect the blots according to the manufacturer's instructions.

Statistical analysis. Data were expressed as the means \pm SE. Student's t-test was used to evaluate the differences between various experimental groups. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

VEGF-C expression is inhibited by SAHA in breast cancer cell lines. As shown in Fig. 1A, we found that all the cell lines, except SKBR3, investigated in this study expressed higher levels of VEGF-C than the normal breast epithelial cell line M10. SAHA dose-dependently downregulated the expression of VEGF-C in MDA-MB-231, MCF-7, MDA-MB-453 and BT-474 cells (Fig. 1B).

Reduction of VEGF-C production by SAHA. We next investigated whether production of the VEGF-C protein was indeed inhibited. Since MDA-MB-231 cells expressed the highest amount of VEGF-C, we used this cell line as a model in the subsequent experiments. Two approaches were performed. First, cellular VEGF-C protein level was determined by western

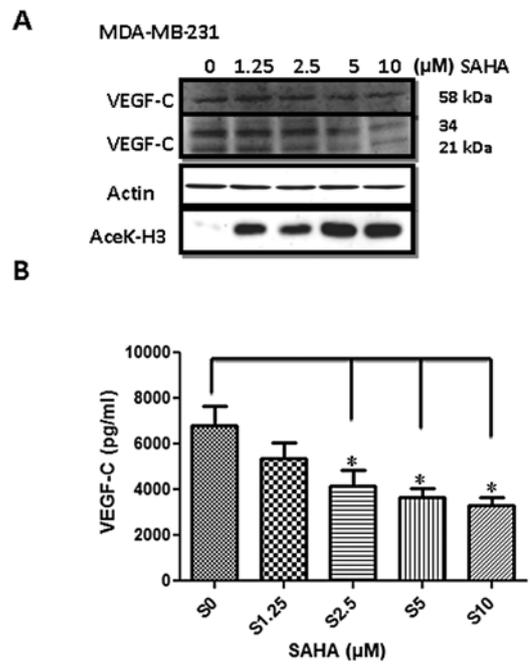


Figure 2. Inhibition of VEGF-C protein expression and production by SAHA. (A) MDA-MB-231 cells were treated with different concentrations of SAHA for 24 h and cellular proteins and histones were extracted. VEGF-C isoforms with a molecular weight of 58, 34 and 21 kDa were detected by western blotting. Acetylation status of histone H3 was also investigated by anti acetyl-lysine H3 antibody. (B) MDA-MB-231 cells were incubated under various concentrations of SAHA in L15 medium for 24 h. The conditioned medium was collected and VEGF-C concentration was measured using ELISA assay. * $p < 0.05$, compared to the control group.

blotting. As shown in Fig. 2A, three isoforms of VEGF-C with a molecular weight of 58, 34 and 21 kDa were reduced by SAHA in a dose-dependent manner. We also found that histone H3 acetylation was significantly increased indicating SAHA at these concentrations indeed exerted potent inhibitory effects on HDACs. In addition, VEGF-C released into the conditioned medium detected by ELISA assay was also reduced accordingly. Treatment of 10 μ M SAHA reduced VEGF-C concentration by 50% (Fig. 2B).

SAHA represses VEGF-C promoter activity via the -185/+38 of the promoter region. Since SAHA reduces VEGF-C mRNA levels, this drug might directly inhibit VEGF-C transcription. We cloned human VEGF-C promoter region between -1046/+38 bp region from translational start site and generated different deletion promoter-luciferase constructs (Fig. 3A). These constructs were transfected into MDA-MB-231 and the effect of SAHA was examined. Our data demonstrated that SAHA at the concentration of 10 μ M inhibited the full-length (-1046/+38) promoter by 30-40% (Fig. 3B). Deletion of promoter to -185 region did not affect SAHA-induced inhibition indicating the responsive elements were located between -185/+38 region.

SAHA reduces Sp1-mediated VEGF-C expression. Bioinformatics search revealed several potential transcription factor binding sites including Sp1, AP-2 and NF- κ B within this region. Notably, we found that SAHA caused reduction of Sp1 but not Sp3 and NF- κ B protein levels in MDA-MB-231 cells

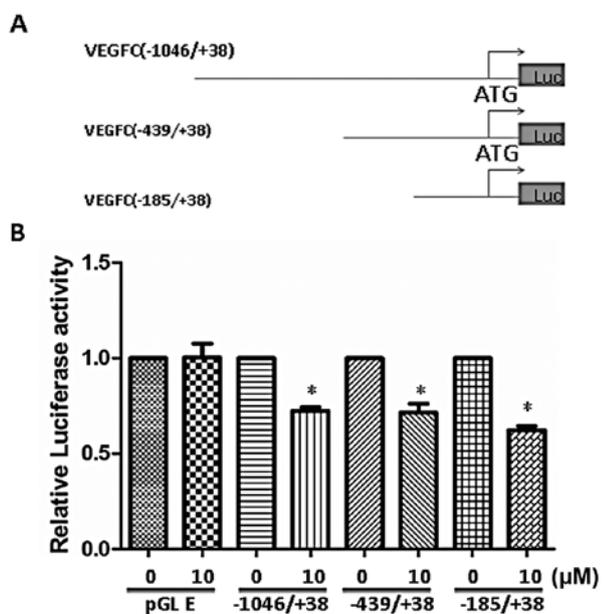


Figure 3. SAHA inhibits human VEGF-C gene promoter activity. (A) Human VEGF-C gene promoter (-1046/+38) was amplified from MCF-7 cells and subcloned into pGL3 reporter vector. Two additional deletion mutant reporters were generated. (B) MDA-MB-231 cells were seeded into 6-well plates and transfected with 1 μ g of serial VEGF-C promoter-luciferase plasmids. After 24 h, cells were incubated without or with SAHA (10 μ M) for an additional 24 h. Luciferase activity was detected by the reporter assay system and normalized by protein concentration in cell lysates. * p <0.05, compared to the control group.

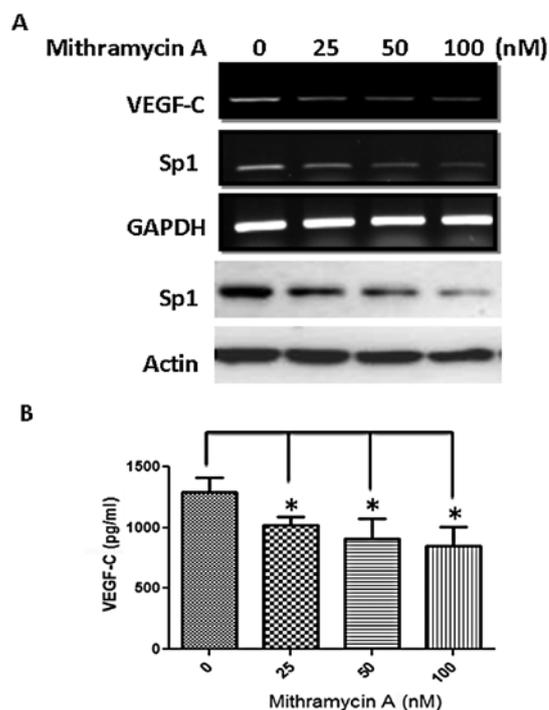


Figure 5. Inhibition of Sp1 reduces VEGF-C expression and production. (A) MDA-MB-231 cells were treated with different concentrations of Sp1 inhibitor mithramycin A for 24 h. Expression of Sp1 and VEGF-C was studied. (B) Conditioned medium was also collected and VEGF-C concentration was examined. * p <0.05, compared to the control group.

(Fig. 4A). Our data demonstrated that the Sp1 protein level was significantly decreased at 12 h after SAHA addition. However, no significant change at the mRNA level of Sp1, Sp3 and NF- κ B was found until a 24-h treatment suggesting SAHA inhibited Sp1 via a post-translational regulation (Fig. 4B). To

confirm the importance of Sp1, we treated cells with an Sp1 inhibitor mithramycin A and found that this inhibitor reduced Sp1 protein and attenuated VEGF-C mRNA expression dose-dependently (Fig. 5A). The amount of VEGF-C in the conditioned medium was also significantly reduced (Fig. 5B).

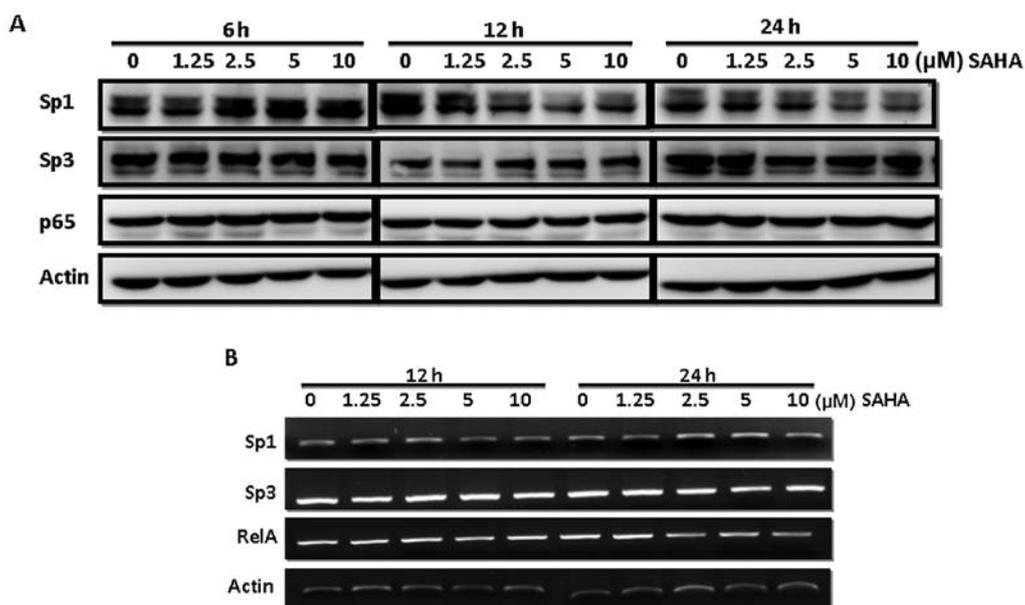


Figure 4. SAHA downregulates Sp1 protein but not mRNA levels. (A) MDA-MB-231 cells were treated with different concentrations of SAHA for 6, 12 or 24 h. Cellular proteins were harvested for western blot analysis. Protein levels of Sp1, Sp3 and p65 were determined. (B) Cellular mRNAs were also harvested at 12 or 24 h after SAHA treatment and mRNA levels of Sp1, Sp3 and RelA were examined by RT-PCR.

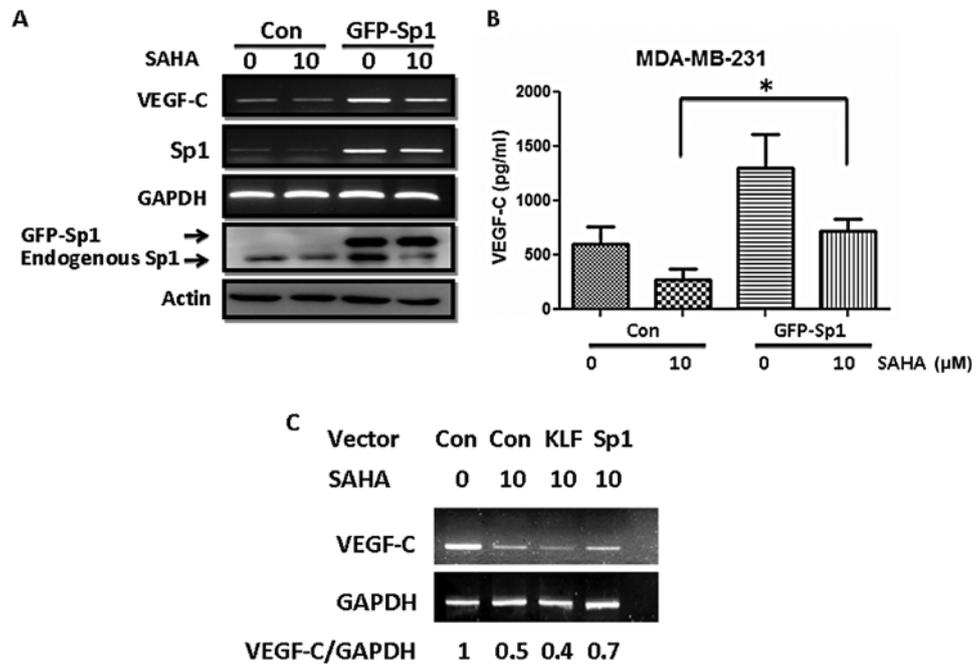


Figure 6. Enforced expression of Sp1 partly reverses the inhibition of VEGF-C by SAHA. (A) MDA-MB-231 cells were transfected with control (Con) or GFP-Sp1 vector for 24 h and were then treated without or with 10 μM of SAHA for another 24 h. Cellular mRNAs and proteins were harvested and expression of VEGF-C and Sp1 was examined. (B) Conditioned medium was also collected and VEGF-C concentration was examined. *p<0.05 when the results of two experimental groups were compared. (C) MDA-MB-231 cells were transfected with Con, KLF10 (KLF) or GFP-Sp1 (Sp1) vector for 24 h and then treated without or with 10 μM of SAHA for another 24 h. VEGF-C mRNA level was examined by RT-PCR and was normalized to GAPDH. The ratio of the densitometric signal of VEGF-C/GAPDH is indicated.

Enforced expression of Sp1 partly reverses the inhibition of VEGF-C by SAHA. The aforementioned data suggest that SAHA inhibited VEGF-C expression partly via Sp1. Consistent with this hypothesis, enforced expression of Sp1 in MDA-MB-231 cells partly reversed the inhibition of VEGF-C by SAHA (Fig. 6A). The VEGF-C released into the medium was also restored by Sp1 overexpression (Fig. 6B, lanes 2 and 4). Ectopic expression of Sp1 alone increased VEGF-C mRNA expression and the amount of VEGF-C in the conditioned medium. However, this upregulation was still attenuated by SAHA as this drug caused significant reduction of endogenous Sp1 protein as shown in Fig. 6A. The effect of Sp1 is specific since expression of KLF10, another member of the Kruppel-like/Sp1 gene family, could not antagonize the inhibition of VEGF-C by SAHA (Fig. 6C). Collectively, our results suggest that SAHA induced downregulation of Sp1 protein via a post-translational mechanism which led to reduction of Sp1-driven VEGF-C expression in breast cancer cells.

Discussion

Pathological studies have demonstrated that expression of VEGF-C and its receptor VEGFR3 is associated with angiogenesis, lymphangiogenesis and lymph node metastasis in breast cancer (22,23). By using an orthotopic transplantation model, Skobe *et al* clearly showed that induction of lymphangiogenesis by VEGF-C promotes breast cancer metastasis (24). Therefore, targeting VEGF-C/VEGFR3 signaling axis is important for breast cancer therapy. Since VEGFR3 is a tyrosine kinase, tyrosine kinase inhibitors are potential candidates

to inhibit VEGFR3 activity. Sorafenib was initially identified as a potent inhibitor of c-RAF. However, it also inhibits VEGFR2 and VEGFR3 at concentrations of approximately 6-10 nM (25). This drug is now used in the clinic for the treatment of solid tumors. Other kinase inhibitors including sunitinib, AMG706, axitinib, and AZD2171 have also shown inhibitory activity against VEGFR3 and are now under different phases of clinical trials (26). Another strategy to suppress VEGFR3 activity is by blocking antibodies. A recombinant bispecific antibody has been developed to neutralize the biological activities of VEGFR2 and VEGFR3 (27). Roberts *et al* also demonstrated that inhibition of VEGFR3 activation with the antagonistic antibody potently suppresses lymph node and distant metastasis in an orthotopic spontaneous breast cancer model (28). An antibody that inhibits homodimerization of VEGFR3 and its heterodimerization with VEGFR2 has recently been shown to suppress both angiogenesis and lymphangiogenesis *in vivo* (29).

The targeting of VEGF-C has not received significant attention in the past decade. By using antibody phage-display, Rinderknecht *et al* identified a VEGF-C-blocking antibody which could effectively inhibit the interaction between VEGF-C and VEGFR3 and suppress its downstream signaling (30). The underlying mechanism mediating the upregulation of VEGF-C in cancer cells is largely unclear. A pioneer study of the genomic organization of human and mouse VEGF-C genes revealed that the upstream sequences contain conserved putative binding sites for Sp1, AP-2, and NF-κB transcription factors but not TATA box (31). In the present study, we demonstrated for the first time that a clinically used histone

deacetylase inhibitor SAHA was able to inhibit Sp1-mediated expression of VEGF-C in human breast cancer cells and reduced VEGF-C concentration in the conditioned medium. Results of this study suggest that multiple transcription factors may be simultaneously involved in the regulation of VEGF-C. In addition, we demonstrated that the Sp1 specific inhibitor mithramycin A also attenuated VEGF-C expression suggesting a critical role of Sp1 in VEGF-C transcription. Our results provide a new strategy to suppress the expression of a lymphangiogenic factor in cancer cells by using HDAC inhibitors. Reduction of the production of VEGF-C will attenuate lymphangiogenesis and lymphatic metastasis *in vivo*. Since SAHA has already been approved for cancer treatment in the clinic, it will be of benefit to test whether this drug, in addition to its cytotoxic effect, also shows anti-lymphangiogenic activity in animals and patients.

Sp1 is a ubiquitous transcription factor expressed in mammalian cells and was initially recognized as a constitutive activator of housekeeping genes. However, recent studies demonstrated that Sp1 is involved in the control of tissue-specific and inducible genes and these target genes play critical roles in proliferation, differentiation, apoptosis and oncogenesis (32). In addition, increased Sp1 protein was found in various types of human cancer suggesting a possible oncogenic role (33,34). Our finding that SAHA causes downregulation of Sp1 protein is noteworthy. Although the underlying mechanism remains unclear, two potential mechanisms may be involved. First, a previous study identified lysine-703 as a major acetylation site of Sp1. Inhibition of HDAC activity by SAHA may directly affect the acetylation status of Sp1 and subsequently change protein stability (35). Second, heat shock protein 90 (hsp90) is an important regulator of Sp1 stability (36). SAHA has been demonstrated to inhibit the HDAC6-hsp90 chaperone complex to induce the degradation of client proteins such as mutant p53 (37). Therefore, it seems possible that SAHA may inhibit hsp90 and then affect Sp1 degradation.

In the present study, we demonstrated that SAHA is a potent inhibitor of VEGF-C expression in breast cancer cells and we showed that the inhibition is mediated by repression of Sp1. Our results also suggest that SAHA may exert anti-lymphangiogenic activity in cancer treatment.

Acknowledgements

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