Epigenetic modulators hydralazine and sodium valproate act synergistically in VEGI-mediated anti-angiogenesis and VEGF interference in human osteosarcoma and vascular endothelial cells

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Abstract. Vascular endothelial growth inhibitor (VEGI; also referred to as TNFSF15 or TL1A) is involved in the modulation of vascular homeostasis. VEGI is known to operate via two receptors: Death receptor-3 (DR3) and decoy receptor-3 (DcR3). DR3, which is thus far the only known functional receptor for VEGI, contains a death domain and induces cell apoptosis. DcR3 is secreted as a soluble protein and antagonizes VEGI/DR3 interaction. Overexpression of DcR3 and downregulation of VEGI have been detected in a number of cancers. The aim of the present study was to investigate the effects of sodium valproate (VPA), a histone deacetylase inhibitor, in combination with hydralazine hydrochloride (Hy), a DNA methylation inhibitor, on the expression of VEGI and its related receptors in human osteosarcoma (OS) cell lines and human microvascular endothelial (HMVE) cells. Combination treatment with Hy and VPA synergistically induced the expression of VEGI and DR3 in both OS and HMVE cells, without inducing DcR3 secretion. In addition, it was observed that the combination of VPA and Hy significantly enhanced the inhibitory effect on vascular tube formation by VEGI/DR3 autocrine and paracrine pathways. Furthermore, the VEGI/VEGF-A immune complex was pulled down by immunoprecipitation. Taken together, these findings suggest that DNA methyltransferase and histone deacetylase inhibitors not only have the potential to induce the re-expression of tumor suppressor genes in cancer cells, but also exert anti-angiogenic effects, via enhancement of the VEGI/DR3 pathway and VEGI/VEGF-A interference.

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Introduction

Osteosarcoma (OS) is a highly malignant and aggressive bone tumor that mostly occurs in children and adolescents. OS is characterized by early lung metastasis and a poor prognosis (1). The 5-year survival rate of patients with localized OS has remained stable at 60-70% in recent years due to advances in chemotherapy. However, the development of metastasis reduces the survival rate to 20% (2).

Epigenetic processes play a key role in the regulation of gene expression by affecting chromatin structure. DNA methylation and histone modification are important epigenetic mediators of transcriptional suppression, and they are essential for biological processes (3). Aberrant epigenetic regulation, such as DNA hypermethylation and histone deacetylation, is a frequent event within the promoters of tumor suppressor genes in cancer cells (4) and contributes to cancer development, progression and metastasis (5). DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors synergistically reactivate epigenetically silenced tumor suppressor genes and induce growth inhibition and apoptosis of tumor cells (6). Recently, DNMT and HDAC inhibitors were shown to directly inhibit endothelial cell growth and tumor angiogenesis (7,8). Angiogenesis is characterized by the formation of new blood vessels and is required for fundamental physiological processes, including embryonic development and tissue repair (9); it also has the potential to promote tumor progression and the development of metastasis (10). Tumor cells secrete pro-angiogenic factors, such as vascular endothelial growth factor (VEGF). VEGF, also referred to as VEGF-A, belongs to a large family of proteins. VEGFs and VEGF-Rs are important for vessel formation in healthy individuals, as well as tumor angiogenesis, and the interaction between receptors and ligands mediates the survival and proliferation of malignant cells (11).

Vascular endothelial growth inhibitor (VEGI), also referred to as TL1A or TNFSF15, is a member of the tumor necrosis factor (TNF) superfamily (12). VEGI is found in endothelial cells and acts as an endogenous angiogenic inhibitor. VEGI in endothelial cells inhibits cell growth and migration (13). VEGI is known to operate via two receptors: Death receptor-3 (DR3) and decoy receptor-3 (DcR3). DR3 is a member of the TNF

receptor superfamily and is also known as TNFSF25; it contains a death domain in the cytoplasm that is associated with the induction of apoptosis and nuclear factor-κB activation (14). DcR3 is a decoy receptor for VEGI and is a secreted soluble protein (14-16) that acts as an antagonist to VEGI/DR3 interaction. DcR3 is expressed in a wide range of human tissues and is overexpressed in several tumors (17).

We previously demonstrated that VPA and Trichostatin A (TSA), which were histone deacetylase inhibitors, increased the expression of VEGI while exerting little effect on its receptor, DR3, and sensitized both OS and human microvascular endothelial (HMVE) cells to apoptosis via the VEGI/DR3 autocrine and paracrine pathways (18). The aim of the present study was to further investigate the effect of the combination of DNMT and HDAC inhibitors on VEGI and DR3. We observed that the combination of the DNA methylation inhibitor Hy with VPA increased the expression of VEGI and markedly increased the expression of DR3 in OS and HMVE cells compared with monotherapy, and also enhanced the production of soluble VEGI without enhancing the production of DcR3. Combination treatment of OS cell culture media significantly inhibited vascular tube formation. Furthermore, the physical interaction of VEGI and VEGF-A was observed by immunoprecipitation. These findings may provide evidence of additional VEGI-mediated anti-angiogenic machinery.

Materials and methods

Cell culture and drugs. U-2 OS and SaOS-2 human OS cells were purchased from the American Type Culture Collection and Riken BRC Cell Bank, respectively. The U-2 OS and SaOS-2 cells were cultured in McCoy's 5A modified medium (Invitrogen; Thermo Fisher Scientific, Inc.). Primary normal HMVE cells were purchased from the Cell Systems Corporation and cultured using a CS-C medium kit (DS Pharma Biomedical Co., Ltd.). All media contained 10% fetal bovine serum (FBS) (MP Biomedical), penicillin (100 U/ml) and streptomycin (100 μg/ml). All the cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37˚C. VPA was purchased from Wako Pure Chemical Industries, Ltd., and Hy was purchased from Sigma-Aldrich; Merck KGaA.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. U-2 OS, SaOS-2 cells and HMVE cells were cultured with or without 20 μM Hy and 1.0 mM VPA. The culture medium was changed on day 3. Total RNA was isolated from each cell culture dish on days 3 and 7 using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and 2.0 μg was reverse-transcribed using a High-Capacity RNA-to-cDNA kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR using TaqMan Gene Expression assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) was performed to detect VEGI, DR3 and DcR3 mRNA expression. The primer sets were Hs00270802_ml for VEGI mRNA, Hs00600930_ml for DR3 mRNA, and Hs00187070_ml for DcR3 mRNA (Applied Biosystems; Thermo Fisher Scientific, Inc.). The amount of GAPDH mRNA (as an internal reference) was estimated using human GAPDH endogenous control (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative gene expression was analyzed and calculated via the $2^{-\Delta\Delta Cq}$ method (19), and the mRNA expression levels of VEGI, DR3 and DcR3 were normalized to GAPDH.

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed using the EZ-Magna ChIP™ A kit (EMD Millipore; cat. no. 17-408) according to the manufacturer's instructions. The ChIP samples were obtained from SaOS-2 cells cultured with or without 20 μM Hy and 1.0 mM VPA for 3 days. In brief, nuclear lysate containing protein-DNA complexes was prepared from cross-linking proteins bound to DNA after formaldehyde treatment. An aliquot of the nuclear lysate was used to immunoprecipitate acetylated histone-DNA complexes with an anti-acetyl-histone H3 rabbit polyclonal antibody (kit supplied by EMD Millipore; cat. no. 06-599B). DNA was extracted from the precipitated acetylated histone-DNA complexes. The VEGI gene promoter sequence was amplified by a PCR using Takara Ex Taq™ DNA polymerase (Takara Bio, Inc.) and the promoter region from -1515 to -953 with the following primers: Sense, 5'-GTTCCAACAC CACCTCTTTC-3' and antisense, 5'-AGTTCTAAATCACG GCTTGG-3', for the VEGI promoter. The initial denaturation and final extension of the PCR were performed at 95˚C for 5 min and at 72˚C for 7 min, respectively, and the PCR conditions were 95˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min for 35 cycles. The amplified fragments were resolved by electrophoresis in a 1.5% agarose gel with ethidium bromide staining.

Methylation-specific polymerase chain reaction (MSP). Bisulfite-converted genomic DNA was obtained from SaOS-2 cells after 3 days of Hy and/or VPA treatment using a Cells-to-CpG™ bisulfite conversion kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The bisulfite-converted genomic DNA was used as a template for MSP using a GC-rich PCR system (Roche Diagnostics). The primer pairs for the amplified methylated DNA for the region from -55 to +143 were as follows: Sense, 5'-TTACGACGGGTAGAGAGTACG-3' and antisense, 5'-ACT TAAAATAAAAACGCGCCC-3'. The primer pairs for the amplified unmethylated DNA for the region from -51 to +150 were as follows: Sense, 5'-GGAATTATGATGGGTAGAGAGT ATG-3' and antisense, 5'-CAATAAAACTTAAAATAAAAAC ACACCC-3'. These primers were designed with the MethPrimer software program (available at www.urogene.org/methprimer2). The initial denaturation and final extension steps of the PCR were performed at 95˚C for 5 min and at 72˚C for 10 min, respectively. The amplification conditions of the PCR were 35 cycles at 95˚C for 1 min, at 57˚C for 1 min, and at 72˚C for 2 min. The amplified fragments were resolved by electrophoresis on 1.5% agarose gels with ethidium bromide staining.

Enzyme-linked immunosorbent assay (ELISA). U-2 OS and SaOS-2 cells were seeded at $2.0x10^5$ cells/dish in 10-cm tissue culture dishes containing 5 ml of medium per dish. HMVE cells were seeded at $1.0x10^4$ cells/dish in 6-cm tissue culture dishes containing 2 ml of medium per dish. After 24 h (Day 0), 20 μM Hy and/or 1.0 mM VPA were added to the medium and cultured for 7 days. The medium was changed after 3 days (Day 3), and the remaining dishes were cultured for a further 4 days (until Day 7). To detect soluble VEGI, 96-well plates were

coated with the capture antibody (anti-human VEGI mouse monoclonal antibody, 2.0 μg/ml; Santa Cruz Biotechnology, Inc.; cat. no. sc-53975) overnight at room temperature, and then washed three times with washing buffer (PBS containing 0.05% Tween-20). Standard protein, recombinant human TL1A/TNFSF15 (R&D Systems, Inc.; cat. no. 1319-TL-010) and samples were incubated in each well for 2 h at room temperature. After washing, biotin-conjugated anti-human VEGI rabbit polyclonal antibody (0.5 μg/ml, Abcam; cat. no. ab84233) was added to the wells and incubated for 2 h at room temperature. Following 20 min incubation at room temperature with horseradish peroxidase-conjugated streptavidin, substrate solution (both from R&D Systems, Inc.) was added to the wells and incubated for 20 min at room temperature. The detection of soluble DcR3 was performed using an ELISA development system (R&D Systems, Inc.) according to the manufacturer's instructions. To determine the optical density of each well, the absorbance at 450 nm was measured against a reference wavelength of 570 nm using a microplate reader (Bio-Rad Laboratories, Inc.). The effect of Hy or VPA was evaluated by determining the mean value of the soluble forms per 10^4 viable cells in the treated cultures, and was expressed as the ratio of the mean value in the untreated control cultures.

Plasmid construction and transfection. VEGI cDNA plasmid (pVEGI) was obtained from RT-PCR fragments amplified from U-2 OS cell total RNA (18). The siRNA designed for VEGI mRNA was 5'-ACCUGACAGUUGUGAGACAtt-3' (sense strand) and was synthesized by Applied Biosystems; Thermo Fisher Scientific, Inc. For transfection, U-2 OS and SaOS-2 cells were seeded at $1x10^5$ cells/well in 6-well tissue culture plates and cultured in 2 ml of medium for 24 h. The culture medium was changed to Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.), and the cells were transfected with 2.0 μg of plasmid DNA or 20 nM of siRNA using Lipofectamine™ 2000 and RNAiMAX (both from Invitrogen; Thermo Fisher Scientific, Inc.), respectively, according to the manufacturer's instructions. Whole-cell lysate and cell culture medium were collected 48 h after transfection. Whole-cell lysis was performed and the findings were analyzed by an immunoprecipitation assay. In addition, the cell culture medium was subjected to an *in vitro* tube formation assay.

Western blot analyses. SaOS-2 and HMVE cells with or without Hy and VPA treatment and pVEGI-transfected OS cells were washed twice with PBS and lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS] supplemented with a complete protease inhibitor cocktail (Roche Diagnostics). In brief, the cell lysates were first incubated on ice for 30 min and then sonicated three times (5 sec each time) prior to centrifugation at 7,700 x g for 10 min at 4˚C. The supernatant was collected and the protein concentration was measured using NanoDrop (Thermo Fisher Scientific, Inc.). An aliquot of the supernatant (equivalent to 30 μg protein) was mixed with a 3-fold volume of SDS sample buffer (BioLab) containing 10% β-mercaptoethanol, heated to 95˚C for 10 min, and electrophoresed on a 4-12% Bis-Tris gel with the MES SDS Running Buffer (Invitrogen; Thermo Fisher Scientific, Inc.) before being transferred onto a nitrocellulose membrane. The membrane was blocked for 30 min at room temperature in blocking buffer containing 5% skimmed milk in Tris-buffered saline with Tween-20 [TBST; 10 mM Tris, 150 mM NaCl (pH 7.4), 1% Tween-20] and then incubated for 90 min at room temperature with anti-human VEGI mouse monoclonal antibody (1:200 dilution; Santa Cruz Biotechnology, Inc., cat. no. sc-53975) or anti-human DR3 mouse monoclonal antibody (1:500 dilution; Santa Cruz Biotechnology, Inc.; cat. no. sc-374203) in TBST buffer, after which time the membrane was washed with TBST three times (10 min each time). The membrane was then incubated for 90 min at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG antibody for VEGI and DR3 (1:5,000 dilution; Santa Cruz Biotechnology, Inc.; cat. no. sc-2005) and was detected and visualized with SuperSignal™ West Pico PLUS chemiluminescence substrate (Thermo Fisher Scientific, Inc.). The same membrane was then stripped using stripping buffer (Thermo Fisher Scientific, Inc.) and re-probed using actin, which was detected with anti-actin rabbit polyclonal antibody (Sigma-Aldrich; Merck KGaA; cat. no. A2066) at a 1:200 dilution in TBST buffer.

HMVE cell proliferation assay. HMVE cells ($1x10^3$ cells per well) were seeded onto 96-well tissue culture plates containing 100 μl of CS-C complete medium in each well. After 24 h (Day 0), 20 μM Hy and 1.0 mM VPA were added to the medium and cultured for 7 days. The medium was changed after 3 days (Day 3) and the remaining dishes were cultured for a further 4 days (until Day 7). The number of viable cells in each well was estimated using a CellTiter 96® AQueous One solution cell proliferation assay kit (Promega Corporation) according to the manufacturer's instructions, and the findings are presented as the ratio to the mean level of optical density in control cultures.

In vitro tube formation assay. HMVE cells were subjected to an *in vitro* tube formation assay using a Cultrex® *in vitro* angiogenesis assay tube formation kit (Trevigen Inc.), according to the manufacturer's instructions. In brief, $1.0x10^4$ HMVE cells were seeded onto BME gel pre-coated 96-well plates and cultured with HMVE cell culture media in the absence of angiogenesis mediators and FBS. After 1 h, Hy and/or VPA was added, or the medium was changed to Hy- and/or VPA-treated OS cell culture media after centrifugation for 1 min at 800 x g at 4˚C, and then incubation was extended for another 16 h. Calcein AM staining was performed after washing twice with 1X PBS by gentle pipetting. The endothelial cells and tubes were examined using a fluorescence microscope (Nikon Corporation). The ratio of the mean value was estimated by counting the number of complete tubular shapes in four independent experiments by four independent researchers.

Immunoprecipitation assay. A total of 200 μg cell lysate obtained from U-2 OS and SaOS-2 cells ($1x10^6$ cells) treated with VPA or transfected with pVEGI was pre-cleaned with 20 μl of protein G-Sepharose beads (Santa Cruz Biotechnology, Inc.) for 3 h at 4˚C and washed twice with 1X immunoprecipitation (IP) buffer [50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.2 mM NaF, 0.2 mM Na_3VO_4, 1 mM PMSF and 0.1% NP-40]. Following centrifugation at

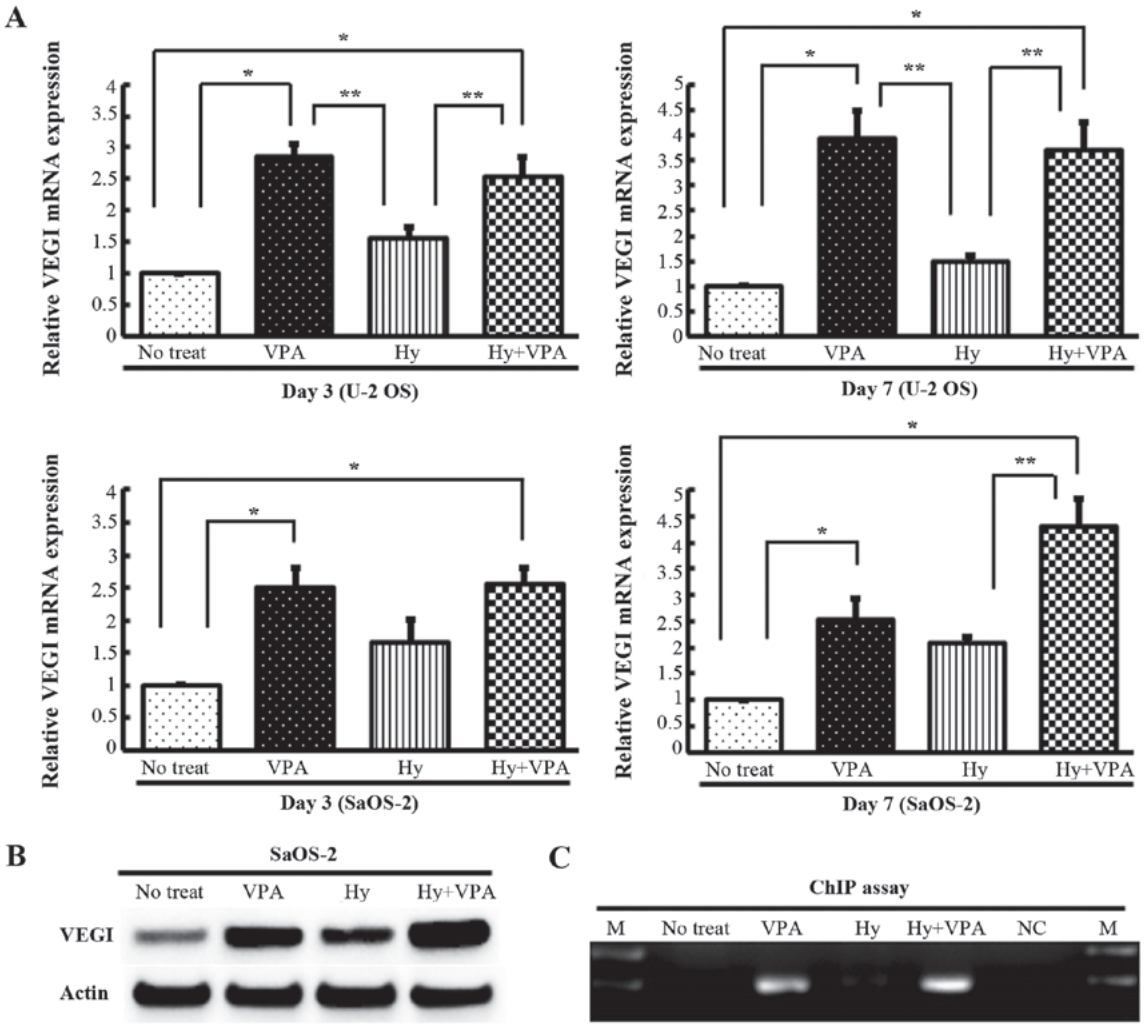

Figure 1. Effects of Hy and VPA on the expression of VEGI and the analysis of the acetylation status of VEGI gene promoters in OS cell lines. U-2 OS and SaOS-2 cells were treated with 20 μM Hy and/or 1.0 mM VPA for 3 (Day 3) or 7 days (Day 7). The medium was changed on Day 3. (A) VEGI mRNAs was quantitated using qPCR analysis. The values are expressed as the ratio to the average value in the no treat as a control. Each bar indicates the mean ± SE of values from four independent experiments of each samples, in three sets of condition cultures. *P<0.001, significant difference in comparison to no treat. **P<0.01, significant difference in comparison to Hy. (B) The VEGI protein expression was examined by western blotting. (C) A ChIP assay was performed with 20 μM Hy and/or 1.0 mM VPA treatment on Day 3 in SaOS-2 cells using anti-acetyl histone H3 antibodies. DNA bound to acetylated histones was amplified using specific primers for the VEGI promoter region. M, molecular marker; NC, negative control; Hy, hydralazine; VPA, sodium valproate; VEGI, vascular endothelial growth inhibitor; OS, osteosarcoma; qPCR, quantitative polymerase chain reaction; SE, standard error; ChIP, chromatin immunoprecipitation.

11,100 x g for 1 min at 4˚C, 100 μl supernatant was collected and incubated with protein G-Sepharose beads (Bio-Rad Laboratories, Inc.) coated with 20 μg anti-VEGF-165 mouse monoclonal antibody (BioLegend; cat. no. 662702) in 500 μl 1X IP buffer overnight at 4˚C. The beads were then gently washed four times with 1X IP buffer, and the proteins bound to the beads were eluted with 3X SDS sample buffer containing 10% β-mercaptoethanol. The protein samples obtained from IP were subjected to western blotting to detect anti-V5-Tag horseradish peroxidase-conjugated antibody (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. R961-25), as described above. Opposite detection was performed with protein G-Sepharose beads coated with 20 μg anti-TL1A rabbit polyclonal antibody (Abcam; cat. no. ab85566) and was detected by western blotting using anti-VEGF-165A mouse monoclonal antibody (Abcam; cat. no. ab69479. The membrane was then incubated for 90 min at room temperature with anti-mouse IgG TrueBlot® ULTRA (1:1,000 dilution; Rockland Immunochemicals, Inc.; cat. no. 18-8817-31) as a secondary antibody.

Statistical analysis. Data are presented as the mean ± standard error. The data of three or more groups were analyzed by one-way ANOVA followed by the Newman-Keuls test for multiple comparisons. P-values <0.05 were considered to indicate statistically significant differences.

Results

Effects of Hy and VPA on VEGI mRNA and protein expression in OS cells. U-2 OS and SaOS-2 cells were cultured with or without 20 μM Hy and 1.0 mM VPA for 3 or 7 days, and the degree of VEGI mRNA transcription was quantitated by qPCR. The VEGI mRNA expression in both cell lines was increased ~2.5- to 4.0-fold by VPA treatment (P<0.001) and 2.5- to 4.3-fold by combination treatment with Hy and VPA

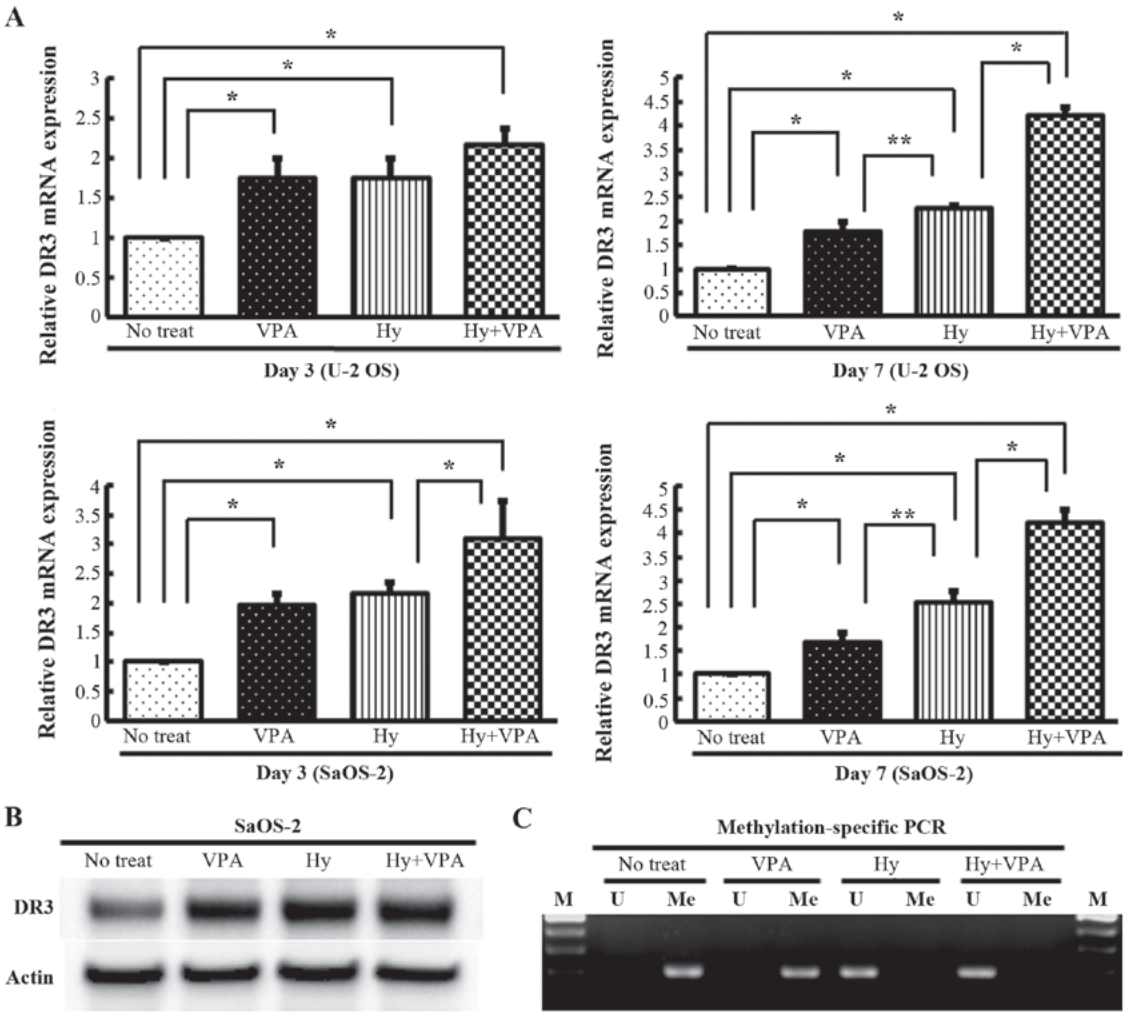

Figure 2. Effects of Hy and VPA on the DR3 and analysis of the methylation status on the DR3 gene promoter in OS cell lines. U-2 OS and SaOS-2 cells were treated with 20 μM Hy and/or 1.0 mM VPA for 3 (Day 3) or 7 days (Day 7). The medium was changed on day 3. (A) DR3 mRNAs was quantitated using qPCR analysis. The values are expressed as the ratio to the average value in the no treat as a control. Each bar indicates the mean ± SE of values from four independent experiments for each sample, in three sets of culture conditions. *P<0.01, significant difference in comparison to no treat and to Hy. **P<0.05, significant difference in comparison to Hy. (B) The DR3 protein expression was examined by western blotting. (C) Methylation-specific PCR (MSP) was performed with 20 μM Hy and/or 1.0 mM VPA treatment on day 3 in SaOS-2 cells. Bisulfited genomic DNA amplified by unmethylated-specific (U) and methylated-specific (Me) primers designed in the CpG island of the DR3 promoter region. M, molecular marker. Hy, hydralazine; VPA, sodium valproate; DR3, death receptor-3; OS, osteosarcoma; qPCR, quantitative polymerase chain reaction; SE, standard error.

(P<0.001). Hy treatment also increased VEGI expression 1.5- to 2.0-fold. However, there was no significant difference compared with no treatment as a control (P>0.05) (Fig. 1A). The protein translation of VEGI under these conditions was confirmed by western blotting (Fig. 1B). These results suggest that the VEGI expression was more prominently enhanced by exposure to VPA rather than to Hy. SaOS-2 cells were examined to confirm the effect of VPA on the VEGI expression by a ChIP assay. The result demonstrated that acetylated histone bound to the VEGI promoters after VPA treatment and combination treatment (Fig. 1C).

Effects of Hy and VPA on the mRNA and protein expression of the VEGI-related receptor DR3 in OS cells. U-2 OS and SaOS-2 cells were cultured with or without 20 μM Hy and 1.0 mM VPA for 3 or 7 days, and the mRNA transcription of DR3 was quantitated by qPCR. The DR3 mRNA expression in both cell lines was increased 1.6- to 1.9-fold by VPA treatment (P<0.01) and 1.7- to 2.5-fold by Hy treatment (P<0.01). Combination treatment with Hy and VPA increased the expression 2.1- to 4.2-fold (P<0.01) (Fig. 2A). The translation of DR3 under these conditions was confirmed in SaOS-2 cells by western blotting (Fig. 2B). These results indicate that the expression of DR3 was more prominently enhanced by exposure to Hy rather than to VPA. SaOS-2 cells were examined by an MSP assay to confirm the effects of Hy on DR3 expression. Demethylation of the promoter region of DR3 was verified by the conversion of cytosine to uracil following Hy treatment and combination treatment (Fig. 2C).

Effects of Hy and VPA on the DcR3 expression levels and the expression of soluble forms of VEGI in OS cells. To determine the effects of Hy and VPA on DcR3 expression and the production of soluble VEGI, U-2 OS and SaOS-2 cells were cultured with and without 20 μM Hy and 1.0 mM VPA for 7 days. The DcR3 mRNA transcription was quantitated by qPCR. The DcR3 mRNA expression was unchanged in both cell lines after 7 days of culture (Fig. 3A). The secreted DcR3

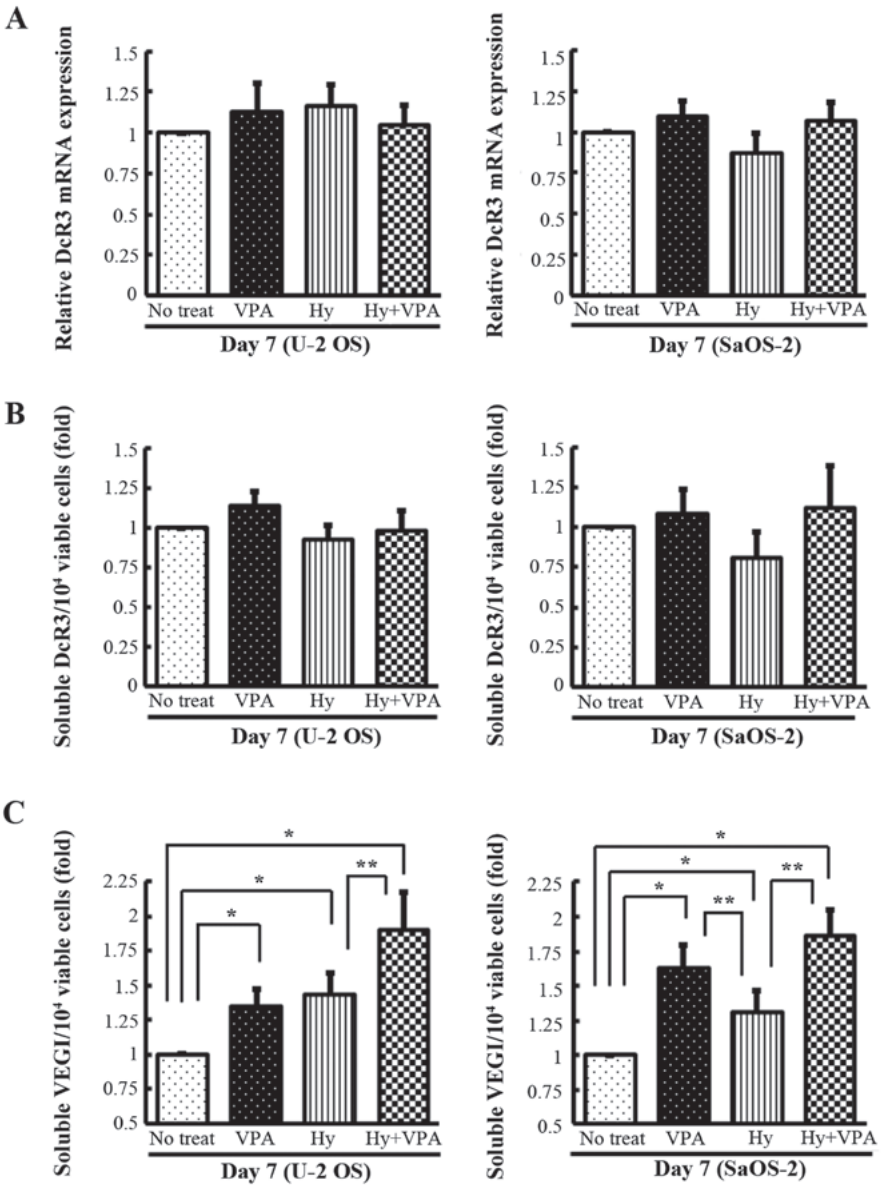

Figure 3. Effects of Hy and VPA on the DcR3 expressions in the OS cell lines and the soluble VEGI production in the OS cell culture media. U-2 OS and SaOS-2 cells were treated with 20 μM Hy and/or 1.0 mM VPA for 7 days (Day 7). The medium was changed on Day 3. (A) DcR3 mRNAs was quantitated on Day 7 by a using qPCR. (B) Soluble DcR3 and (C) soluble VEGI were detected on Day 7 by ELISA. The values are the indicated ratio of the mean amounts of soluble DcR3 and VEGI per 10^4 viable cells with the amounts in no treat set at 1. Each bar indicates the mean ± SE of values obtained from eight independent experiments for each sample under four sets of culture conditions. *P<0.01, significant difference in comparison to no treat; **P<0.01, significant difference in comparison to Hy. Hy, hydralazine; VPA, sodium valproate; DcR3, decoy receptor-3; OS, osteosarcoma; VEGI, vascular endothelial growth inhibitor; qPCR, quantitative polymerase chain reaction; SE, standard error.

in the culture medium was analyzed by ELISA and found to be essentially unchanged compared with no treatment (Fig. 3B). The accumulation of soluble VEGI was increased 1.3- to 1.6-fold by VPA treatment (P<0.001). A statistically significant 2.0-fold increase in soluble VEGI was observed following combination treatment in both types of OS cells on day 7 (P<0.001) (Fig. 3C).

Effects of Hy and VPA on the transcription of VEGI, its related receptors and their soluble forms in HMVE cells. qPCR analysis of HMVE cells revealed that VEGI mRNA transcription was increased ~2.5-fold following VPA treatment (P<0.01) (Fig. 4A), while that of DR3 was increased ~1.9-fold by Hy treatment (P<0.001) (Fig. 4C). The combination of Hy and VPA increased the VEGI and DR3 gene expression ~2.8-fold (P<0.001) (Fig. 4A and C). The protein expression profiles reflected the trends in gene expression (Fig. 4B and D). A 1.5-fold increase in soluble VEGI levels was observed after treatment with 1.0 mM VPA (P<0.001), while combination treatment resulted in a 2.0-fold increase (P<0.001) (Fig. 4G). However, the DcR3 gene expression and secretion did not differ to a statistically significant extent from no treatment (control; P>0.05) (Fig. 4E and F).

Effects of Hy and VPA on cell proliferation and effects of Hy and VPA inhibitor treatment of OS cell culture media on vascular tube formation. An analysis of the HMVE cell growth revealed that 20 μM Hy and 1.0 mM VPA treatment was not

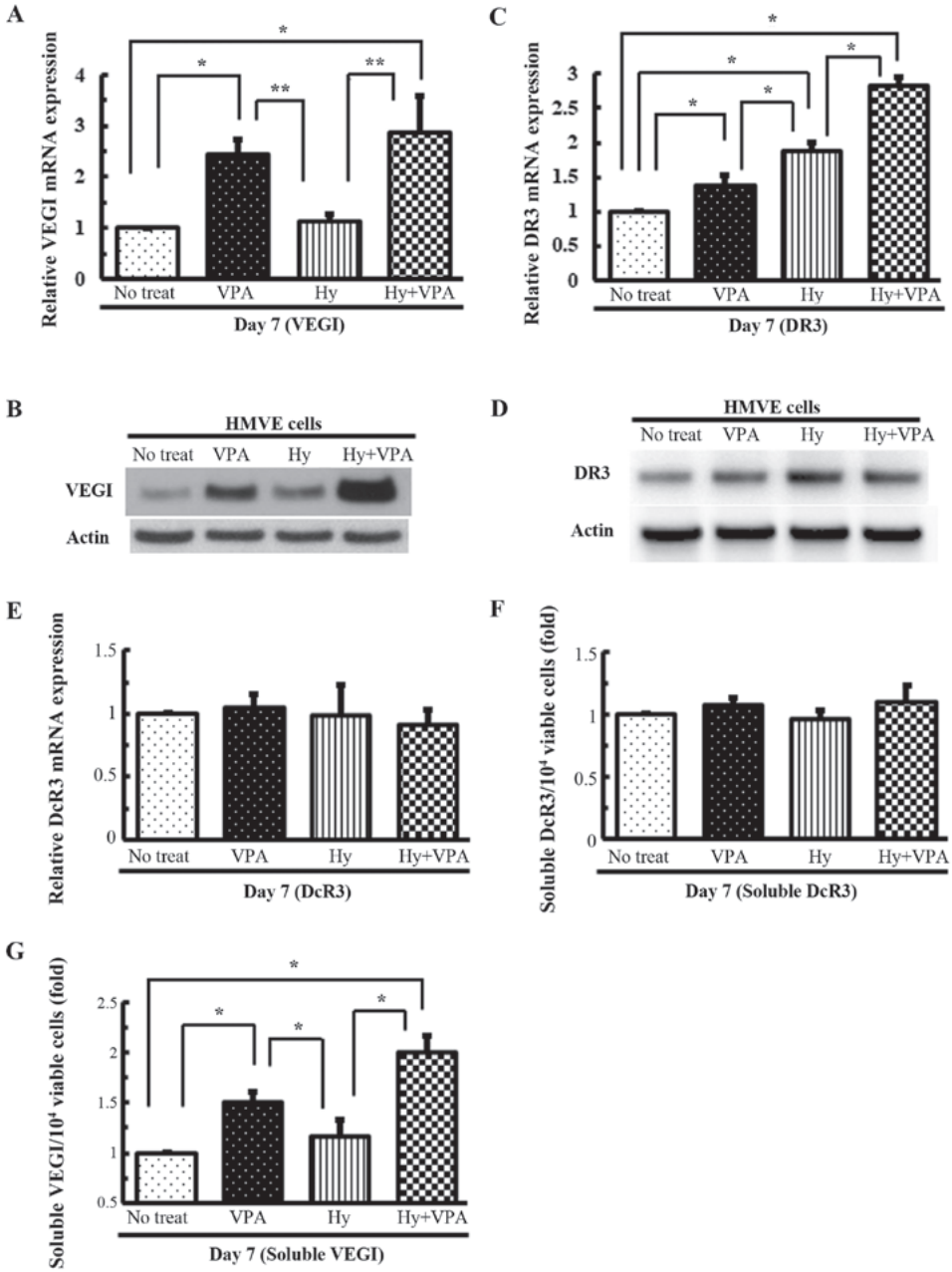

Figure 4. Effects of Hy and VPA on VEGI and its related receptors on transcription and the production of their soluble forms in the HMVE cells. HMVE cells were cultured in medium with 20 μM Hy and/or 1.0 mM VPA for 7 days (Day 7). The medium was changed on day 3. The gene expression levels of (A) VEGI, (C) DR3 and (E) DcR3 were quantitated using qPCR. The values are expressed as the ratio to the mean value in the no treat as a control. Each bar indicates the mean ± SE of values obtained from four independent experiments for each sample, under three sets of culture conditions. *P<0.01, significant difference in comparison to no treat and to Hy. **P<0.05, significant difference in comparison to Hy. The (B) VEGI and (D) DR3 protein expression levels were examined using western blotting. The amount of (F) soluble DcR3 and (G) VEGI in the medium was determined on Day 7. The values are indicated as the ratio of the mean amounts of soluble DcR3 and VEGI per 10^4 viable cells in no treat is expressed as 1.0. Each bar indicates the mean ± SE of values obtained from four independent experiments for each sample, under three sets of culture conditions. *P<0.001, significant difference in comparison to no treat and to Hy. Hy, hydralazine; VPA, sodium valproate; VEGI, vascular endothelial growth inhibitor; HMVE, human microvascular endothelial; DR3, death receptor-3; DcR3, decoy receptor-3; qPCR, quantitative polymerase chain reaction; SE, standard error.

associated with notable changes compared with no treatment as a control. However, their combination markedly inhibited HMVE cell growth (P<0.001) (Fig. 5A-a and -b). We previously demonstrated that VEGI from VPA-treated OS culture medium markedly inhibited HMVE tube formation (18). To confirm whether soluble VEGI in the culture medium of OS cells treated with Hy alone or combination treatment with VPA and Hy can inhibit neovascularization, we performed the same experiment as described in our previous study (18). The results demonstrated that treatment with Hy and/or VPA alone slightly affected tube formation (P<0.001) (Fig. 5B-d and -e), whereas combination treatment and the treatment of OS cell medium markedly inhibited tube formation in HMVE cells (P<0.001) (Fig. 5B-f, B-i and C).

VEGI directly interferes with VEGF-A in vitro. U-2 OS and SaOS-2 cells were cultured with or without 20 μM Hy and 1.0 mM VPA for 3 or 7 days, and the VEGF-A mRNA transcription

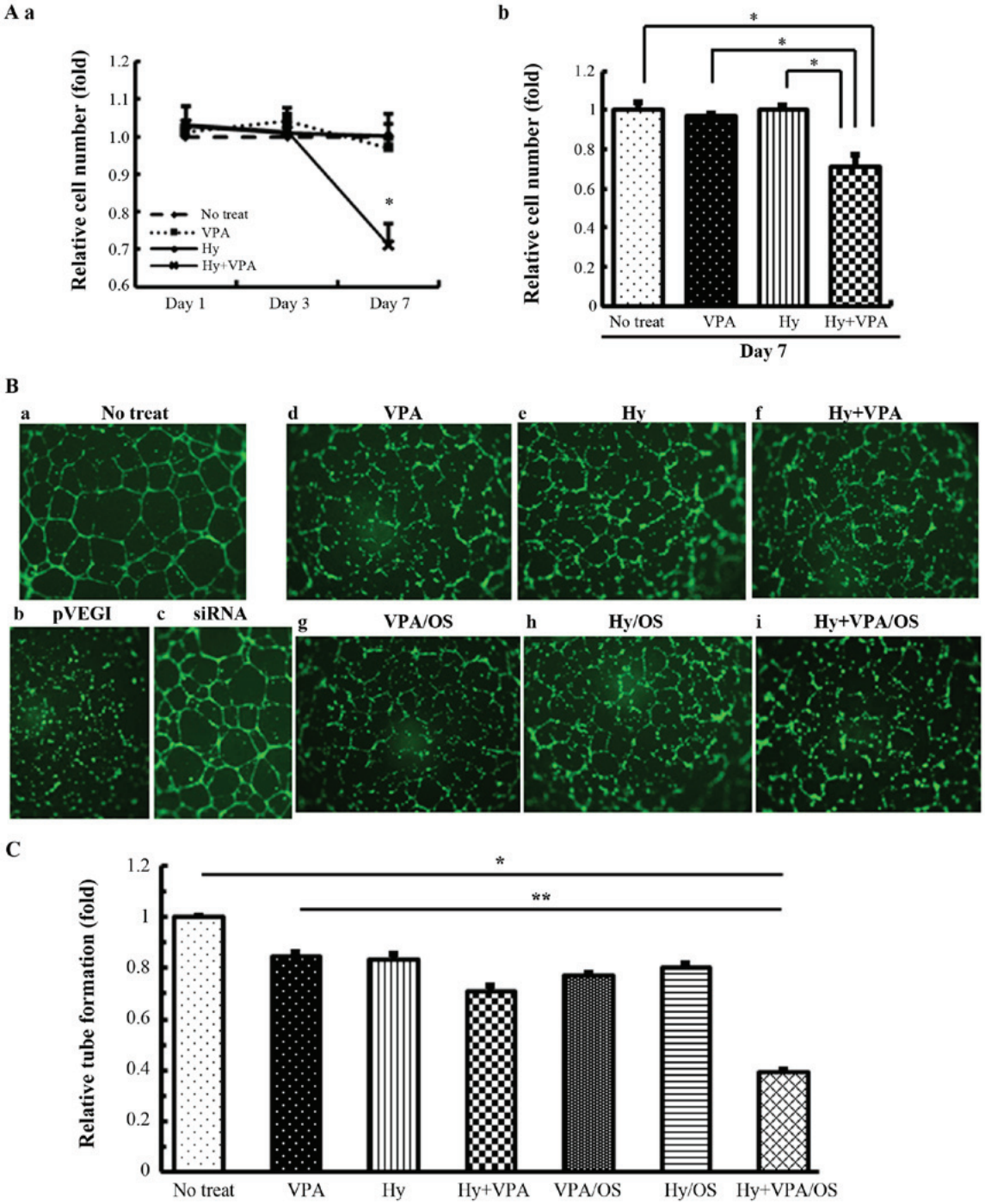

Figure 5. Effects of Hy and VPA on HMVE cell proliferations and the effects of Hy and VPA-treated OS cell culture media on vascular tube formation. (A) The growth of HMVE cells was analyzed with 20 μM Hy and/or 1.0 mM VPA on the indicated days. (a) Cell growth on Day 1, 3 and 7 of treatment is indicated on the polygonal line graph. (b) Cell growth on Day 7 of treatment with Hy and/or VPA. The values are the indicated ratio, with the mean optical density of no treat set at 1. Each bar indicates the mean ± SE of 8 wells of separate experiments, performed in triplicate. *P<0.001, significant difference in comparison to Hy + VPA. (B) HMVE cells were cultured or harvested under each condition: (a) No treat (control); (b) pVEGI (OS culture media transfected plasmid VEGI); (c) siRNA (OS culture media transfected siRNA for VEGI); (d) 1.0 mM VPA; (e) 20 μM Hy; (f) 20 μM Hy and 1.0 mM VPA; (g) VPA/OS (1.0 mM VPA-treated OS cell culture medium); (h) Hy/OS (20 μM Hy-treated OS cell culture medium); (i) Hy + VPA/OS (20 μM Hy and 1.0 mM VPA-treated OS cell culture medium). OS culture media treated with Hy and/or VPA was obtained from U-2 OS cells after 7 days of culturing in media. The pVEGI and siRNA medium was obtained from plasmid VEGI- or the siRNA of VEGI-transfected U-2 OS cells cultured for 48 h. The endothelial cell tube formation was examined by fluorescence microscopy. (C) Tube formation was counted based on the identification of a complete tubular shape in four independent experiments. Counting was performed by four independent researchers. The values are the indicated ratio of the mean amounts of complete tubular shape in no treat is expressed as 1.0. Each bar indicates the mean ± SE of values. *P<0.001, significant difference in comparison to no treat. **P<0.001, significant difference in comparison to Hy + VPA/OS. Hy, hydralazine; VPA, sodium valproate; HMVE, human microvascular endothelial; OS, osteosarcoma; SE, standard error; VEGI, vascular endothelial growth inhibitor; SE, standard error.

was quantitated by qPCR. The VEGF-A mRNA expression of both cell lines was significantly decreased under all treatment conditions (P<0.05) (Fig. 6A). To determine the mechanism underlying the VEGI-mediated anti-angiogenesis without

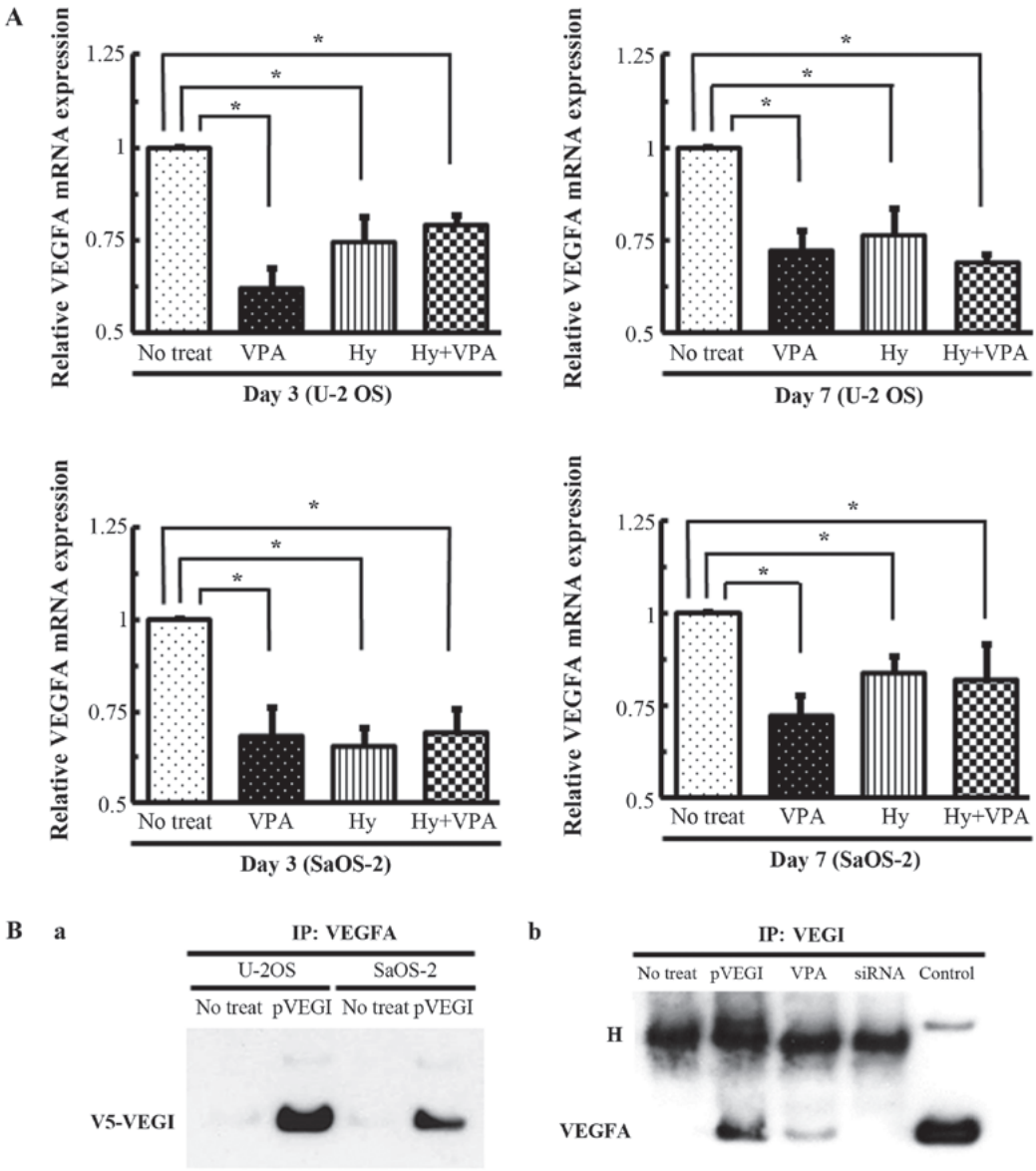

Figure 6. Anti-angiogenic effect of Hy and VPA on VEGI. (A) U-2 OS and SaOS-2 cells were treated with 20 μM Hy and/or 1.0 mM VPA for 3 (Day 3) or 7 days (Day 7) with medium changed on day 3. The VEGF-A mRNAs was quantitated by qPCR. The values are expressed as the ratio to the average value in no treat as a control. Each bar indicates the mean ± SE of values obtained from four independent experiments for each samples under three sets of condition cultures. *P<0.05, significant difference in comparison to no treatment. (B) An IP assay was performed with OS cell lysis. (a) V5-His-tagged VEGI expression vector and siRNA transfected into U-2 OS and SaOS-2 cells. IP with VEGF-A was detected with V5-His-tag antibody by western blotting. (b) SaOS-2 cell lysate of VPA treatment after 7 days was performed IP with VEGI-coated beads and was detected by western blotting using VEGF-A antibody. H, heavy chain; Hy, hydralazine; VPA, sodium valproate; VEGI, vascular endothelial growth inhibitor; VEGF, vascular endothelial growth factor; qPCR, quantitative polymerase chain reaction; SE, standard error; IP, immunoprecipitation; OS, osteosarcoma.

VEGI/DR3-induced apoptosis, a VEGI/V5-His-tag expression vector was transfected into OS cells, and cell lysis was performed via an immunoprecipitation assay. The results demonstrated that the V5-His-tagged VEGI bound to VEGF-A-coated beads and precipitated the VEGF-A/VEGI immune complex (Fig. 6B-a). OS cell lysis after VPA treatment also created VEGI/VEGF-A immune complexes (Fig. 6B-b). These results indicate that VEGI may exert one aspect of its anti-angiogenic effects by directly binding to VEGF-A and inhibiting neovascularization.

Discussion

Our previous study demonstrated that the histone deacetylase inhibitors VPA and TSA increased the expression of VEGI with little effect on its receptor (DR3), and sensitized both OS and HMVE cells to apoptosis via the VEGI/DR3 autocrine and paracrine pathways (18). In the present study, the combination of the DNA methyltransferase inhibitor Hy and the histone deacetylase inhibitor VPA induced the expression of DR3 and VEGI more efficiently compared with either monotherapy, without inducing DcR3 secretion. DNMT or HDAC inhibitors usually activate gene expression within minutes/hours. The expression of VEGI, DR3 and DcR3 were analyzed at 12, 24 and 48 h of Hy and/or VPA treatment. These genes displayed similar degrees of fluctuation to that observed at 3 and 7 days (Fig. S1). It was also found that their combination inhibited HMVE cell proliferation and, subsequently, vascular tube formation by HMVE cells in

comparison to Hy or VPA alone. However, perhaps the most notable finding of the present study was that VEGI bound directly to VEGF-A, and precipitated the VEGI/VEGF-A immune complex as determined by to immunoprecipitation studies.

In recent years, HDAC inhibitors have been tested against various cancers in clinical trials (20). However, HDAC inhibitors alone are not sufficiently effective, as several trials have demonstrated increased histone acetylation in tumor samples, despite there being only a slight clinical effect (21,22). DNMT and HDAC inhibitors have been reported to act synergistically against cancer development through regulating the expression of tumor suppressor genes and oncogenes (23). Chavez-Blanco *et al* investigated the growth inhibitory effect of VPA; however, Hy did not exert a similar effect on cervical, colon and breast cancer, sarcoma, glioma, or head and neck cancer cell lines. By contrast, Hy in combination with VPA significantly inhibited cell growth in all cell lines, and this combination improved the efficiency of treatment with current anticancer agents (24). Bauman *et al* conducted a phase I trial of the combination of Hy and VPA to determine the maximum tolerated dose. The combination of Hy and VPA was found to be non-toxic, and may be appropriate for patients with resistance to anticancer drugs, or in combination with other cancer treatments (25). Capobianco *et al* suggested that 5-aza-dc and TSA inhibited cell growth and induced reprogramming towards osteoblast differentiation in cases of OS with multidrug resistance, through the induction of the re-expression of several epigenetically silenced genes. These agents exhibited greater efficacy in combination than either did as monotherapy (26). In our previous study, VPA and TSA alone were unable to increase the DR3 expression in HMVE cells (18). Takami *et al* reported that DR3 contained several CpG motifs in the promoter region, and was hypermethylated in synovial cells in patients with rheumatoid arthritis (RA). Thus, RA synovial cells may become resistant to apoptosis as a result of DR3 downregulation (27). However, in the present study, we demonstrated that even Hy treatment alone was able to enhance DR3 expression in HMVE cells, and its combination with VPA further increased the expression of DR3 in both OS and HMVE cells. These results suggest that the upregulation of the DR3 expression after Hy treatment is mediated by promoter demethylation and is associated with a decrease in the expression of DNMT. Hellebrekers *et al* were the first to demonstrate that DNMT and HDAC inhibitors directly inhibited endothelial cell growth and angiogenesis by inducing the re-expression of growth suppression genes in endothelial cells (7). Our *in vitro* vascular tube formation assay also revealed only a mild effect of VPA on HMVE cells, but the combination with Hy synergistically prevented vascular tube formation.

As regards the clinicopathological study of angiogenesis in OS, the expression of VEGF was detected in 63% of 27 primary OS biopsy samples. Patients with VEGF-positive tumors exhibited higher rates of cancer recurrence and poorer survival in comparison to those with VEGF-negative tumor (28). In fact, pulmonary metastasis was confirmed in ~82% of the VEGF-positive samples (28). Qu *et al* demonstrated that a notable reduction in the VEGF expression after chemotherapy was correlated with a good prognosis (29). These reports suggest that VEGF may exert a paradoxical effect in OS: It is associated with a poor outcome, but can also contribute to a better response to chemotherapy. The anti-angiogenic effects of HDAC inhibitors downregulate VEGF expression via suppression of hypoxia-inducible factor 1α activity (30,31). VPA inhibits angiogenesis by inducing the expression of endogenous anti-angiogenic proteins, such as thrombospondin-1, activin A and VEGI (18,32). Thus, we assessed the effect of Hy and VPA on the VEGF-A gene expression by qPCR analysis. Treatment of OS cells with Hy and/or VPA significantly suppressed the VEGF-A gene expression. VEGI, also referred to as TNFSF15, and VEGF interfere with each other in the modulation of angiogenesis (33-36). VEGI is capable of inhibiting the VEGFR1 and VEGFR2 activity of vascular endothelial cells (33,34). VEGI treatment of endothelial progenitor cells leads to the accelerated degradation of membrane-bound VEGFR1 (mFlt1) and the enhanced production of soluble VEGFR1 (sFlt1), thus inhibiting VEGF-stimulated blood vessel growth in experimental animals (34). Zhang *et al* demonstrated that VEGI was able to suppress the VEGF gene expression, thereby inducing miR-29b as result of JNK-GATA3 activation (35). We herein assessed the effect of Hy and VPA on the miR-29b and GATA3 expression by qPCR. Treatment with VPA induced the expression of the miR-29b and GATA3 genes in both OS and HMVE cells (data not shown). Thus, epigenetic modification was able to control VEGF expression and may have also occurred through the TNFSF15 gene expression. These observations prompted us to investigate other options by comparing VEGF to VEGI. Immunoprecipitation was performed using either VEGF-A- or VEGI-coated beads. Surprisingly, we observed that VEGI physically interacted with VEGF-A on pVEGI transfected OS cell lysis and that VPA treatment induced VEGI expression. This finding suggests that suppression of VEGF-A production is controlled by the physical interaction of VEGF-A and VEGI. While further studies are required, this finding may indicate a novel mechanism for interfering with VEGF-driven neovascularization.

In conclusion, the findings from our present and previous studies suggest that the DNMT and HDAC inhibitors Hy and VPA, respectively, not only induce the re-expression of tumor suppressor genes in cancer cells, but also exert anti-angiogenic effects, leading to the enhancement of the VEGI/DR3 pathway, and that their combination synergistically enhances the VEGI and DR3 gene expression and promotes HMVE cell death. Moreover, the precipitation of the VEGI/VEGF-A immune complex may constitute evidence of an additional VEGI-mediated anti-angiogenic machinery. Although further studies with *in vivo* assays and clinical samples are required, these findings may indicate a novel mechanism for interfering with VEGF-driven neovascularization. Hy and VPA are administered long-term in anti-hypertensive therapy and for the treatment of epilepsy and bipolar disorder, respectively. Importantly, our results demonstrated that Hy and VPA exerted their effects at concentrations that are attainable in the sera of patients undergoing oral treatment with the respective agents, without serious side effects (37,38). Thus, Hy and VPA may achieve better results with regard to reducing the population of vascular endothelial cells in OS, while also reducing host toxicity. There is a possibility that the benefit is due to an unknown activity of Hy and VPA (i.e., other than their activity as DNMT and HDAC inhibitors). The targeting of epigenetic modifications is currently being pursued in clinical studies.

The findings of the present study suggest that Hy and VPA may prevent hematogenous pulmonary metastasis and support the need for further investigation of epigenetic modifications as a novel therapeutic approach to OS.

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Availability of data and materials

All the datasets generated and analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

KY, HF and SY designed the study and participated in the discussion during the preparation of the manuscript. SK, KK and KY performed and contributed to all the experiments. YF and HF performed OS cell experiments. HN and KN performed HMVE cell experiments. SK, KK and KY performed IP experiments. SK and KY analyzed the data and wrote the manuscript. All the authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests to disclose.

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