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Original Article

Emodin inhibits HMGB1-induced tumor angiogenesis in human osteosarcoma by regulating SIRT1

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Abstract: The anti-cancer effects of emodin, including inhibition of proliferation, invasion, metastasis and angiogenesis, were confirmed by various previous studies. However, the specific mechanisms were not clear. In this study, we investigated emodin's anti-angiogenesis effect and focused on the mechanisms in human osteosarcoma (OS). OS cells were implanted to nude mice to form OS xenografts. Immunofluorescence assay was used to assess vWF expression in tumor tissue. MTT assay was employed to screen proper emodin concentrations unrelated with proliferation inhibition. siRNA technique was utilized to silence SIRT1 expression in OS cells. Expression levels of SIRT1 and VEGF were investigated by real-time PCR and western blotting. H4-k16Ac expression which indicated the deacetylation activity of SIRT1 was also detected by western blotting. As in results, HMGB1 treatment exacerbated OS angiogenesis both *in vivo* and *in vitro*. Emodin administration attenuated angiogenesis in both OS and HMGB1 treated OS *in vivo* and *in vitro*. After emodin treatment, the expression level and deacetylation activity of SIRT1 were dramatically enhanced. HMGB1-induced angiogenesis was more striking in SIRT1 silenced OS cells. SIRT1 silencing also impaired the anti-angiogenesis effect of emodin in OS cells. In conclusion: SIRT expression and deacetylation activity elevation are involved in emodin's anti-angiogenesis effect in human OS.

Keywords: Osteosarcoma, angiogenesis, SIRT1, HMGB1, emodin

Introduction

In adolescents and children, osteosarcoma (OS) is considered the most frequent malignant bone tumor which is characterized by osteoid tissue generation or immature bone formation [1]. The incidence of OS was reported 5/million. As the anti-tumor treatment has been developing rapidly in recent decades, the 5-year survival rate of OS is currently 60%-70%, the prognosis of OS is still poor because of its aggressive malignancy [2]. Most patients with OS were diagnosed until obvious clinical manifestations such as bone fractures and local pain were observed, thus the OS was often found at advanced stage [3]. The indications for curative surgical treatment are limited because of metastasis [4]. Therapeutic effects of other conventional therapies including chemotherapy or radiotherapy are also undermined due to metastasis, chemoresistance, side effects and dyscrasia. Thus, effective novel agents inhibiting metastasis without serious general side effects are favorable and promising in OS clinical treatment.

It is generally accepted the notion that the progression and development of malignant tumor are largely associated with angiogenesis [5]. In physiological conditions, the novel vessel formation is restricted. In pathological conditions, such as acute inflammation and wound healing, the angiogenesis is also localized. However, in malignant tumors, the angiogenesis is usually uncontrolled in order to fit the blood supply to the boosting tumor growth [6, 7]. Blood vessel extension, irregularity and circuitry are often found. The tumor angiogenesis is also found highly associated with tumor invasion and metastasis [8, 9]. Thus, drugs or agents with anti-angiogenesis activity are promising for malignant tumor treatment and prevention.

Emodin, also referred as 1,3,8-trihydroxy-6-methylanthraquinone, is plant original, which is derived from rhizome of *Rheum palmatum L*

[10]. In traditional medicine in eastern and southern Asia, from ancient times, *Rheum palmatum L.* has been used as an effective agent in treatment of peptic ulcer [11], indigestion, hemorrhoid, and several infectious diseases [12]. In recent decades, studies found anti-cancer activities of emodin, characterized by inhibition of tumor growth [13], invasion [14] and metastasis [15]. Also, emodin was reported to suppress tumor angiogenesis by blocking vascular endothelial growth factor (VEGF) signaling in cancer cells [16]. However, further interpretations are still needed to elucidate the exact mechanisms.

The sirtuin (SIRT) family (SIRT1-SIRT7) was reported associated with regulation of multiple pathophysiological events and has been drawing more and more attention recently [17, 18]. SIRT1 is the most studied SIRT member and is known for its association with cancer [19]. It was suggested that the activation of SIRT1 played a promoting role in cancer development and progression [20]. Several well-known cancer suppressors, such as p53 and HIC1, were supposed the down-stream molecules in SIRT1 signaling [21]. A recent study pointed out that the angiogenesis was suppressed after SIRT1 activation which down-regulated VEGF transcription by inhibiting HMGB1 induced hypoxia-induced factor (HIF)-1 regulated angiogenesis [22].

In the present study, we investigated the possible involvement of SIRT1 signaling pathway in anti-angiogenesis effect of emodin in OS. We hypnotized that: (1) Emodin could inhibit VEGF expression in OS; (2) Emodin attenuates angiogenesis in OS by regulating SIRT1/HMGB1 signaling pathway. We believe that results in this study would not only broaden our knowledge of mechanisms of angiogenesis in OS, but also provide ground for possible clinical application of emodin in OS treatment in the future.

Materials and methods

Cell lines, culturing and treatment

Human osteosarcoma cell lines SOSP-9607, MG63 and SAOS-2 which were purchased from China Center for Type Culture Collection (CCTCC, China) were used in this study. SOS-9607 and MG63 cells were maintained in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 2.0 mM L-glutamine (Sigma-Aldrich, USA) and 1% antibiotic solution (Sigma-Aldrich, USA, containing 150 μmol/L streptomycin and 100 U/ml penicillin). SAOS-2 cells were maintained in DMEM medium (Gibco, USA) supplemented with 10% FBS, 2.0 mM L-glutamine and 1% antibiotic solution. Cells were cultured in humidified condition with 5% CO_2 and 95% fresh air at 37°C. In some cases, cells were pre-treated with exogenous HMGB1 protein (HMG Biotech, Italy) at 40 ng/ml and emodin at serial diluted concentrations (0, 15, 30, 45, 60 μmol/L).

In vivo animal study

Male BALB/c nude mice were purchased from Animal experimental center of Fourth Military Medical University. Cell suspension containing 1×10^6 SOSP-9607/MG63/SAOS-2 cells were injected into left dorsa region of mice subcutaneously. Mice were treated by HMGB1 protein by single intramuscular injection (800 ng/mouse) or/and emodin (300 μg/Kg bodyweight) by 14-day continuous intraperitoneal injections. 4 weeks after injection, xenograft tumor tissue was harvested and prepared for Western blotting, real-time PCR and immunofluorescence assays.

Cell proliferation determination

Proliferation of SOSP-9607, MG63 and SAOS-2 cells was determined by colorimetric 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. Same amount of cells (1×10^4/mL) were seeded into wells in a 96-well plate and then cultured for 24 hours. After being washed by PBS, 150 μL dimethylsulfoxide (DMSO, Sigma-Aldrich) was used to incubate cells in each well. A plate reader (Bio-Rad) was utilized to detect the absorbance at 540 nm (A_{540}). The proliferation inhibition rate was calculated by "[1-A_{540} (experimental well)/A_{540} (control well)] ×100%".

Small interfering RNA (siRNA) transfection

Sequence of siRNA against human SIRT1 gene was designed by TaKaRa (Tokyo, Japan) and synthesized accordingly. Specifically, the sequence was: sense 5'-CCAAGCAGCUAAGAGUAAUTT-3', antisense 5'-AUUACUCUUAGCUGCUUGGTT-3'. SOSP-9607, MG63 and SAOS-2 cells

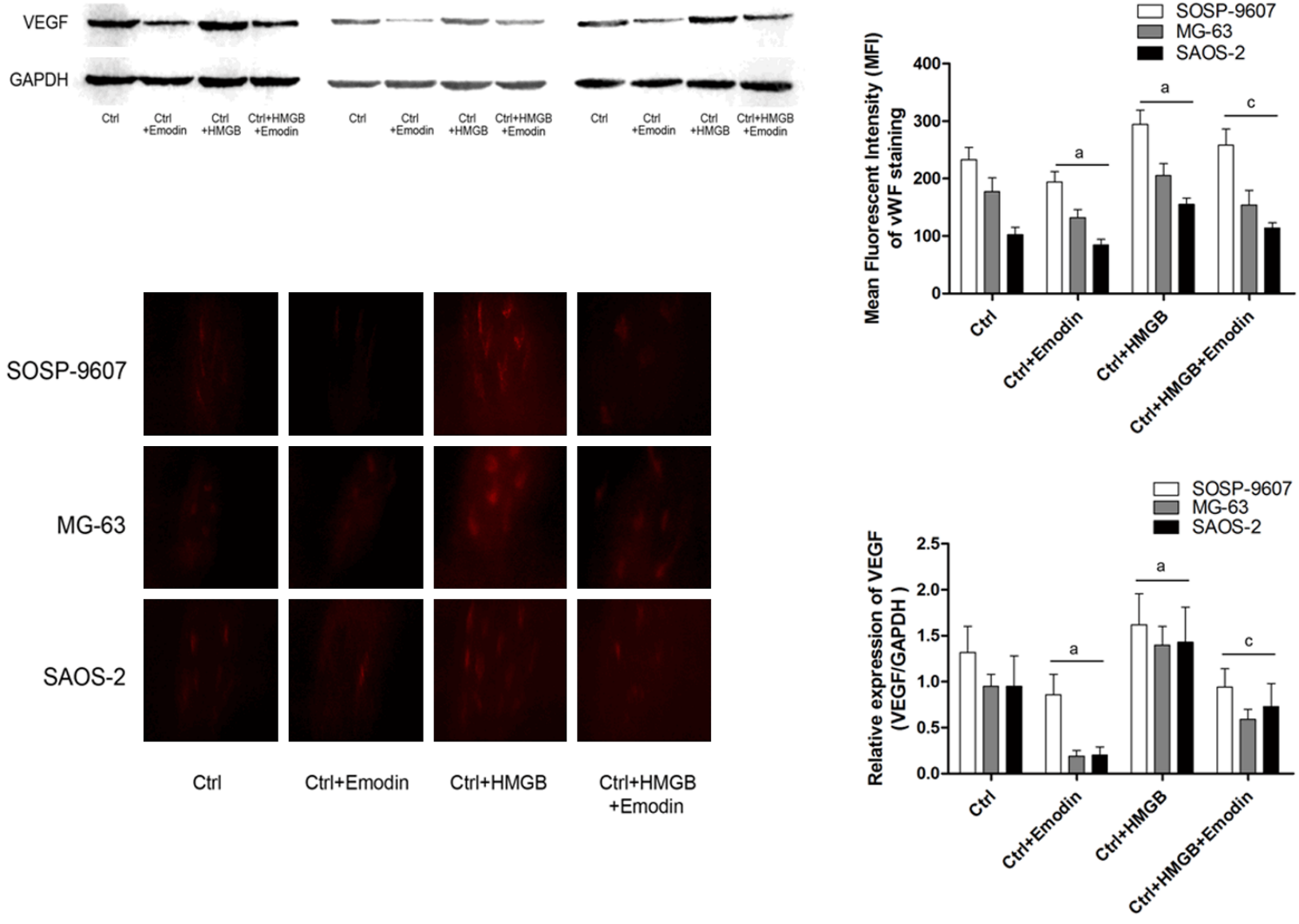

Figure 1. Emodin's anti-angiogenesis effect in human OS graft tumor *in vivo*. Upper part of this figure demonstrated the immunoblots of VEGF in human OS graft tumors (SOSP-9607, MG-63 and SAOS-2) harvested from nude mice. Lower part of this figure showed captured images of immunofluorescent staining of vWF in graft

tumors. Ctrl: tumor bearing nude mice, Ctrl+Emodin: tumor bearing nude mice treated with emodin; Ctrl+HMGB: tumor bearing nude mice treated with HMGB; tumor bearing nude mice received co-administration of emodin and HMGB. [a]Differences were significant when compared with Ctrl; [c]differences were significantly from Ctrl+Emodin.

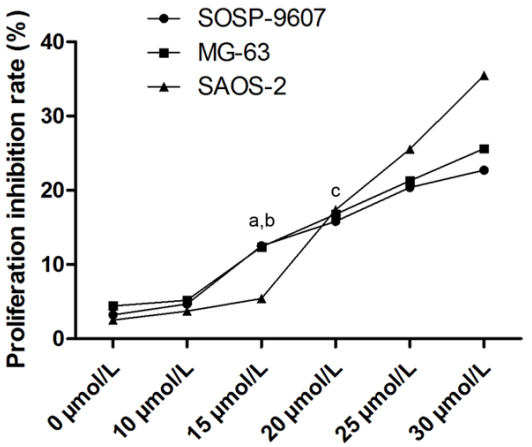

Figure 2. Effects of emodin incubation on proliferation of human OS cells. This chart showed proliferation inhibitory effect of emodin on SOSP-9607, MG-63 and SAOS-2 cells at serial concentrations ranging from 0 to 30 μmol/L. [a]Differences were significantly from 10 μmol/L in SOSP-9607 cells; [b]differences were significantly from 10 μmol/L in MG-63 cells; [c]differences were significantly from 15 μmol/L in SAOS-2 cells.

were incubated for 24 hours and then the above siRNA and vehicle control (GenePharma, Shanghai) was transfected into cells by using PepMute™ siRNA Transfection Reagent (SignaGen) per se the manufacturer's instructions.

Real-time RT-PCR

The total RNA was extracted from cells by using TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. SuperScript III Reverse Transcriptase (Invitrogen) was used to perform the reverse transcription and cDNA synthesis. Finally, by using All-in-one qPCR kit (Gene Copoeia), the quantitative real-time PCR was carried out. Primers were provided by TaKaRa, specifically, for SIRT1 was: sense 5'-CAGCAAGGCGAGCATAAA-3', antisense 5'-TTCAGAACCACCAAAGCG-3'; for GAPDH (internal reference) was: sense 5'-TTGCCATCAATGACCCCTTCA-3', antisense 5'-CGCCCCACTTGATTTTGGA-3'.

Western blotting

By using RIPA lysis buffer system (Beyotime) and Protein Extraction kit (Beyotime), total protein from tissue and cultured cells was extracted. A BCA protein assay kit (Solarbio) was used to detect protein concentration. 50 μg protein sample was loaded and then separated by vertical electrophoresis in SDS-polyacrylamide gels (8%-10%) and transferred to PVDF membranes (Millipore) electronically. Antibodies against SIRT1 (Abcam), acetylated histone 4 lysine 16 residue (H4-k16Ac, Invitrogen), VEGF (Abcam) and GAPDH (internal reference, Abcam) were used to incubate the membranes. SuperSignal West Pico kit (Peirce) was used to detect the immunoblots which were the analyzed by ImageJ2x software.

Von Willebrand factor (vWF) immunofluorescence assay

Harvested tumor tissue was fixed, dehydrated and then embedded in paraffin. 5-μm thick slice were made and incubated with anti-vWF antibody (Abcam) for 1 hour at 37°C. After washing and incubation with second antibody (Santa Cruz) for 30 minutes at 37°C, the fluorescence was detected and images were captured by a fluorescence microscope (Nikon).

Statistical considerations

The data in this study was presented as (mean ± SD). Differences between two groups were assessed performed with two sample two-tailed *t* test. Differences among multiple groups were assessed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant. The statistical analysis was performed by software SPSS (version 16.0, SPSS).

Results

Emodin significantly attenuated HMGB1-associated VEGF-induced angiogenesis in vivo

As shown in **Figure 1**, after nude mice were implanted with human osteosarcoma cells (SOSP-9607, MG63 and SAOS-2), exogenous HMGB1 administration was found playing a positive role in promoting role in tumor angiogenesis which was evidenced by increased VEGF and vWF expression. However, after administrated by emodin, both of VEGF and

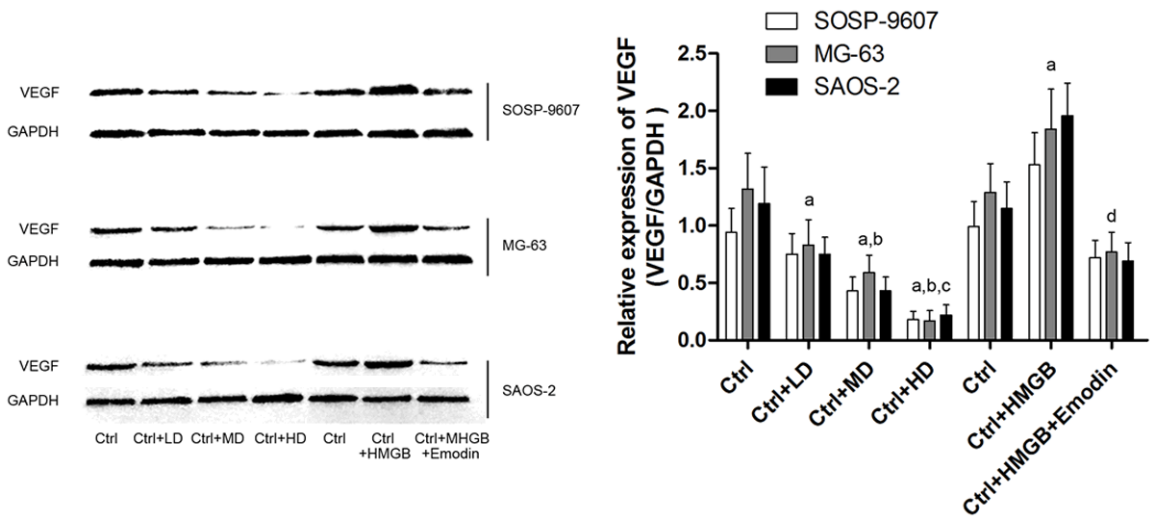

Figure 3. Effects of emodin and HMGB incubation on VEGF expression in human OS cells. Left part of this figure demonstrated the immunoblots of VEGF in human OS cells including SOSP-9607, MG-63 and SAOS-2 cells respectively. Ctrl: human OS cells; Ctrl+LD: human OS cells incubated with 2.5 μmol/L emodin; Ctrl+MD: human OS cells incubated with 5 μmol/L emodin; Ctrl+HD: human OS cells incubated with 10 μmol/L emodin. [a]Differences were significantly from Ctrl; [b]differences were significantly from Ctrl+LD; [c]differences were significantly from Ctrl+MD; [d]differences were significantly from Ctrl+HMGB.

vWF expression were suppressed in xenografted tumor tissue harvested from nude mice.

Proliferation of cultured human osteosarcoma cells was inhibited by emodin

Emodin incubation at serial diluted concentrations inhibited the proliferation of SOSP-9607, MG63 and SAOS-2 cells in a concentration-dependent manner (**Figure 2**). Emodin showed significant inhibitory effect on proliferation at 15 μmol/L for SOSP-9607 and MG63 cells; at 20 μmol/L for SAOS-2 cells manner (**Figure 2**). Thus, concentration below 15 μmol/L and 20 μmol/L were selected for anti-angiogenesis evaluation in SOSP-9607/MG63 and SAOS-2 cells respectively for the subsequent experiments.

Emodin dramatically reduced HMGB1 induced VEGF production in cultured human osteosarcoma cells

In cultured SOSP-9607, MG63 and SAOS-2 human osteosarcoma cells, emodin treatment significantly decreased VEGF expression in these cells in a concentration-dependent manner (**Figure 3**). Exogenous HMGB1 administration was found significantly induced cellular VEGF production which is considered fundamental in tumor angiogenesis. After emodin incubation, however, also in a concentration-dependent manner, HMGB1-induced VEGF production was found reduced dramatically.

Emodin elevated SIRT1 expression level and SIRT1 mediated deacetylase activity in cultured human osteosarcoma cells

SIRT1 was considered to execute the role as a deacetylase on multiple nuclear transfactors. Previously, H4-k16 was thought one of the substrates of SIRT1. Expression of acetylase from of H4-k16 (H4-k16Ac) is considered an indicator of deacetylase activity of SIRT1. In this study, we found both of SIRT1 expression level and deacetylase activity were elevated after emodin treated OS cells (**Figure 4**). We also found that in HMGB1 incubated human osteosarcoma cells, emodin significantly increased both SIRT1 expression level and deacetylase activity (**Figure 4**). In all, emodin increased SIRT1 expression and deacetylase activity in human OS cells, which was not affected by HMGB1 incubation.

SIRT1 silencing impaired emodin's anti-VEGF production activity in cultured human osteosarcoma cells

After SIRT1 was silenced by siRNA, compared with counterpart transfected with siRNA vehicle

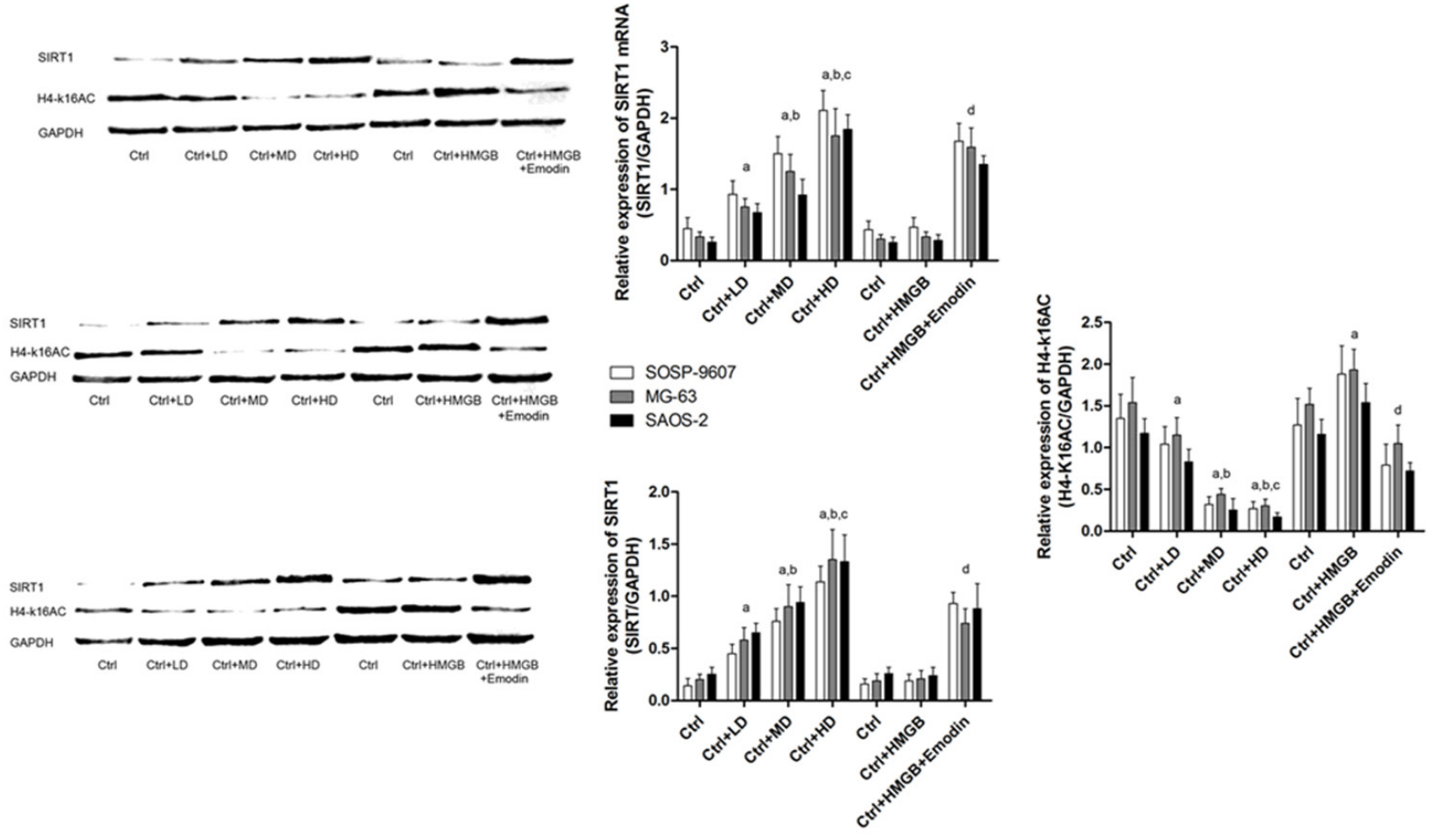

Figure 4. Effects of emodin and HMGB incubation on SIRT1 expression and deacetylase activity in human OS cells. Left part of this figure demonstrated the immunoblots of SIRT1 and H4-k16AC in human OS cells including SOSP-9607, MG-63 and SAOS-2 cells respectively. Upper part of the right part of this figure showed the mRNA levels in human OS cells. Ctrl: human OS cells; Ctrl+LD: human OS cells incubated with 2.5 μmol/L emodin; Ctrl+MD: human OS cells incubated with 5 μmol/L emodin; Ctrl+HD: human OS cells incubated with 10 μmol/L emodin. [a]Differences were significantly from Ctrl; [b]differences were significantly from Ctrl+LD; [c]differences were significantly from Ctrl+MD; [d]differences were significantly from Ctrl+HMGB.

control, we found that HMGB1 incubation dramatically increased VEGF expression in human OS cells (**Figure 5**). Furthermore, compared with vehicle siRNA control, SIRT1 silencing significantly impaired emodin's ability of reducing VEGF expression which was believed crucial in malignant tumor angiogenesis in human OS cells (**Figure 5**).

Discussion

In this study, both of *in vivo* and *in vitro* investigations were implemented to explore the possible mechanisms of anti-angiogenesis activity of emodin. In the *in vivo* study, OS cells were planted to nude mice to form xenograft tumor. We found that HMGB1 administration aggravated the tumor associated angiogenesis which was then significantly attenuated by emodin administration. In the *in vitro* part, three kinds of human OS cell lines, namely SOSP-9607, MG63 and SAOS-2 cells were used. HMGB1 incubation induced over-expression of VEGF which was attenuated by emodin treatment. Moreover, the anti-angiogenesis activity of emodin was found associated with its promoting effect on SIRT1 expression and de acetylase activity in HMGB1 incubated OS cells. This association was then testified by the impairment of the inhibitory effect of emodin on VEGF over-expression in HMGB1-incubated OS cells.

OS is originated from mesenchymal stem cell (MSC) and considered as the most common cancer of bone, especially in adolescents [23]. It was reported that the overall 5-year survival rate of OS was 60%-70% [24]. The prognosis of OS is pessimistic due to its poor response to chemotherapy and high rate of relapse and metastasis. Except for cell proliferation and invasion, angiogenesis is also considered as one of the remarkable characteristics of malignant tumor including OS [25]. In addition, to some extent, it was believed that angiogenesis was the biological basis for cancer cell proliferation and invasion [26]. In this process, endothelial cells were recruited and stimulated to proliferate. These cells would migrate through basement membrane and extracellular matrix (ECM) and eventually form novel tubular blood vessels [27]. Therefore, on one hand, more blood supply would be introduced to support cancer cell proliferation; on the other hand, cancer cells would have more chance of invasion and metastasis because of more contact with blood vessels.

Many molecules were described involved in the process of cancer-associated angiogenesis, which were called angiogenic molecules, such as basic fibroblast growth factor (bFGF) [28], placental growth factor (PLGF) [29], epidermal growth factor (EGF) [30], VEGF [31] and so on. In previously studies, VEGF was reported positively correlated with tumor angiogenesis [32]. Ubiquitously distributed in cell nucli, HMGB1 is exerting massive biological functions. Previous studies have reviled the association of HMGB1 in the occurrence and development of malignant tumors. Over-expression of HMGB1 was found indicating the development and prognosis in several human cancers [33, 34]. HMGB1 could interact with multiple down-stream molecules such as NF-κB [35], mitogen-activated protein kinase (MAPK) [36] and receptors for advanced glycation end products (RAGE) [37] to activate signaling pathways which play parts in tumor growth, invasion, metastasis and angiogenesis. In the present study, we found that in nude mice bearing human OS xenograft tumor, HMGB1 administration significantly increased angiogenesis in tumor tissue, which was indicated by elevated expression of VEGF and vWF. In the *in vitro* study, similar observation was obtained that HMGB1 treatment induced VEGF production in OS cells.

Angiogenesis inhibition could be a promising therapeutic strategy for malignant tumor prevention and treatment. Reagents with this anti-angiogenic effect are of great clinical significance. Emodin is one of the natural anthraquinones extracted from *Rheum palmatum L*. Literature records reporting emodin's anti-cancer activity could be found as early as 1970s. Following studies found emodin could suppress cancer proliferation, invasion and metastasis in a variety of cancer cells including gastric cancer, cervical cancer, hepatic cancer and so on [38-40]. A recent study reported that emodin inhibited angiogenesis in pancreatic cancer by regulating NF-κB-associated angiogenic factors [41]. It was reported in another study that emodin suppressed VEGF-induced angiogenesis in human [42]. In this study, we found that in HMGB1 administrated nude mice, emodin treatment significantly attenuated tumor angiogenesis in tumor tissue. We further confirmed that emodin also dramatically down-regulated VEGF in HMGB1 incubated human OS cells.

Additionally, in our current study, possible mechanisms were also investigated. Some pre-

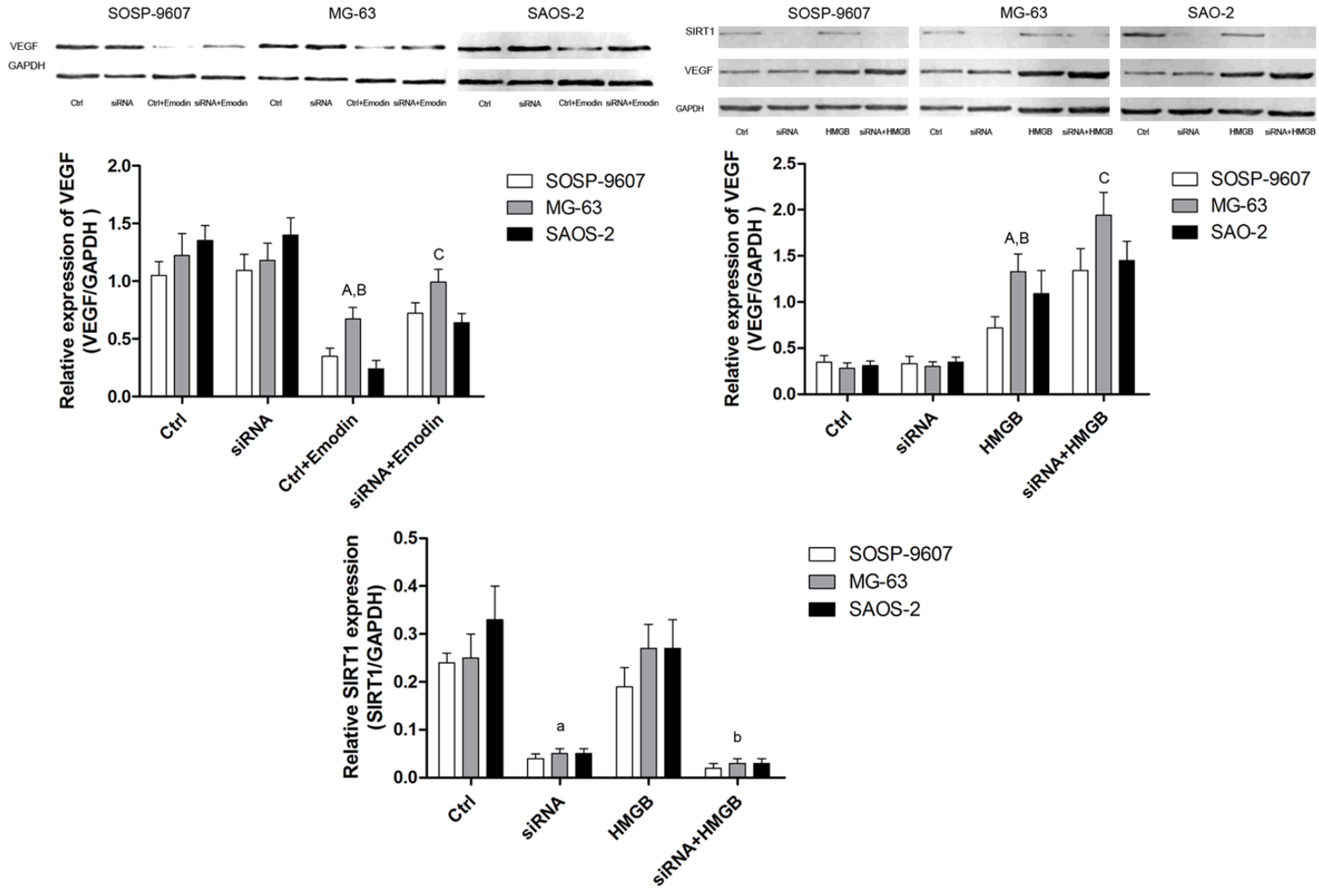

Figure 5. Effects of HMGB-incubated SIRT1-/-human OS cells on VEGF expression. Upper part of this figure demonstrated the immunoblots of VEGF and SIRT1 in HMGB-incubated SIRT1/human OS cells. Ctrl: human OS cells; siRNA: human OS cells incubated with SIRT1 siRNA; Ctrl+Emodin: human OS cells incubated with emodin; siRNA+Emodin: SIRT1/human OS cells incubated with emodin. [A]Differences were significantly from Ctrl, [B]differences were significantly from siRNA; [C]differences were significantly from Ctrl+Emodin. [a]Differences were significantly from Ctrl; [b]differences were significantly from HMGB.

vious studies pointed out that VEGF gene transcription was regulated by its upper stream nuclear transfector-HIF-1 [43]. The HIF-1 activity is largely dependent on the acetylation degree of its alpha subunit, namely HIF-1α. It was reported that HMGB1 induced tube formation by elevating acetylation of HIF-1α, while SIRT1 was the main molecule inducing HIF-1α deacetylation directly [44]. Thus, SIRT1 is the possible regulator in HMGB1-induced angiogenesis in OS and target of emodin. In the present study, we tried to testify this presupposition. In HMGB1-incubated OS cells, emodin significantly elevated SIRT1 expression level and its deacetylation activity. Thus, HMGB1-induced HIF-1α acetylation is attenuated by emodin-regulated SIRT1-induced HIF-1α deacetylation. As a result, HIF-1α-induced VEGF production was decreased by emodin administration. Furthermore, siRNA technique was also employed to silence SIRT1 expression in OS cells. We found that in SIRT1 silenced OS cells, HMGB1 treatment increased cellular VEGF production compared with wild type. Moreover, we also found that, in OS cells, SIRT1 silencing impaired emodin's inhibiting effect against VEGF production. These results indicated that SIRT1 was the molecular target of emodin when exerting its anti-angiogenesis effect in OS.

In conclusion, our results demonstrate that HMGB1 administration exacerbates tumor angiogenesis by inducing VEGF in human OS. Emodin alleviates tumor angiogenesis by reducing cellular VEGF production in human OS. Furthermore, SIRT expression and deacetylation activity elevation are involved in emodin's anti-angiogenesis effect in human OS.

Disclosure of conflict of interest

None.

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