

Original Article

Vorinostat (SAHA) promotes osteoblast differentiation and bone formation via Dkk-1 suppression

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Abstract: Objective: This study aims to elucidate the therapeutic effect of Vorinostat (SAHA), one of the histone deacetylase inhibitor (HDIs), against osteoporosis in vitro and in vivo, and its mechanism in Wnt signal pathway. Method: In the study, western blotting, cytochemistic and histochemistry staining and CHIP-PCR methods were used to explore whether SAHA induces MCT3T3-E1 cells osteogenic differentiation, and its action in Wnt pathway activation. The authors also build an ovariectomized rat model to mimic osteoporosis disease. ELISA was employed to study HDIs or saline on osteoporosis prevention in ovariectomized rats. Results: It showed that SAHA increased ALP activity and promoted MC3T3-E1 cells towards terminal osteoblast differentiation in vitro. Additionally, SAHA induced genomic-wide H3 and H4 acetylation and activated the Wnt/beta-catenin signal pathway. RT-PCR results indicated that TCF1 expression level increased significantly after SAHA treatment ($P < 0.01$). In ovariectomized rat study, serum Dkk-1 level dropped significantly in SAHA treatment (i.p.) compared with DNSO group ($P < 0.01$). The bone mature biomarkers, ALP and RUNX2, increased in bone marrow cells. The accumulation of beta-catenin within cells indicates SAHA activates Wnt pathway to promote osteoblast osteogenic differentiation. Conclusion: SAHA promotes osteoblasts osteogenic differentiation through reducing Dkk-1 expression and activating Wnt/beta-catenin signal pathway. It could be regarded as a potential agent for the prevention and therapy against osteoporosis.

Keywords: Vorinostat, Dickkopf-1, osteoblast, differentiation, osteoporosis

Introduction

Osteoporosis has become a major threat to health of the elderly people in aging societies, causing bone fragility, and increased fracture risk. It is defined as a bone metabolic disease with bone mass lost and microarchitecture destroyed [1]. Primary osteoporosis is generally related with osteoblasts disability, senescence and abnormal function of mesenchymal stem cells (MSCs). The main reason is persistent bone mineral lost and the death of osteoblasts and osteocytes, as well as osteoclast over activation. In the procedure of osteoblast differentiation towards mature bone cells, the main bone formation markers include the serum bone formation markers total osteocalcin (OC) and the alkaline phosphatase (ALP) bone isoenzyme. At the same time, transcription factor Runx2 is also regarded as a biomarker during osteoblast differentiation.

Wnt/beta-catenin signaling shows the key impact in generation, differentiation of pluripo-

tential MSCs toward osteoblasts. It also participates in bone homeostasis, including bone matrix formation, osteoblast and osteoclast balance [2]. Additionally, Wnt proteins prevent the apoptosis of mature osteoblasts, and thereby prolong their lifespan. Wnt signaling in bone is fine-tuned by several secreted glycoproteins that act as Wnt antagonists. The most potent and best recognized of these are sclerostin, Wise, and Dickkopf proteins [3]. It is reported Dickkopf-1 (Dkk-1) acts as a blocker of canonical Wnt signaling pathway, and has been associated with the progression of osteolytic bone metastases by impairing osteoblast activity.

Numerous studies reported histone deacetylase inhibitor (HDIs) induce cell cycle arrest, differentiation, and/or apoptosis of tumor cell lines in vitro and inhibit tumor growth in vivo. The tumor-selective effects of HDIs make them promising anticancer agents and several are currently in phase I and II clinical trials for leukemia and solid tumors [4, 5]. In addition to

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metastatic and primary cancers, HDIs hold promise as therapeutics for other diseases such as rheumatoid arthritis and cardiocyte hypertrophy because of their ability to alter gene expression and reprogram cellular activities. Many studies reported that HDIs accelerate bone matrix production and bone maturation, induce the expression of osteoblast genes in osteogenic cell lines, primary calvarial cells, calvarial organ cultures, and mesenchymal progenitor cells [6, 7]. The positive effects of HDIs on inducing osteoblast maturation are largely due to promote the activity of beneficial transcription factor, such as Run2X [8]. As one of the HDIs, Vorinostat (SAHA) has been reported to impair immature osteoblasts and cause bone loss [9], thus does not fit for osteoporosis therapy, but other reports testify SAHA did not cause bone loss [10, 11]. Although previous studies focused on SAHA and osteoporosis, SAHA and ovariectomized rat were not involved. The mechanism involved in the HDIs-mediated bone formation and osteoblast differentiation are quite complicated. Thus, we elucidate the effect of SAHA in inducing MC3T3 cell osteogenic formation. In another aspect, we explore whether SAHA could benefit ovariectomized osteoporosis rat in bone formation or osteoblast differentiation in vivo.

Materials and methods

Chemicals, cell line and reagents

SAHA was purchased from Selleck Inc. Antibodies of Wnt3a, Wnt5, beta-catenin, LRP6 and phospho-LRP6 were from Wnt Signaling Antibody Sampler Kit (Cellsignal Inc. #2915). MC3T3-E1 was purchased from ATCC institution (Chian distributor agency). Antibodies of Ac-Histone H3, Ac-Histone H4 were from Millipore company. Other chemicals were from Sigma-Aldrich if not specifically indicated.

ALP activity measurement

On days 2, 7 and 14, the cultured cells of each treatment group were washed twice with PBS, and 0.25% trypsin was used to digest the cells, followed by the addition of 1 ml of culture medium to terminate the digestion. After centrifugal washing, cell lysis solution was added, and ALP activity was assayed according to the kit manufacturer's instructions. OD was measured with Synergy 2 multimode plate-reader (Biotek, Winooski, VT, USA) at a wavelength of 520 nm.

Mineralization

Osteoblasts were seeded in 6-well plates, and cultured for 21 days in minimal medium containing 10 nM SAHA or DMSO to investigate bone nodule formation. Extracellular matrix calcium deposits were stained with Alizarin red dye as reported. Osteoblasts were fixed with ice-cold 70% (v/v) ethanol and then stained with Alizarin Red S (20 mM) for 30 min. Cell mineralization was evaluated by the microscopy and image processing software.

Western blotting

Briefly, cells were lysed in RIPA buffer containing protease inhibitors (0.1 mg/ml PMSF, 10-fold diluted Aprotinin). Protein extracts (less than 50 µg) were electrophoresed on 8~15% SDS-PAGE gels and transferred onto Hybond membranes (GE Healthcare, Arlington Heights, IL) by electroblotting at 100 V for 1 h~2 h. The membranes were blocked in 5% nonfat milk and then were incubated with the designated primary antibodies for 2 hours, followed by incubation with a HRP-conjugated secondary antibody for another hour. After the membrane was washed with Tris-buffered saline containing Tween-20, the reaction was visualized by means of the ECL reagent kit (GE Healthcare). The membrane was exposed to films for a period ranging from 30 sec to 5 min.

RT and real time PCR

Total mRNA was extracted by a commercial mRNA extraction kit (Thermo). Two µg of RNA were reverse-transcribed into cDNA using oligo dT primers. Real-time PCR primers for TCF-1 were synthesized by Lifetechnologies Inc.. Forward primer: CTCCAAGGACTGGAGAACA-CTT; Reverse primer: CAGGCCAGCGTGGCTGGCTGT. Real time PCR was carried out at 94°C for 30 s, 55°C for 30 s and 72°C for 45 s for 30 cycles, using ABI prism 7700 sequence detector and software to analyze the data (Applied Biosystems, CA).

Chromatin precipitation (ChIP) and PCR

Chromatin immunoprecipitation was performed based on the protocol previously published [12]. Briefly, cells were treated with 1% final concentration of formaldehyde for 10 min on

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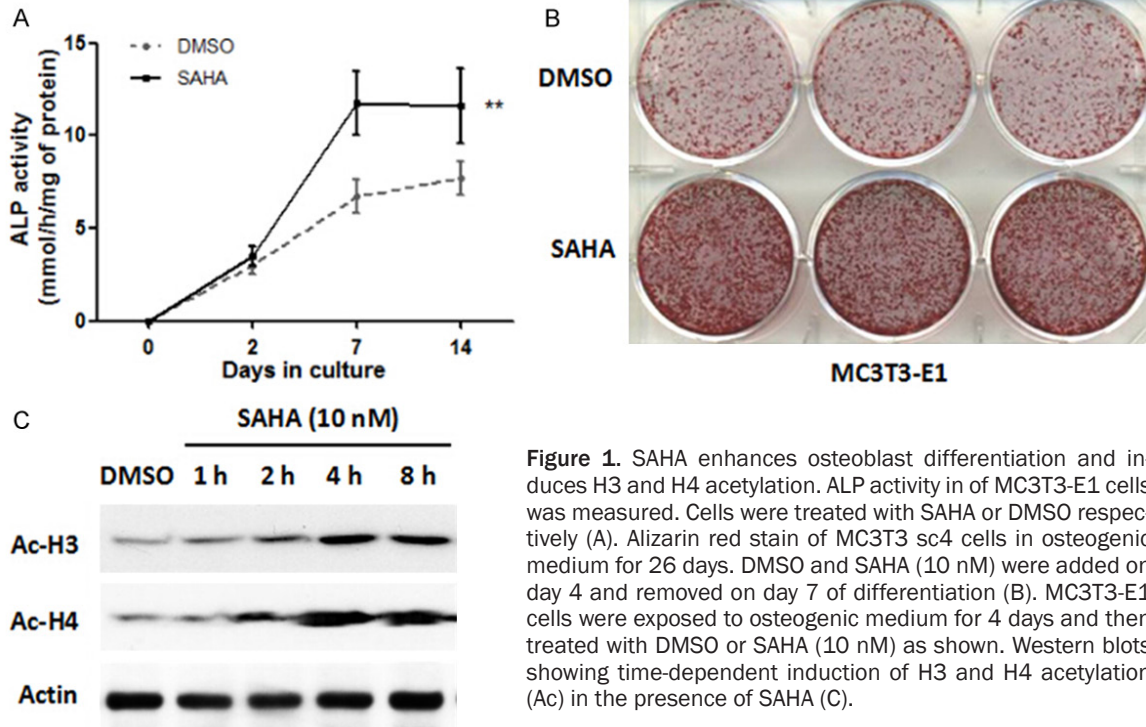


Figure 1. SAHA enhances osteoblast differentiation and induces H3 and H4 acetylation. ALP activity in of MC3T3-E1 cells was measured. Cells were treated with SAHA or DMSO respectively (A). Alizarin red stain of MC3T3 sc4 cells in osteogenic medium for 26 days. DMSO and SAHA (10 nM) were added on day 4 and removed on day 7 of differentiation (B). MC3T3-E1 cells were exposed to osteogenic medium for 4 days and then treated with DMSO or SAHA (10 nM) as shown. Western blots showing time-dependent induction of H3 and H4 acetylation (Ac) in the presence of SAHA (C).

the plate. Cell lysates were sonicated to let chromatin break approximately 500 bp size. Chromatin from 107 cells was incubated with 1 μ g antibody. Antibody-chromatin complexes were pulled down with protein A agarose, and washed. Formaldehyde crosslinks were reversed. Antibodies used for ChIP were anti-AC-H4, IgG control (normal goat IgG, Santa Cruz). One part of precipitated was used for Western blotting analysis. The other part was used by PCR confirmation of TCF1 promoter.

Serum Dkk-1 ELISA

Rat peripheral blood samples collected into anticoagulant-free tubes were centrifuged and stored at -70°C prior to testing. Using commercial ELISA kit to performed serum DKK-1 quantification assays (R&D Systems), according to manufacturer's instruction. Briefly, 96-well Corning ELISA high binding ability plates were coated with the Dkk-1 monoclonal antibody supplied with the kit and incubated overnight at 4°C . The reaction was blocked with 1% BSA. The biotinylated goat anti-human Dkk-1 detection antibody was incubated for 2 h at 37°C , followed by streptavidin-horseradish peroxidase for 20 min. Color development was initiated with 3,3',5,5'-tetramethylbenzidine and

hydrogen peroxide followed by 1 M sulphuric acid to stop the reaction. The optical density was measured at 450 nm and referenced to 570 nm on a Synergy 2 multimode plater reader (Biotek, Winooski, VT, USA).

Ovariectomized rat model

Eighteen female rats were carried out ovaries excision under anesthesia status. Three months later, rats were administered SAHA (100 mg/kg), stilbestrol (50 mg/kg) or DMSO (equal volume to SAHA) every other day for 2 weeks. After the rats were sacrificed, bone formation was assessed based on serum ALP level, bone marrow cell RUN2 expression level and histomorphometric staining analysis.

Statistical analysis

Results are showed in the form of mean with standard error (SEM). All the data were analyzed using PRISM software. One-way ANOVA was used to compare means of three or more samples. Specifically, in vitro and in vivo data were analyzed using the Mann-Whitney U test to determine the difference between means. *P* values of less than 0.05 were considered statistically significant.

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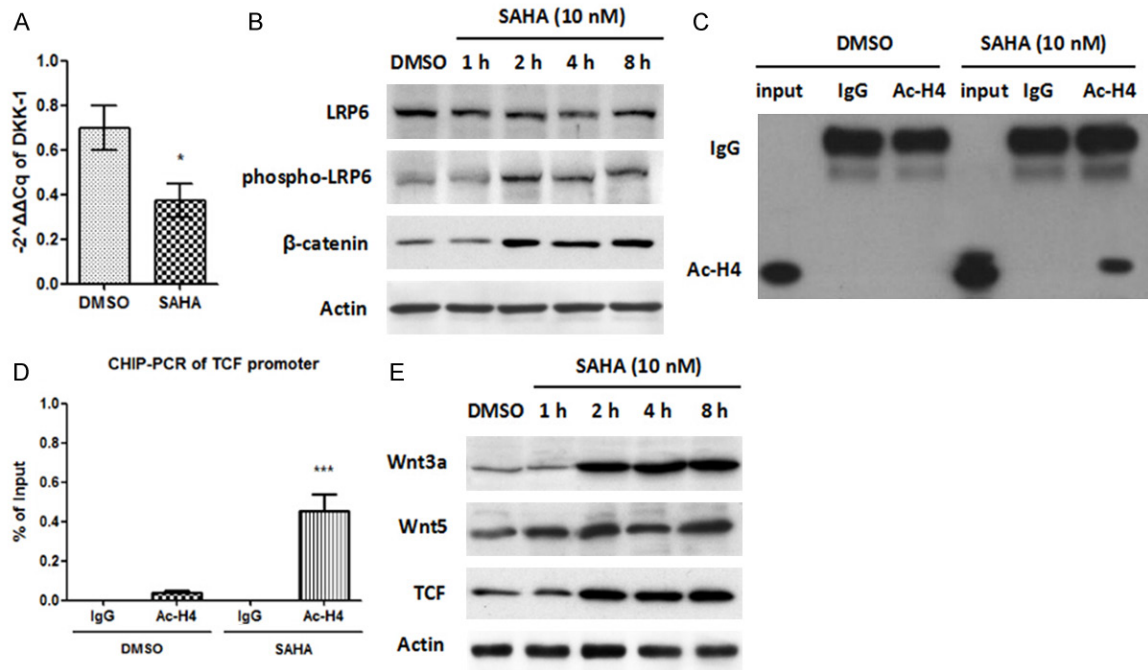


Figure 2. SAHA suppressed Dkk-1 expression. RT-PCR method was used to show Dkk-1 transcription level in SAHA or DMSO treated cells (A). SAHA stimulated LRP6 phosphorylation activation, and increase beta-catenin accumulation with cytoplasm (B). MC3T3-E1 cells were differentiated in osteogenic medium for 4 days and treated with DMSO or SAHA (20 μ m) for 4 h before the ChIP assay was performed with anti-acetylated H4 and IgG antibodies. Western blot from ChIP assay showing successful pull-down of acetylated H4 in the presence of SAHA (C). ChIP-PCR showing H4 acetylation of the TVF1 promoter with and without SAHA (D, n = 3, Data are normalized to input DNA). SAHA increased Wnt3a, Wnt5 and TCF1 protein expression (E).

Results

SAHA promoted osteoblasts differentiation and induced global histone H3 and H4 acetylation

ALP activity represents osteoblasts differentiation ability, which was quantified by commercial ELISA kit. In this study, the measurement of ALP activity in MC3T3 cells treated with SAHA or DMSO on days 2, 7 and 14 days, was employed to investigate the effect of osteoblast differentiation ability. Compared to its control group, the osteoblasts treated with SAHA exhibited higher ALP activity than cells treated with DMSO, as of day 2, day 7 and day 14 (**Figure 1A**).

To determine the mineralizing effects of SAHA on the differentiation induction of MC3T3 E1 cells, we added SAHA (10 nM) or equal volume of DMSO to osteogenic cultures on days 4-7 of a 26-day period. Consistent with those published results [12], SAHA enhances matrix calcification of MC3T3 E1 cells (**Figure 1B**). Mean-

while, we found that SAHA induced global histones H3 and H4 hyperacetylation within 4 h (**Figure 1C**).

SAHA suppressed Dkk-1 expression was related with increased histones acetylation

Many reporters found serum Dkk-1 levels were significantly higher in osteoporotic patients and rat models than in control group, indicating the important role of Dkk-1 in osteoporosis development. Dkk-1 acts as a blocker of canonical Wnt signaling pathway to inhibit bone formation. To assess if SAHA-induced histones hyperacetylation could decrease Dkk-1 expression, RT-PCR method was used. We found SAHA treated cells had less Dkk-1 transcription than DMSO treated cells (**Figure 2A**). Consequently, SAHA activated WNT/beta-catenin pathway (**Figure 2B**). These finding may explain why SAHA induces MC3T3 cell differentiation and bone formation.

In the chromatin immunoprecipitation study, acetylated H4 was precipitated by the anti-H4

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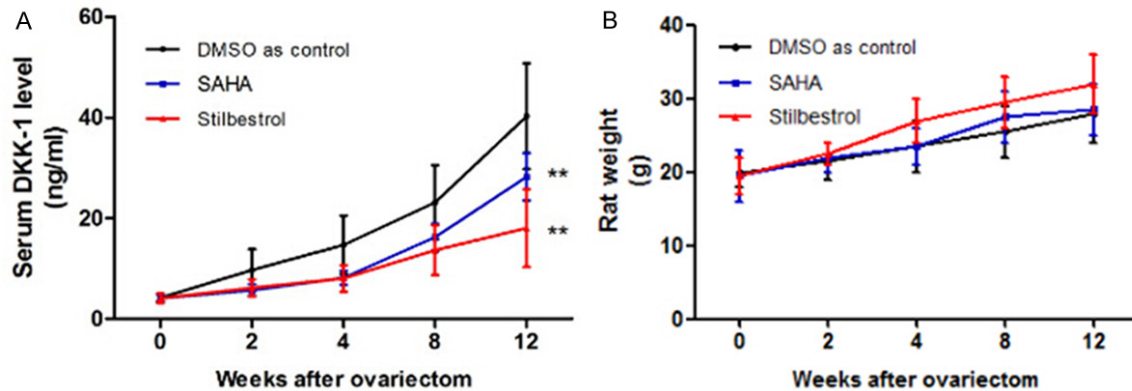


Figure 3. SAHA decreases ovariectomized rat serum Dkk-1 level. In the beginning of the ovariectomy, Dkk-1 level did not change, but increase gradually within three months (A). After one week of SAHA and stilbestrol treatment, Dkk-1 level drop slightly while saline group continue to increase. One month later, Dkk-1 level in SAHA and stilbestrol groups were greatly lower than DMSO treatment group (A, **represent $P < 0.01$). Weight change difference was not significant (B).

antibody from SAHA-treated and DMSO treated cells. The nonspecific IgG acts as control. Western blot analysis confirmed SAHA treated group has more acetylated H4 than control group, indicating that the anti-H4 antibody strictly and efficiently precipitates acetylated H4 protein (Figure 2C). We also explore the acetylation status of acetylated H4 on TCF1 promoter region in the presence of SAHA or DMSO. Hyperacetylated H4 was detected on the TCF-1 promoter in SAHA treated cells and control cells. Results showed SAHA treated cell was 10-fold higher than DMSO treated cells (Figure 2D). Western blotting results also suggested SAHA increase Wnt3a, Wnt5, and TCF1 overexpression (Figure 2E).

SAHA decreased ovariectomized rat serum Dkk-1 level

A total of 18 rats were ovariectomized, and randomly separated into SAHA treated group, osteoporosis drug treatment group and DMSO administrative group. The surgical excision of ovaries were referred to published study [13]. Regarding Dkk-1 as a potent inhibitor of bone formation, we monitored serum Dkk-1 level before and after ovary surgical removal, and the time of SAHA treatment. Three months later, SAHA and stilbestrol were administrated to rats by i.p. injection respectively, while control group was given DMSO. During the time, the body weight and serum Dkk-1 level were showed in Figure 3A and 3B. Dkk-1 monitoring is an important marker to evaluate osteoporosis

and bone health status. In the beginning of the ovariectomy, Dkk-1 level did not change, but increased gradually within three months. After one week of SAHA and stilbestrol treatment, Dkk-1 level dropped slightly while saline group continued to increase. One month later, Dkk-1 level in SAHA and stilbestrol groups were greatly lower than DMSO treatment group (**represent $P < 0.01$).

SAHA induced ovariectomized rat bone formation and maturation

Tissue pathology inspection of femur bone was carried out after rats sacrifice. Femur bone sections showed cavities with obvious bone lost in DMSO treated group, indicating that the rat model of osteoporosis was established successfully (Figure 4A). SAHA and stilbestrol treated groups showed less bone lost and more comprehensive microarchitecture compared with controls (Figure 4A). As a master factor of osteoblast differentiation, RUNX2 transcription factor expression level was quantified using RT-PCR method. mRNA was extract from bone marrow cells using commercial kit. Results showed SAHA or stilbestrol treated rats increase Runx2 expression (Figure 4B). SAHA also significantly increased ALP activity compared to the control group (Figure 4C).

Discussion

HDIs (SAHA) promote terminal osteoblast differentiation, bone maturation and extracellular

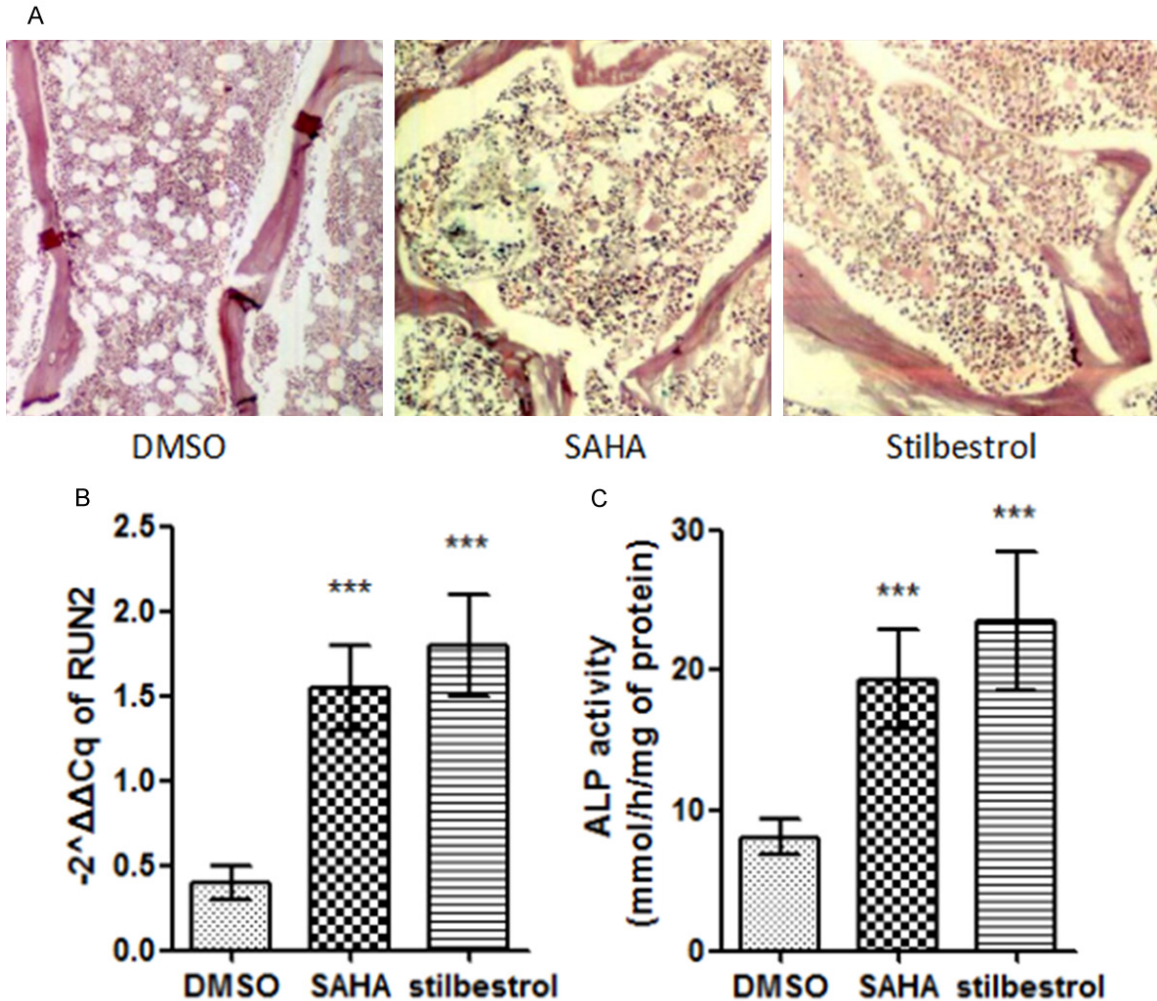


Figure 4. SAHA induces ovarioectomized rat bone formation and maturation. Tissue pathology inspection of femur bone was carried out after rats sacrifice (A). RUN2 transcription factor expression level was quantified using RT-PCR method. mRNA was extract from bone marrow cells using commercial kit (B). SAHA also significantly increased ALP activity compared to the control group (C).

matrix production in culture, which will help to prevent osteoporosis. Drug treatment by HDIs might enhance the ability of osteogenic differentiation and increase the number of osteoblasts.

In the study of Dkk-1 expression level, including mRNA and protein level (western blotting data not shown), we found SAHA-induced histones hyperacetylation and decreased Dkk-1 expression (Figure 2A). Consequently, reduced Dkk-1 activated Wnt/beta-catenin pathway due to the negative feedback regulation loop. We also showed that SAHA treated MC3T3 cells increased TCF1 promoter region histones acetylation (Figure 2C). Histone hyperacetylation of gene promoter is usually related to gene

upregulated expression [14]. In the present study, although global histone acetylation was observed, the Dkk-1 expression level surprisingly decreased. We supposed that other Dkk-1 inhibitory factors might also increase expression, and thus suppressed Dkk-1 expression finally. It is reported that SAHA could also promote Dkks feedback signal protein increased expression [15], and thus Dkk-1 is also a target of the Wnt/beta-catenin/TCF pathway [16]. To test the hypothesis, we examined TCF1 gene promoter H4 acetylation status and expression level. CHIP-PCR and western blotting results supports the hypothesis. These biological effects are exerted by activation of the Wnt/beta-catenin/TCF increased transcription. Taken together, it finally decreases DKK-

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1 expression, and subsequently promotes osteoblast differentiation and bone maturation.

SAHA increased MC3T3 cells ALP activity in the culture, and activated Wnt/beta-catenin pathway. These findings may explain why SAHA induces MC3T3 cell differentiation and bone formation in vitro. In the in vivo study, we elucidated that SAHA induced ovariectomized rat bone formation and maturation. Firstly, we showed femur bone sections of drug treatment to make sure that the success of ovariectomized rat is the meaningful model. SAHA and stilbestrol treated groups showed less bone loss compared with control (**Figure 4A**). Runx2, a key transcription factor that binds several histone deacetylases (HDAC), benefits osteoblast osteogenesis and bone formation. Runx2 has been demonstrated to up-regulate osteoblast-related genes such as Col1A1, ALP, BSP, BGLAP and OCN [17]. In the study, RUNX2 transcription factor expression level was quantified using RT-PCR method. mRNA was extracted from bone marrow cells using commercial kit. Results showed SAHA or stilbestrol treated rats increased Runx2 expression (**Figure 4B**). SAHA also significantly increased ALP activity compared to the control group (**Figure 4C**). Our findings were consistent with the previous studies on HDIs, including TSA [18] and sodium butyrate [19].

Studies found serum Dkk-1 levels were significantly higher in osteoporotic patients and rat models than in control group [20, 21], indicating the important role of Dkk-1 in osteoporosis development. Dkk-1 acts as a blocker of canonical Wnt signalling pathway to inhibit bone formation. When Dkk-1 binds to Kremen and LRP5, it undergoes internalization and therefore becomes unable to bind Wnt [22]. Finally it leads to degradation of beta-catenin and to inhibition of bone formation. To assess if the ovariectomized rat model fits the clinical observation, we examined the serum before and after the drug treatment. From the sections of femur, morphologically, SAHA caused less bone mass loss and at the same time maintain the bone microarchitecture. On the other hand, in agreement with previous studies of Dkk-1, Dkk-1 level did not change, but increase gradually within three months. However, Dkk-1 level in SAHA and stilbestrol groups were greatly lower than DMSO treatment group. This

implies SAHA cured against osteoporosis via suppressed Dkk-1 expression. However, more studies were needed to further clarify the mechanism of this suppression effect of SAHA on Dkk-1.

In conclusion, SAHA, as one of the HDIs, reduced Dkk-1 expression and activated Wnt/beta-catenin signal pathway, and promoted osteoblasts osteogenic differentiation, which consequently alleviated bone loss in ovariectomized rat. Therefore, SAHA might be a potential promising agent for the prevention and therapy against osteoporosis.

Disclosure of conflict of interest

None.

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References

- [1] Garnero P. New developments in biological markers of bone metabolism in osteoporosis. *Bone* 2014; 66: 46-55.
- [2] Rodda SJ and McMahon AP. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* 2006; 133: 3231-3244.
- [3] Manolagas SC. Wnt signaling and osteoporosis. *Maturitas* 2014; 78: 233-237.
- [4] Terranova-Barberio M, Roca MS, Zotti AI, Leone A, Bruzzese F, Vitagliano C, Scogliamiglio G, Russo D, D'Angelo G, Franco R, Budiillon A and Di Gennaro E. Valproic acid potentiates the anticancer activity of capecitabine in vitro and in vivo in breast cancer models via induction of thymidine phosphorylase expression. *Oncotarget* 2016; 7: 7715-31.
- [5] Evens AM, Balasubramanian S, Vose JM, Harb W, Gordon LI, Langdon R, Sprague J, Sirisawad M, Mani C, Yue J, Luan Y, Horton S, Graef T and Bartlett NL. A Phase I/II Multicenter, Open-Label Study of the Oral Histone Deacetylase Inhibitor Abexinostat in Relapsed/Refractory Lymphoma. *Clin Cancer Res* 2016; 22: 1059-1066.
- [6] Schroeder TM and Westendorf JJ. Histone deacetylase inhibitors promote osteoblast maturation. *J Bone Miner Res* 2005; 20: 2254-2263.

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- [7] Di Bernardo G, Squillaro T, Dell'Aversana C, Miceli M, Cipollaro M, Cascino A, Altucci L and Galderisi U. Histone deacetylase inhibitors promote apoptosis and senescence in human mesenchymal stem cells. *Stem Cells Dev* 2009; 18: 573-581.
- [8] Jensen ED, Schroeder TM, Bailey J, Gopalakrishnan R and Westendorf JJ. Histone deacetylase 7 associates with Runx2 and represses its activity during osteoblast maturation in a deacetylation-independent manner. *J Bone Miner Res* 2008; 23: 361-372.
- [9] McGee-Lawrence ME, McCleary-Wheeler AL, Secreto FJ, Razidlo DF, Zhang M, Stensgard BA, Li X, Stein GS, Lian JB and Westendorf JJ. Suberoylanilide hydroxamic acid (SAHA; vorinostat) causes bone loss by inhibiting immature osteoblasts. *Bone* 2011; 48: 1117-1126.
- [10] Xu S, De Veirman K, Evans H, Santini GC, Vande BI, Leleu X, De Becker A, Van Camp B, Croucher P, Vanderkerken K and Van Riet I. Effect of the HDAC inhibitor vorinostat on the osteogenic differentiation of mesenchymal stem cells in vitro and bone formation in vivo. *Acta Pharmacol Sin* 2013; 34: 699-709.
- [11] Dudakovic A, Evans JM, Li Y, Middha S, McGee-Lawrence ME, van Wijnen AJ and Westendorf JJ. Histone deacetylase inhibition promotes osteoblast maturation by altering the histone H4 epigenome and reduces Akt phosphorylation. *J Biol Chem* 2013; 288: 28783-28791.
- [12] Dudakovic A, Evans JM, Li Y, Middha S, McGee-Lawrence ME, van Wijnen AJ, Westendorf JJ. Histone deacetylase inhibition promotes osteoblast maturation by altering the histone H4 epigenome and reduces Akt phosphorylation. *J Biol Chem* 2013; 288: 28783-28791.
- [13] Park SB, Park SH, Kim NH and Chung CK. BMP-2 induced early bone formation in spine fusion using rat ovariectomy osteoporosis model. *Spine J* 2013; 13: 1273-1280.
- [14] Lakshmaiah KC, Jacob LA, Aparna S, Lokanatha D and Saldanha SC. Epigenetic therapy of cancer with histone deacetylase inhibitors. *J Cancer Res Ther* 2014; 10: 469-478.
- [15] Li C, Wei G, Gu Q, Wang Q, Tao S and Xu L. Proliferation and differentiation of rat osteoporosis mesenchymal stem cells (MSCs) after telomerase reverse transcriptase (TERT) transfection. *Med Sci Monit* 2015; 21: 845-854.
- [16] Niida A, Hiroko T, Kasai M, Furukawa Y, Nakamura Y, Suzuki Y, Sugano S and Akiyama T. DKK1, a negative regulator of Wnt signaling, is a target of the beta-catenin/TCF pathway. *Oncogene* 2004; 23: 8520-8526.
- [17] Florencio-Silva R, Sasso GR, Sasso-Cerri E, Simoes MJ and Cerri PS. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. *Biomed Res Int* 2015; 2015: 421746.
- [18] Azechi T, Kanehira D, Kobayashi T, Sudo R, Nishimura A, Sato F and Wachi H. Trichostatin A, an HDAC class I/II inhibitor, promotes Pi-induced vascular calcification via up-regulation of the expression of alkaline phosphatase. *J Atheroscler Thromb* 2013; 20: 538-547.
- [19] Jung HM, Song GA, Lee YK, Baek JH, Ryoo HM, Kim GS and Woo KM. Modulation of the resorption and osteoconductivity of alpha-calcium sulfate by histone deacetylase inhibitors. *Biomaterials* 2010; 31: 29-37.
- [20] Tian J, Xu XJ, Shen L, Yang YP, Zhu R, Shuai B, Zhu XW, Li CG, Ma C and Lv L. Association of serum Dkk-1 levels with beta-catenin in patients with postmenopausal osteoporosis. *J Huazhong Univ Sci Technolog Med Sci* 2015; 35: 212-218.
- [21] Gifre L, Ruiz-Gaspa S, Monegal A, Nomdedeu B, Filella X, Guanabens N and Peris P. Effect of glucocorticoid treatment on Wnt signalling antagonists (sclerostin and Dkk-1) and their relationship with bone turnover. *Bone* 2013; 57: 272-276.
- [22] McGraw HF, Culbertson MD and Nechiporuk AV. Kremen1 restricts Dkk activity during posterior lateral line development in zebrafish. *Development* 2014; 141: 3212-3221.