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Histone deacetylase 8 suppresses osteogenic differentiation of bone marrow stromal cells by inhibiting histone H3K9 acetylation and RUNX2 activity

Yu Fu[a,b], Ping Zhang[a], Jie Ge[b], Jie Cheng[a], Weijie Dong[a,b], Hua Yuan[a], Yifei Du[a], Mifang Yang[b], Ruoxing Sun[b], Hongbing Jiang[a,b,*]

[a] Department of Oral and Maxillofacial Surgery, School of Stomatology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
[b] Institute of Stomatology, School of Stomatology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

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ABSTRACT

Bone marrow stromal cells (BMSCs) are multipotent progenitor cells with capacities to differentiate into the various cell types and hold great promise in regenerative medicine. The regulatory roles of histone deacetylases (HDACs) in osteoblast differentiation process have been increasingly recognized; however, little is known about the precise roles of HDAC8 in the osteogenic differentiation of BMSCs. Herein we aimed to investigate the roles of HDAC8 in the osteogenic differentiation of rat BMSCs by pharmacological and genetic manipulations in vitro. During osteogenic differentiation of BMSCs, pharmacological inhibition of HDAC8 by HDAC inhibitor valproic acid (VPA) promoted the level of histone H3 lysine 9 acetylation (H3K9Ac) and significantly enhanced the expression of osteogenesis-related genes Runx2, Osterix, osteocalcin (OCN), osteopontin (OPN) and alkaline phosphatase (ALP). Similarly, knockdown of HDAC8 using short interfering RNA triggered H3K9Ac and enhanced osteogenic differentiation of BMSCs, largely phenocopied the effects of VPA-mediated HDAC8 depletion. However, enforced expression of HDAC8 significantly reduced the level of H3K9Ac and inhibited osteogenic differentiation of BMSCs, which can be attenuated by VPA addition. Mechanistically, HDAC8 suppressed osteogenesis-related genes expression by removing the acetylation of histone H3K9, thus leading to transcriptional inhibition during osteogenic differentiation of BMSCs. Importantly, we found that HDAC8 physically associated with Runx2 to repress its transcriptional activity and this association decreased when BMSCs underwent osteogenic differentiation. Taken together, these results indicate that epigenetic regulation of Runx2 by HDAC8-mediated histone H3K9 acetylation is required for the proper osteogenic differentiation of BMSCs.

1. Introduction

Bone marrow stromal cells (BMSCs) are progenitor cells as defined by their self-renewal capabilities and multilineage differentiation potentials that give rise to various tissues and organs both in vitro and in vivo (Undale et al., 2009). Under certain conditions, BMSCs are able to differentiate into osteoblasts and hold significant promise for clinical applications, especially for bone regeneration in skeletal defects, largely due to easy harvest, accessibility and lack of immunogenicity (Caplan and Bruder, 2001; Kon et al., 2012; Wagers and Weissman, 2004). Previous studies have been focused on endeavoring to promote the capability of BMSCs to undergo osteogenic differentiation via epigenetic modifications, such as histone acetylation, which may contribute to differentiation of BMSCs into osteoblasts lineage (Hu et al., 2013; McGee-Lawrence and Westendorf, 2011; Teven et al., 2011). However, the detailed molecular mechanisms underlying these processes remain incompletely recognized.

Histone acetylation is a reversible epigenetic process modulated by the combined and delicate activities of histone acetylases (HATs) and histone deacetylases (HDACs). Hyperacetylation contributes to relaxing the chromatin structure and allows the binding of DNA sequences by transcription factors, thereby activating transcription, whereas histone deacetylation results in transcriptional silencing (Cheung et al., 2000; Choudhary et al., 2009; Teven et al., 2011). HDACs regulate transcriptional process by affecting

* Corresponding author at: Department of Oral and Maxillofacial Surgery, School of Stomatology, Nanjing Medical University, 136 Hanzhong Road, Nanjing, Jiangsu Province 210029, China. Tel.: +86 25 85031914; fax: +86 25 85031910.
E-mail address: jhb@njmu.edu.cn (H. Jiang).

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the structure of chromatin and activities of relevant transcription factors under diverse biological contexts. Upon removal of negatively charged acetyl groups (CH3CO-) from the ε-amino groups of lysine residues by HDACs, such histone changes result in chromatin condensation, thus decreasing the accessibility of DNA-binding transcription factors (Choudhary et al., 2009; Glozak et al., 2005). During the differentiation of stem cells, the relative abundance of epigenetic marks usually reflect the activation or repression of genes which further guide cells development toward a particular cell lineage. Modified histone domains such as acetylated histone H3 lysine 9 (H3K9) are considered as key epigenetic signatures as indicative of transcriptional activation (Zheng et al., 2013). Although histone modifications play crucial roles in the transcriptional regulation, the relationship between the differentiation of specific cell types and histone modifications is not completely understood. Therefore, it is of great interest and importance to investigate the effects of chromatin modifications by individual HDACs on the osteogenic differentiation of BMSCs, which may advance our understanding of stem cell fate decision and facilitate the clinical translation of BMSCs-mediated bone repair.

The HDACs are grouped into four subclasses based on structural and functional similarities (Hewitson et al., 2013). A line of evidence has indicated the inhibitory roles of HDAC1–7 in osteoblast differentiation by regulating histone acetylation or interacting with RUNX2, a master transcription factor for osteoblast differentiation (Jensen et al., 2007, 2009;Lee et al., 2006; Li et al., 2009; Schroeder, 2004; Shimizu et al., 2010; Westendorf et al., 2002). HDAC inhibitors (HDIs), such as valproic acid (VPA) and sodium butyrate, are often utilized to promote osteoblast differentiation and maturation (Cho et al., 2005; Iwami and Moriyama, 1993; Lee et al., 2006). HDAC8 is a unique member of class I HDACs, as it exhibits a special crystal structure (Gantt et al., 2010) and lacks the conserved C-terminal domain (Somoza et al., 2004), suggesting a distinct and specific biological function for HDAC8 in various pathophysiological processes. Interestingly, knockdown of HDAC8 in cultured neuroblastoma cells has no effect on histone acetylation (Oehme et al., 2009), whereas the class I HDACs 1–3 are potent regulators of histone acetylation in osteoblasts. Moreover, HDAC8 specifically controls craniofacial skeletal patterning by repressing a subset of transcription factors in cranial neural crest cells upon conditional HDAC8 deletion, whereby HDAC8 is shown to regulate these genes at the chromatin level (Haberland et al., 2009). Therefore, an interesting question arises whether HDAC8 has some uncharacterized key roles during osteogenic differentiation of BMSCs.

In this study, we aimed to investigate the biological roles of HDAC8 on the osteogenesis of BMSCs and the associated regulatory mechanisms. Our findings indicated that HDAC8 functioned as a transcriptional repressor via regulating the level of H3K9Ac and interacting with RUNX2 during the specification of BMSCs to the osteoblasts lineage in vitro, suggesting that therapeutic inhibition of HDAC8 might be beneficial for bone repair and regeneration.

2. Materials and methods

2.1. Cell culture and differentiation

All experiments and animal protocols were performed with the approval of the Ethics Committee and Animal Care Committee of Nanjing Medical University. Primary BMSCs were obtained from the tibia bone marrow of three-week-old male Sprague Dawley rats as we described before (Zhang et al., 2012), and used between passages 3 and 5. To induce osteogenic differentiation of BMSCs in vitro, cells were cultured in osteogenic medium consisting of DMEM supplemented with 50 μM ascorbic acid (Sigma, St. Louis, MO, USA), 10 mM β-glycerophosphate (Sigma) and 10^{-7} M dexamethasone (Sigma).

2.2. Lentiviral vector construction and transfection

The molecular cloning technique was applied to construct the recombinant lentiviral expression vector pGC-FU-HDAC8-GFP (LeV-HDAC8). The recombinant rat HDAC8 expression plasmid was transfected into 293 T cells with pHelper 1.0 and pHelper 2.0. After 48 h, the viral supernatant was collected and filtered. BMSCs were transfected with LeVs carrying GFP (LeV-GFP) and overexpressing HDAC8 (LeV-HDAC8) at a multiplicity of infection (MOI) of 3 pfu/cell. The transfection efficiency was determined via phase contrast microscopy. At 48 h after transfection, cells were then cultured with DMEM containing 10% FBS in the presence of 1 mg/ml G418 for 7 days. Stable clones of cells were selected and used in the following experiments.

2.3. RNA interference

Small interfering RNA (siRNA) targeting HDAC8 and scrambled siRNA were purchased from RiboBio Co. Ltd. (Guangzhou, China). Three sequences of siRNAs targeting rat HDAC8 were listed as follows: siRNA1, 5′-GGACGGUACUACAGUGUCA dTdT-3′,3′-dTdT CCUGCCAUGAUGUCACAGU-5′; siRNA2,5′-GCAAGUGUCUGAAGUAUGU dTdT-3′,3′-dTdT CGUUCACAGACUUCAUACA-5′; siRNA3,5′-CAUCUAAAGUUAUGACUGU dTdT-3′,3′-dTdT GUAGAUUUCAAUACUGACA-5′. All of these siRNA duplexes (final concentration 50 nM) were transfected into BMSCs using Lipofectamine 2000 (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Cells were harvested 48 h later, and the knockdown efficiency was further determined via real-time PCR and Western blot analyses.

2.4. Protein extraction and immunoblotting

Total protein lysates were obtained from harvested cells in protein RIPA lysis buffer (Beyotime, Shanghai, China) containing 10 mM phenylmethylsulphonyl fluoride as a protease inhibitor (PMSF; Beyotime) on ice for 30 min. Extraction of nucleoproteins was performed following the manufacturer's instruction (KGP150; Nanjing KeyGen Biotech, Nanjing, China). The concentrations of both total proteins and nucleoproteins were measured via Coomassie blue staining. The total proteins and nucleoproteins were separated on 10% SDS-PAGE gels and transferred to 0.45 μm Immobilon-P Transfer Membranes (Millipore Corporation, Billerica, MA, USA). The membranes were blocked in 5% skim milk dissolved in Tris-buffered saline containing Tween and then immunoblotted with primary antibodies, as indicated in Supplemental Table 1.

2.5. Alkaline phosphatase (ALP) activity assay and Alizarin Red staining

ALP activity assay was performed as we described before (Zhang et al., 2012). Briefly, BMSCs transfected with LeV-HDAC8 or LeV-GFP, and non-transfected BMSCs were plated in 96-well plates at a density of 1×10^3 cells/well and cultured in osteogenic medium for 9 days. ALP activity was determined using an ALP activity assay kit (Biosino, Beijing, China). The mineralization potential of the cells was assessed via Alizarin Red staining when cells were cultured with osteogenic medium for 14 days.

2.6. Immunofluorescence

Immunocytochemical analyses of HDAC8 and H3K9Ac were performed using a standard protocol. After cultured in osteogenic medium for 3, 7 or 14 days, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 (Sigma) for 15 min, followed by incubation at 4 °C overnight with primary antibodies against HDAC8 (Ab39664; Abcam, Cambridge, MA, USA) (1:50) and H3K9Ac (#9649; Cell Signaling Technology, Danvers, MA, USA) (1:200). Then, cells were further incubated with anti-mouse FITC tagged secondary antibody (Bioworld; Louis Park, MN, USA) (1:50) for 30 min at 37 °C. Nuclei were stained with DAPI (Beyotime). Immunofluorescent images were captured using a fluorescence microscope (Leica Microsystems, Mannheim, Germany).

2.7. Cell viability assay

Cell viability was determined via the MTT assay. Briefly, cells were plated at a density of 5×10^3 cells/well in 96-well plates, and various concentrations of VPA (0–3.5 mM) were added on the second day. After 72 h, cells were treated with 5 mg/ml MTT (Sigma) and then incubated for 4 h at 37 °C. The absorption at 490 nm was measured in an automatic enzyme-linked immunosorbent assay reader (ELx 800; BioTek Instruments Inc., Winooski, VT, USA). Each experiment was carried out at least three times.

2.8. Bromodeoxyuridine (BrdU) cell proliferation assay

Cells were plated on coverslips at a density of 1×10^4 cells/ml. After cultured in serum free medium for 24 h, cells were replaced with medium containing 10% FBS. Cells were exposed to 10 μM BrdU for 2 h and fixed with 4% paraformaldehyde for 20 min, and then stained with anti-BrdU antibody (ZSGB-BIO, Beijing, China) overnight according to the manufacturer's instructions. Nuclei were stained with DAPI (Beyotime) (1:1000), and images were obtained in five random chosen fields from three independent samples. Percentage of BrdU positive cells was then calculated, and the results are presented as the mean ± SD.

2.9. Real-time quantitative-PCR analysis

Reverse transcription and real-time PCR were performed using the PrimeScript RT reagent kit and real-time SYBR Premix Ex Taq™ kit (TaKaRa Holdings Inc., Kyoto, Japan). The primers for each genes of interest were synthesized according to the sequences listed in Supplemental Table 2. All values were normalized to β-actin, and the relative quantification of gene expression was performed using the $2^{-\Delta\Delta CT}$ method.

2.10. Immunoprecipitation

The nucleoproteins used for immunoprecipitation were extracted following the manufacturer's instructions (KGP150; Nanjing KeyGen Biotech, Nanjing, China). Cells lysates were incubated with normal rabbit IgG (sc-2027, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), an anti-HDAC8 antibody (ab39664; Abcam) or an anti-RUNX2 antibody (#12556; Cell Signaling Technology) overnight at 4 °C. Immunoprecipitates were collected via incubation with Protein A/G-plus agarose beads (sc-2003; Santa Cruz) for 4 h at 4 °C, followed by washing three times with either RIPA lysis buffer (Beyotime) containing 0.3% BSA (Sigma) or RIPA lysis buffer (Beyotime), boiling in 2× SDS-PAGE loading buffer (Beyotime) and detection via immunoblotting as described.

2.11. Chromatin immunoprecipitation (ChIP)

The ChIP analysis was carried out using EZ-ChIP (Millipore, Billerica, MA, USA). Rabbit IgG was used as a negative control. Briefly, BMSCs of osteogenic induction were cross-linked in 1% formaldehyde for 10 min and lysed in SDS lysis buffer and then sonicated to shear DNA. Lysates after diluted with ChIP dilution buffer were immunoprecipitated with rabbit IgG and anti-H3K9Ac antibody (#9649; Cell Signaling Technology) overnight at 4 °C. Antibody–chromatin complexes were precipitated with ChIP blocked protein G agarose for 1 h at 4 °C and then washed and eluted. After reverse crosslink of protein-DNA complexes, DNA was purified using spin columns and analyzed by real-time PCR. Primer sequences for real-time PCR were listed in Supplemental Table 3.

2.12. Statistical analyses

All data showed here are expressed as the mean ± SD ($n = 3$). Statistical analyses were conducted using Student's t-test or ANOVA unless otherwise stated. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Histone H3K9 is hyperacetylated and associated with reduced expression of HDAC8 in osteogenic differentiation of BMSCs

To investigate the potential roles of HDAC8 in osteogenic differentiation of BMSCs, we initially determined the temporospatial expression pattern of HDAC8 in rat BMSCs cultured with osteogenic medium. The formation of mineralized nodules was readily observed on day 7 and increased significantly on day 14 as assessed by Alizarin Red staining (Fig. 1A). The level of RUNX2 protein increased gradually from day 3 to day 7 and then declined on day 14. At the same time, two representative osteogenic markers OCN and OPN, also downstream targets of Runx2, exhibited increased expression from day 7 to day 14 (Fig. 1B). The expression of HDAC1 and 2 appeared to remain largely unchanged in early stage osteogenic differentiation of BMSCs and decreased significantly on day 14, while HDAC8 abundance was reduced gradually during the osteogenic induction (Fig. 1C). More importantly, as shown in Fig. 1C, simultaneously decreased HDAC8 and increased H3K9Ac occurred, suggesting possible association between these two molecules in osteogenic differentiation of BMSCs. These trends were also evidenced by our immunofluorescence staining data (Fig. 1D and E). Together, these results revealed an increased level of histone H3 (K9) acetylation and decreased HDAC8 expression during the osteogenic differentiation of BMSCs, raising the possibility that epigenetic regulation of HDAC8-mediated H3K9Ac may be implicated in osteogenic differentiation of BMSCs.

3.2. VPA promotes the osteogenic differentiation of BMSCs by inhibiting HDAC8 expression and enhancing histone H3K9 acetylation

Accumulating evidence has indicated that HDAC inhibitors have the capacity to remarkably enhance the osteogenic differentiation of BMSCs, most likely due to HDACs-mediated epigenetic modifications (Gurvich et al., 2004). We further sought to address whether similar changes occurred in HDAC8 and H3K9Ac levels during the VPA-induced osteogenic differentiation of BMSCs. Because VPA caused growth arrest and induced the hyperacetylation of histone H3 and H4 with a low potency (Ki ≈ mM) (Gurvich et al., 2004), we first determined the appropriate concentration of VPA exposure for BMSCs. Our data revealed that cell viability displayed

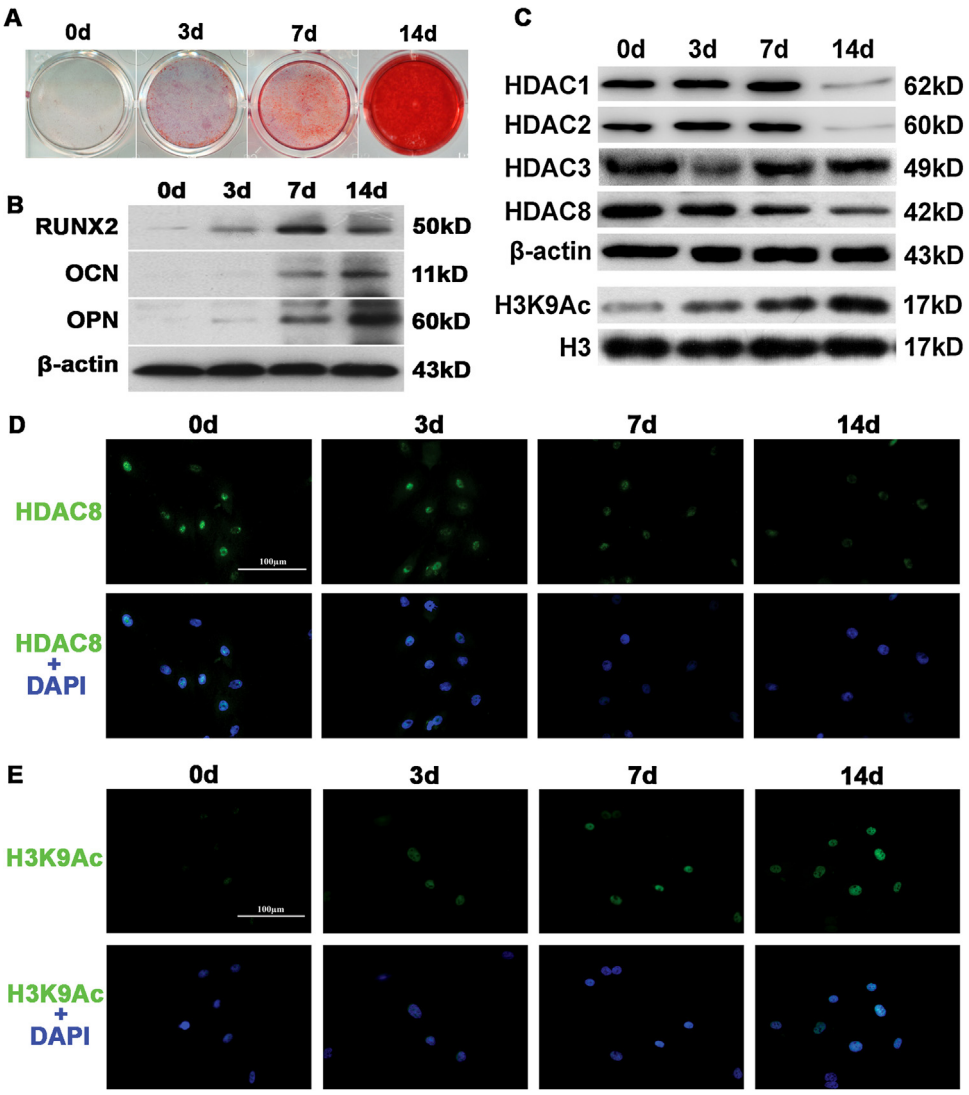

Fig. 1. Expression patterns of HDAC8 and histone acetylation during the osteogenic differentiation of BMSCs. (A) Osteogenic mineralization was assessed via Alizarin Red S staining on days 0, 3, 7 and 14 of culture in osteogenic medium. (B) The expression patterns of RUNX2, OPN and OCN on days 0, 3, 7 and 14 were analyzed by Western blot. (C) The changes in HDAC1, -2, -3, -8 and histone acetylation were analyzed using total proteins and nucleoproteins, respectively, on days 0, 3, 7 and 14 by Western blot (D) and (E) representative figures for HDAC8 and H3K9Ac expression on days 0, 3, 7 and 14 were determined by immunofluorescence staining. Magnification bar is 100 μm.

no significant change when VPA was added between 0.1–1 mM, while gradually declined with increased concentrations (Fig. 2A). Moreover, it was reported that low concentrations of VPA (up to 1 mM) did not reduce cell viability significantly (Paino et al., 2014; Xu et al., 2009). Thus, we then chose 1 mM VPA in the following experiments unless otherwise specified. As shown in Fig. 2B, different BMSCs proliferation was observed between the VPA-treated group and control group in BrdU assay, indicating that VPA can inhibit cell proliferation of BMSCs during a relative long treatment course.

To assess the effects of VPA on HDAC8 expression, the levels of HDAC8 protein of untreated BMSCs and BMSCs treated with 1 mM VPA for 0.5, 1, 12 or 24 h were measured using Western blot analysis. The results revealed that HDAC8 was gradually reduced while H3K9Ac was concomitantly increased upon VPA addition for 24 h (Fig. 2C). To assess the effects of VPA on the osteogenic differentiation of BMSCs, we found that the VPA-treated BMSCs displayed stronger retention of Alizarin Red S than vehicle-treated BMSCs after cells were cultured in osteogenic medium for 2 weeks. This result suggested that VPA treatment accelerated the osteogenic mineralization of BMSCs (Fig. 2D). Real-time PCR assay further revealed that the mRNA levels of multiple osteogenic markers including Runx2, Osterix, ALP, OPN and OCN were increased by approximately 2.1-, 3.5-, 5.5-, 3.7-, and 4.3-fold, respectively, in VPA-treated BMSCs compared to control (Fig. 2E). Similar results were also found regarding protein expression levels as assessed by Western blot analysis (Fig. 2F). Collectively, these results showed that VPA had capacity to promote osteogenic differentiation of BMSCs via inhibiting HDAC8 expression and enhancing H3K9Ac level in BMSCs.

3.3. *HDAC8 knockdown promotes the osteogenic differentiation of BMSCs*

To further verify the functions of HDAC8 in the osteogenic differentiation of BMSCs, the osteogenic differentiation of BMSCs followed HDAC8 knockdown using small interfering RNA (siRNA) was determined. The knockdown efficiency was confirmed by the fact that HDAC8 siRNA treatment induced significant reduction of HDAC8 mRNA by approximately 2.4-fold as compared to scramble siRNA (Fig. 3A). The level of HDAC8 protein decreased significantly, with the HDAC1–3 remained largely unaltered (Fig. 3B).

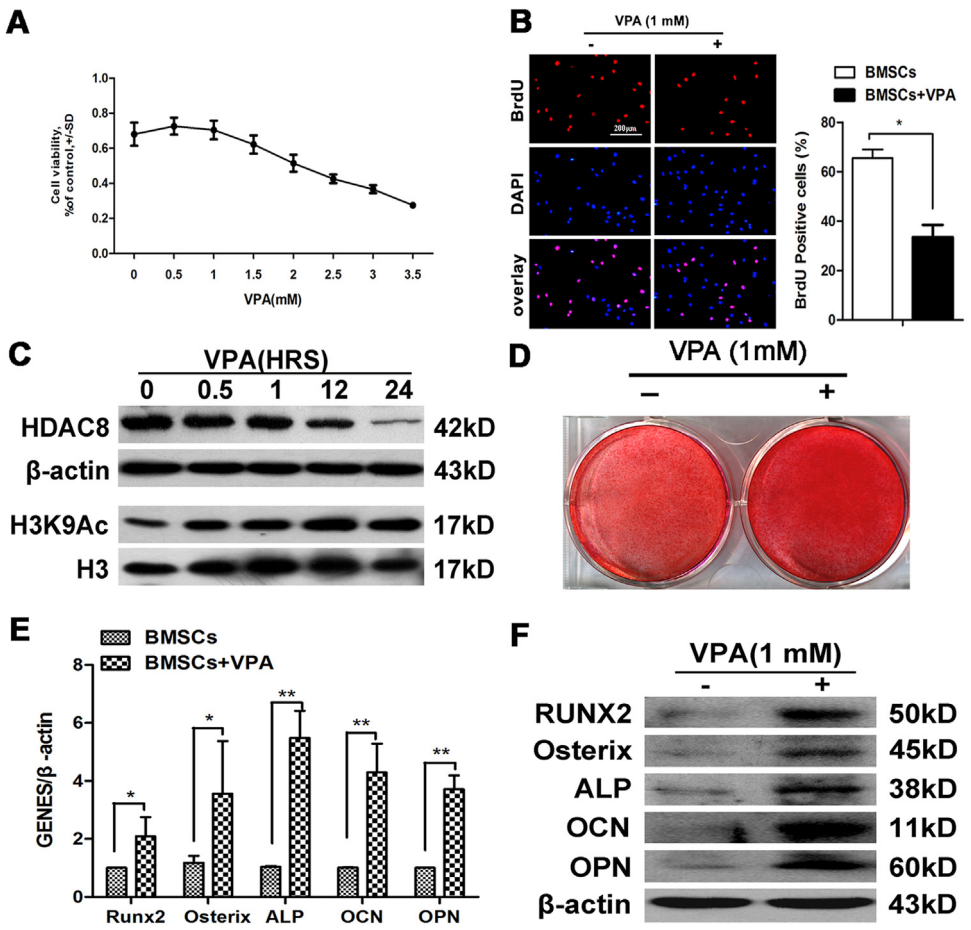

Fig. 2. VPA promotes the osteogenic differentiation of BMSCs. (A) Cell viability was analyzed following the addition of VPA at concentrations ranging from 0 to 3.5 mM in MTT assay. (B) The difference in proliferation between BMSCs cultured in DMEM versus DMEM with 1 mM VPA was analyzed in BrdU assay. Left was representative micrographs and right was quantification of BrdU incorporation by the indicated cells. (C) The changes in HDAC8 and H3K9Ac expression at 0, 0.5, 1, 12 and 24 h after the addition of 1 mM VPA were analyzed by Western blot. (D) The difference between the osteogenic mineralization of BMSCs cultured in osteogenic medium and in osteogenic medium with 1 mM VPA was assessed by Alizarin Red S staining after 14 days. (E) The patterns of Runx2, Osterix, ALP, OPN and OCN mRNA expression in BMSCs cultured in osteogenic medium (with or without 1 mM VPA) for 7 days were determined by real-time PCR. (F) The patterns of RUNX2, Osterix, ALP, OPN and OCN expression at the protein level were analyzed in BMSCs cultured in osteogenic medium for 7 days via Western blot. $^{*}p < 0.05$; $^{**}p < 0.01$. Magnification bar is 200 μm.

As expected, the level of acetylated histone H3K9 was significantly increased following HDAC8 depletion, compared to the other lysine sites of histones (Fig. 3C). Furthermore, HDAC8 silence significantly enhanced the osteogenesis-related genes expression of BMSCs. The mRNA levels of Runx2, Osterix, ALP, OPN and OCN in the HDAC8 siRNA group were increased by approximately 1.5-, 3-, 3.4-, 29.3- and 7.5-fold, respectively, compared to the scramble group (Fig. 3D). Similar results were shown in Fig. 3E, as determined by Western blot analysis. Collectively, these results clearly revealed that HDAC8 knockdown promoted the osteogenic differentiation of BMSCs, suggesting a negative role of HDAC8 in the osteogenic differentiation of BMSCs.

3.4. HDAC8 overexpression inhibits histone H3K9 acetylation in BMSCs

To further confirm whether HDAC8 was associated with histone H3K9 acetylation in BMSCs, the BMSCs with stable ectopic HDAC8 expression were generated using a recombinant lentiviral expression vector (Fig. 4A). Compared to the LeV-GFP group, the mRNA and protein level of HDAC8 increased markedly in the LeV-HDAC8 group (Fig. 4C and D). The result of BrdU cell proliferation assay displayed no pronounced difference between the LeV-HDAC8 group and the LeV-GFP group (Fig. 4B). Importantly, the decreased level of histone H3K9 acetylation was the most significant, compared to the change of other histone (including H3 and H4) lysine sites acetylation (Fig. 4D). Combined with the above results regarding HDAC8 knockdown, our data indicated that HDAC8 directly regulated the acetylation of histone H3K9 in BMSCs.

3.5. HDAC8 overexpression inhibits the osteogenic differentiation of BMSCs

We next characterized the effects of HDAC8 overexpression on the osteogenic differentiation of BMSCs. The BMSCs transfected with LeV-HDAC8 exhibited much lower retention of Alizarin Red S compared to the non-transfected BMSCs and the LeV-GFP group after osteogenic induction for 14 days (Fig. 5A). The LeV-HDAC8 group obviously showed a lower amount of calcium nodules compared to the non-transfected BMSCs and the LeV-GFP group (Fig. 5B). Moreover, the ALP activity in the LeV-HDAC8 group was significantly lower than those in the non-transfected BMSC and LeV-GFP group from day 5 to day 9 (Fig. 5C). Real-time PCR and Western blot data revealed that overexpression of HDAC8 significantly inhibited the expression of Runx2, Osterix, ALP, OCN and OPN (Fig. 5D and E). Together, these results showed that HDAC8 overexpression inhibited the osteogenic differentiation of BMSCs.

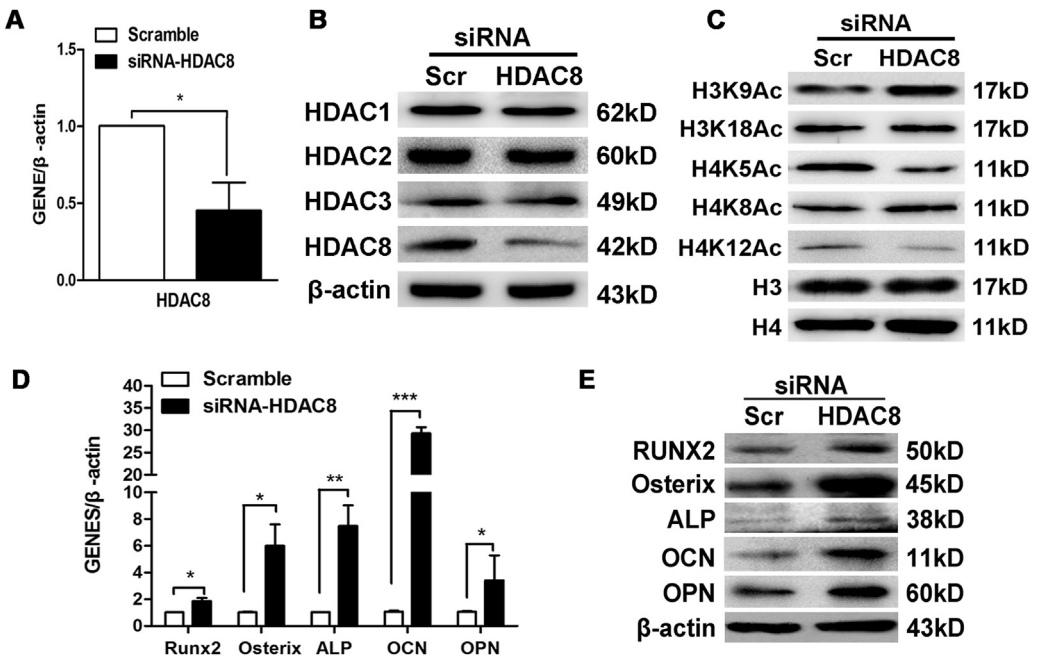

Fig. 3. Effects of HDAC8 knockdown on the osteogenic differentiation of BMSCs. (A) The interference efficiency was determined at the mRNA level via real-time PCR. (B) The protein expression levels of HDAC1, -2, -3, -8 were analyzed at the protein level by Western blot. (C) The changes in HDAC8, H3K9Ac, H3K18Ac, H4K5Ac, H4K8Ac and H4K12Ac were analyzed at the protein level by Western blot. (D) The patterns of Runx2, Osterix, ALP, OPN and OCN mRNA expression following culture with scramble siRNA or siRNA-HDAC8 in osteogenic medium for 7 days were determined by real-time PCR. (E) The patterns of RUNX2, Osterix, ALP, OPN and OCN expression following culture of BMSCs with scramble siRNA or siRNA-HDAC8 in osteogenic medium for 7 days were analyzed by Western blot. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.00$.

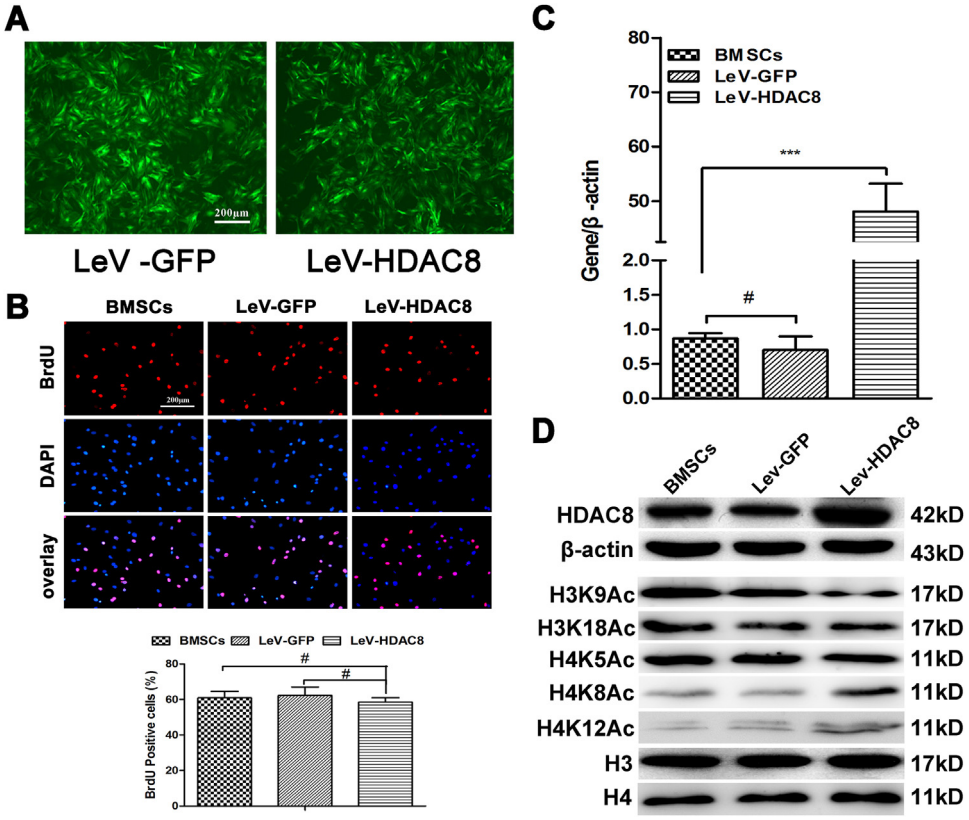

Fig. 4. Enforced HDAC8 overexpression inhibits histone H3K9 acetylation in BMSCs. (A) BMSCs were infected with a lentiviral vector containing an exogenous HDAC8 gene or the green fluorescent protein (GFP) gene. The transfection efficiency was determined using an inverted fluorescence microscope. (B) The differences in cell proliferation between non-transfected BMSCs and LeV-HDAC8- or LeV-GFP-transfected BMSCs were analyzed by BrdU assay. The upper image was representative micrographs and the lower image was quantification of BrdU incorporation by the indicated cells. (C) The efficiency of transfection was analyzed at the HDAC8 mRNA level by real-time PCR. (D) The changes in HDAC8, H3K9Ac, H3K18Ac, H4K5Ac, H4K8Ac and H4K12Ac were analyzed at the protein level by Western blot. $^{***}p < 0.00$; $^{\#}p > 0.05$. Magnification bar is 200 μm.

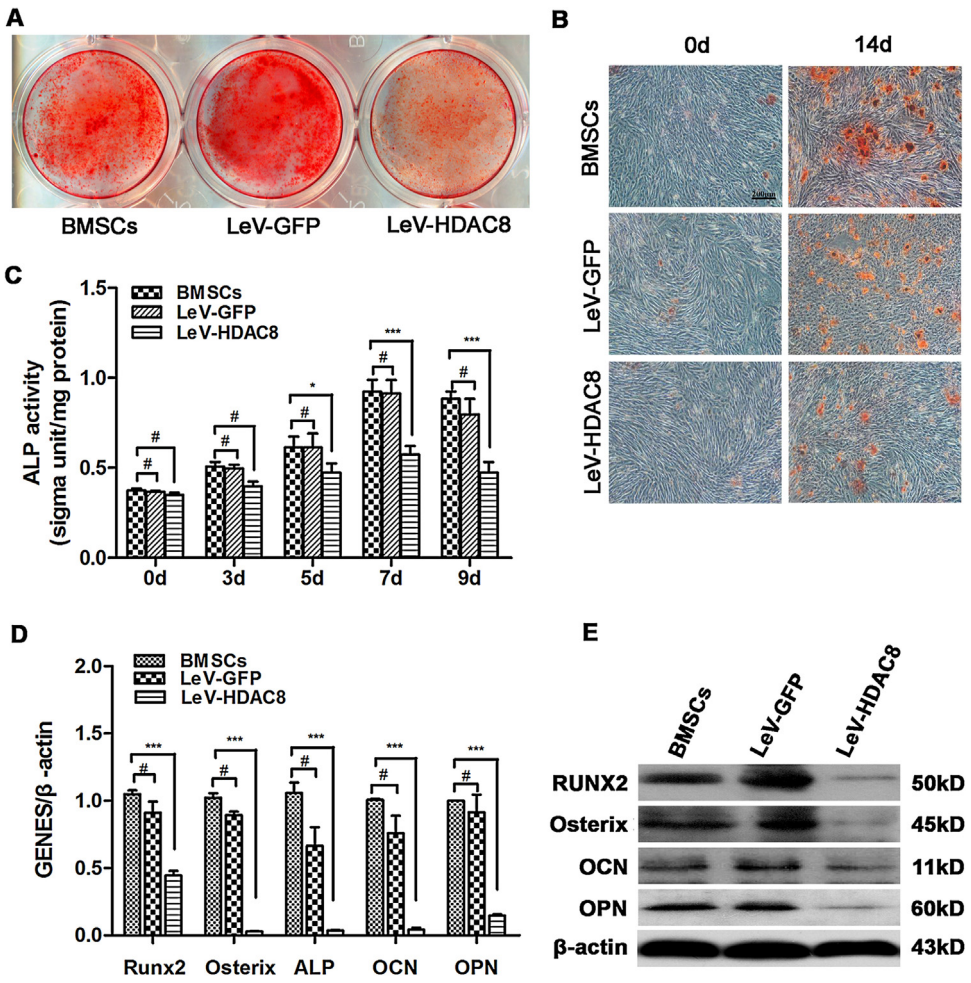

Fig. 5. Effects of enforced HDAC8 overexpression on the osteogenic differentiation of BMSCs. (A) The osteogenic mineralization of non-transfected BMSCs and LeV-HDAC8- or LeV-GFP-transfected BMSCs was measured by Alizarin Red S staining after being cultured in osteogenic medium for 14 days. (B) Calcium nodes were observed under an inverted phase contrast microscope. (C) ALP activity was measured on days 0, 3, 5, 7 and 9 of cultured in osteogenic medium. (D) The mRNA levels of Runx2, Osterix, ALP, OPN and OCN in the three groups of BMSCs cultured in osteogenic medium for 7 days were analyzed by real-time PCR. (E) The protein levels of RUNX2, Osterix, ALP, OPN and OCN in the three groups of BMSCs cultured in osteogenic medium for 7 days were analyzed by Western blot. $^{*}p < 0.05$; $^{***}p < 0.00$; $^{\#}p > 0.05$. Magnification bar is 200 μm.

3.6. VPA inhibits the effect of HDAC8 overexpression on the osteogenic differentiation of BMSCs

To explore whether the inhibitory effect of HDAC8 overexpression on the osteogenic differentiation of BMSCs could be relieved by HDIs, HDAC8-overexpressed BMSCs cultured in the presence or absence of 1 mM VPA were compared. The results revealed that VPA treatment significantly increased the expression of Runx2, Osterix, ALP, OCN and OPN in HDAC8-overexpressed BMSCs compared to the group without VPA addition after 7 days of osteogenic induction (Fig. 6A and B). After 14 days of osteogenic induction, the HDAC8-overexpressed BMSCs treated with VPA exhibited higher retention

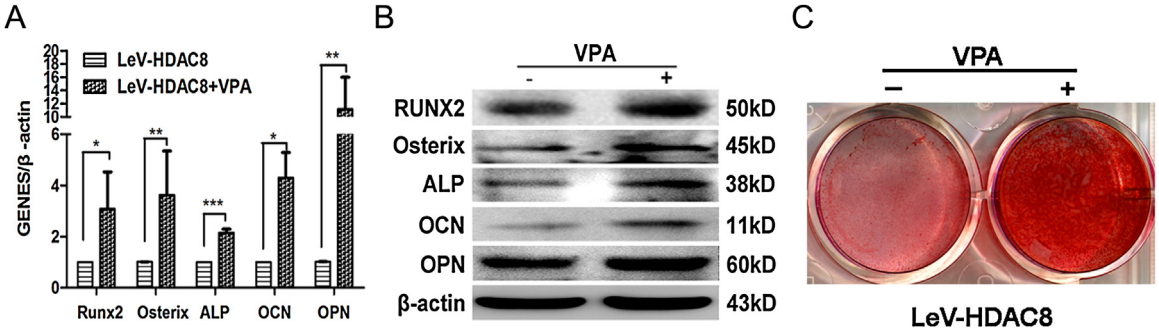

Fig. 6. VPA attenuates the inhibitory effect of HDAC8 overexpression on the osteogenic differentiation of BMSCs. (A) The mRNA levels of Runx2, Osterix, ALP, OPN and OCN in the LeV-HDAC8 and LeV-GFP group treated with 1 mM VPA were analyzed by real-time PCR after BMSCs cultured in osteogenic medium for 7 days. (B) The protein levels of RUNX2, Osterix, ALP, OPN and OCN in these cells were analyzed by Western blot. (C) The osteogenic mineralization of two groups of BMSCs was measured by Alizarin Red S staining after BMSCs cultured in osteogenic medium for 14 days. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.00$.

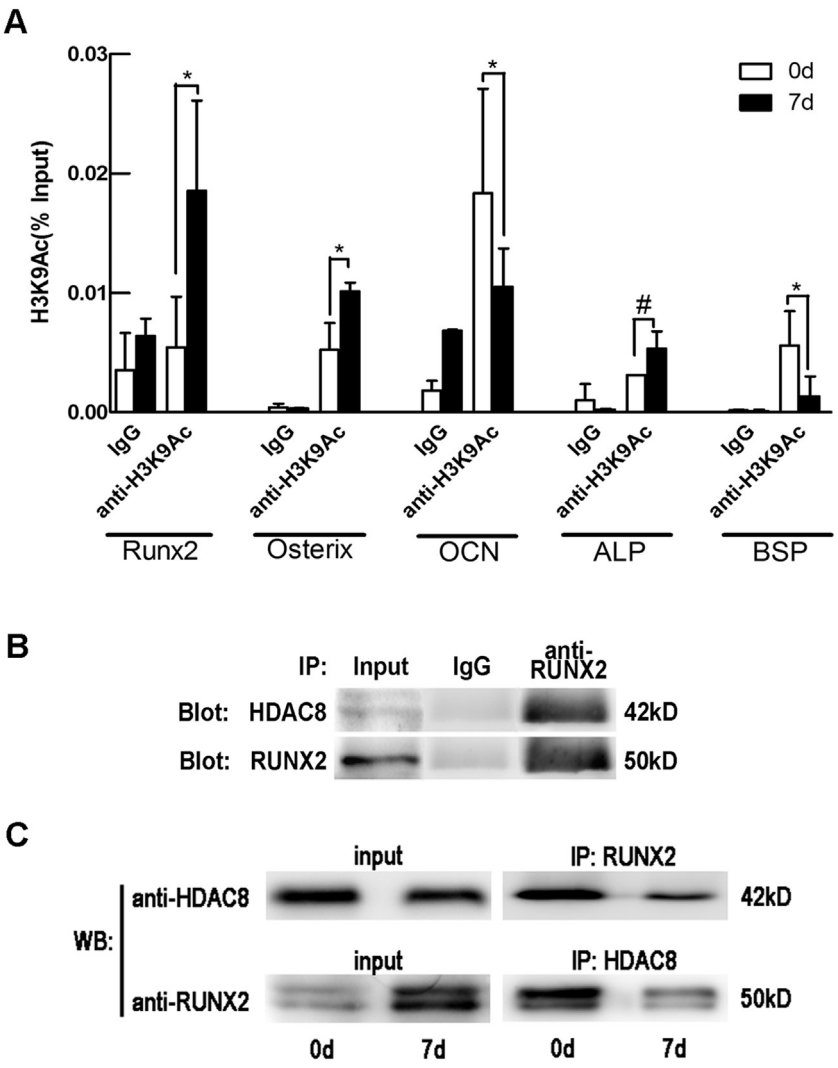

Fig. 7. HDAC8 suppresses osteogenic differentiation of BMSCs by removing H3K9Ac and inhibiting transcriptional activity of RUNX2. (A) The level of H3K9Ac in the promoter regions of Runx2, Osterix, ALP, OCN and BSP was analyzed in BMSCs and BMSCs with osteogenic induction for 7 days by ChIP assay. $^{*}p < 0.05$, $^{\#}p > 0.05$. (B) HDAC8 immunoprecipitated with RUNX2 and the protein level of HDAC8 and RUNX2 were analyzed by Western blot. The nuclear proteins were extracted from BMSCs with osteogenic induction for 7 days. (C) The level of HDAC8 and RUNX2 in the immunoprecipitation was determined respectively with anti-HDAC8 antibody and anti-RUNX2 antibody by Western blot analysis. Immunoprecipitation assay was performed with BMSCs and BMSCs with osteogenic induction for 7 days.

of Alizarin Red S compared to the group without VPA addition (Fig. 6C). These results indicated that the inhibitory effect of HDAC8 overexpression on the osteogenic differentiation of BMSCs could be rescued by HDIs treatment.

3.7. *HDAC8 suppresses the osteogenic differentiation of BMSCs by removing H3K9Ac and inhibiting the transcriptional activity of Runx2*

It is well established that acetylation of histone H3 lysine 9 correlates with gene activation. Our results showed that the epigenetic regulation of HDAC8-mediated histone H3K9 acetylation was associated with the osteogenic differentiation of BMSCs (Figs. 1–6). To further uncover the mechanism of HDAC8 underlying osteogenic differentiation of BMSCs, we investigated whether the acetylation of histone H3K9 was associated with active transcription of osteogenesis-related genes through ChIP analysis. Although the level of histone H3K9 acetylation in promoter regions of OCN and BSP decreased and no significant change in ALP promoter region, the enhanced acetylation of histone H3K9 at the promoter regions of Runx2 and Osterix genes, the master transcription factors for osteoblast differentiation, were detected during osteogenic differentiation of BMSCs (Fig. 7A).

Previous studies have suggested that HDAC1–3 physically interact with RUNX2 and suppress its transcriptional activity during osteoblast differentiation (Lee et al., 2006; Schroeder et al., 2004). So, we determined whether such interaction also existed in HDAC8 and RUNX2. Our results showed that both HDAC8 and RUNX2 were present in the anti-RUNX2 or anti-HDAC8 nuclear immunoprecipitate (Fig. 7B and C), implying that endogenous HDAC8 and RUNX2 physically interact as a transcriptional regulatory complex in the nucleus. To investigate the binding change between HDAC8 and RUNX2 during the osteogenic differentiation of BMSCs, we further immunoprecipitated nuclear extracts of undifferentiated and differentiated BMSCs. As shown in Fig. 7C, our data revealed that the level of HDAC8 protein was down-regulated in the anti-RUNX2 immunoprecipitate during osteogenic differentiation of BMSCs. Additionally, the RUNX2 protein level in differentiated BMSCs was lower than those in undifferentiated BMSCs in the anti-HDAC8 immunoprecipitate. This finding implied that the binding between HDAC8 and RUNX2 weakened during the osteogenic differentiation of BMSCs, and this change derepressed transcriptional activity

of RUNX2 to facilitate its regulation on downstream osteogenesis-related genes.

4. Discussion

The fate decision and lineage differentiation of BMSCs is a complex biological process orchestrated by multiple layers of regulation including epigenetic, transcriptional and post-transcriptional ways. It is becoming increasingly clear that the chromatin modifications play crucial roles during these processes (Teven et al., 2011). Among them, histone acetylation is a widely studied type of chromatin modifications and correlates with transcriptional activation, and histone deacetylases (HDACs) are responsible for the level of histone acetylation (Gurvich et al., 2004). For example, acetylation of histone H3K9 is a common chromatin mark identified at genes that are actively transcribed (Collas et al., 2008). In the present study, we found that the level of H3K9Ac increased with HDAC8 redcution during the osteogenic differentiation of BMSCs. Furthermore, HDAC8 was negatively associated with the acetylation level of histone H3K9 in BMSCs differentiation. Together, our findings highlighted that HDAC8-mediated chromatin modification played an important role in the osteogenic differentiation of BMSCs.

Accumulating evidence has showed that several HDACs contribute to normal skeletal development and bone formation as evidenced that HDAC2-mutant mice and HDAC3 knockout mice exhibit severe bone defects such as reduced body size resulting from impaired endochondral bone formation (Razidlo et al., 2010; Zimmermann et al., 2007). Moreover, conditional deletion of HDAC8 hinders intramembranous ossification through upregulating homeobox transcription factors such as Lhx1 and Otx2, which are normally repressed in cranial neural crest cells in vivo (Haberland et al., 2009). These in vivo results clearly show that class I HDACs are required for proper bone development pattern by regulating relevant targets. Many in vitro studies have revealed that HDAC1–7 play inhibitory roles in osteoblast differentiation and maturation (Jensen et al., 2007, 2009; Lee et al., 2006; Li et al., 2009; Schroeder, 2004; Shimizu et al., 2010; Westendorf et al., 2002). However, little is known about the role of HDAC8 in osteoblast differentiation in vitro. Here, we provide evidence that HDAC8 has an inhibitory effect on osteogenic differentiation of BMSCs. First, HDAC8 expression decreased gradually during the osteogenic process of BMSCs. Second, osteogenic differentiation and mineralization of BMSCs were enhanced when HDAC8 was pharmacological inhibited by VPA. Third, HDAC8 overexpression significantly enhanced osteogenesis, whereas HDAC8 depletion by siRNA induced the opposite effects in BMSCs. The acetylation of histone H3K9 was reduced in BMSCs associated with HDAC8 overexpression while increased in HDAC8-knockdown BMSCs. Moreover, we further verified the inhibitory effect of exogenous HDAC8 on the osteogenic differentiation of BMSCs can be reversed by VPA.

HDACs regulate transcriptional activity through epigenetic mechanisms mainly in two ways. The one is that HDACs limit the binding of transcription factors and DNA by removing the acetyl groups from histones. The other is they deacetylate non-histone proteins, such as RUNX2 (Jeon et al., 2006; McGee-Lawrence and Westendorf, 2011). In the present study, we not only revealed that exogenous HDAC8 was able to regulate deacetylaion of histone H3K9 but also demonstrated that histone H3K9 was acetylated at the promoter regions of osteogenic genes (*i.e. Runx2* and *Osterix*) by ChIP assay. These results implied that HDAC8 suppressed the expression of several master osteogenic transcription factors via histone H3K9 acetylation. Several studies have showed that H3K9Ac is enriched at gene promoters and is highly correlated with gene expression, as well as with other histone marks (such as H3K9me3), suggesting a coordinated regulation of active histone marks (Hezroni et al., 2011; Park et al., 2011; Sridharan et al., 2013; Zheng et al., 2013). Therefore, further work is needed to verify whether the deacytylation action of HDAC8 has substrate specificity of histone lysine residue. Intriguingly, RUNX2 interacts with numerous transcriptional co-repressors including HDAC3 and HDAC6 to modulate downstream targets under diverse circumstances (Schroeder et al., 2004; Vega et al., 2004; Westendorf et al., 2002). Consistent with this notion, our study showed that HDAC8 interacted with RUNX2 to regulate osteogenic differentiation of BMSCs as a physical nuclear interaction between HDAC8 and RUNX2 was detected. Together, these data indicate that HDAC8 is an inhibitory regulator of osteogenic gene expression during osteogenic differentiation of BMSCs.

HDACs have recently emerged as important therapeutic targets for cancer and epilepsy in the clinic (Glaser et al., 2003; Gurvich et al., 2004; Hezroni et al., 2011). Recent studies also suggested the promising use of HDAC inhibitors to promote bone regeneration (McGee-Lawrence and Westendorf, 2011; Xu et al., 2009). Our data indicated that VPA and knockdown HDAC8 clearly enhanced osteogenic differentiation of BMSCs, thus suggested that epigenetic modification by chemical-like VPA might have beneficial effects for bone repair and regeneration. However, given the functions of HDACs and the pleiotropic effects of pan-HDAC inhibitors (McGee-Lawrence et al., 2011), clinical use of HDIs should be approached with caution.

In summary, the present study indicates that HDAC8 plays an inhibitory role in the osteogenic differentiation of BMSCs by regulating histone acetylation and repressing Runx2 activity. The collaborative function of HDAC isoforms in osteogenic differentiation of BMSCs is warranted to be explored in future. Rational design of HDACs selective inhibitors and especially HDAC8-targeting agents will further advance our knowledge of HDAC8 in BMSCs and represent a novel therapeutic strategy in clinical bone repair.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.07.003.

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