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Phorbaketal A stimulates osteoblast differentiation through TAZ mediated Runx2 activation

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ABSTRACT

Osteoporosis arises from an imbalance between osteoblastic bone formation and osteoclastic bone resorption. In this study, we screened molecules from marine natural products that stimulate osteoblast differentiation. We found that phorbaketal A significantly stimulates osteoblast differentiation in mesenchymal cells. Increased interaction of TAZ and Runx2 stimulated phorbaketal A-induced expression of osteoblastic marker genes. The activation of ERK was important for the stimulation of differentiation because an inhibitor of ERK blocked phorbaketal A-induced osteogenic differentiation. Taken together, the results showed that phorbaketal A stimulates TAZ-mediated osteoblast differentiation through the activation of ERK.

Structured summary of protein interactions:
TAZ physically interacts with **RUNX2** by pull down (View interaction)

1. Introduction

Osteoporosis is a common skeletal disease that results in decreased bone mass and has serious consequences, such as the promotion of bone fracture and disability. Bone mass in adults is maintained by the balance between osteoclastic bone resorption and osteoblastic bone formation. This bone homeostasis is dynamic, and the current drug development for osteoporosis has primarily focused on identifying molecules that can regulate the process by increasing osteoblastic activity or decreasing osteoclastic activity [1,2].

Osteoblast differentiation is a key event in bone formation, and it is known that cell type-specific gene regulation plays an important role in the differentiation process [3,4]. Runx2 is a critical transcription factor that regulates the target genes involved in osteoblast differentiation [5,6]. Runx2 is a member of runt, a pair-rule gene of the Drosophila family of transcription factors, and controls the expression of osteoblast-specific genes, such as osteocalcin. Deletion of the Runx2 gene in mice leads to a phenotype that completely lacks ossification, suggesting that Runx2 is an essential factor for osteoblast differentiation [5,6]. Several studies have shown that Runx2 can interact with many other transcription factors and co-regulators that enhance or inhibit Runx2 function. Among these co-regulators, HDAC3, HDAC4, TLE and YAP inhibit the transcriptional activity of Runx2, but Grg5, Rb and TAZ activate Runx2 (for a review, see [7]).

TAZ (Transcriptional coactivator with PDZ-binding motif) is a 14-3-3-binding protein that regulates cell differentiation, proliferation and development. For its function, TAZ interacts with several transcription factors, including Runx2, PPARγ, TEADs, TTF-1/Nkx2.1, Tbx5, Pax3 and Smad2/3-4 complexes and MyoD [8–16]. The nuclear localization of TAZ and its interaction with these transcription factors regulates the transcription of their target genes. Certain signaling pathways, including the Hippo and TGFβ pathways, regulate the localization and activity of TAZ. The Hippo signaling pathway plays an important role in TAZ-mediated cell proliferation and tumorigenesis [17,18]. TAZ also modulates mesenchymal stem cell differentiation, with the activation of osteoblast and myoblast

Abbreviations: TAZ, transcriptional coactivator with PDZ-binding motif; Runx2, runt-related transcription factor 2; OC, osteocalcin; ALP, alkaline phosphatase; MSCs, mesenchymal stem cells; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase

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differentiation and the inhibition of adipocytes [8,16]. TAZ interacts with Runx2 and stimulates Runx2-mediated gene transcription for the stimulation of osteoblastic differentiation, but the interaction with TAZ and PPARγ inhibits adipocyte differentiation [8].

Here, we report that phorbaketal A [19] stimulates Runx2-mediated gene transcription in osteoblast differentiation. We found that increased TAZ expression and ERK activity facilitate the osteoblast differentiation induced by phorbaketal A.

2. Materials and methods

2.1. Isolation of phorbaketal A

The marine sponge, *Phorbas* sp. (collected from Gageo Island, South Korea), was extracted twice with MeOH at room temperature. This methanolic extract was partitioned between CH_2Cl_2 and H_2O solvents, and the organic layer was then repartitioned between *n*-hexane and 15% aqueous MeOH for the removal of lipids. The MeOH fraction was separated using vacuum column chromatography and eluted with seven different solvent mixtures of MeOH and water. The fraction from the MeOH/H_2O (90/10) solvent contained a large amount of phorbaketal A (Fig. 1A). For the purification of this compound, this fraction was subjected to reversed-phase preparative HPLC and eluting with AcCN/H_2O (65/35).

2.2. Cell culture and osteoblast differentiation

C3H10T1/2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). To induce osteoblast differentiation, the C3H10T1/2 cells were seeded on 24-well culture plates at a density of 2×10^4 cells/cm^2; 48 h later, the culture media were changed with DMEM containing 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and 10% FBS (differentiation media) for 6 days. The differentiation media was replaced every 2 days. Bone marrow-derived human mesenchymal stem cells were purchased from Lonza.

For additional methods, see supplementary materials and methods.

3. Results

3.1. Phorbaketal A is isolated from the marine sponge Phorbas sp.

In our search for compounds inducing osteoblast differentiation, we screened more than 200 extracts of marine organisms with calcium deposition activity. Among them, the extracts from the marine sponge, *Phorbas* sp., showed significantly increased calcium deposition activity, and the activity-guided separation allowed for the isolation of phorbaketal A (Fig. 1A) [19]. Next, to study its dose-dependent activity in osteoblast differentiation, C3H10T1/2 cells were incubated with increasing amounts of phorbaketal A, and the cells were then induced to differentiate into osteoblasts with differentiation media. The calcium deposition and alkaline phosphatase activity were then analyzed for the differentiation analysis. As shown in Fig. 1B and C, a dose-dependent induction of calcium deposition and alkaline phosphatase activity was observed, suggesting that phorbaketal A stimulates osteoblast differentiation in a dose-dependent manner.

3.2. Phorbaketal A stimulates the marker genes of osteoblast differentiation

To analyze the activity of phorbaketal A further, osteoblast marker gene expression was analyzed. C3H10T1/2 cells were incubated with osteoblast differentiation media in the absence or presence of phorbaketal A. After 6 days of differentiation, osteoblastic marker genes, including osteocalcin, Dlx5, and alkaline phosphatase, were

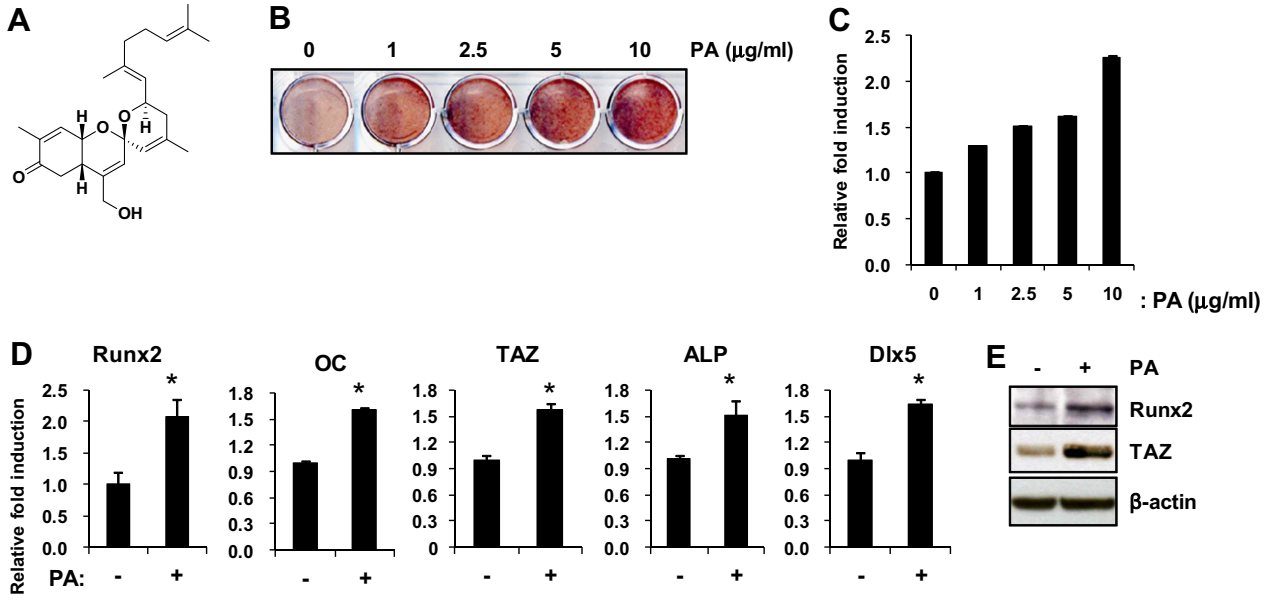

Fig. 1. Phorbaketal A stimulates osteoblast differentiation. (A) Structure of phorbaketal A (5α, 8α-epidioxy-24(*S*)-methylcholesta-6,22-dien-3β-ol). (B) Phorbaketal A (PA) increases calcium deposition in a dose-dependent manner. C3H10T1/2 cells were incubated with osteoblastic differentiation media in the presence of phorbaketal A at the indicated concentration. After 6 days of differentiation, the cells were stained with Alizarin Red S solution. Increased calcium deposition activity was indicated by a dark-red color. (C) Phorbaketal A (PA) increases alkaline phosphatase activity in a dose-dependent manner. Alkaline phosphatase activity in (B) was analyzed. (D) Phorbaketal A stimulates the expression of osteoblastic marker genes. C3H10T1/2 cells were incubated with osteoblastic differentiation media in the presence of 10 μg/ml of phorbaketal A. After 6 days of differentiation, the cells were harvested, and total RNA was obtained. Using quantitative real-time PCR (qRT-PCR), the expression of osteocalcin (OC), alkaline phosphatase (ALP), Dlx5, Runx2, and TAZ were analyzed. Their relative expression was calculated after normalization to the GAPDH level. *indicates a *P* value; * for $P < 0.01$ by the *t*-test. (E) C3H10T1/2 cells were treated with phorbaketal A and induced to differentiate for 6 days. Whole cell extracts were harvested and resolved by SDS–PAGE, and then subjected to immunoblot analysis for Runx2, TAZ, or β-actin.

analyzed by quantitative RT-PCR (qRT-PCR). In these experiments, an increased expression of these markers was observed (Fig. 1D), further indicating that this compound has osteogenic potential. We also observed significant expression of Runx2, a key transcription factor of osteoblast differentiation, and TAZ protein, a transcriptional co-regulator (Fig. 1E), indicating that the increased expression of the proteins is important for phorbaketal A-induced osteogenic differentiation.

3.3. Phorbaketal A stimulates Runx2-mediated gene transcription

Runx2 is a key transcription factor which regulates the promoter of osteoblastic marker genes including osteocalcin. To test whether phorbaketal A induces Runx2-mediated gene transcription, luciferase reporter constructs containing Runx2 binding sites were transfected into 293T cells with the Runx2 expression plasmid, and the reporter activity was analyzed in the presence of phorbaketal A. As shown in Fig. 2A, Runx2 stimulated reporter activity, and the activity was further increased by approximately 4-fold in the presence of phorbaketal A, which suggests that phorbaketal A activates Runx2-mediated gene transcription.

It is known that TAZ physically interacts with Runx2 and activates Runx2-mediated gene transcription [8]. Next, to test the role of TAZ in the phorbaketal A induced luciferase reporter activity, TAZ expression plasmids were introduced into cells with Runx2 expression plasmids and the luciferase reporter plasmids. As shown in Fig. 2B, TAZ stimulated Runx2-mediated reporter gene activity, and the activity also increased by approximately 35% in the presence of phorbaketal A. These results suggest that TAZ mediates phorbaketal A-induced osteogenic potential through the activation of Runx2.

Next, to study whether TAZ induces endogenous osteocalcin gene expression through the Runx2-binding site in the presence of phorbaketal A, chromatin immunoprecipitation analysis was assessed with Flag-tagged TAZ (Flag-TAZ)-overexpressing cells. As shown in Fig. 2C, Flag-TAZ was recruited into the Runx2-binding site of the osteocalcin gene promoter, and a 2.4-fold increase in the recruitment of Flag-TAZ was observed in the presence of phorbaketal A. The results showed that phorbaketal A stimulates osteocalcin gene expression through the recruitment of TAZ into the osteocalcin promoter in differentiating cells.

3.4. Phorbaketal A stimulates the physical interaction between TAZ and Runx2

To understand the mechanism of TAZ-mediated transcriptional activation in the presence of phorbaketal A, we studied the effects of phorbaketal A on the physical interaction between TAZ and Runx2. As shown in Fig. 3A, phorbaketal A significantly enhanced the physical interaction of TAZ with Runx2, indicating that the increased interaction between TAZ and Runx2 stimulates Runx2-mediated osteogenic gene transcription. Notably, we observed that ectopically expressed Runx2 was significantly stabilized in phorbaketal A treated cells (Fig. 3A), indicating the positive effect of phorbketal A in osteogenic differentiation.

In Fig. 2C, increased occupancy of TAZ on the osteocalcin promoter suggested that the localization of TAZ in the nucleus increases in the presence of phorbaketal A. To analyze the cellular distribution of TAZ after phorbaketal A treatment, immunocytochemical analysis was assessed and TAZ localization was revealed by green fluorescence signal. In Fig. 3B, increased fluorescence signal was observed in phorbaketal A treated cells, indicating increased expression of TAZ. We also observed increased nuclear localization of TAZ in phorbaketal A treated cells. The results suggested that phorbaketal A facilitates nuclear localization of TAZ somehow.

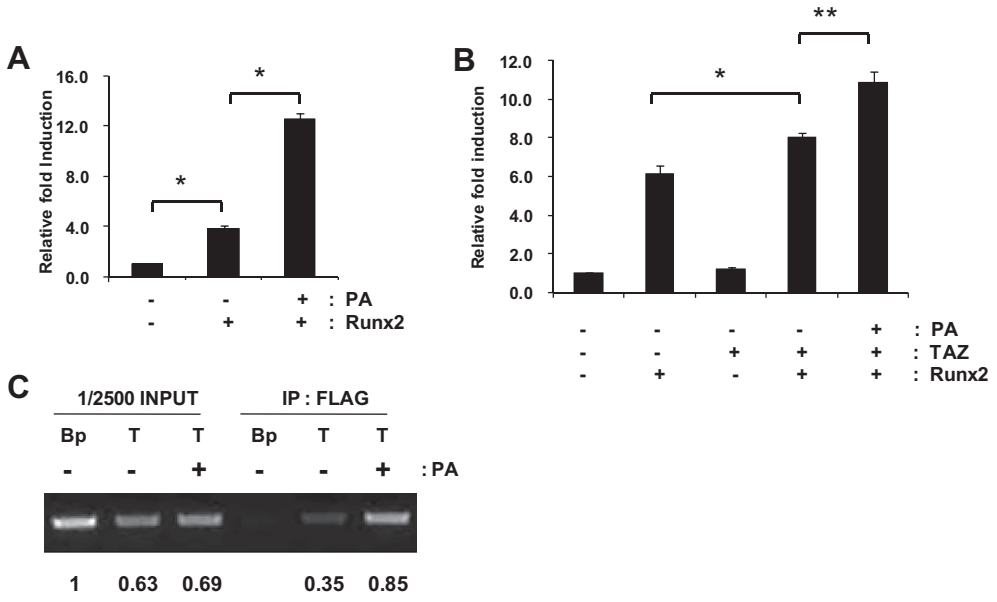

Fig. 2. Phorbaketal A stimulates Runx2-mediated osteoblast differentiation. (A) Stimulation of Runx2-driven gene expression by phorbaketal A (PA). The Runx2 expression plasmid (0.2 μg/well) was transfected into 293T cells with the 6XOSE2-luc reporter construct (0.05 μg/well), which contains six copies of the Runx2-binding site in the osteocalcin promoter. After 24 h of transfection, the cells were incubated with 10 μg/ml of phorbaketal A. After 24 h, cell lysates were prepared for analyzing the luciferase activities. Differences in the transfection efficiency were adjusted by normalizing the *Firefly* luciferase activity to that of *Renilla* luciferase. The luciferase activity was calculated and expressed as fold induction. $^{*}P < 0.01$, *t*-test. (B) 293T cells were transfected with a vector encoding a reporter gene containing the 6XOSE2-luc reporter and Runx2 and TAZ expression vectors, and subsequently treated with the indicated concentrations of phorbaketal A for 24 h. The luciferase activity was normalized to the level of *Renilla* luciferase activity and expressed as the fold induction compared to the mock control. $^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test. (C) Chromatin immunoprecipitation of TAZ with the endogenous osteocalcin promoter in response to phorbaketal A. Stable Flag-tagged TAZ-expressing C3H10T1/2 cells (T) were treated for 4 days with osteogenic differentiation media in the presence of 10 μg/ml phorbaketal A, and the immunoprecipitates of anti-Flag antibodies were analyzed for osteocalcin promoter occupancy by PCR. Bp indicates control cells. The numbers indicate the relative intensities.

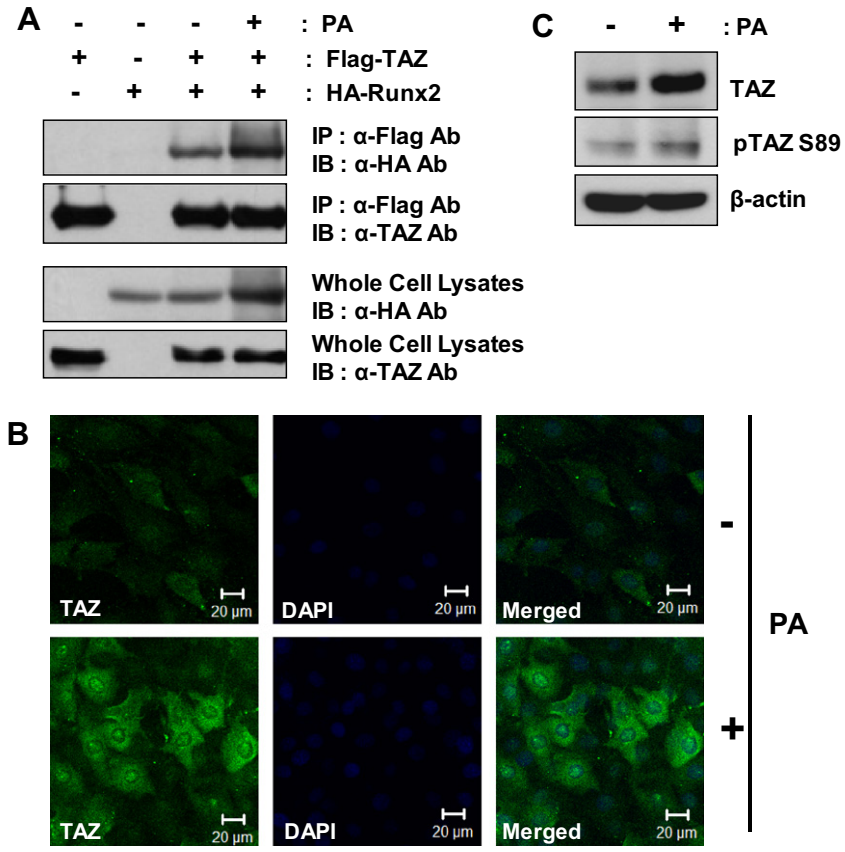

Fig. 3. Phorbaketal A stimulates Runx2 and TAZ interaction and facilitates the recruitment of TAZ onto the osteocalcin promoter. (A) 293T cells were transfected with HA-tagged Runx2 and/or Flag-tagged TAZ expression plasmids and incubated with 10 μg/ml phorbaketal A (PA) for 24 h. Whole cell lysates (WCL) were precipitated with Flag-M2 agarose beads. The precipitates and WCL were analyzed by immunoblot analysis with antibodies against HA and TAZ. (B) Phorbaketal A increases nuclear localization of TAZ. Serum-deprived C3H10T1/2 cells were incubated with 10 μg/ml phorbaketal A. After 24 h, the cells were fixed, and the cellular location of TAZ was analyzed by immunocytochemistry. A FITC-conjugated secondary antibody was used for the green fluorescence signal. DAPI staining indicates the nuclei of the cells. The arrows in merged section indicate the cells that show enriched TAZ expression in the nucleus. (C) C3H10T1/2 cells were treated for 2 days with osteogenic differentiation media in the absence or presence of 10 μg/ml phorbaketal A, cell lysates were prepared, and the phosphorylation status of TAZ at serine 89 was analyzed with a phospho-specific TAZ antibody.

It is known that the phosphorylation status of TAZ is important for nuclear localization. The phosphorylation of TAZ at serine 89 induces 14-3-3 binding and the cytosolic sequestration of TAZ, and dephosphorylated TAZ is not subjected to 14-3-3 binding and can be found in the nucleus [27]. Thus, we studied the phosphorylation status of TAZ at serine 89 using a phospho-specific antibody. In the presence of phorbaketal A, a significantly increased level of TAZ was observed, but the phosphorylation status of TAZ at serine 89 was unchanged (Fig. 3C). Thus, the unphosphorylated form of TAZ at serine 89 significantly increases in the presence of phorbaketal A, suggesting that phorbaketal A increases the level of nuclearly localized TAZ and facilitates the physical interaction of TAZ and Runx2.

3.5. TAZ depletion significantly decreases phorbaketal A-mediated osteogenic stimulation

To study whether TAZ is required in phorbaketal A-mediated osteoblast differentiation, TAZ knockdown C3H10T1/2 cells were generated using TAZ-specific shRNA-producing retroviruses. The control and TAZ knockdown cells were then incubated with osteoblast differentiation media in the presence of phorbaketal A (Fig. 4A). In these experiments, the control cells showed increased TAZ, Runx2, and Dlx5 gene expression in the presence of phorbaketal A; however, the knockdown cells did not show increased expression even in the presence of phorbaketal A (Fig. 4B), indicating that TAZ plays an important role in phorbaketal A-stimulated osteoblast differentiation.

3.6. Introduction of TAZ in TAZ depleted cells recovers phorbaketal A-mediated osteogenic marker genes expression

To further study the role of TAZ in phorbaketal A-mediated osteoblast differentiation, TAZ expression plasmids were introduced into TAZ knockdown C3H10T1/2 cells and recovered TAZ expression in TAZ depleted cells was observed in Fig. 5A. Next, phorbaketal A-mediated osteogenic differentiation activity was analyzed in control, TAZ-depleted, and TAZ-introduced TAZ-depleted cells. In Fig. 5B, the TAZ-introduced TAZ-depleted cells showed increased TAZ, Runx2, and Dlx5 gene expression compare to TAZ-depleted cells or control cells. These results indicate that TAZ is an important factor in phorbaketal A stimulated osteoblast differentiation.

3.7. Phorbaketal A stimulates osteoblast differentiation of human mesenchymal stem cells

To rule out the possibility that the osteogenic potential of phorbaketal A is not a cell type-specific response, we studied whether phorbaketal A stimulates osteoblast differentiation of human mesenchymal stem cells, which are the origin of osteoblasts in adult humans. Human mesenchymal stem cells were incubated with

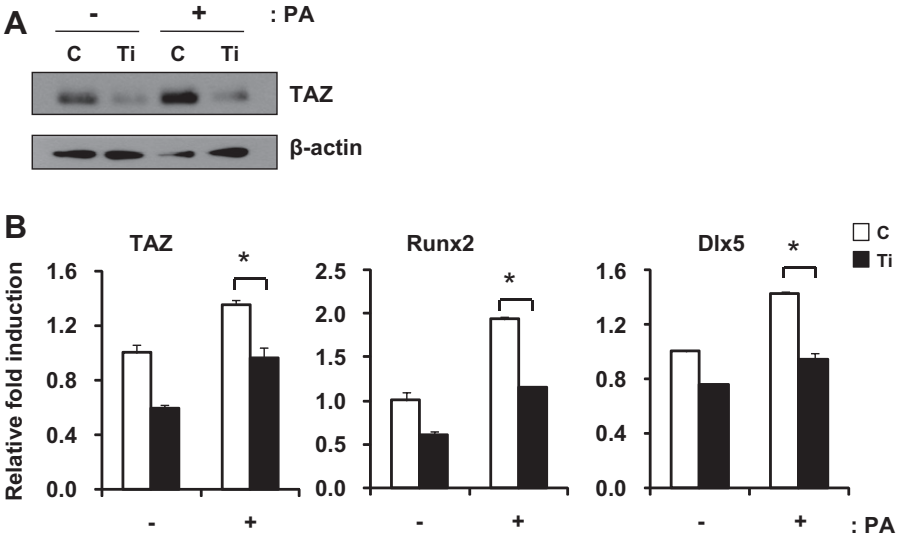

Fig. 4. TAZ depletion inhibits phorbaketal A-mediated osteoblastic marker gene expression. (A) C3H10T1/2 cells were infected with a vector control retrovirus or TAZ short hairpin RNA (shRNA)-producing retrovirus. After puromycin selection for 7 days, the cell population was amplified. The selected C3H10T1/2 control (C) and TAZ knockdown (Ti) cells were incubated with osteoblast differentiation media in the absence or presence of 10 μg/ml phorbaketal A (PA). After 6 days of differentiation, the cell lysates were analyzed for the depletion of endogenous TAZ by immunoblotting. (B) Total RNA of the cells in (A) was prepared at 6 days after differentiation, and the TAZ, Runx2, and Dlx5 expression levels were analyzed by qRT-PCR. The level of GAPDH was analyzed for the control. $^{*}P < 0.01$, *t*-test.

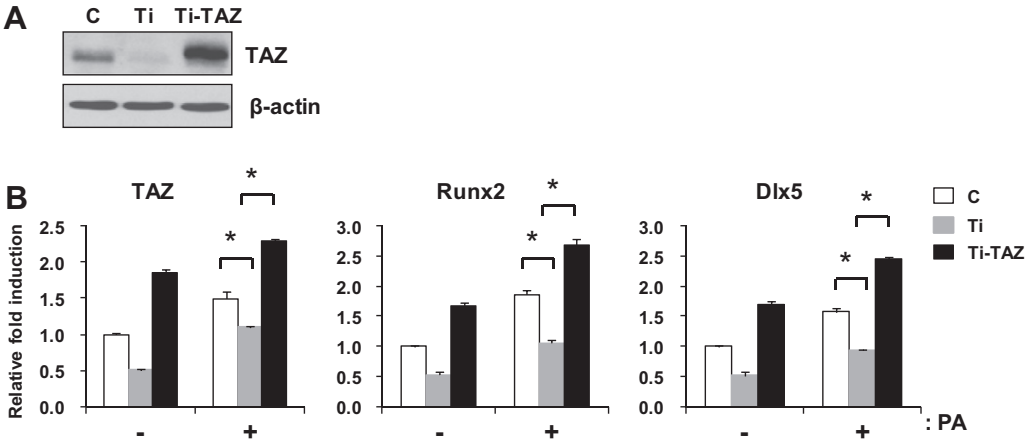

Fig. 5. Introduction of TAZ in TAZ depleted C3H10T1/2 cells recovers phorbaketal A-mediated osteoblastic marker gene expression. (A) TAZ depleted C3H10T1/2 cells (Ti) were infected with TAZ expressing retrovirus. After puromycin selection for 7 days, the cell population was amplified (Ti-TAZ cells). The C3H10T1/2 control (C), TAZ knockdown (Ti), and TAZ recovered (Ti-TAZ) cells were prepared and the cell lysates were analyzed for the expression of TAZ by immunoblotting. (B) The Cells in (A) were incubated with osteoblast differentiation media in the absence or presence of 10 μg/ml phorbaketal A (PA). After 6 days of differentiation, total RNA of the above cells was prepared and the TAZ, Runx2, and Dlx5 expression levels were analyzed by qRT-PCR. The level of GAPDH was analyzed for the control. $^{*}P < 0.01$, *t*-test.

osteogenic differentiation media in the presence of phorbaketal A. As shown in Fig. 6A and B, phorbaketal A significantly activated the osteogenic differentiation of human mesenchymal stem cells, as indicated by increased calcium deposition and osteogenic marker gene expression. These results indicated that the effect of phorbaketal A is not a cell type-specific effect, and suggested that phorbaketal A might be a potential lead compound for the treatment of osteoporosis.

3.8. Phorbaketal A stimulates osteoblast differentiation through the activation of extracellular signal regulated kinase (ERK)

MAPK plays an important role in osteoblast differentiation. To study the role of phorbaketal A in the signals for osteoblast differentiation, we analyzed the cellular MAPK activity. As shown in Fig. 7A, the ERK and c-Jun N-terminal kinase (JNK) activities were significantly increased, but p38 MAPK was not stimulated. Next, to test whether the increased kinase activity is important for the phorbaketal A-induced osteoblast differentiation, U0126 (an MEK inhibitor) and SP600125 (a JNK inhibitor) were co-treated with phorbaketal A. As shown in Fig. 7B, U0126 significantly inhibited calcium deposition, but SP600125 did not show significant inhibition of calcium deposition. Thus, the results showed that ERK, not JNK, activity induced by phorbaketal A is a critical factor for osteoblast differentiation. To further study the effect of U0126 treatment, the expression of osteoblastic marker genes, including TAZ, Dlx5 and osteocalcin were analyzed, and decreased expression of them was observed (Fig. 7C). Also, phorbaketal A induced TAZ expression was also significantly inhibited by the treatment of U0126 (Fig. 7D). Taken together, the results suggest that ERK activity is important for phorbaketal A-induced osteoblast differentiation.

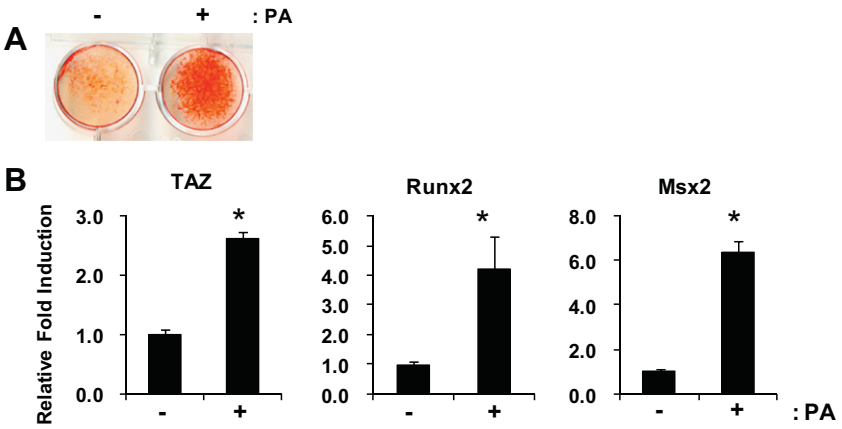

Fig. 6. Phorbaketal A stimulates the osteogenic differentiation of human mesenchymal stem cells. (A) Bone marrow-derived human mesenchymal stem cells were incubated with osteogenic differentiation media in the absence or presence of 10 μg/ml phorbaketal A (PA). After 12 days of differentiation, the cells were stained with Alizarin Red S solution. (B) The total RNA of the cells in (A) was prepared, and the expression of osteoblastic marker genes, including TAZ, Runx2, and Msx2, was analyzed by qRT-PCR. $^{*}P < 0.01$.

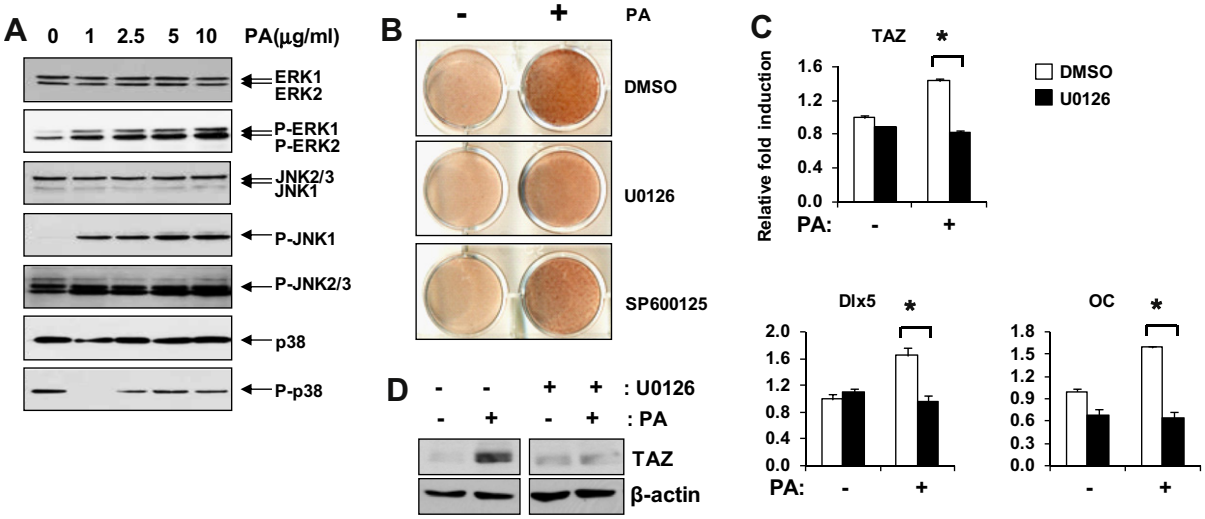

Fig. 7. Phorbaketal A stimulates ERK for osteoblast differentiation. (A) C3H10T1/2 cells were incubated with the indicated amount of phorbaketal A (PA); 30 min later, the cells were lysed, and the activity of cellular ERK, JNK, and p38 kinases were analyzed by immunoblot analysis. The activation status of the kinases was analyzed using their phospho-specific antibody. (B) C3H10T1/2 cells were incubated with osteogenic differentiation media in the absence or presence of 20 μM U0126, a MEK inhibitor, or 20 μM SP600125, a JNK inhibitor; 6 days later, the osteogenic potential was analyzed by calcium deposition activity through Alizarin Red S staining. (C) The total RNA of the cells treated with DMSO or 20 μM U0126 was prepared, and the expression of the osteoblastic marker genes, TAZ, Dlx5, and osteocalcin (OC), were analyzed by qRT-PCR. $^{*}P < 0.01$. (D) The U0126-treated cell lysates in (B) were prepared, and the TAZ expression was analyzed by immunoblot analysis.

4. Discussion

Many research groups are currently attempting to identify molecules that stimulate osteoblast differentiation for the development of drugs for osteoporosis, and natural-product compounds are frequently used for drug screening. In this study, we screened the natural compounds of marine organisms that stimulate osteoblast differentiation. One of the compounds was identified as phorbaketal A, which displayed a strong osteoblastic potential based on calcium deposition activity and the expression of osteoblastic marker genes. In particular, phorbaketal A increases levels of TAZ, a transcriptional regulator, in osteoblast differentiation, and TAZ is required for phorbaketal A-mediated osteoblast differentiation. For its signal transduction, phorbaketal A stimulates the ERK pathway, and disruption of the signal inhibits its phorbaketal A-induced osteoblastogenesis. Taken together, the results show that phorbaketal A is a strong inducer of osteoblast differentiation.

It was shown that MAPK activation could induce Runx2 phosphorylation and Runx2-dependent expression of the osteocalcin gene [20,21]. An examination of the osteocalcin promoter for MAPK-responsive sequence elements identified two Runx2-binding sites [22]. Indeed, an increased skeletal size and calvarial mineralization was observed in mice that have constitutively active MAPK/ERK in their osteoblasts [23]. The FGF2-induced ERK activation increased the phosphorylation of Runx2 at serine 301 and the level of the Runx2 protein [24]. Thus, increased ERK activity is a notable effect of phorbaketal A-mediated osteogenic differentiation. In our result ERK signal increased TAZ expression and the expression was inhibited by the treatment of ERK inhibitor (Fig. 7D). We also previously observed that increased TAZ expression stimulates osteoblast differentiation. Thus, it suggests that increased TAZ expression and interaction with Runx2 is important for ERK signal-mediated osteogenic stimulation.

JNK is required for the late-stage differentiation of preosteoblasts and BMP-2-induced differentiation of preosteoblasts and

pluripotent cells [25]. The activation of JNK1 increases the cellular responsiveness to BMP-2 and decreases the binding of inhibitory Smad6 to the type I BMP receptor [26]. These results suggest that JNK activity stimulates osteoblast differentiation. We observed that phorbaketal A stimulates JNK activity; however, JNK activity is not critical for phorbaketal A-mediated osteoblastic differentiation because a chemical inhibitor of JNK did not significantly inhibit osteoblastic differentiation. Presently, we do not know the direct target molecule of phorbaketal A and speculate that it could be a cellular receptor or signaling molecule(s) for ERK and JNK activation. Further study should address the identification of the target molecule.

Our results showed that phorbaketal A regulates cellular differentiation through TAZ. It has previously been shown that TAZ interacts with Runx2 [8] and activates its target genes through the recruitment of transcriptional co-activators [13]. Thus, the increased interaction between TAZ and Runx2 mediated by phorbaketal A may facilitate the recruitment of transcriptional co-activators to the promoters of osteoblastic genes. Indeed, we observed that phorbaketal A stimulates localization of TAZ on the promoter of osteocalcin (Fig. 2C).

TAZ is a 14-3-3 binding protein and is sequestered in the cytosol after binding with 14-3-3 proteins, and phosphorylation at serine 89 of mouse TAZ is important for 14-3-3 binding and cytosolic sequesteration of TAZ [27]. Recently it is revealed that Lats kinase, a component of Hippo signal, phosphorylates the site and induces proteolytic degradation of TAZ [17]. Thus, the phosphorylation status of TAZ was analyzed to study the mechanisms by which phorbaketal A regulates the function of TAZ. Interestingly, we observed that phorbaketal A treatment resulted in a significant induction of unphosphorylated TAZ at serine 89 (Fig. 3C), producing the unbound form of TAZ in the interaction of 14-3-3. These results suggest that phorbaketal A stimulates induction and nuclear localization of TAZ for osteogenic differentiation. At this moment, we do not know whether phorbaketal A regulates the activity of Lats kinase and effect of the kinase on osteogenic differentiation, but the study should be assessed for further understanding the effects of phorbaketal A.

TAZ depletion induced significant reduction of osteoblastic marker genes expression after phorbaketal A treatment, but it did not produce complete reduction (Fig. 4), indicating that there are another mechanisms for phorbaketal A induced osteogenic differentiation. Indeed, there are other transcriptional co-activators such as p300 for Runx2-mediated gene transcription and they may also be involved in phorbaketal A induced osteogenic differentiation. Further study should be addressed for understanding the mechanism.

Phorbaketal A can stimulate osteoblast differentiation of human mesenchymal stem cells (Fig. 6), suggesting that phorbaketal A is a possible candidate for therapeutic reagents for osteoporosis. Thus, we are currently investigating whether it can induce bone formation in normal and ovariectomized animal model and developing several phorbaketal A derivatives with modified side chains to increase its biological effect.

In summary, we report that phorbaketal A, which was isolated from the marine sponge, *Phorbas* sp., stimulated osteoblast differentiation through the induction of TAZ and the activation of ERK, revealing a novel stimulator for osteoblast differentiation.

Acknowledgements

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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.febslet.2012.03.008.

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