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A New Synthetic Compound, SST-VEDI-1, Inhibits Osteoblast Differentiation with a Down-Regulation of the Osterix Expression

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SST-VEDI-1(VEDI-1) is a new synthetic compound which is synthesized from tryptamine. However, the effect of VEDI-1 on various bio-phenomena in cells has not yet been examined. Tryptamine is one of the known trace amines. Trace amines are present in the central nervous system at very low concentrations and they are generally considered to have potent sympathomimetic actions. On the other hand, SSH-BM-I and SSH-BM-II-type compounds have been demonstrated to stimulate osteoblast activity in the cultured scales of goldfish. These compounds are also synthesized from tryptamine. VEDI-1 has a similar chemical structure to that of SSH-BM-I and SSH-BM-II-type compounds. Therefore, this study examined the effect of VEDI-1 on osteoblastic differentiation. VEDI-1 inhibited the osteoblast differentiation identified by mineralization, which was accompanied by the down-regulation of the expression of an osteogenic transcription factor, Osterix (OSX). Furthermore, as well as VEDI-1-treatment, the suppression of the OSX expression by stable-transfection with OSX/shRNA decreased the formation of mineralized nodules. These results suggest a possibility that VEDI-1 inhibits the osteoblast differentiation by suppressing the OSX expression.

Key words: differentiation, mineralization, osteoblast, osterix, SST-VEDI-1.

Abbreviations: shRNA, short hairpin RNA; Id-1, inhibitor of DNA binding 1; cAMP, cyclic AMP.

SST-VEDI-1(VEDI-1) is a new synthetic compound which is synthesized from tryptamine according to a synthetic method reported in a previous paper (*1*, *2*; Fig. 1). Tryptamine is one of the known trace amines (*3*). Trace amines are present in the central nervous system at very low concentrations and they are generally considered to have potent sympathomimetic actions (*4*). On the other hand, SSH-BM-I and SSH-BM-II-type compounds have been demonstrated to stimulate the osteoblast activity, while suppressing the osteoclast activity in the cultured scales of goldfish (*5*). These compounds are also synthesized from tryptamine. VEDI-1 is not only a new synthetic compound which has a similar chemical structure to that of SSH-BM-I and SSH-BM-II-type compounds but also a synthetic intermediate for them (Fig. 1). However, the effect of SST-VED-I-1 on osteoblast differentiation has not yet been examined. Therefore, this study examined the effect of VEDI-1 on osteoblastic differentiation in mesenchymal progenitor-like cells and mature osteoblast-like cells.

Mesenchymal cells differentiate into several specialized cell types, including osteoblasts and adipocytes (*6–8*). The developmental process from the mesenchymal progenitors to particular cell types can be divided into two stages, i.e. commitment and terminal differentiation (*9*, *10*).

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This differentiation process is regulated by several cytokines. Bone morphogenetic protein 2 (BMP-2) is one of the most powerful cytokines which promotes the differentiation of mesenchymal cells into osteoblasts *in vitro* and also induces bone formation *in vivo* (*11*). BMP-2 exerts its effects through forming complexes with type I and II serine/threonine kinase receptors, which in turn results in the activation of receptor-Smads (R-Smads), including Smad1 5 and 8 (*12*, *13*). Activated R-Smads dissociate from the receptors and form complexes with common-Smad (Co-Smad), Smad4, which are then subsequently translocated into the nucleus where they regulate the transcription of osteogenic genes (*14*).

Runt-related transcription factor 2 (Runx2), Osterix (OSX), Distal-less homeobox protein 5 (Dlx5) and Msh homeobox protein 2 (Msx2) are osteogenic transcription factors that are necessary for early and late osteoblast differentiation (*15–25*). Runx2 plays an essential role in the regulation of osteoblast marker genes (*15*). Runx2-knockout mice display a complete absence of bone due to arrested osteoblast maturation (*16*). OSX is specifically expressed in all developing bones and the OSX null-mutation in mice results in insufficient bone mineralization (*17*). Dlx5 is a bone-inducing transcription factor that is expressed in differentiating osteoblasts (*18*). The forced expression of Dlx5 leads to a fully mineralized matrix in cell culture (*19*). Moreover, Dlx5 deficient mice demonstrate severe craniofacial abnormalities with delayed ossification of the cranium and abnormal osteogenesis (*20*). The functional role of Msx2

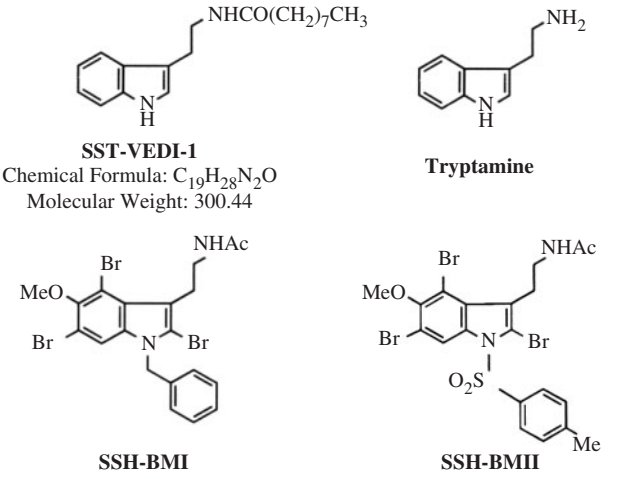

Fig. 1. **Chemical structure of SST-VEDI-1 (VEDI-1), Triptamine, SSH-BMI and SSH-BMII.**

in osteoblasts appears to be complex, as several studies have reported that Msx2 positively and negatively controls osteoblast differentiation (*21–23*). However, genetic studies have indicated that Msx2 plays a critical role in bone formation and osteoblast differentiation (*24*). Furthermore, a recent study has demonstrated the overexpression of Msx2 to induce the expression of OSX in Runx2-deficient cells (*25*).

The study of the biological effects on cell differentiation requires *in vitro* models using phenotypically stable cultured cell lines, in which the direct effect on the individual cell types can be investigated. Therefore, to investigate the effect of VEDI-1 on osteoblast differentiation, two types of cultured cell lines, ROB-C26 and ROS17/2.8 were used. ROB-C26 is a clonal mesenchymal progenitor-like cell line isolated from newborn rat calvaria. ROB-C26 cells are pre-committed cells and they are capable of differentiating into osteoblastic cells with BMP-2 treatment (*26*), however, they differentiate into adipocytes with dexamethasone (Dex) treatment (*8*). ROS17/2.8 cells are rat osteoblast-like osteosarcoma cells which have been committed to osteoblasts. ROS17/2.8 cells have mature osteoblast phenotypes (*27*) and are able to form bone-like nodules during terminal osteoblast differentiation (*28*).

In this study, VEDI-1 inhibited the osteoblast differentiation identified by mineralization, which was accompanied by the down-regulation of the OSX expression, but no decrease in the Runx2, Dlx5 or Msx2 expression.

MATERIALS AND METHODS

Cell Culture and Reagents—The cells were precultured in 12-well plates until they reached confluence in the growth media consisting of α-MEM, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Subsequently, the cells were cultured for the indicated periods under the indicated culture condition in the media supplemented with 50 μg/ml L-ascorbate phosphate (Sigma) and 10 mM β-glycerophosphate (Sigma). The culture medium was changed at an interval of 2 days. The antibiotics and cell culture media were obtained from Gibco or Wako respectively. FBS was purchased from Japan Bioserum Co Ltd Dex and rhBMP-2 were acquired from Sigma or R&D SYSTEMS respectively. SST-VEDI-1 was synthesized according to the method in a previous report (*2*). VEDI-1 was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 10^{-3} M, which was then diluted in culture medium to obtain the desired concentration.

Alizarin Red S Staining—The cells cultured under the indicated conditions were rinsed twice and fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for 30 min. Subsequently, Alizarin Red S solution was added. Following 10 min incubation, the plates were washed with distilled water.

Ca^{2+} Releasing Assay—After removing supernatants, cells were washed with 10 mM Tris–HCl (pH 7.2) solution and 1 N HCl solution was added into each well, and then incubated until drying. Twenty microliters of distilled water were added to each well, and the amount of Ca^{2+} was determined using the Calcium E-test (Wako Co.) according to manufacturer's instructions.

Oil Red O Staining—ROB-C26 cells were cultured under the indicated conditions. The adipocyte-like cells were identified using Oil Red O to stain cytoplasmic lipid droplets in the cells. Oil Red O (0.5 g) was dissolved in 100 ml of isopropanol, diluted 3:2 with distilled water, and filtered through a Whatmann #1 filter before use. ROB-C26 cell cultures were fixed with 10% formalin for 30 min, stained with Oil Red O for 1 h, rinsed repeatedly with distilled water to remove residual stain, and examined with a light microscope.

Real-Time RT–PCR and Western Blot Analysis—Real-time RT–PCR and western blot analysis were performed as described previously (*28*). Sense and antisense primers used for real-time RT–PCR were as follows: Runx2 (forward, 5′-ACA ACC ACA GAA CCA CAA G-3′; reverse, 5′-TCT CGG TGG CTG GTA GTG A-3′), Dlx5 (forward, 5′-TAC AAC CGA GTC CCG AGT-3′, reverse, 5′-AAT AGT CCT GGG TTT ACG-3′), OSX (forward, 5′-GGA GGT TTC ACT CCA TTC CA-3′, reverse, 5′-TAG AAG GAG CAG GGG ACA GA-3′). Id-1 (forward, 5′-CTG AAC GGC GAG ATC AGC TT-3′, reverse, 5′-CCA TCT GGT TCC TCA GTG C-3′), β-actin (forward, 5′-CTT TCT ACA ATG AGC TGC GTG-3′, reverse, 5′-ATG GCT GGG GTG TTG AAG G-3′). Messenger RNA expression levels were normalized by values obtained by that of β-actin mRNA. The antibodies used for western blot, against Runx2 (sc-10758), OSX (sc-22538), Dlx5 (sc-18152), Msx2 (sc-13771) and actin (sc-1615), were obtained from Santa Cruz Biotechnology and against phosphorylated Smad1/5/8 (#9511) was obtained from Cell Signaling Technology. As an internal control, an antibody specific for actin was used.

Cell Proliferation Assay—The cells were seeded onto 96-well plates (1200 cells per well) and cultured for the indicated periods with or without VEDI-1. After performing a culture in order to assess the cell proliferation, the cell number was measured using a colorimetric cell

counting assay kit (Cell counting kit 8: Dojin Co.) according to the manufacturer's instructions.

Suppression of OSX Expression—The ROS17/2.8 cells were plated at a density of 5 x 10^5 cells per well on a 6-well plate and they were allowed to grow under normal culture conditions for 18 h. Subsequently, the cells were incubated in 2 ml of α-MEM containing 10 μl of lipofectamine with 2 μg of the OSX/shRNA plasmid (SuperArray; KR53023P: Clone ID 1) or the negative control/shRNA plasmid (SuperArray; KR53023P). After transfection, the cells with stable DNA integration were selected by culturing with 2 μg/ml Puromycin (Sigma). Ten surviving cell colonies were randomly selected from the OSX/shRNA transfected culture and the control/shRNA transfected culture respectively. Colonies selected from control/shRNA transfected culture exhibited similar expression levels of OSX in comparison to each other and two colonies (#Co1 and #Co2) were chosen for the following experiments. The two colonies that exhibited the lowest expression level of OSX (#Ox1 and #Ox6) were chosen from OSX/shRNA transfected colonies for the following experiments.

RESULTS

Effect of VEDI-1 on Cell Differentiation into Osteoblast and Adipocyte, and on Cell Proliferation in ROB-C26—To investigate the effect of VEDI-1 on the cell differentiation of pluripotent cells for osteoblasts, the formation of the mineralization in ROB-C26 culture was examined by Alizarin red S staining and a Ca^{2+} releasing assay. The cells were cultured with or without VEDI-1 in the presence or absence of BMP-2 for 28 days. After culture, alizarin red S staining and the Ca^{2+} releasing assay were performed and the results are shown in Fig. 2A and B. Alizarin red S-positive bone-like mineralization

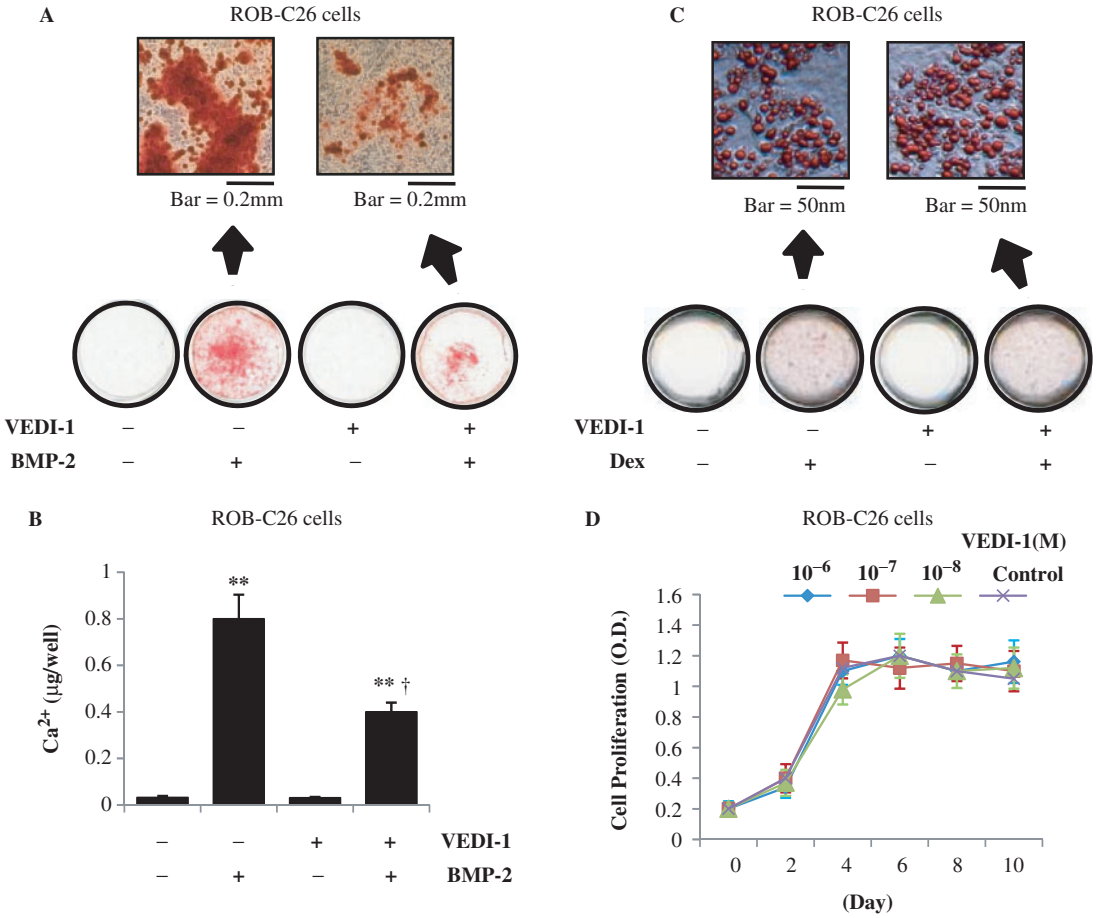

Fig. 2. **Effect of VEDI-1 on cell differentiation and cell proliferation in ROB-C26 cells.** (A) Effect of VEDI-1 on the formation of mineralized bone-like nodules: Cells were cultured with or without VEDI-1 (10^{-6} M) in the presence or absence of BMP-2 (100 ng/ml) for 28 days and then Alizarin Red S staining was performed and observed under a light microscope (upper). (B) Quantification of calcium deposition: Cells were cultured under the same conditions indicated in A. The calcium contents of the cell layers were measured by the *o*-cresolphthalein complexone method. [Mean ± SD ($n = 3$; $P < 0.05$). **Differentiated from VEDI-1(+)/BMP-2(–) and VEDI-1(–)/BMP-2(–). †Different from VEDI-1(–)/BMP-2(+)]. (C) Effect of VEDI-1 on Dex-induced lipid-droplet formation: The cells were cultured with or without VEDI-1 (10^{-6} M) in the presence or absence of Dex (10^{-7} M) for 21 days and then were stained with Oil Red O and observed under a light microscope (upper). (D) Effect of VEDI-1 on the cell proliferation: Cells were cultured with or without the indicated concentration of VEDI-1 for the indicated days and the number of cells was measured as the O.D. at each time point.

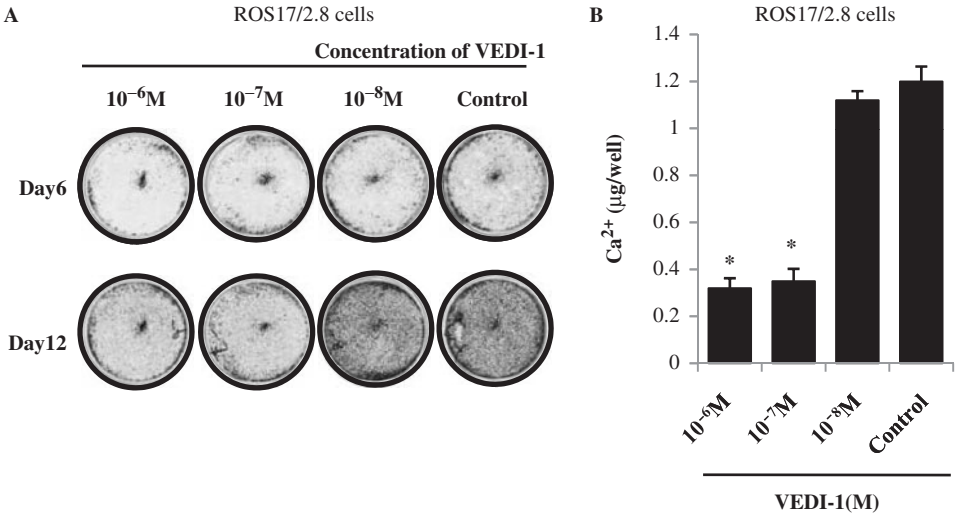

Fig. 3. **Effect of VEDI-1 on the mineralization in ROS17/2.8 cells.** (A) Effect of VEDI-1 on the formation of mineralized bone-like nodules: Cells were cultured with or without the indicated concentration of VEDI-1 for 6 days and 12 days and then were stained with Alizarin Red S. (B) The quantification of calcium deposition: Cells were cultured under the same conditions indicated in A for 12 days. The calcium contents of the cell layers were measured by the *o*-cresolphthalein complexone method. [Mean ± SD ($n = 3$; $P < 0.05$). *Differentiated from control (no treated)].

nodules were observed in both (BMP-2)- and (BMP-2 + VEDI-1)-treated cultures. However, bone-like nodules decreased in the (BMP-2 + VEDI-1)-treated culture in comparison to that in the (BMP-2)-treated culture. No bone-like nodules were observed in either the (VEDI-1)-treated cultures or the no-treated control cultures. The results of Alizarin red S staining were confirmed by the results of the Ca^{2+} releasing assay.

The amount of Ca^{2+} released from the bone-like nodules was also determined for the (BMP-2)- and (BMP-2 + VEDI-1)-treated cultures, and the Ca^{2+} level decreased in the (BMP-2 + VEDI-1)-treated culture in comparison to that in the (BMP-2)-treated cultures.

Next, to determine the effect of VEDI-1 on cell differentiation of pluripotent cells for adipocytes, the formation of lipid-droplets in ROB-C26 culture was examined by Oil Red O staining. The cells were cultured with or without VEDI-1 in the presence or absence of Dex for 21 days. After culture, oil Red O staining was performed and the results are shown in Fig. 2C. The lipid-droplet formation level in the (Dex)-treated cultures was similar to that in the (Dex + VEDI-1)-treated culture. However, no lipid-droplet formation was observed in either the (VEDI-1)-treated or the no-treated control culture.

To determine whether VEDI-1 affects cell proliferation, which is closely related to cell differentiation (*26*), ROB-C26 cells were cultured with or without VEDI-1 for the indicated days and then the cell number in each culture was measured as O.D. (Fig. 2D). No difference was observed in each cell proliferation curve during the culture time period.

Effect of VEDI-1 on the Terminal Osteoblast Differentiation—To investigate whether the treatment with VEDI-1 inhibits the terminal osteoblast differentiation, ROB17/2.8 cells were cultured with or without the indicated concentration of VEDI-1 for 6 and 12 days and then Alizarin red S staining and Ca^{2+} releasing assays were performed. The results are shown in Fig. 3A and B. Treatment with 10^{-6} M and 10^{-7} M VEDI-1 similarly decreased the formation of the bone-like nodules and the Ca^{2+} level in comparison to those in no-treated control cultures at Day 12. However, the treatment with the lower concentration of VEDI-1 (10^{-8} M) no longer decreased the formation of bone-like nodules and the Ca^{2+} level in comparison to those in the control cultures at Day 12.

Effect of VEDI-1 on the Expression of the Osteogenic Transcription Factors in ROS17/2.8 Cells—The mRNA and protein expression of osteogenic transcription factors, Runx2, OSX, Dlx5 and Msx2 were analyzed with the ROS17/2.8 cells treated with or without VEDI-1 for the indicated days by real time RT-PCR and Western blotting. The results are shown in Fig. 4A and B, respectively. The treatment of VEDI-1 suppressed the OSX mRNA and protein expression in comparison to those in the no-treated control cultures on Day 1 and Day 12, although it had no effect on the mRNA and protein expression of Runx2, Dlx5 and Msx2.

Next, to determine whether the treatment of VEDI-1 affects the BMP-2-induced expression of these transcription factors in ROB-C26 cells, the cells were cultured with or without VEDI-1 in the presence or absence of BMP-2 for the indicated days. After culture, real-time RT–PCR and western blotting were performed and the results are shown in Fig. 5A and B. OSX mRNA and protein expression were induced in the (BMP-2)- and (BMP-2 + VEDI-1)-treated cells although they were not detectable in the (VEDI-1)-treated and the no-treated control cells at Day 1 and Day 28. However, the OSX mRNA and protein expression level in the (BMP-2 + VEDI-1)-treated cells were much lower than those in the (BMP-2)-treated cells at Day 1 and Day 28. On the other hand, although Runx2, Dlx5 and Msx2 also

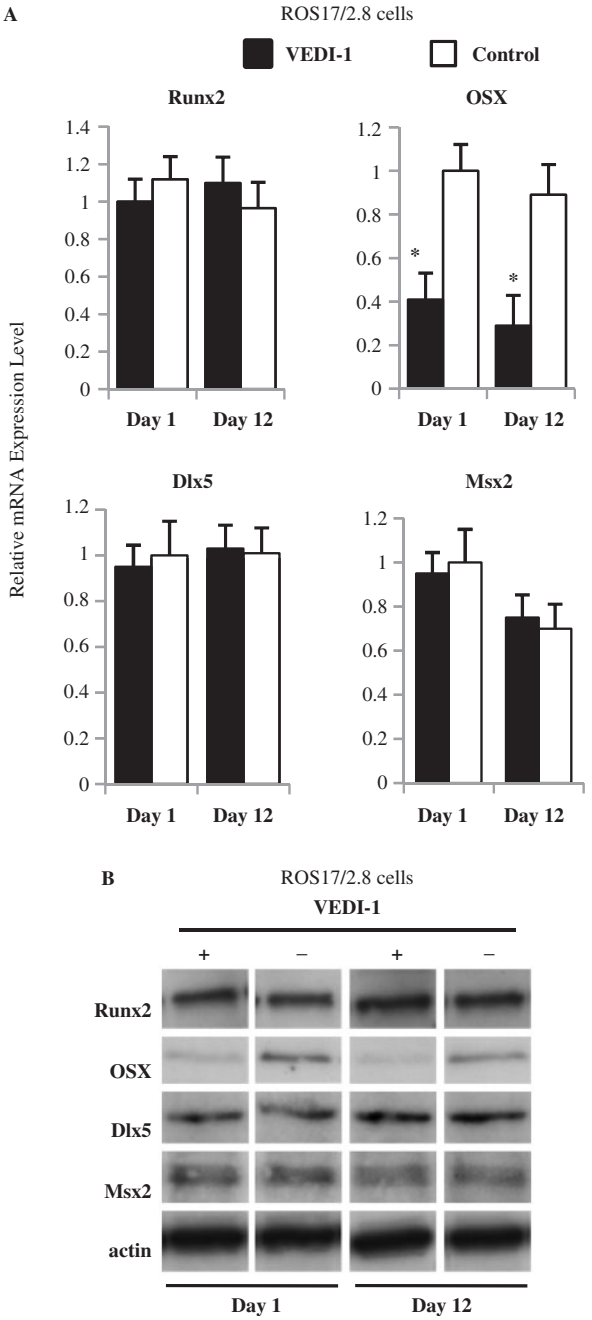

Fig. 4. **Effect of VEDI-1 on the expression of the osteogenic transcription factors in ROS17/2.8 cells.** (A) Effect of VED-I-1 on the mRNA expression of osteogenic transcription factors: Cells were cultured in the absence or presence of 10^{-6} M VEDI-1 for 1 day and 12 days. mRNA expression was determined by real-time RT-PCR. [Mean ± SD ($n = 3$; $P < 0.05$). *Differentiated from control (no treated) at each time point]. (B) Effect of VED-I-1 on the protein expression of osteogenic transcription factors: Cells were cultured in the absence or presence of 10^{-6} M VEDI-1 for 1 day and 12 days. The protein expression was determined by a western blot analysis.

increased their mRNA and protein expression in the (BMP-2)- and (BMP-2 + VEDI-1)-treated cells in comparison to those in the (VEDI-1)-treated cells and the no-treated control cells at each time point, no difference was observed in the expression of these transcription factors between the (BMP-2)- and (BMP-2 + VEDI-1)-treated cells.

Effect of VEDI-1 on the Smad Signalling—To examine whether VEDI-1 affects the Smad signalling activated by BMP-2, the BMP-2-induced phosphorylation of Smad1/5/8 and the expression of an inhibitor of DNA-binding protein 1 (Id-1), a faithful target of activated Smad signalling (*29*), were analysed. ROB-C26 cells were cultured with or without VEDI-1 in the presence or absence of BMP-2 for the indicated times. After culture, Western blotting using antibody against phosphorylated Smad1/5/8 and real time RT–PCR were performed, and the results are shown in Fig. 6A and B, respectively. Although the Phosphorylated Smad1/5/8 (P-Smads) expression level increased in the (BMP-2)- and (BMP-2 + VEDI-1)-treated cells in comparison to those in the (VEDI-1)-treated cells and the no-treated control cells, no differences were observed in those protein expression levels between the (BMP-2)- and (BMP-2 + VEDI-1)-treated cells. Similarly, the Id-1 mRNA expression was induced by the BMP-2 treatment and no effect of the VEDI-1 treatment on the BMP-2 induced Id-1 expression was seen during the culture time periods.

Effect of the Suppression of OSX Expression on the Mineralization—In order to investigate whether VEDI-1 inhibited the mineralization through down-regulating the OSX expression, ROS17/2.8 cells were stable-transfected with either the OSX/shRNA vector or with the negative control/shRNA vector. These cells were cultured for 12 days and then each analysis was performed. First, the expression level of OSX, Runx2, Dlx5 and Msx2 was analyzed by real-time RT–PCR and western blotting. OSX mRNA and protein expression in the OSX/shRNA transfected cells (#Ox1 and #Ox6) were remarkably suppressed in comparison to those in the control/shRNA transfected cells (#Co1 and #Co2; Fig. 7A and B). There was no difference between the OSX/shRNA transfected cells and the control/shRNA transfected cells in mRNA and the protein expression of the other three transcription factors. Next, the mineralization level was examined by Alizarin red S staining and a Ca^{2+} releasing assay. The formation of the bone-like nodule and the Ca^{2+} level in both #Ox1 and #Ox6 decreased in comparison to those in #Co1 and #Co2 (Fig. 7C).

DISCUSSION

This is the first direct demonstration of the effect of VEDI-1 on osteoblast differentiation. First, the effect of VEDI-1 on the formation of bone-like nodules in ROB-C26 cultures was investigated. Since SSH-BM-I and SSH-BM-II-type compounds stimulate the osteoblast activity, it was expected that VEDI-1 played a positive role in osteoblast differentiation. However, contrary to expectations, treatment with VEDI-1 remarkably decreased the BMP-2-induced bone-like nodule formation and the Ca^{2+} level in comparison to the control culture (Fig. 2A and B).

The differentiation of osteoblasts had generally been considered to be competitively balanced with that of adipocytes, because both osteoblasts and adipocytes

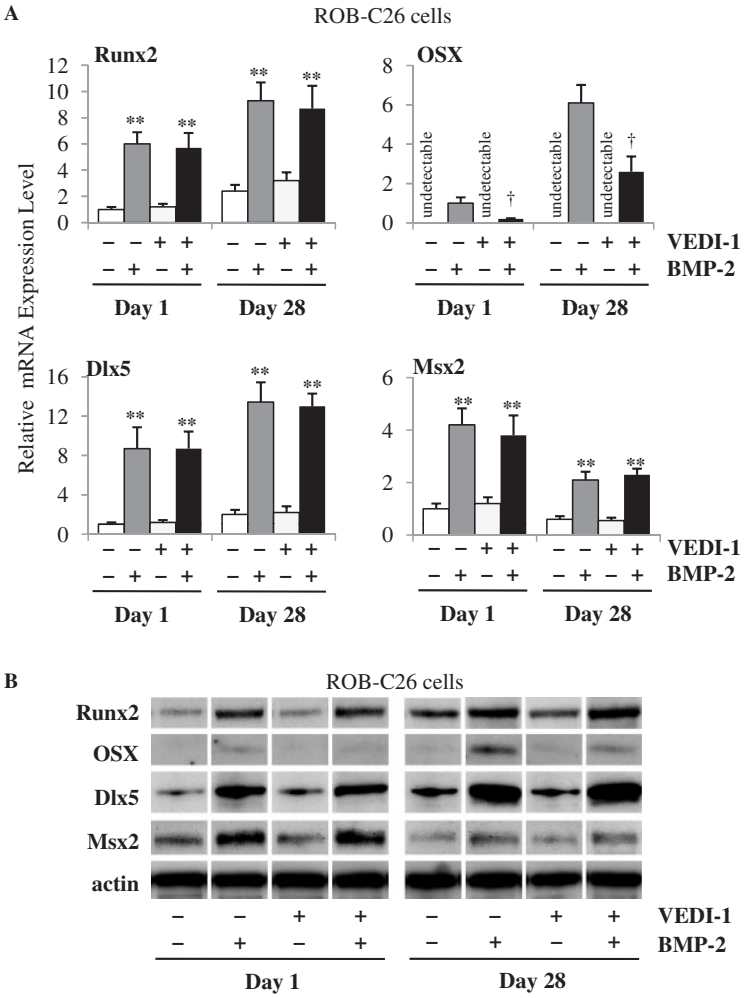

Fig. 5. **Effect of VEDI-1 on the BMP-2-induced osteogenic transcription factors in ROB-C26 cells.** (A) Effect of VED-I-1 on BMP-2 induced mRNA expression of osteogenic transcription factors, Runx2, OSX, Dlx5 and Msx2: Cells were cultured with or without BMP-2 (100 ng/ml) in the presence or absence of VEDI-1 (10^{-6} M) for 1 day and 28 days. After culture, the mRNA expression was determined by real-time RT-PCR. [Mean ± SD ($n = 3$; $P < 0.05$). **Differentiated from both (VEDI-1)-treated and control (no treated) at each time point. †Different form VEDI-1(−)/BMP-2(+). (B) Effect of VED-I-1 on BMP-2-induced protein expression of osteogenic transcription factors: The cells were cultured under the same conditions as indicated in A. The protein expression was determined by a western blot analysis.

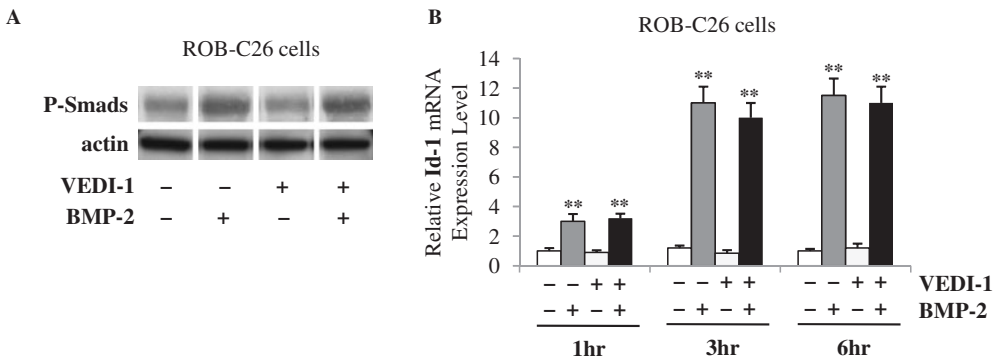

Fig. 6. **Effect of VEDI-1 on the Smad signalling.** (A) Effect of the VEDI-1 on the BMP-2-induced phosphorylation of Smad1/5/8: ROB-C26 cells were cultured with or without VEDI-1 (10^{-6} M) in the absence or presence of BMP-2 (100 ng/ml) for 1hr and then western blotting using antibody against phosphorylated Smad1/5/8 was performed. (B) Effect of the VEDI-1 on the BMP-2-induced Id-1 mRNA expression: cells were cultured under the same conditions as indicated in A for the indicated times and then real-time PCR was performed. [Mean ± SD ($n = 3$; $P < 0.05$). **Differentiated from both (VEDI-1)-treated and control (no treated) at each time point].

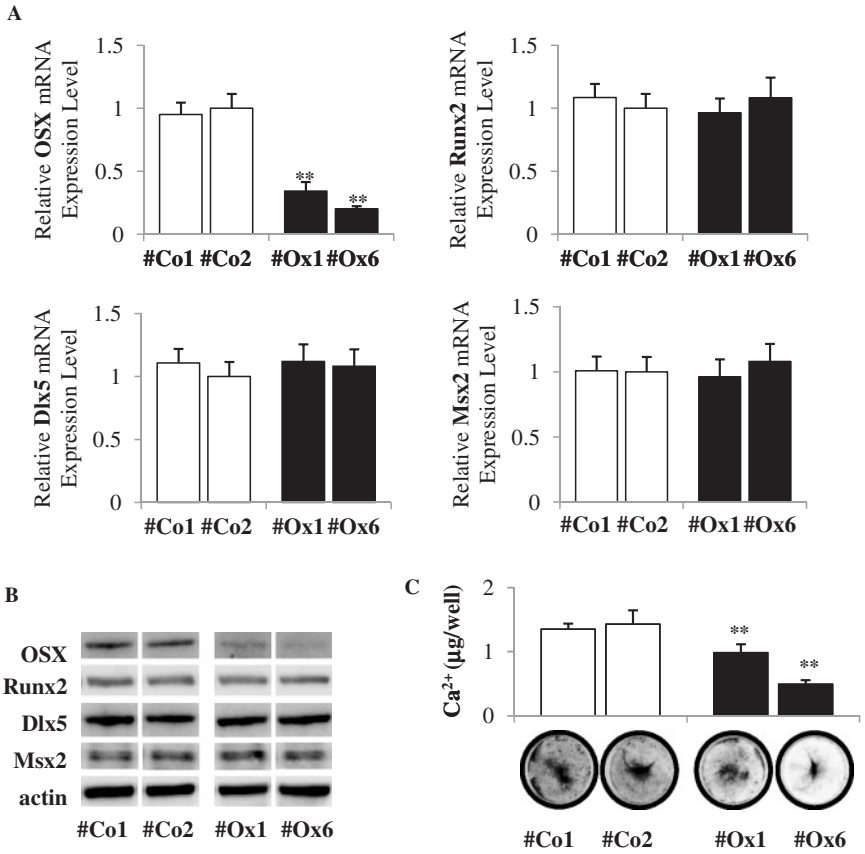

Fig. 7. **Effect of the suppression of OSX expression by OSX/shRNA on osteoblast differentiation in ROS17/2.8 cells.** (A) Effect of the stable-transfection with OSX/shRNA on mRNA expression of osteogenic transcription factors: Cells were stably transfected with OSX/shRNA vector (#Ox1 and #Ox6) and or control/shRNA vector (#Co1 and #Co2) were cultured for 1 day and 12 days and then real time RT-PCR was performed at each time point. [Mean ± SD ($n = 3$; $P < 0.05$). **Differentiated from #Co1 and #Co2 at each time point.] (B) Effect of the stable-transfection with OSX/shRNA on the protein expression of osteogenic transcription factors: Cells (#Ox1, #Ox6, #Co1 and #Co2) were cultured for 1 day and 12 days and then Western blotting was performed at each time point. (C) The effect of the suppression of OSX expression by OSX/shRNA on the mineralization: Cells (#Ox1, #Ox6, #Co1 and #Co2) were cultured for 12 days and then Ca^{2+} releasing assay (upper) and Alizarin Red S staining (lower) were performed. [Mean ± SD ($n = 3$; $P < 0.05$). **Differentiated from #Co1 and #Co2.].

differentiate from common progenitor cells. We therefore next investigated the effect of VEDI-1 on adipocyte differentiation. VEDI-1 had no effect on the Dex-induced lipid-droplet formation in ROB-C26 culture (Fig. 2C). These results indicate that VEDI-1 has a suppressive effect on the cell differentiation of progenitor cells into osteoblasts although it has no effect on the differentiation of adipocytes.

Next, the effect of VEDI-1 on the cell proliferation, which is closely related to cell differentiation (*26*), was investigated. The proliferation assay revealed that the cell number in VED1-1-treated cultures and that in the control cultures increased with similar growth curves until they reached confluence and maintained a similar level during the culture time period after confluence (Fig. 2D). This indicates that, VEDI-1 did not inhibit osteoblast differentiation by regulating the cell proliferation.

Secondly, to determine whether VEDI-1 inhibited the terminal osteoblast differentiation, mature osteoblast like ROS17/2.8 cells were treated with or without various concentrations of VEDI-1. The treatment with VEDI-1 decreased the formation of bone-like nodules and the Ca^{2+} level at concentrations of 10^{-6}M and 10^{-7}M (Fig. 3).

The development of mesenchymal progenitors into osteoblasts can be divided into two stages, namely commitment and terminal differentiation (*9*, *10*). ROS17/2.8 cells have been committed to becoming osteoblasts and they are able to form bone-like nodules during terminal osteoblast differentiation (*27*, *28*). In contrast, ROB-C26 cells are pre-committed cells, which require commitment into osteoblasts for the formation of bone-like nodules (*26*). Taking both previous evidence and the current results into consideration, it is possible that VEDI-1 inhibits the formation of bone-like nodules by acting in both the commitment stage and the terminal differentiation stage or by acting only during the terminal differentiation stage.

OSX has been suggested to play a role mainly on the terminal differentiation stage in osteogenesis, while Runx2 plays a role in the commitment for the common progenitor cells into osteoblasts (*17*). In this report, as described below, VEDI-1 suppressed OSX expression but

not Runx2, Dlx5 and Msx2 expression. Therefore, it is suggested that VEDI-1 may inhibit bone-like nodule formation by acting on the terminal osteoblast differentiation stage rather than acting on the commitment stage for osteoblasts.

Thirdly, we attempted to determine the osteogenic transcription factors that were involved in the mechanism of VEDI-1-inhibited bone-like nodule formation. The results of real-time RT-PCR and a Western blot analysis showed that the OSX mRNA and protein expressions decreased after VEDI-1 treatment in ROS17/2.8 (Fig. 4). Similarly, BMP-2-induced OSX mRNA and protein expression were suppressed by the treatment with VEDI-1 in ROB-C26 cells (Fig. 5). This may suggest a possibility that VEDI-1 inhibited the formation of bone-like nodules by suppressing OSX expression.

Whereas OSX deficient mice express Runx2, OSX is not expressed in Ruxn2 deficient mice (*17*). Furthermore, Runx2 overexpression induces OSX (*16*). Therefore, it is natural to suggest that OSX functions downstream of Runx2. On the other hand, an overexpression of Msx2 induced OSX expression in the Runx2-deficient cells (*25*). The knockdown of Msx2 clearly inhibited the induction of OSX by BMP-2 in the Runx2-deficient mesenchymal cells (*25*). These reports suggest that the OSX expression is independently induced by two distinct transcription factors, Runx2 and Msx2. Furthermore, the forced expression of Dlx5 antisense RNA completely blocks the BMP2-stimulated OSX expression and Dlx5 overexpression strongly activates Runx2 and OSX expression (*23*, *25*). These reports suggest that Dlx5 is also an indispensable mediator of OSX expression. Therefore, it was expected that VEDI-1 suppressed the OSX expression by down-regulating the Runx2, Dlx5 and/or Msx2 expression. However, the treatment with VEDI-1 failed to regulate the mRNA and protein expression of Runx2, Dlx5 and Msx2 (Figs 4 and 5). This indicates that VEDI-1 suppressed the OSX expression by a Runx2, Dlx5 and Msx2 independent mechanism.

Lastly, to support the possibility that VEDI-1 inhibited osteoblast differentiation by suppressing the OSX expression, the effect of the suppression of OSX expression by OSX/shRNA transfection on the bone-like nodule formation and the Ca^{2+} level in ROS17/2.8 cells was investigated. Stable transfection with OSX/shRNA decreased OSX mRNA expression level down to approximately 20–30% of the level of that in the control cells without changing the Runx2, Dlx5 and Msx2 expression level (Fig. 7A and B). This OSX-suppression pattern was similar to that exhibited by the VEDI-1 treatment. The formation of bone-like nodules and the Ca^{2+} level in the cells transfected with OSX/shRNA (#Ox1 and #Ox6) decreased in comparison to those in the control cells (#Co1 and #Co2; Fig. 7C). Although the results of further studies are needed, these results strongly support the possibility that VEDI-1 inhibits osteoblast differentiation by suppressing the OSX expression. However, it is important to note that our results do not rule out the possibility that other factors may play a major role in VEDI-1-inhibitted osteogenesis.

In this study, in order to determine how VEDI-1 inhibited Osteoblast differentiation and suppressed OSX expression, we investigated whether VEDI-1 affects Smad signalling because BMP-2 induced osteoblast differentiation through the activation of Smad signalling by phosphorylating Smad1/5/8 (*12*, *13*). The results of Western blotting using antibody against phosphorylated Smad1/5/8 and real time RT-PCR revealed that VEDI-1 was not effective on either the BMP-2-induced Smad1/5/8 phosphorylation or the mRNA expression of the target of Smads, Id-1 (Fig. 6). These results suggested that VEDI-1 did not inhibit Smad signalling.

VEDI-1 is synthesized from tryptamine which is a member of trace amines (*3*, *4*). A family of G protein-coupled receptors (GPCRs) has been reported as trace amine-associated receptor (TAAR1) (*30*). Some amines derived from thyroid hormone decarboxylation and deiodination, known as thyronamines, have been shown to be endogenous compounds that stimulate cAMP production via the activation of TAAR1 (*30*). Cyclic AMP signalling regulates osteogenic events including OSX transcription (*31*, *32*). For example, parathyroid hormone (PTH) regulates the OSX expression predominantly through cAMP-dependent protein kinase A (PKA) signalling in osteoblast-like cells (*33*) and the overexpression of PKA inhibitor γ (PKIγ) suppressed the OSX expression in C2C12 cells (*34*). Based on the findings of these reports, it is therefore possible that the cAMP-dependent signalling pathway via TAAR1 may play an important role in the mechanism by which VEDI-1 inhibits osteoblast differentiation and suppresses the OSX expression.

In conclusion, this study demonstrated that VEDI-1 inhibited osteoblast differentiation identified by mineralization, which was accompanied by the suppression of the OSX expression by a Runx2, Dlx5 and Msx2-independent mechanism. These findings therefore characterized the activity of VEDI-1, and contribute to a better understanding of the molecular basis by which progenitor cells differentiate into osteoblasts.

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CONFLICT OF INTEREST

None declared.

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