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A NOVEL VITAMIN D DERIVATIVE ACTIVATES BONE MORPHOGENETIC PROTEIN (BMP) SIGNALING IN MCF10 BREAST EPITHELIAL CELLS

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Running Title: Activation of BMP signaling by vitamin D

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The abbreviations used are: BMP, bone morphogenetic protein; R-Smad, receptor-regulated Smad; DAPI, 4,6-diamidino-2-phenylindole; TGF-□, transforming growth factor-□

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Abstract

We investigated the action of 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) as well as a novel Gemini vitamin D_3 analog Ro-438-3582 [1α,25-dihydroxy-20S-21(3-hydroxy-3-methyl-butyl)-23-yne-26,27-hexafluorocholecalciferol; Ro3582] and a classical vitamin D_3 analog Ro-26-2198 (1α,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor-cholecalciferol; Ro2198) in modulating the transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) system in MCF10 immortalized breast epithelial cells. We found that 1α,25$(OH)_2D_3$, Ro3582 and Ro2198 all enhanced BMP/Smad signaling by increasing the phosphorylation of receptor-regulated Smads. Ro3582 was more active than Ro2198 but both were considerably more active than 1α,25$(OH)_2D_3$. Ro3582 enhanced BMP/Smad signaling by (a) inducing the phosphorylation of receptor-regulated Smads (Smad1/5), (b) increasing the accumulation of phosphorylated Smad1/5 in the nucleus, and (c) activating BMP-mediated transcription in MCF10 breast epithelial cells. Furthermore, Ro3582 induced the synthesis of BMP-2 and BMP-6 mRNA and protein, and the expression of Smad6 mRNA in MCF10 breast epithelial cells was inhibited by Ro3582. The induction of phospho-Smad1/5 by Ro3582 was inhibited by treatment with the BMP antagonist, Noggin, while neutralizing antibody to TGF-β did not block the induction of phospho-Smad1/5 by Ro3582. Treatment with Noggin also blocked the effect of Ro3582 on nuclear accumulation of phospho-Smad1/5 and the induction of BMP-2 and BMP-6 mRNA synthesis. These results indicate that the activation of BMP/Smad signaling by the Gemini vitamin D_3 analog Ro3582 may be through the production of BMP ligands including BMP-2 and BMP-6 and/or down-regulation of the inhibitory Smad6. This is the first report to show that 1α,25$(OH)_2D_3$ and its derivatives activate BMP/Smad specific signaling in human breast epithelial cells.

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Introduction

The transforming growth factor-β (TGF-β) superfamily, including TGF-βs, activins, and bone morphogenetic proteins (BMPs), are multifunctional cytokines that affect inflammation, immune responses, cell growth, differentiation, apoptosis, and development as well as carcinogenesis (Ten Dijke et al., 2002; Derynck and Zhang, 2003). BMPs are members of the TGF-β superfamily regulating a large variety of biologic responses in many different cells and tissues during embryonic development and postnatal life (Kawabata et al., 1998; Miyazono et al., 2005). BMPs exert their biologic effects via binding to two types of serine/threonine kinase BMP receptors, activation of which leads to phosphorylation and translocation into the nucleus of intracellular signaling molecules, including Smad1, Smad5, and Smad8 (Kawabata et al., 1998). Upon BMP receptor activation, the BMP receptor-regulated Smads are phosphorylated in the C-terminal of the MH2 domain and recruit the common partner Smad, Smad4, to the nucleus to mediate BMP-dependent target gene expression (Kawabata et al., 1998; Miyazawa et al., 2002; Miyazono et al., 2005).

Although BMPs belong to the TGF-β family and have mainly been known to stimulate bone formation, they are now identified as multifunctional molecules regulating growth, differentiation, and apoptosis in many target cells. The biologic effects and mechanisms by which BMPs function in breast cancer cells have not been well defined. Importantly, BMPs are known to play roles as potent growth inhibitors in many epithelial cells (Ghosh-Choudhury et al., 2000; Pardali et al., 2005). This growth inhibition is lost with mutations and the associated loss of expression of genes for molecules of the BMP signaling pathway, which results in uncontrolled cell growth with tumor formation, suggesting the possible role of BMP as a tumor suppressor (Horvath et al., 2004; He et al., 2004; Haramis et al., 2004). BMP-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells (Ghosh-Choudhury et al., 2000). The mechanism of BMP-induced p21 promoter activation involves BMP receptors and BMP Smads (Pouliot and Labrie, 2002). Also, it has been shown that upregulation of BMP signaling is associated with inhibition of proliferation and *in vivo* tumor growth of androgen-insensitive prostate carcinoma cells (Miyazaki et al., 2004). However, it has also been reported that the BMP/Smad pathway is activated in breast cancer and may contribute to

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breast cancer progression and dedifferentiation in estrogen receptor-positive breast cancer (Helms et al., 2005).

The hormonally active metabolite of vitamin D_3, 1α,25$(OH)_2D_3$, functions in the maintenance of calcium homeostasis through regulation of genes in the intestine, kidney and bone, and it also controls immune cells and hormone secretion (Sutton and MacDonald, 2003). Interestingly, nuclear receptor ligands, such as retinoic acid or vitamin D_3 analogs, have been shown to induce the synthesis of ligands and receptors for both TGF-β and BMP in many epithelial cell lines as well as in myeloid leukemia cells (Falk et al., 1991; Koli and Keski-Oja, 1995; Hatakeyama et al., 1996). Among the studies with TGF-β/BMP signaling and nuclear receptors, 1α,25$(OH)_2D_3$ has been shown to induce an interaction with intracellular effectors of TGF-β, the Smad3/4 complex, and the vitamin D receptor (VDR) in the nucleus, and to potentiate VDR-dependent transcription (Yanagisawa et al., 1999; Yanagi et al., 1999) Since BMP signaling is known to be involved in bone formation and bone metastasis from breast cancer (Reinholz et al., 2002; Helms et al., 2005; Raida et al., 2005), it is of great interest to investigate the role of vitamin D derivatives for the activation of the BMP system and its further downstream signaling in breast cancer.

In the present study, we have focused on studying the effects of 1α,25$(OH)_2D_3$, the classical vitamin D_3 analog Ro2198 and the novel Gemini analog Ro3582 (Fig. 1) in modulating TGF-β/BMP signaling in a breast epithelial cell line. Among many vitamin D analogs we have tested, we selected two synthetic vitamin D analogs, Ro2198 and Ro3582, to investigate their activity in enhancing BMP signaling. Although Ro2198 has potent anti-proliferative activity, this analog is not optimal for *in vivo* studies due to its hypercalcemic toxicity. The novel Gemini analog, Ro3582, has a distinctive chemical structure with two bulky side chains that may contribute to its very low hypercalcemic effect. Gemini analogs showed markedly enhanced potency with low hypercalcemic toxicity when compared with 1α,25$(OH)_2D_3$, possibly due to different properties of the liganded VDR facilitating selectivity of cofactor binding and selectively modulating levels of transcription (Uskokovic et al., 2001; Weyts et al., 2004). We have investigated the action of synthetic vitamin D_3 analogs in modulating the TGF-β/BMP signaling in MCF10 immortalized breast epithelial cells. The MCF10 human breast epithelial cell lines were established from an immortalized normal MCF10A cell line originally derived from benign breast tissue from a woman with fibrocystic disease (Miller, 2000). The series of MCF10 cell lines was established by transfecting MCF10A

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normal breast epithelial cells with H-ras (MCF10AT1) and subsequently selecting by growth for several generations in mice (MCF10DCIS.com and MCF10CA1a) (Santner et al., 2001). This series of MCF10 cell lines provides a unique progressive breast cancer model with different stages of progression, such as MCF10A (immortalized), MCF10AT1 (premalignant), MCF10DCIS.com (invasive potential), and MCF10CA1a (malignant, metastatic) cell lines (Miller, 2000; Santner et al., 2001; Tang et al., 2003; Tian et al., 2003). Interestingly, the late stage metastatic MCF10CA1a cells are resistant to TGF-β-induced growth inhibition, while premalignant MCF10AT1 cells are still responsive to TGF-β for growth inhibition (Santner et al., 2001; Tang et al., 2003).

In this report, we have examined the effects of classical vitamin D_3 analogs and a novel Gemini analog on the activation of Smad signaling and TGF-β/BMP-induced transcriptional activation in MCF10AT1 breast epithelial cells. We report here on the ability of the vitamin D_3 analogs to activate BMP signaling by (a) inducing the phosphorylation of Smads 1/5, (b) increasing its localization in the nucleus, (c) enhancing BMP/Smad-mediated transcription, and (d) increasing synthesis of BMP-2 and BMP-6 mRNAs and proteins in MCF10 breast epithelial cells. This is the first study to show that vitamin D_3 analogs activate BMP/Smad signaling in breast epithelial cells.

Materials and Methods

Reagents. All vitamin D_3 analogs including 1α,25-dihydroxyvitamin D_3, Ro-26-2198 (1α,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor-cholecalciferol; Ro2198), and Ro-438-3582 [1α,25-dihydroxy-20S,21(3-hydroxy-3-methylbutyl)-23-yne-26,27-hexafluorocholecalciferol; Ro3582] (> 95% purity) were synthesized and provided by Dr. Milan Uskokovic at Hoffmann-La Roche Inc (Nutley, NJ). BMP-2 (> 95% purity), BMP-6 (> 95% purity) and Noggin (> 90% purity) were obtained from R & D systems (Minneapolis, MN). TGF-β1 and monoclonal anti-TGF-β1, β2, β3 (clone 1D11) were obtained from R & D systems (Minneapolis, MN). Fugene6 and okadaic acid were obtained from Roche Diagnostics (Indianapolis, IN) and Sigma (St. Louis, MO), respectively. All vitamin D_3 analogs were dissolved in dimethylsulfoxide (DMSO) before addition to cell cultures; final concentrations of DMSO were 0.1% or less. Controls with DMSO alone were run in all cases.

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Cell culture. Human breast MCF10AT1 cell line was developed and provided by Dr. Fred Miller's group at the Barbara Ann Karmanos Cancer Institute. MCF10AT1 cells were grown in complete media (DMEM/F12 supplemented with 5% horse serum, 1% penicillin/streptomycin, 10 μg/ml insulin, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, and 100 ng/ml cholera toxin) at 37°C, 5% CO_2.

Western Blot Analysis. These procedures have been described previously (Suh et al., 1998). The primary antibodies against VDR (Affinity BioReagents, Golden, CO), actin (Sigma, St. Louis, MO), phospho-Smad3 (Cell Signaling Technology Inc., Beverly, MA), phospho-Smad2 (Chemicon Inc, Temecula, CA), Smad2 (BD Biosciences, San Jose, CA), phospho-Smad1/5/8 (Cell Signaling Technology Inc., Beverly, MA), Smad3 (Zymed Laboratories, South San Francisco, CA), Smad4 (Santa Cruz Biotech., CA), and secondary antibodies (Santa Cruz Biotech., CA) were used. Cells were treated with test compounds and harvested at the time indicated in the Figure legends.

Quantitative RT-PCR Analysis. Total RNA was isolated from cultured cells using Trizol method from Invitrogen (Carlsbad, CA). One □g of total RNA is reverse-transcribed to cDNA using the random primers and Applied Biosystems' High Capacity cDNA Archive Kit in a 96-well format Mastercycler Gradient from Eppendorf North America (Westbury, NY). Quantitative real time PCR was performed using Applied Biosystems Taqman Gene Expression Assay reagents on an ABI Prism 7000 Sequence Detection System (Foster City, CA). Relative quantification was analyzed by the ABI Prism 7000 SDS software (Foster City, CA). The conditions were: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 seconds and 60°C for 1 min. Labeled primers for BMP-2, BMP-6, Smad6 and GAPDH were obtained from Applied Biosystems (Foster City, CA).

Immunoassay for BMP-2 and BMP-6. MCF10AT1 cells were plated in 100 mm dish and starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with the vitamin D analog Ro3582 (1 and 10 nM) in 0.1% BSA/DMEM/F-12 medium for 48 hr. The supernatant was collected and stored as aliquots at -20°C. The supernatant was concentrated by using Amicon Bioseparations Centricon YM-10 from Millipore Corporation (Bedford, MA) for 90 min. The BMP-2 and BMP-6 protein secreted into cell culture supernates was detected by Quantikine□ BMP-2 immunoassay and DuoSet□ Human BMP-6 from R & D systems (Minneapolis, MN), according to the manufacturer's instruction.

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Plasmids and Transfection Assays. 3GC2-Lux (Ishida et al., 2000) was provided by Dr. Kohei Miyazono (The University of Tokyo, Japan); pCMV-β-gal was provided by Dr. David Mangelsdorf (University of Texas Southwestern Medical School at Dallas, Texas). For transient transfection assays, cells (40,000 cells/well in 24 well plates) were plated and transfected with a total of 200 ng of DNA vectors, such as 3GC2-Lux (100 ng/well) or pCMV-β-gal (50 ng). Cells were transfected using Fugene6 in serum-free medium for 6 hr, and then replaced with fresh medium (0.1% BSA/DMEM) with test compounds, as previously published (Suh et al., 2003). Twenty-four hr later, cells were washed with PBS and lysed with 100 µl of 1X reporter lysis buffer (Promega Corp., Madison, WI). Luciferase values were analyzed using Veritas Microplate Luminometer (Turner Biosystems, CA) and normalized for β-galactosidase activity.

Phosphatase Assay. The serine/threonine phosphatase assay was performed according to the manufacturer's instruction (Promega Corp., Madison, WI). Briefly, MCF10AT1 cells were grown in growth medium to subconfluency, collected, centrifuged, and homogenized in cell extraction buffer (50 mM Tris-HCl, pH 7.0, 0.1% (v/v) β-mercaptoethanol, 0.1 mM EDTA, 0.1 mM EGTA, 25 µg/ml leupeptin, and 25 mg/ml aprotinin) on ice for 30 seconds. The homogenized lysate was centrifuged at 100,000 x g at 4^{o}C for 1 hr, and endogenous inorganic phosphate from the supernatant was removed using a Sephadex G-25 resin column. Test compounds were then added to the cell lysate, incubated for 10 min at 30^{o}C, mixed with molybdate dye/additive solution, and inorganic phosphate was determined by measuring the absorbance at 595 nm.

Fluorescence microscopy. Plates were coated with poly-D-lysine (0.1 mg/ml) overnight at 37^{o}C. Then, cells (30-50% confluent) were plated in appropriate medium as indicated. After incubation of cells with compounds for 24 hr, cells were fixed in 4% paraformaldehyde (1x PBS, pH 7.4) for 20 min at room temperature. Cells were then washed with PBS twice and blocked for 1 hr with 10% bovine serum albumin/0.5% Triton-X/1x PBS solution. The primary antibody solution (1:100 dilution for phospho-Smad1/5) was added to the plates, and the cells were incubated at 4^{o}C overnight or 3 hr at room temperature. The cells were then washed with PBS for 5 to 10 min. Fluorophore-conjugated secondary antibody (1:200 dilution, Alexa Fluor® 488 goal anti-rabbit IgG, Molecular Probes, OR) against the species of the primary antibody was added to the cells and incubated for 1-2 hr at room temperature. The

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cells were washed with 1x PBS three times for 15 min and irradiated with a green laser (488 nm). Fluorescence was viewed with a microscope. For DAPI staining, plain UV light (364 nm) was used.

Statistical Analysis. The statistical analysis was performed using the Students' t-test.

Results

Phosphorylation of Smad1/5 is increased by vitamin D_3 analogs in MCF10AT1 breast epithelial cells. Since TGF-β/BMP signal transduction is mediated by Smad proteins, we first evaluated the effects of 1α,25$(OH)_2D_3$ and vitamin D_3 analogs on the activation of Smad signaling. Receptor-regulated Smads (R-Smads), Smad2 or Smad3, are phosphorylated by the TGF-β type I receptor kinase, while Smad2 is also activated by the activin receptor. Other R-Smads, such as Smad1, Smad5 and Smad8, are phosphorylated by the BMP receptor kinase. Once R-Smads are activated by receptor kinases, they can form a heteromeric complex with the common partner Smad, Smad4, and the complex is then translocated into the nucleus to induce transcriptional activation of TGF-β, activin, or BMP specific genes. In our study on the effect of vitamin D_3 analogs on Smad signaling, we treated MCF10AT1 cells for 24 hr before harvest. For the BMP pathway, a single antibody that recognizes pSmad1(Ser463/465), pSmad5(Ser463/465), and pSmad8(Ser426/428) was used. All three of these Smads are direct targets of the BMP receptor kinase-mediated pathway. Therefore, measuring the phosphorylation of Smads1,5 and/or 8 together (Smad1/5/8) with a single antibody provides a measure of the activity of the BMP receptor kinase-mediated pathway. In the present study, the antibody for Smad1, 5, and/or 8 is measuring Smad1/5 since Smad8 has a different mobility on SDS gels. We found that treatment of MCF10AT1 cells with synthetic vitamin D_3 analogs, Ro2198 and Ro3582, had marked stimulatory effects on the phosphorylation of Smad1/5 at 24 hr that was dose-dependent (Fig. 2A). Both synthetic vitamin D_3 analogs caused a strong induction of phospho-Smad1/5 at 0.1, 1, and 10 nM, while the naturally occurring and active metabolite, 1α,25$(OH)_2D_3$, had a small increase at 10 nM (Fig. 2A). Furthermore, Ro3582 began to induce phosphorylation of Smad1/5 at 12 hr and the effect was much stronger at 24 hr (Fig. 2B). Activation of Smad2 or Smad3 was also measured with an antibody that recognizes phospho-Smad2 (Serine 465 and 467) or an antibody to phospho-Smad3 (Serine 433 and 435). Both

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phosphorylation sites in Smad2 and Smad3 are direct targets of the TGF-β type I receptor kinase. Using antibodies to Smad1/5 and Smad2/3, we determined that the vitamin D_3 analogs induced the phosphorylation of BMP R-Smads, Smad1/5, but not the phosphorylation of Smad2 or Smad3 (TGF-β/activin R-Smads), in MCF10AT1 cells (data not shown).

Upregulation of BMP signaling by the vitamin D_3 analog Ro3582 is not blocked by TGF-β neutralizing antibody but by a BMP antagonist, Noggin. As shown in Fig. 3A, we used the TGF-β antibody (1D-11) to determine whether the effects of Ro3582 are mediated by an increase in TGF-β synthesis in MCF10AT1 breast epithelial cells. The antibody to phospho-Smad3 used (Cell Signaling Technology Inc.) recognizes both phospho-Smad3 (lower band) and phospho-Smad1/5 (upper band). The phosphorylation of Smad3 was induced by TGF-β_1 (1 ng/ml), which is blocked by TGF-β neutralizing antibody (1D11, 50 μg/ml). However, we show here that the induction of the phospho-Smad1/5 by the vitamin D_3 analog Ro3582 (10 nM) or BMP-2 (100 ng/ml) is not blocked by the neutralizing antibody to TGF-βs. This suggests that the phosphorylation of Smad1/5 is not mediated by an effect of the vitamin D_3 analog on stimulating the synthesis of TGF-βs or to activate the TGF-β system. More importantly, we evaluated the effect of a specific antagonist of BMP, Noggin, to determine whether a BMP antagonist blocks the action of vitamin D derivatives. As shown in Fig. 3B, the phosphorylation of Smad1/5 induced by the vitamin D_3 analog Ro3582 (10 nM) or BMP-2 (100 ng/ml) was inhibited by the antagonist, Noggin [+, 60 ng/ml or ++, 300 ng/ml]. In contrast, the induction of phospho-Smad3 (the lower band) by TGF-β_1 (1 ng/ml) was not blocked by Noggin, while the induction of pSmad1/5 (the upper band) by Ro3582 was completely abolished by Noggin, confirming that induction of R-Smads by the vitamin D_3 analog Ro3582 is specific to BMP signaling.

Vitamin D-induced increase in phospho-Smad1/5 is not due to the inhibition of phosphatase activity. As described above and in Fig. 4A, we observed an increased amount of phospho-Smad1/5 in MCF10AT1 cells treated with the vitamin D_3 analog. Since it was reported that vitamin D_3 analogs might regulate phosphatase activity (Bettoun et al., 2004), we determined whether the increased amount of phospho-Smad1/5 might be due to inhibition of phosphatase activity in these cells. A serine/threonine phosphatase enzyme inhibitor, okadaic acid, is known to suppress the activity of protein phosphatases, mostly isotype PP2A, which accounts for most of the cellular activity. It has been shown that treatment of

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HL-60 leukemia cells with okadaic acid increased the level of phospho-Smad2 (Cao et al., 2003). In our study, okadaic acid did not significantly increase the phosphorylation of Smad2, Smad3, or Smad1/5 in MCF10AT1 cells, while the phosphorylation of Smad1/5 induced by the vitamin D_3 analog Ro3582 (10 nM) or BMP-2 (100 ng/ml) was shown (Fig. 4A). Secondly, we performed *in vitro* serine/threonine phosphatase enzyme assays to determine whether the vitamin D_3 analog Ro3582 inhibits phosphatase activity in MCF10AT1 cells. Using MCF10AT1 cell lysate as the source for the enzyme, Ro3582 (10, 100, and 1000 nM) or okadaic acid (OA, 10, 100, and 1000 nM) was added to the cell lysate and tested for their ability to inhibit phosphatase enzyme activity. Non-radioactive specific phosphopeptide RRA(pT)VA was used as a peptide substrate for serine/threonine protein phosphatases 2A, 2B and 2C. Okadaic acid inhibited the phosphatase activity dose dependently, whereas the vitamin D_3 analog Ro3582 did not inhibit the activity even at 1 μM (Fig. 4B).

Nuclear localization of phospho-Smad1/5 is induced by vitamin D_3 analog Ro3582 in MCF10AT1 breast epithelial cells. Nuclear localization of activated Smad1/5 by the vitamin D_3 analog Ro3582 in MCF10AT1 breast epithelial cells is shown in Fig. 5. The phosphorylation of Smad1/5 and its localization in the nucleus is markedly increased by treatment with the vitamin D_3 analog Ro3582. As shown in Fig. 5, there is very low staining of phospho-Smad1/5 in the control (DMSO treated) or Noggin control (300 ng/ml). However, when cells are treated with BMP-2 (100 ng/ml) or Ro3582 (1 nM), the phospho-Smad1/5 is mainly localized in the nucleus in the cells. Furthermore, when cells were treated with BMP-2 (100 ng/ml) or Ro3582 (1 nM) in the presence of the BMP antagonist Noggin, the accumulation of phospho-Smad1/5 in the nucleus induced by Ro3582 or BMP-2 was blocked. DAPI staining was used to recognize the nuclear morphology in cells.

The vitamin D_3 analog Ro3582 enhances BMP transcriptional activation in MCF10AT1 breast epithelial cells. Next, we determined whether increased phospho-Smad1/5 induced by the vitamin D_3 analog can lead to activation of BMP-mediated transcription, using a transfection assay with a GC binding element linked to luciferase. This construct, 3GC2-Lux, contains three repeats of the GC-rich sequence derived from the proximal BMP response element in the Smad6 promoter (Ishida et al., 2000), and is specific for response to BMPs. Human breast MCF10AT1 cells were transiently transfected with 3GC2-Lux vector and pCMV-β-gal. In the experiments shown in Fig. 6, cells were then treated with either DMSO

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or the vitamin D_3 analog Ro3582 (1, 0.1, or 0.01 nM), and incubated for 24 hr before assaying for luciferase activity. When the BMP-dependent response to Ro3582 was determined using 3GC2-Lux vector, the vitamin D_3 analog increased this activity by 3-fold at 1 nM.

The vitamin D_3 analog, Ro3582, not only induces the synthesis of mRNA and protein of both BMP-2 and BMP-6, but also inhibits the expression of Smad6 mRNA. In Fig. 7A, MCF10AT1 cells were treated with the vitamin D_3 analog Ro3582 (1 nM) for 4 or 12 hr, total RNA was harvested, and BMP-2, BMP-6 and Smad6 mRNA production was measured by quantitative PCR. We found that the vitamin D_3 analog Ro3582 (1 nM) induced BMP-2 and BMP-6 mRNA up to 3-fold and 10-fold, respectively, at 12hr. Further, it down-regulated mRNA for Smad6, an inhibitor of BMP-mediated R-Smads, compared to the DMSO control (Fig. 7A). We also determined that the vitamin D_3 analog Ro3582 induced the synthesis of BMP-2 and BMP-6 protein in MCF10AT1 cells, as shown by immunoassay for BMP-2 and BMP-6 (Fig. 7B). Approximately 130 and 360 picogram of BMP-2 protein, and 360 and 400 picogram of BMP-6 protein were synthesized from MCF10AT1 cells treated with the vitamin D_3 analog Ro3582 at 1 and 10 nM for 48 hr, respectively (Fig. 7B). We also determined whether the synthesis of BMP-2 and BMP-6 mRNA induced by Ro3582 may be blocked by Noggin. The induction of both BMP-2 mRNA and BMP-6 mRNA production was significantly reversed by Noggin at 24 hr (Fig. 7C).

Discussion

BMPs are members of the TGF-β superfamily that have multiple functions in development, bone formation, and tissue remodeling (Reinholz et al., 2002; Miyazono et al., 2005). Recently, BMPs have been identified as multifunctional regulators of proliferation, differentiation and apoptosis, and the role of the BMP/Smad pathway in cancer is now being discussed intensively (Villanueva et al., 1998; Ghosh-Choudhury et al., 2000; Reinholz et al., 2002; Miyazaki et al., 2004). In this report, we investigated the action of vitamin D derivatives on Smad signaling proteins, which are known to be important cytoplasmic mediators of signals from the TGF-β/activin/BMP receptor serine/threonine kinases. We showed that the synthetic vitamin D_3 analog Ro3582 activated the BMP-specific Smad signaling system in MCF10 breast epithelial cells. The vitamin D_3 analog increased phosphorylation of receptor-regulated Smad1/5 (Fig. 2),

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translocated phosphorylated Smad1/5 into the nucleus (Fig. 5), and enhanced Smad1/5-dependent activation of BMP-mediated gene transcription (Fig. 6). We also found that the vitamin D derivative induced the synthesis of mRNA and protein of BMP-2 and BMP-6, and reduced the expression of Smad6 mRNA (Fig. 7). Furthermore, our results showed that Noggin reversed the phosphorylation of Smad1/5 induced by Ro3582 (Fig. 3), and inhibited the nuclear localization of phospho-Smad1/5 by the vitamin D_3 analog Ro3582 (Fig. 5). In addition, the induction of BMP-2 and BMP-6 mRNA synthesis was blocked by Noggin (Fig. 7C). This is the first report demonstrating the activation of Smad1/5 by vitamin D_3 analogs as well as cross-talk between BMP and vitamin D signaling pathways in breast epithelial cells.

Interactions between the nuclear receptor family and TGF-β superfamily have been investigated for many years, and multiple levels of interactions between steroid receptors and the TGF-β superfamily have been identified. Steroids, such as vitamin D_3 analogs and retinoids, are known to enhance the response to TGF-β/BMP by inducing the synthesis of more TGF-β/BMP ligands and their receptors (Falk et al., 1991; Koli and Keski-Oja, 1995; Hatakeyama et al., 1996; Hallahan et al., 2003). Furthermore, there is now an abundant literature on the interaction of steroids and their nuclear receptors with Smads (Yanagisawa et al., 1999; Yanagi et al., 1999; Matsuda et al., 2001). In a previously reported study (Cao et al., 2003), it was shown that certain vitamin D_3 analogs induced phospho-Smad2 in myeloid leukemia cells, and that phosphorylation of Smad2 is a critical sensor for the differentiation of these cells. In MCF10 breast epithelial cells, we have shown that vitamin D_3 analogs activated the BMP/Smad system by selectively phosphorylating Smad1/5 and increasing its nuclear localization.

Since it has been shown that C-terminal phosphorylation of R-Smads by the TGF-β type I receptor is a key event in the activation of Smads (Derynck and Zhang, 2003), we have utilized the antibody against Ser433/435 phosphorylation at the C-terminal domain of Smad3 and the antibody to pSmad1(Ser463/465)/ pSmad5(Ser463/465)/ pSmad8(Ser426/428) to determine the effects of the vitamin D_3 analogs on the TGF-β/BMP signaling system in MCF10 epithelial cells. Using these antibodies to the phospho-Smads, we found that the vitamin D_3 analog Ro3582 increased the phosphorylation of R-Smads responsible for activating the BMP pathway, namely Smad1/5, but not R-Smads of the TGF-β/activin pathways, Smad2 and Smad3. In the present study, we showed that activation of R-Smads by a vitamin D derivative was specific to BMP signaling, as its response was abrogated by the BMP antagonist,

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Noggin. The selectivity of vitamin D_3 analogs to increase the phosphorylation of Smad1/5, but not Smad3 or Smad2 in MCF10 breast epithelial cells is of considerable interest, but further research is needed to determine the mechanism of this selectivity.

The vitamin D_3 analogs clearly increased the phosphorylation of Smad1/5 and further induced the translocation of Smad1/5 into the nucleus. It is known that R-Smads, such as Smad3 or Smad5, do not require Smad4 for nuclear translocation, although Smad4 can co-translocate with the R-Smads. In our study, we found that Smad4 also translocated into the nucleus when the nuclear localization of phospho-Smad1/5 was induced by vitamin D_3 analogs (data not shown). While treatment of the cells with TGF-□, BMP-2 or BMP-6 induces the phosphorylation of R-Smads within 30 min and the activation goes down gradually by 24 hr, the vitamin D_3 analog Ro3582 did not induce the phosphorylation of Smad1/5 at 30 min (data not shown). A time course study indicated that the vitamin D_3 analog started to increase Smad1/5 phosphorylation at 12 hr, and increased the level of phosphorylation markedly by 24 hr. These results indicate that activation of Smad1/5 by the vitamin D_3 analogs may not be caused by a direct action on Smad1/5 molecules, but it requires time for the activation or synthesis of the kinase that phosphorylates Smad1/5.

The possible mechanism of activation of Smad1/5 is that the vitamin D derivative functions by inhibiting phosphatases that dephosphorylate phospho-Smad1/5. However, this is not the case for the action of the vitamin D derivative in these MCF10 breast epithelial cells, as shown in Fig. 4. It is more likely that vitamin D analogs activate BMP-specific signaling by increasing the production of BMPs or by down-regulating the inhibitory system of the signaling such as Smad6. The results in Fig. 7 show that Ro3582 not only increased mRNA expression and protein synthesis of BMP-2 and BMP-6, but also reduced the Smad6 mRNA level. We also determined the expression level of BMP-7, but the basal level was too low to be detected by quantitative real time RT-PCR in MCF10AT1 cells (data not shown). These results support the hypothesis that vitamin D_3 analogs activate BMP-specific signaling by increasing the synthesis of BMP-2 and BMP-6, but not BMP-7, as well as by down-regulating Smad6. Smad6 was identified as an inhibitory Smad by inhibiting the BMP/Smad signaling. It binds to BMP receptor type I and blocks the phosphorylation of Smad1/5 (Imamura et al., 1997; Chen et al., 2004), and it also inhibits the complex formation of Smad1 and Smad4 by binding to activated Smad1 (Hata et al., 1998). The activity

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of Smad ubiquitin regulatory factor 1 (Smurf1) is also enhanced through interacting with Smad6 (Murakami et al., 2003).

A potential role for the BMP/Smad pathway during the progression of estrogen receptor-positive breast cancer has been recently reported (Helms et al., 2005). However, the anti-proliferating and pro-apoptotic effects of BMPs are reported in breast cancer cell lines, and significantly decreased expression of BMP-2 has been shown in non-invasive, invasive, and liver metastatic breast tumor tissue compared to normal breast tissue (Reinholz et al., 2002), suggesting a possible role of the BMP-2 as a tumor suppressor. Since we have shown that the vitamin D system interacts with BMP signaling, it will be interesting to determine whether vitamin D_3 analogs induce growth suppression in breast cancer by the activation of BMP signaling, and eventually can be used as potent chemopreventive agents in breast cancer.

In conclusion, the present study provides the first demonstration of effects of 1□,25$(OH)_2D_3$ and vitamin D derivatives to enhance BMP/Smad signaling in breast epithelial cells. This effect occurs by inducing the phosphorylation of Smad1/5 in the cytoplasm followed by its translocation to the nucleus where it may interact with the VDR or other molecules for transcription. Since breast cancer mainly metastasizes to the bone where BMPs play an important role in bone formation and bone metastasis, the enhancement of BMP signaling by vitamin D derivatives may be an important aspect of their anti-proliferative, differentiating, apoptotic or anti-metastatic effects. An understanding of the functional significance of enhanced BMP signaling will require further investigation and may depend on the cell type and whether it is at an early or late stage of carcinogenesis.

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Footnotes

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FIGURE LEGENDS

Figure 1. Structures of 1α,25-dihydoxyvitamin D_3 (VD_3, calcitriol, active vitamin D_3 metabolite), and its synthetic vitamin D_3 analogs, Ro-26-2198 (1α,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor-cholecalciferol; Ro2198, classical vitamin D_3 synthetic analog) and Ro-438-3582 (1α,25-dihydroxy-20S-21(3-hydroxy-3-methyl-butyl)-23-yne-26,27-hexafluorocholecalciferol; Ro3582, synthetic Gemini analog).

Figure 2. Upregulation of BMP signaling by 1α,25-dihydroxyvitamin D_3 and vitamin D_3 analogs in MCF10AT1 breast epithelial cells. **A.** MCF10AT1 cells (5 X 10^5 cells/6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with DMSO, 1α,25$(OH)_2D_3$, Ro2198, or Ro3582 (0.1, 1, 10 nM) in 0.1% BSA/DMEM/F-12 medium for 24 hr. Upregulation of the phospho-Smad1/5 by vitamin D_3 analogs is shown. **B**. MCF10AT1 cells (5 X 10^5 cells/6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with DMSO or Ro3582 (10 nM) in 0.1% BSA/DMEM/F-12 medium for 6, 12, and 24 hr. The phosphorylation of Smad1/5 was upregulated by Ro3582 in a time-dependent manner.

Figure 3. The phosphorylation of Smad1/5 induced by the novel vitamin D_3 analog, Ro3582, is not blocked by TGF-β neutralizing antibody, but by a BMP antagonist, Noggin. **A.** MCF10AT1 cells (5 X 10^5 cells/6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with the indicated compounds in 0.1% BSA/DMEM/F-12 medium for 24 hr. The phosphorylation of Smad3 induced by TGF-β_1 (0.1 and 1 ng/ml) is blocked by TGF-β neutralizing antibody (TGF-β Ab, 1D11, 50 μg/ml). The induction of the phospho-Smad1/5 by Ro3582 (10 nM) or BMP-2 (100 ng/ml) is not blocked by the neutralizing antibody to TGF-β. **B.** MCF10AT1 cells (5 X 10^5 cells/6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with the indicated compounds in 0.1% BSA/DMEM/F-12 medium for 24 hr. The phosphorylation of Smad1/5 induced by the vitamin D_3 analog Ro3582 (10 nM) or BMP-2 (100 ng/ml) was blocked by treatment with Noggin, while the induction of the phospho-Smad3 by TGF-β_1 (1 ng/ml) was not abolished by Noggin (+, 60 ng/ml, ++, 300 ng/ml).

Figure 4. Increased phospho-Smad1/5 is not due to the inhibition of phosphatase activity. **A.** MCF10AT1

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cells (1 X 10^6 cells/dish) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with the indicated compounds in 0.1% BSA/DMEM/F-12 medium for 24 hr. The phosphorylation of Smad1/5 induced by the vitamin D_3 analog Ro3582 (10 nM) or BMP-2 (100 ng/ml) was shown, whereas TGF-β_1 (1 ng/ml) or Okadaic acid (10 and 100 nM) did not increase phospho-Smad1/5 in these cells. **B.** *In vitro* phosphatase assay was carried out using MCF10AT1 cell lysate. Ro3582 (10, 100, and 1000 nM) or okadaic acid (OA, 10, 100, and 1000 nM) was added to the cell lysate and inhibition of phosphatase enzyme activity was determined using non-radioactive specific phosphopeptide RRA(pT)VA, a peptide substrate for serine/threonine protein phosphatases 2A, 2B and 2C.

Figure 5. Nuclear localization of phospho-Smad1/5 induced by the vitamin D_3 analog Ro3582 in MCF10 breast epithelial cells. MCF10AT1 cells (30,000/chamber) were incubated with Ro3582 (1 nM) or BMP-2 (100 ng/ml) in the presence and absence of BMP antagonist, Noggin (300 ng/ml), in 0.1% BSA/DMEM/F-12 medium for 24 hr. The staining for phospho-Smad1/5 is shown as green and DAPI staining for the nucleus is shown as blue. Immunofluorescence microscopy was shown (63 X magnification).

Figure 6. The synthetic vitamin D_3 analog, Ro3582, enhances BMP-dependent transcriptional activation. **A**. MCF10AT1 cells (40,000/well in 24 well plates) were plated and transfected with 3GC2-Lux vector (100 ng/well) for 6 hr and further treated with Ro3582 at concentrations of 1, 0.1, and 0.01 nM for 24 hr. Luciferase values were normalized for β-galactosidase activity.

Figure 7. The vitamin D_3 analog, Ro3582, induces the mRNA and protein synthesis of BMP-2 and BMP-6, and also inhibits the mRNA expression of Smad6. **A.** MCF10AT1 cells (1 X 10^6 cells/100 mm dish) were incubated with the vitamin D_3 analog Ro3582 (1 nM) in DMEM/F-12 medium supplemented with 5% horse serum for 4 or 12 hr. Total RNA was isolated, and the measurement of BMP-2, BMP-6 and Smad6 mRNA was performed as described in the Materials and Methods. GAPDH values were used to normalize the production of the mRNA. Two separate experiments were performed. **B.** MCF10AT1 cells (5 X 10^5 cells/100 mm dish) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with Ro3582 (1 and 10 nM) in 0.1% BSA/DMEM/F-12 medium for 48 hr. The supernatant was collected and stored at –20^{o}C until assayed. The BMP-2 and BMP-6 protein synthesized and secreted into the

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medium were measured by the immunoassay using a BMP-2 and BMP-6 standard. Representative data are shown from two similar experiments. **C.** MCF10AT1 cells (1 X 10^6 cells/100 mm dish) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with the vitamin D_3 analog Ro3582 (10 nM) with or without Noggin (300 ng/ml) in DMEM/F-12 medium supplemented with 0.1% BSA for 12 or 24 hr. Total RNA was isolated, and the measurement of BMP-2 and BMP-6 mRNA was performed as described in the Materials and Methods. GAPDH values were used to normalize the production of the mRNA. Two separate experiments were performed (statistical significance, $^*p< 0.05$, $^{**}p< 0.01$).

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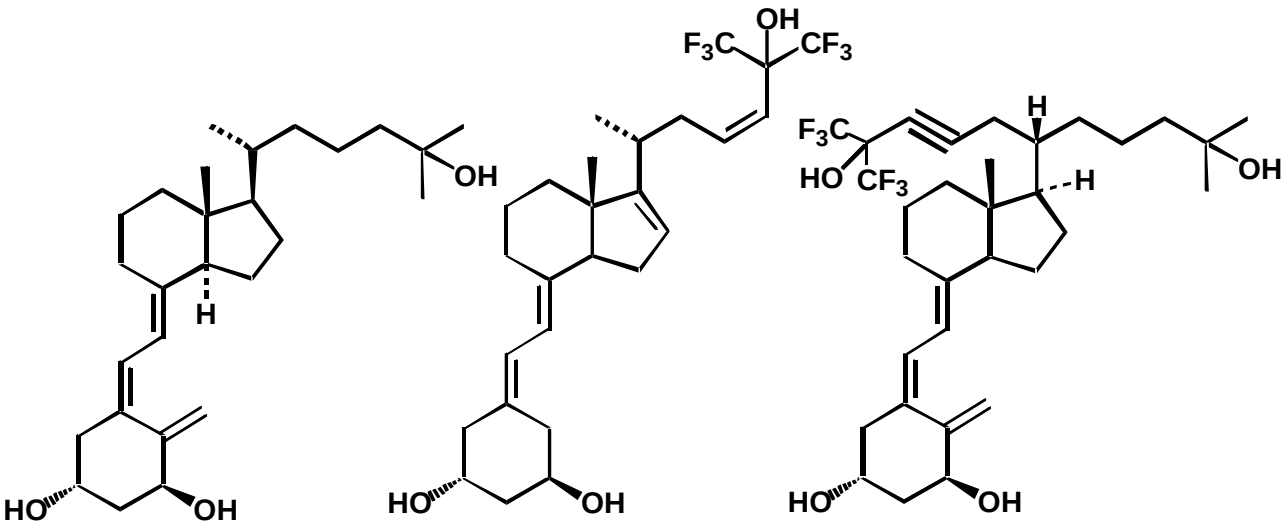

1α,25-(OH)$_2$-D$_3$ (VD$_3$) **Ro-26-2198 (Ro2198)** **Ro-438-3582 (Ro3582)**

Figure 1

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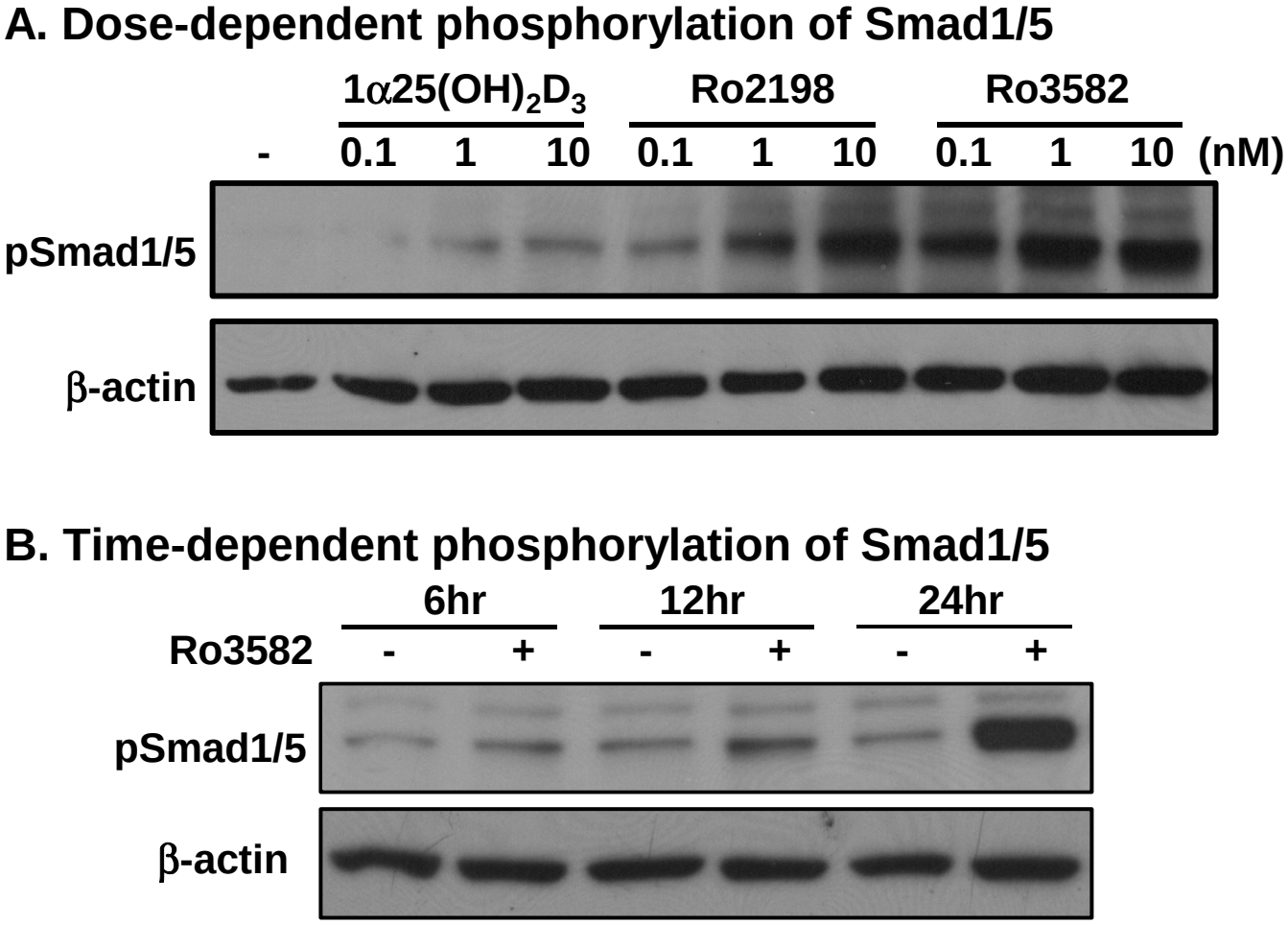

Figure 2

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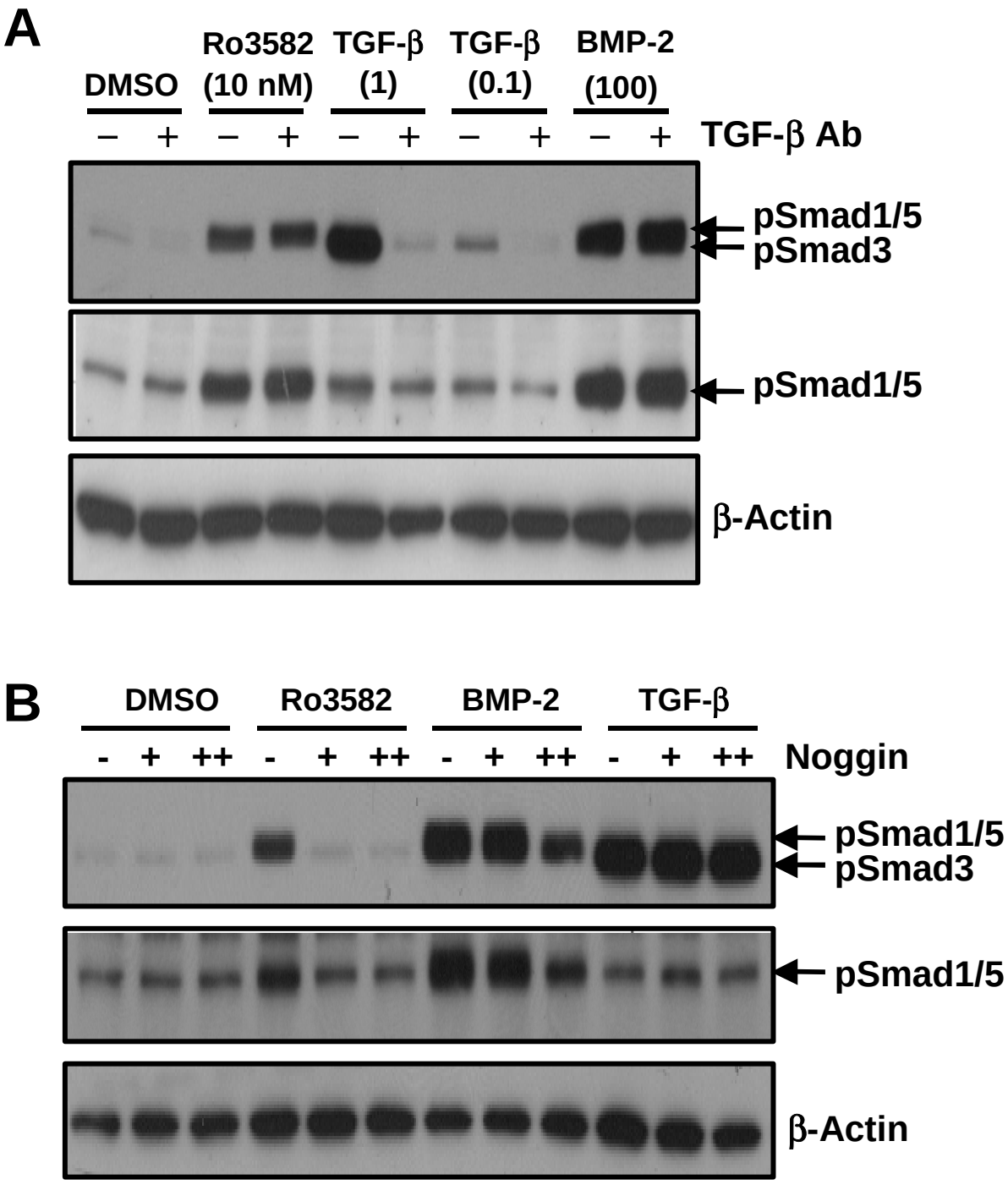

Figure 3

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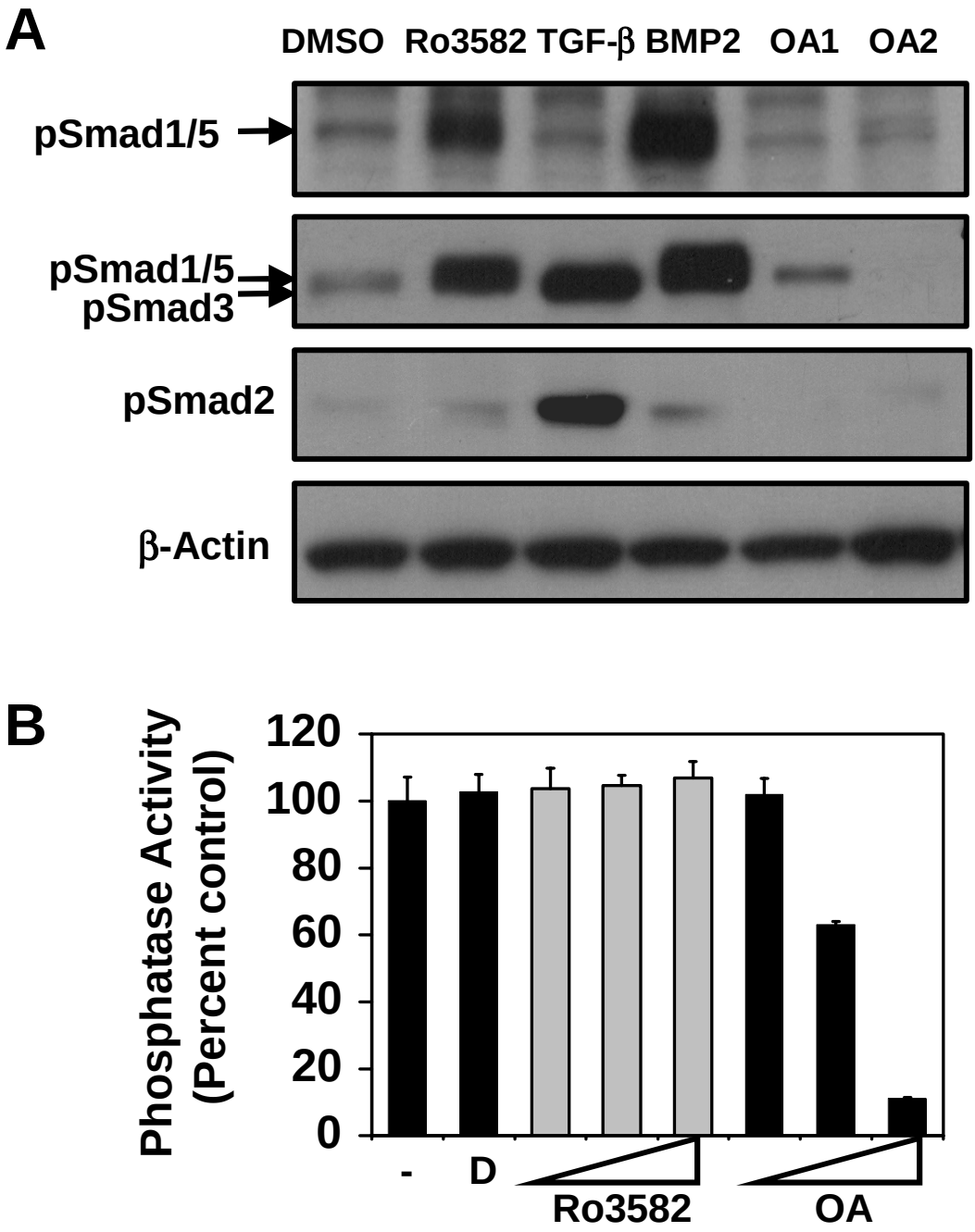

Figure 4

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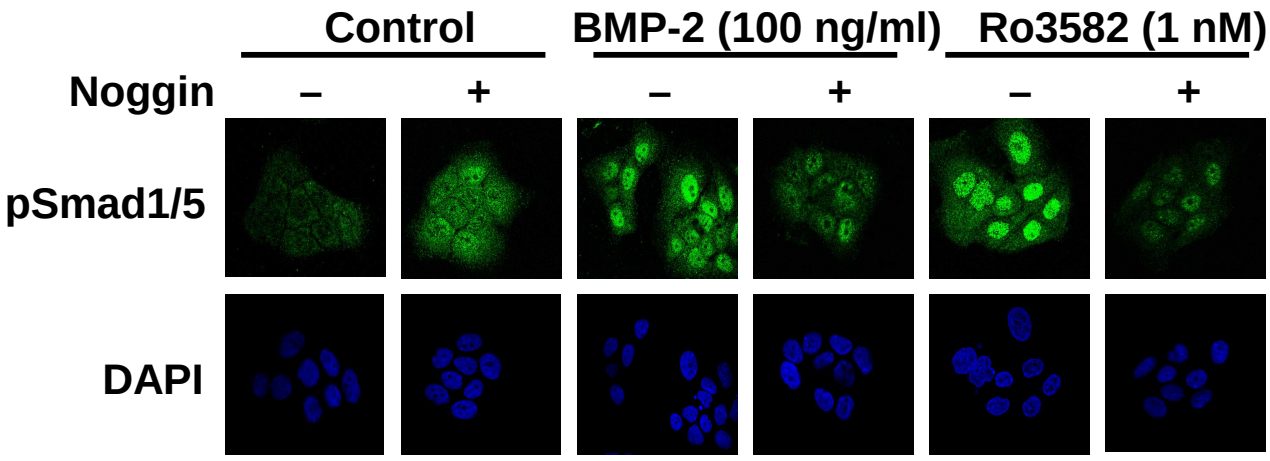

Figure 5

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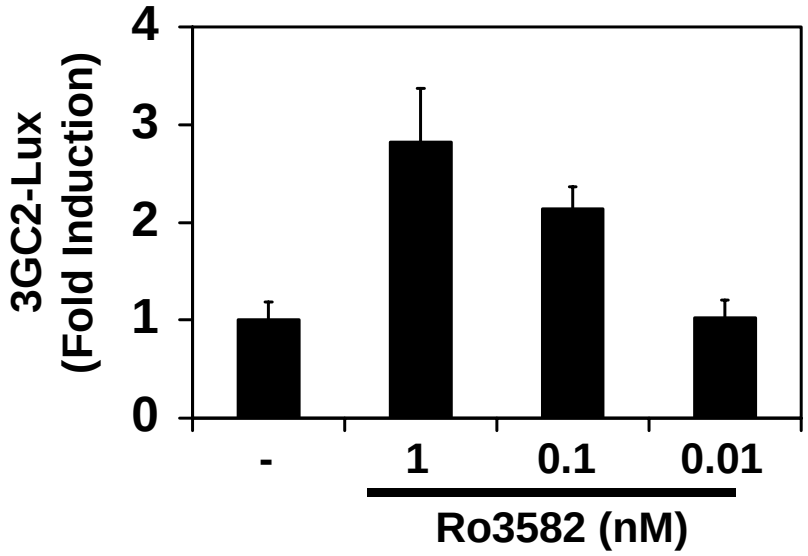

Figure 6

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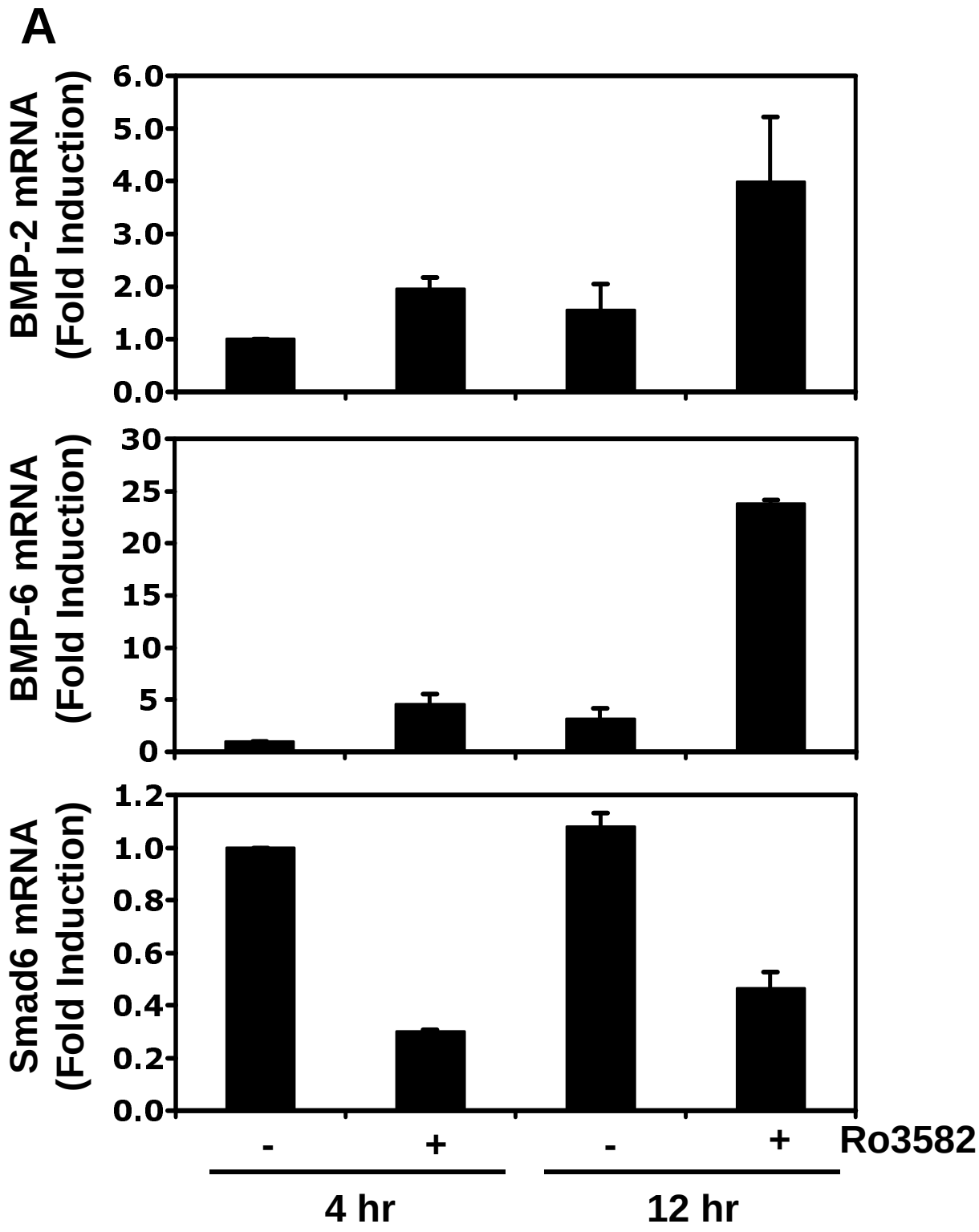

Figure 7

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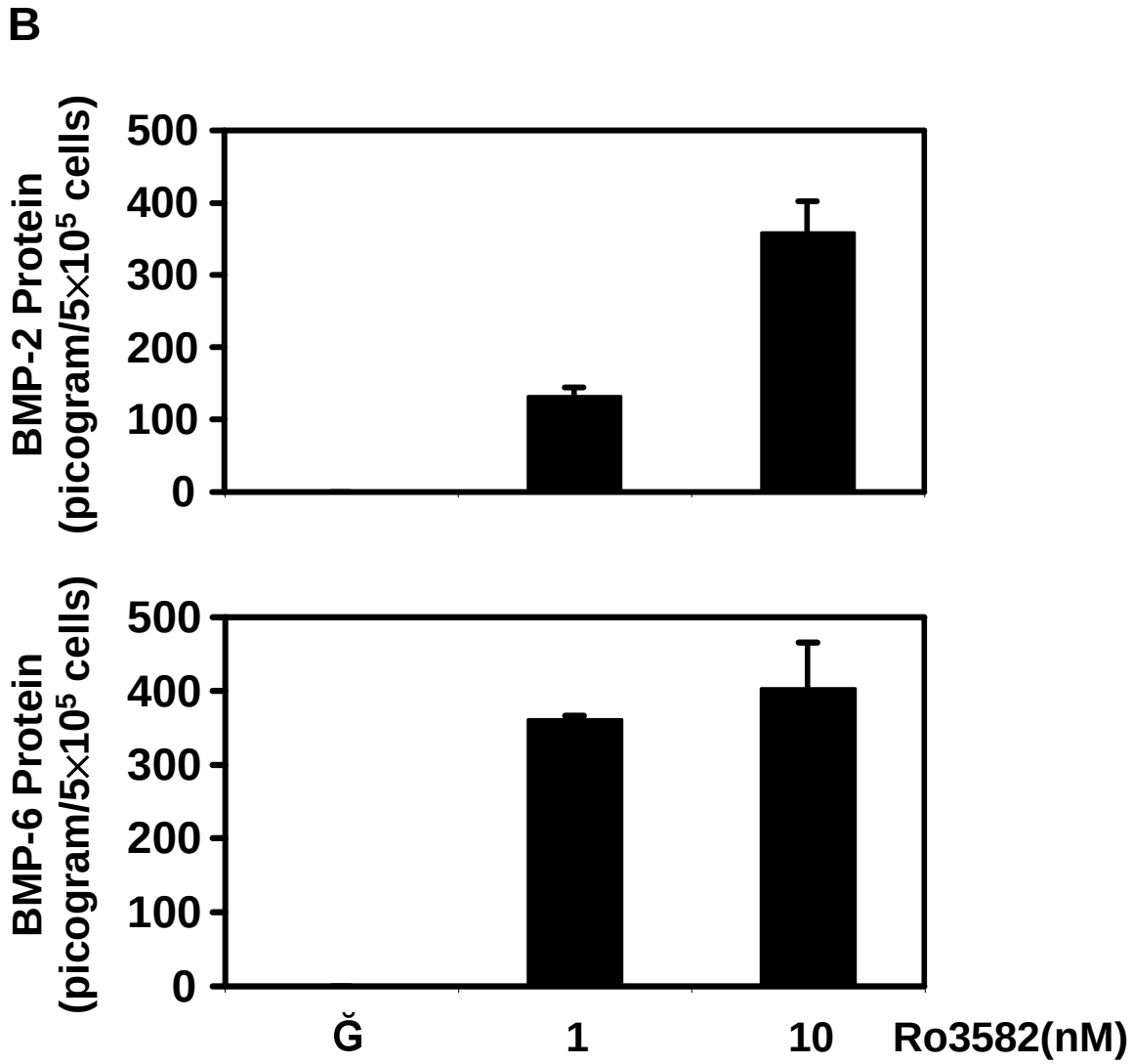

Figure 7

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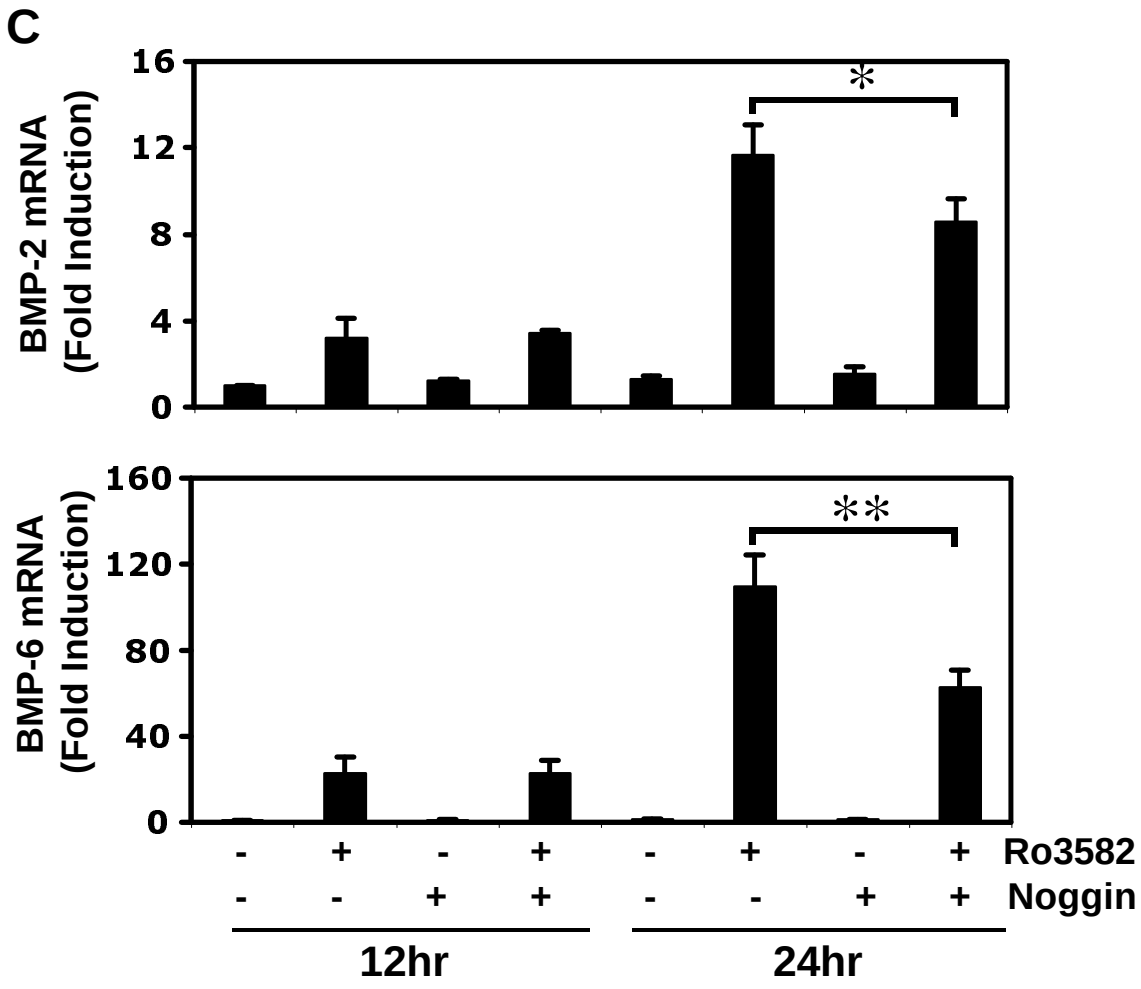

Figure 7