tors (15, 16). To date, five proteins with conserved homology in the Rel domain (p65 (RelA), RelB, cRel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100)) have been identified. The activity of NF-κB that results from cell stimulation is tightly regulated by the shuttling of NF-κB from the cytoplasm to the nucleus. In unstimulated cells, NF-κB is predominantly localized in the cytoplasm as part of a complex with inhibitory IκB proteins, including IκBα, IκBβ, IκBε, and IκBγ. In response to a variety of stimuli, such as TNFα or IL-1β, IκBs are phosphorylated (Ser-32 and Ser-36 for IκBα and Ser-19 and Ser-21 for IκBβ) by the activated IκB kinase (IKK) complex. Phosphorylated IκBs are ubiquitinated and then degraded by the 26 S proteasome. The IKK complex consists of two catalytic kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit, NEMO (NF-κB essential modulator), also called IKKγ (15, 16). IKKβ is most critical for the classical (canonical) NF-κB pathway that depends on IκB degradation. In this pathway, the p50/p65 heterodimer enters the nucleus and binds to NF-κB-responsive elements to regulate the expression of genes that are involved in the regulation of immune and inflammatory responses, proliferation, tumorigenesis, and survival (15, 16).

In contrast to NF-κB, BMP signaling provides anti-proliferative differentiation signaling in osteoblasts and in other tissues (17). Furthermore, BMP, via both Smads and Smad-independent mechanisms, inhibits the cell cycle and increases apoptosis by regulating pro-apoptotic proteins (18, 19). Together, the BMP/Smad and NF-κB signaling systems seem to exert antagonistic effects. Therefore, in this study, we assessed the molecular mechanisms underlying TNFα/NF-κB-mediated regulation of BMP-dependent Smad signaling and BMP2-induced osteoblast differentiation.

EXPERIMENTAL PROCEDURES

Reagents—Purified recombinant human BMP2 was provided by Wyeth Pharmaceuticals (Madison, NJ). Recombinant human TNFα was purchased from PeproTech Inc. (Rocky Hill, NJ). Anti-Smad1 (sc-7965), anti-Smad4 (sc-7154), anti-IκBα (sc-371), anti-HDAC1 (sc-7872), anti-β-actin (sc-8432), anti-IgG antibodies p65siRNA (sc-29411), and siRNA transfection reagent (sc-2528) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). BAY11-7082 and anti-p65 antibodies and anti-phosphorylated-Smad1,5,8 antibodies, were obtained from Biomol (Plymouth Meeting, PA) and Cell Signaling (Beverly, MA), respectively.

Cell Culture Conditions—Mouse osteoblastic cell line MC3T3-E1 cells were maintained in an α-modified minimum essential medium (α-MEM) with 10% heat-inactivated fetal bovine serum (FBS) with 100 units/ml penicillin and 100 μg/ml streptomycin. p65-deficient mouse embryonic fibroblasts ($p65^{-/-}$ MEFs) were prepared from p65-deficient mice (20) and maintained in Dulbecco's modified Eagle's medium with 5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. These cells were cultured under 5% CO_2 at 37 °C.

Alkaline Phosphatase (ALP) Activity and Staining—MC3T3-E1 cells were seeded at a density of 1.0×10^4 cells/well in 96-well plates with α-MEM containing 10% FBS the day before treatment. The cells were pretreated with TNFα (10 ng/ml) for 30 min or left untreated and were subsequently treated with BMP2 (100 ng/ml) for 72 h. After the 72-h treatment, the cells were fixed with an acetone/ethanol mixture (50:50, v/v) and then incubated with a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$, and 10 mg/ml *p*-nitrophenyl phosphate). The reaction was terminated by adding 5 M NaOH, and then the absorbance was measured at 405 nm using a microplate reader (Bio-Rad). To determine ALP activity histochemically, cells were stained for enzyme activity as previously described (5).

Transfection Conditions and Luciferase Assay—MC3T3-E1 cells and $p65^{-/-}$ MEFs were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total amounts of transfected plasmids in each group were equalized by the addition of an empty vector. BMP signaling was monitored using the IdWT4F-luciferase reporter plasmid, which expresses a luciferase protein under the control of a BMP-responsive element in the human *ID1* gene, as described previously (21). The pNF-κB luciferase plasmid was purchased from Promega (Madison, WI). Luciferase activities were measured using a dual luciferase system (Promega).

Immunoprecipitation and Immunoblotting—Cells were lysed in TNT buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol) containing protease inhibitors (Roche, Basel, Switzerland). Cytosolic and nuclear fractions were prepared according to methods described by Dignam *et al.* (21). For co-precipitation experiments, whole cell extracts were incubated for 6 h at 4 °C with anti-Smad1, anti-Smad4, or anti-p65 antibodies coupled to A/G-Sepharose beads. The immune complex was extensively washed with TNT buffer, and then the samples were boiled and analyzed by immunoblotting. Protein content was measured with Pierce reagent following the manufacturer's protocol. Twenty micrograms of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to a polyvinylidene difluoride membrane at 100 V for 1 h at 4 °C. These membranes were incubated with antibodies at dilutions of 1:500 to 1:1,000 in a 5% dry milk solution containing 0.01% azide overnight at 4 °C. Subsequently, the blots were washed in TTBS (10 mM Tris-HCl, 50 mM NaCl, 0.25% Tween 20) and incubated with a horseradish peroxidase-conjugated secondary antibody. The immunoreactive proteins were visualized using ECL (Amersham Biosciences, Piscataway, NJ).

Chromatin Immunoprecipitation (ChIP) Assays—ChIP was performed with a ChIP Assay Kit (Upstate Biotechnology, Waltham, MA), according to the manufacturer's instructions, using antibodies against phosphorylated Smad1,5,8, p65 antibodies or normal IgG. The purified DNA was analyzed by PCR using primers that amplify sequences containing the Id1 promoter, which harbors a BMP2-responsive element (BRE) to which Smad proteins bind (21). The primer pairs for the Id1, Smad6, and IκBα promoters were 5′-TAAGTTGACCCTTGGTCAGC-3′ (forward) and 5′-GACGTCACCCATTCATAAAAC-3′ (reverse), 5′-CATCCCTAGTGTATCCAACAAAGAG-3′ (forward) and 3′-AGCTCAAGACGGTCACGTACTC-3′ (reverse), and 5′-GCTCATCAAAAAGTTCCCTGTGC-3′ (forward) and 5′-TGGCGAGGTCTGACTGTTGTGG-3′ (reverse), respectively.

Electrophoresis Mobility Shift Assay—EMSAs were performed using MC3T3-E1 cells pretreated with, or without, TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for 30 min. Nuclear extracts were prepared from the pretreated cells. The Smad DNA binding activity in the nuclear fractions was measured by EMSA. The double-stranded BRE probe and the κB probe were radiolabeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (New England Nuclear, Boston, MA) using a labeling kit (Takara Shuzo Co.). The sequences of the oligo DNAs we used were as follows: 5′-TCTCCATGGCGACCGCCCGCGCGGCGCCAGCCT-3′ (BRE probe) and 5′-GATCAGAGGGGACTTTCCGAGG-3′ (κB probe). Six micrograms of the nuclear extracts were incubated with the labeled BRE probe. For the supershift experiments, antibodies were added prior to the addition of the probe, and the mixture was incubated for 15 min at room temperature. Antibodies against Smad1 (sc-7154X) and Smad4 (sc-6201X) were obtained from Santa Cruz Biotechnology. The reaction mixture was loaded onto a 5% polyacrylamide gel in 0.5 × TBE (44.5 mM Tris base, 44.5 mM boric acid, and 1 mM EDTA) and resolved by electrophoresis.

RT-PCR—For semi-quantitative RT-PCR, total RNA form MC3T3-E1 cells prepared with TRIzol (Invitrogen) was amplified by Superscript II and Taq polymerase (Invitrogen). Primer sequences were as follows: Id1: 5′-TCCTGCAGCATGTAATCGAC-3′ (forward) and 5′-GAGAGGGTGAGGCTCTGTTG-3′ (reverse), ALP: 5′-ACTGCTGATCATTCCCACGTT-3′ and (forward), 5′-GAACAGGGTGCGTAGGGAGA-3′ (reverse), osterix: 5′-GCGTCCTCTCTGCTTGA-3′ (forward) and 5′-TGTGTTGCCTGGACCTGGTG-3′ (reverse), and osteocalcin : 5′-AAGCAGGAGGGCAATAAGGT-3′ (forward) and 5′-TTTGTAGGCGGTCTTCAAGC-3′ (reverse); GAPDH: 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ (forward) and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (reverse).

Statistical Analysis—Statistical significance was determined using the Student's *t* test. A *p* value of less than 0.05 was considered significant.

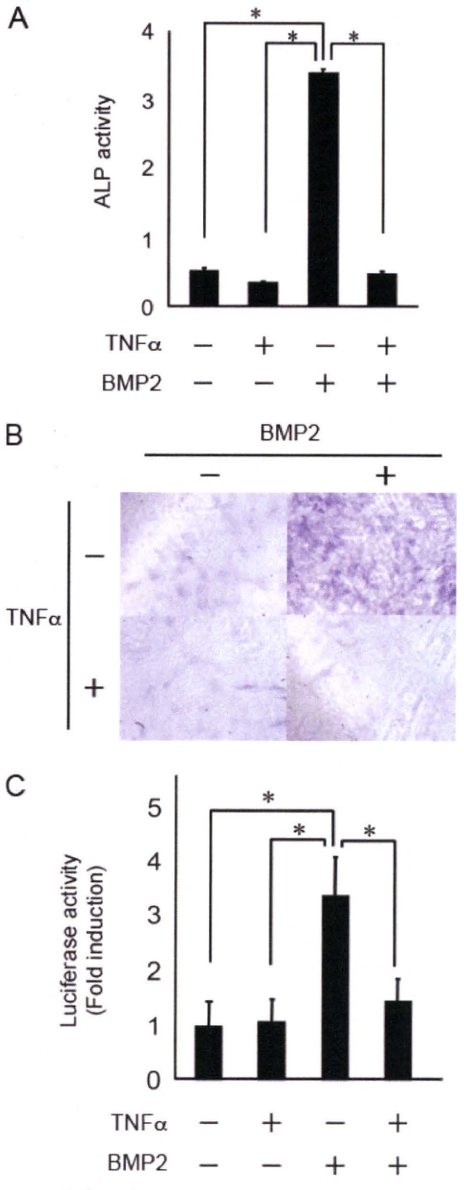

FIGURE 1. **TNFα inhibited BMP2-induced osteoblast differentiation in MC3T3-E1 cells.** *A*, MC3T3-E1 cells were pretreated with or without TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for 72 h. The cells were fixed with an acetone/ethanol mixture (50:50, v/v) and were then incubated with a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$, 10 mg/ml *p*-nitrophenyl phosphate). ALP activity was then determined. *B*, cells were stained for ALP activity. *C*, MC3T3-E1 cells were transiently transfected with the Id1-luc reporter plasmid. After 24 h, the cells were pretreated with or without TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for 24 h. Luciferase activity was determined. Data are means + S.E. ($n = 3$). Similar results were obtained in three independent experiments. *, $p < 0.01$.

RESULTS

TNFα Inhibited BMP2-induced Osteoblast Differentiation—We first examined the effect of TNFα on the BMP2-induced ALP activity, a typical marker of osteoblast differentiation, in MC3T3-E1 cells. BMP2 induced a ~7-fold increase in ALP activity, and there were many more ALP-positive cells compared with cells cultured with the control levels of BMP2. Consistent with previous reports (10, 11, 22), this induction was completely abolished by pretreatment with TNFα (Fig. 1, *A* and *B*).

To precisely determine whether TNFα inhibits BMP signaling via Smad-dependent or Smad-independent mechanisms, we examined the inhibitory effect of TNFα on BMP2-induced Id1-luciferase activity. The Id1-luciferase reporter construct contains a consensus Smad binding site that drives the expression of the luciferase gene (21). BMP2 induced a ~3–4-fold increase in Id1-luciferase activity compared with controls, and pretreatment with TNFα strongly inhibited the luciferase activity induced by BMP2 (Fig. 1*C*). These results demonstrate that TNFα inhibits osteoblast differentiation and BMP2-induced Id1-luciferase activity by inhibiting the Smad-dependent pathway.

TNFα Did Not Affect the Phosphorylation or Nuclear Translocation of Smad1,5,8 Induced by BMP2—Upon binding of the BMP ligand to the type I and type II receptor complexes, the activated type I receptor phosphorylates Smad1,5,8, which then assemble into complexes with Smad4 and translocate into the nucleus. In the nucleus, the Smad complexes regulate the expression of genes related to osteoblast differentiation, such as

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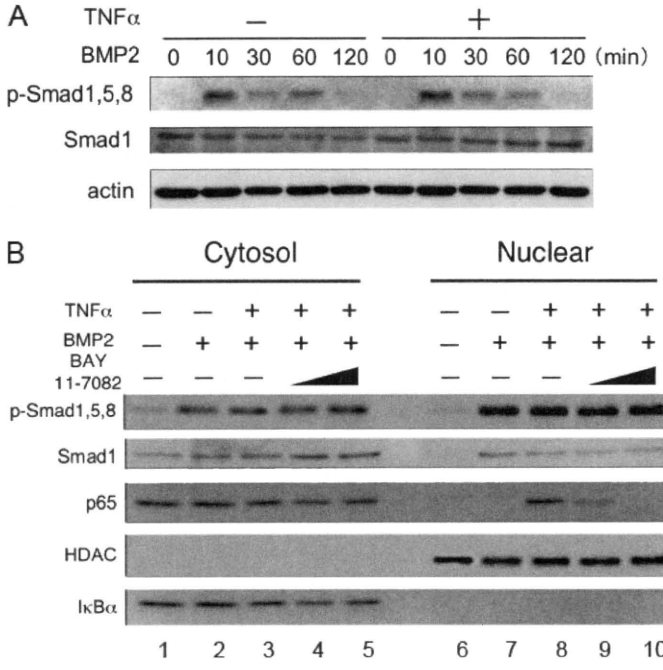

FIGURE 2. **TNFα did not affect the phosphorylation or the nuclear translocation of Smad1,5,8 induced by BMP2.** *A*, MC3T3-E1 cells were pretreated with or without TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for the indicated time. Total cell lysates were immunoblotted with anti-phosphorylated Smad1,5,8 antibodies, and anti-β-actin was used as a loading control. *B*, MC3T3-E1 cells were pretreated with or without TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for 1 h. Cytosolic and nuclear fractions were prepared, and then lysates were immunoblotted with anti-phosphorylated Smad1,5,8, and anti-p65 antibodies. Anti-IκBα antibodies and anti-HDAC antibodies were used to probe the cytosolic fraction and nuclear fraction, respectively. Similar results were obtained in three independent experiments.

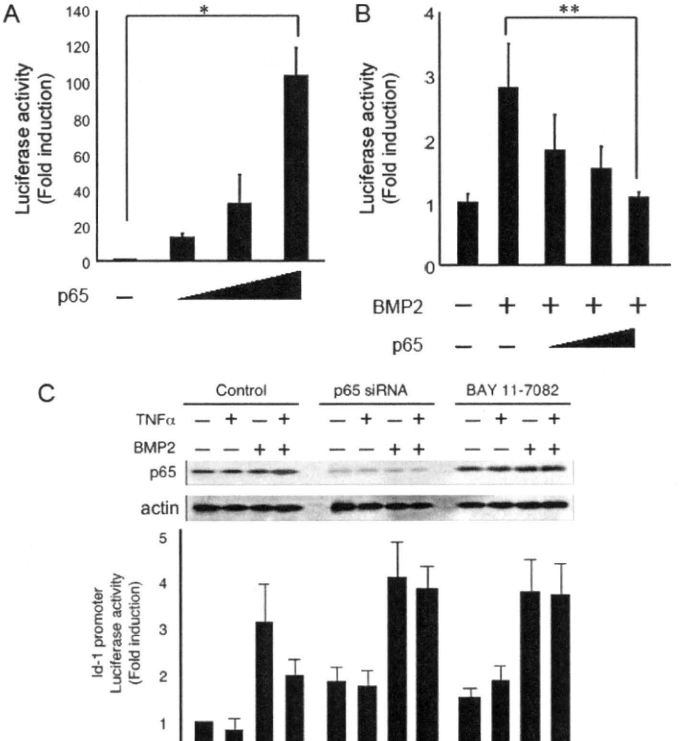

FIGURE 3. **Activation of NF-κB inhibited BMP2-induced Id1-luciferase activity in MC3T3-E1 cells and p65$^{-/-}$ MEFs.** *A*, p65$^{-/-}$ MEFs were transiently transfected with a pNF-κB-luc reporter and increasing concentrations of a p65 expression plasmid and were then assayed for luciferase activity after 24 h. *B*, p65$^{-/-}$ MEFs were transiently transfected with an Id1-luc reporter with or without a p65 expression plasmid for 24 h. The cells were then treated with BMP2 (100 ng/ml) and assayed for luciferase activity after 24 h. *C*, NF-κB activation in MC3T3-E1 cells was inhibited by either p65 siRNA or BAY11-7082, and then the cells were treated with BMP2 (100 ng/ml) with or without pretreatment with TNFα (10 ng/ml). The cells were assayed for Id1- or NF-κB-luciferase activity after 24 h. The expression level of p65 was examined by immunoblotting using anti-p65 antibodies.

ALP and osteocalcin (24, 25). The previous report demonstrated that TNFα inhibited BMP-induced Smad1,5,8 phosphorylation in C2C12 cells (23). In this study, as shown in Fig. 2*A*, phosphorylation of Smad1,5,8 was first detected within 10 min and declined thereafter (Fig. 2*A*). Pretreatment with TNFα did not affect the Smad1,5,8 phosphorylation induced by BMP2 (Fig. 2*A*). Furthermore, the translocation of phosphorylated Smad1,5,8 was not inhibited by pretreatment with TNFα, although TNFα led to nuclear translocation of p65, an NF-κB subunit (Fig. 2*B*).

Activation of NF-κB Inhibited BMP2-induced Id1-luciferase Activity in Both MC3T3-E1 Cells and p65$^{-/-}$ MEFs—We hypothesized that TNFα inhibits BMP/Smad signaling through NF-κB activation because NF-κB regulates the expression of several genes related to inflammation induced by TNFα (15, 16). The most abundant form of NF-κB is a heterodimer of the p50 and p65 subunits that contains a transcriptional activator domain (15, 16). p65$^{-/-}$ MEFs transfected with Id1-luc or pNF-κB-luc were cotransfected together with or without p65 and were treated with BMP2 for 24 h. The transcriptional activity of NF-κB was evaluated in a p65 dose-dependent manner (Fig. 3*A*). The inhibition of BMP2-induced Id1-luciferase activity coincided with the activation of NF-κB in p65$^{-/-}$ MEFs (Fig. 3*B*). To further confirm the possibility that NF-κB activation inhibits BMP-induced Id1-luc activity, we inhibited NF-κB activity in MC3T3-E1 cells by two different approaches: using p65 siRNA to knockdown p65 expression or using the pharmacological NF-κB-specific inhibitor BAY11-7082. BMP2-induced Id1-luc activity was reduced by pretreatment with TNFα in MC3T3-E1 cells (Fig. 3*C*, *lanes 3* and *4*). By contrast, TNFα failed to suppress BMP2-induced Id1-luc activity in MC3T3-E1 cells that had been transfected with p65 siRNA (Fig. 3*C*, *lanes 4* and *8*). NF-κB suppression by BAY11-7082 also led to a recovery of Id1-luc activity (Fig. 3*C*, *lanes 4* and *12*).

Furthermore, we cotransfected cells with expression constructs for Id1-luc, a constitutively active form of FLAG-Smad1 (FLAG-Smad1(DVD)) that exchanges two distal serines that are part of the C-terminal SS*X*S motif for aspartic acids (4), FLAG-Smad4, and/or p65, with or without an expression vector for a dominant-negative form of IκBα (IκBαDN). The overexpression of p65 completely abrogated the Id1-luciferase activity induced by FLAG-Smad1(DVD) and FLAG-Smad4. By

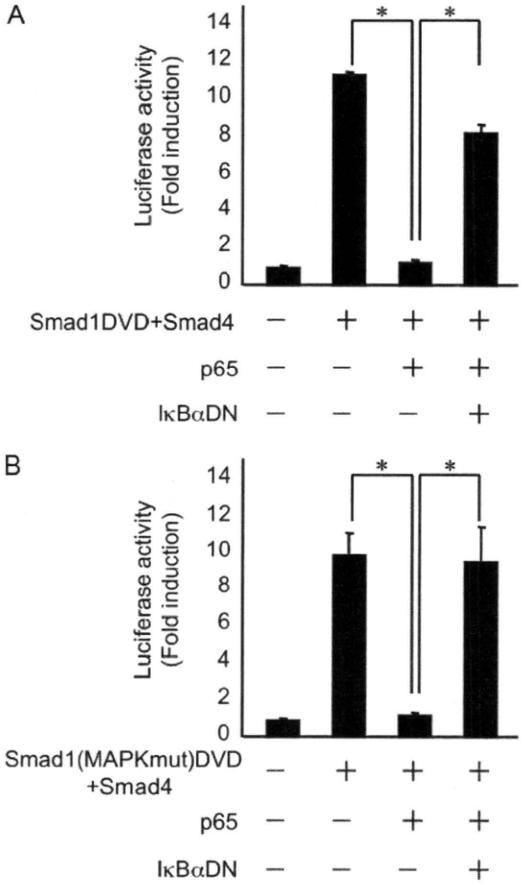

FIGURE 4. **Overexpression of p65 inhibited Id1-luciferase activity induced by the constitutively active form of Smad1-induced in $p65^{-/-}$ MEFs.** $p65^{-/-}$ MEFs were transiently transfected with Id1-luc, FLAG-Smad1(DVD) (*A*) or with FLAF-Smad1(MAPKmut-DVD) (*B*), FLAG-Smad4, p65, and IκBαDN expression plasmids and were assayed for luciferase activity after 24 h. Data are means + S.E. ($n = 3$). Similar results were obtained in three independent experiments. *, $p < 0.01$.

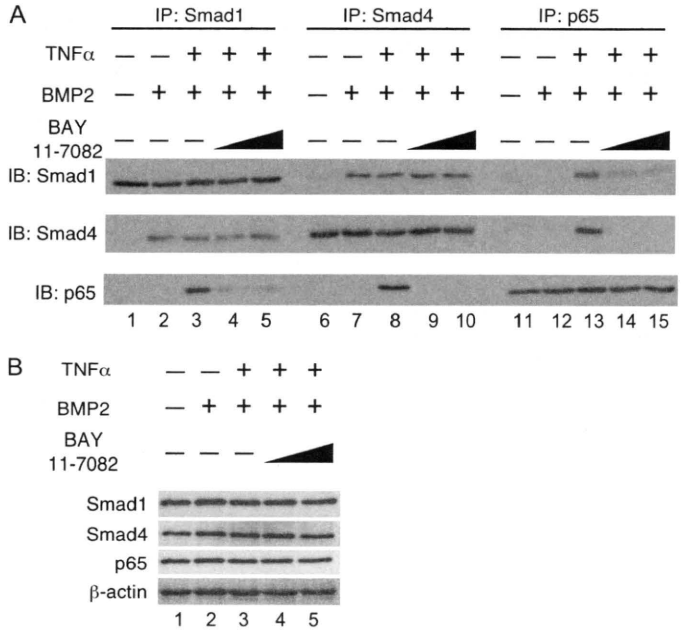

FIGURE 5. **TNFα did not disrupt the Smad1 and Smad4 complex but activated p65 by TNFα associated with Smad1 and Smad4.** *A*, MC3T3-E1 cells were pretreated with or without TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for 1 h in the presence or absence of increasing amounts of BAY11-7082. The whole cell extract was immunoprecipitated with anti-Smad1, anti-Smad4, or anti-p65 antibodies that were coupled to A/G-Sepharose beads; the whole cell extract was then processed for immunoblotting with the indicated antibodies. *B*, part of the whole cell extract was immunoblotted with the indicated antibodies to examine that all treatments did not affect the expression levels of Smad1, Smad4, and p65 and anti-β-actin was used as a loading control. Similar results were obtained in three independent experiments.

contrast, the transient overexpression of IκBαDN, which inhibits NF-κB in the cytoplasm, rescued this inhibition (Fig. 4*A*). TNFα signaling can activate MAPK pathway members, including MEK, ERK, and JNK (14). Recently, MAPK phosphorylation sites were identified in Smad1 at Ser-187, Ser-195, Ser-206, and Ser-214. Subsequently, the phosphorylation of these sites by MAPK negatively regulates BMP signaling (26). Therefore, we mutated all of these serines to alanines to get the FLAG-Smad1(MAPKmut-DVD) construct. The overexpression of p65 strongly inhibited the Id1-luciferase activity induced by FLAG-Smad1(MAPKmut-DVD) and FLAG-Smad4. Furthermore, the transient overexpression of IκBαDN again rescued this inhibition (Fig. 4*B*). These results suggest that cross-talk between NF-κB and BMP/Smad signaling influences BMP2-induced osteoblast differentiation induced by pretreatment with TNFα.

TNFα Did Not Disrupt the Smad1 and Smad4 Complex but Did Activate p65 through the Association of TNFα with Smad1 and Smad4—To confirm the possibility that TNFα interferes with the formation of a Smad1/Smad4 complex, we examined the interactions between Smad1 and Smad4 in cells pretreated with or without TNFα using co-immunoprecipitation assays. Cell extracts were subjected to immunoprecipitation with anti-Smad1, anti-Smad4, or anti-p65 antibodies, followed by immunoblot analysis with individual antibodies. Smad1 and Smad4 formed a complex in a ligand-dependent fashion (Fig. 5*A*, *lanes 2* and *7*). Pretreatment with TNFα failed to disrupt the association of Smad1 and Smad4. However, p65 activated by TNFα did associate with the complex of Smad1 and Smad4 (Fig. 5*A*, *lanes 3*, *8*, and *13*). Pretreatment with BAY11-7082 interfered with the association of p65 with Smad1 and/or Smad4 but did not interfere with the complex of Smad1 and Smad4 without affecting the expression levels of Smad1, Smad4, and p65 (Fig. 5, *A*, *lanes 4*, *5*, *9*, *10*, *14*, and *15* and *B*).

Activation of p65 by TNFα Inhibits the DNA Binding Activities of Smad Proteins Induced by BMP2—We next examined whether TNFα inhibited the DNA binding of Smad complexes induced by BMP2. MC3T3-E1 cells pretreated with or without TNFα were treated with BMP2 for the indicated time. Following pretreatment, genomic DNA was immunoprecipitated with anti-phosphorylated Smad1,5,8 antibodies, or control IgG, and was subsequently subjected to PCR amplification using primers that amplify the Id1 promoter region harboring the BMP2-responsive element. ChIP analysis showed that phosphorylated Smad1,5,8 was recruited to the Id1 promoter after 30 min of BMP2 stimulation, reached a maximum level at 1 h, and declined thereafter (Fig. 6*A*). When cells were pretreated with TNFα, the recruitment of phosphorylated Smad1,5,8 to the Id1 promoter was strongly suppressed compared with BMP2 treatment alone (Fig. 6*A*). p65 still bound to the Id1 promoter under

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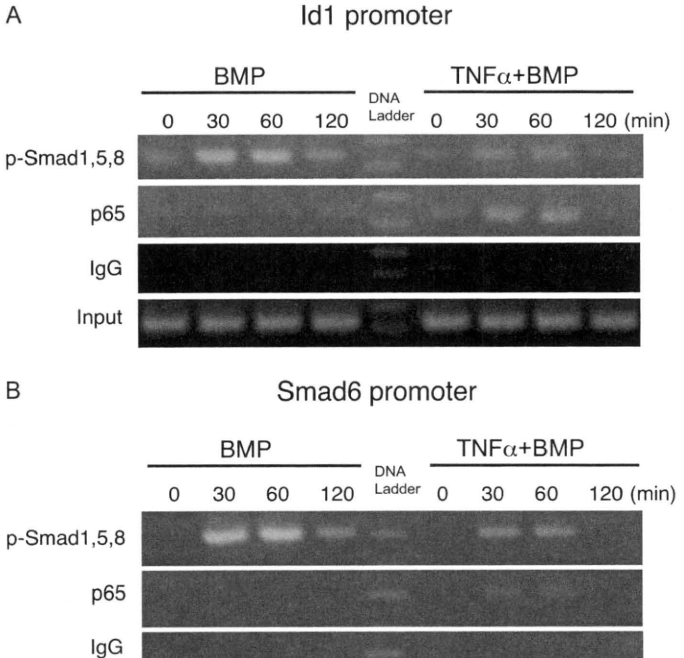

FIGURE 6. **Activation of p65 by TNFα inhibited the BMP-induced Smad1 recruitment to the Id1 and Smad6 promoters.** MC3T3-E1 cells were pretreated with or without TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for the indicated time. Chromatin from individual samples was precipitated using the indicated antibodies or with control IgG. The Id1 promoter (*A*) or the Smad6 promoter (*B*) was amplified by PCR from the precipitated DNA.

these conditions (Fig. 6*A*). Similar results were obtained using Smad6, a BMP target gene (27), promoter (Fig. 6*B*).

To further confirm whether the inhibitory effect of TNFα on the activation of Smad-DNA binding in response to BMP2 is mediated by p65, we performed EMSAs using nuclear extracts prepared from MC3T3-E1 cells. BMP2 induced the formation of DNA-binding protein complexes in MC3T3-E1 cells (Fig. 7*A*, *lane 3*). Consistent with the ChIP analysis, TNFα suppressed BMP2-induced DNA-binding protein complexes (Fig. 7*A*, *lanes 3* and *4*). The addition of anti-Smad1 or anti-Smad4 antibodies to the BMP2-treated nuclear extracts reduced the amount of the complex and induced supershifted bands (Fig. 7*B*, *lanes 3* and *4*). Moreover, when both the anti-Smad1 and anti-Smad4 antibodies were added together, a slower-migrating supershifted band appeared (Fig. 7*B*). We further performed EMSAs using nuclear extracts prepared from $p65^{-/-}$ MEFs. BMP2 induced Smad DNA binding in wild-type and $p65^{-/-}$ MEFs (Fig. 7*C*, *lanes 2* and *5*). TNFα pretreatment in wild-type strongly suppressed the DNA binding activities of Smad proteins induced by BMP2 (Fig. 7*C*, *lanes 3* and *6*). Although TNFα slightly suppressed Smad DNA binding activity, large amount of Smads still bound to DNA in $p65^{-/-}$ MEFs compared with wild-type cells (Fig. 7*C*, *lanes 5* and *6*). TNFα induced NF-κB DNA binding in wild-type but not $p65^{-/-}$ MEFs (Fig. 7*C*, *lanes 9* and *12*). These results suggest that the activation of p65 by TNFα inhibits the DNA binding of Smad proteins normally induced by BMP2.

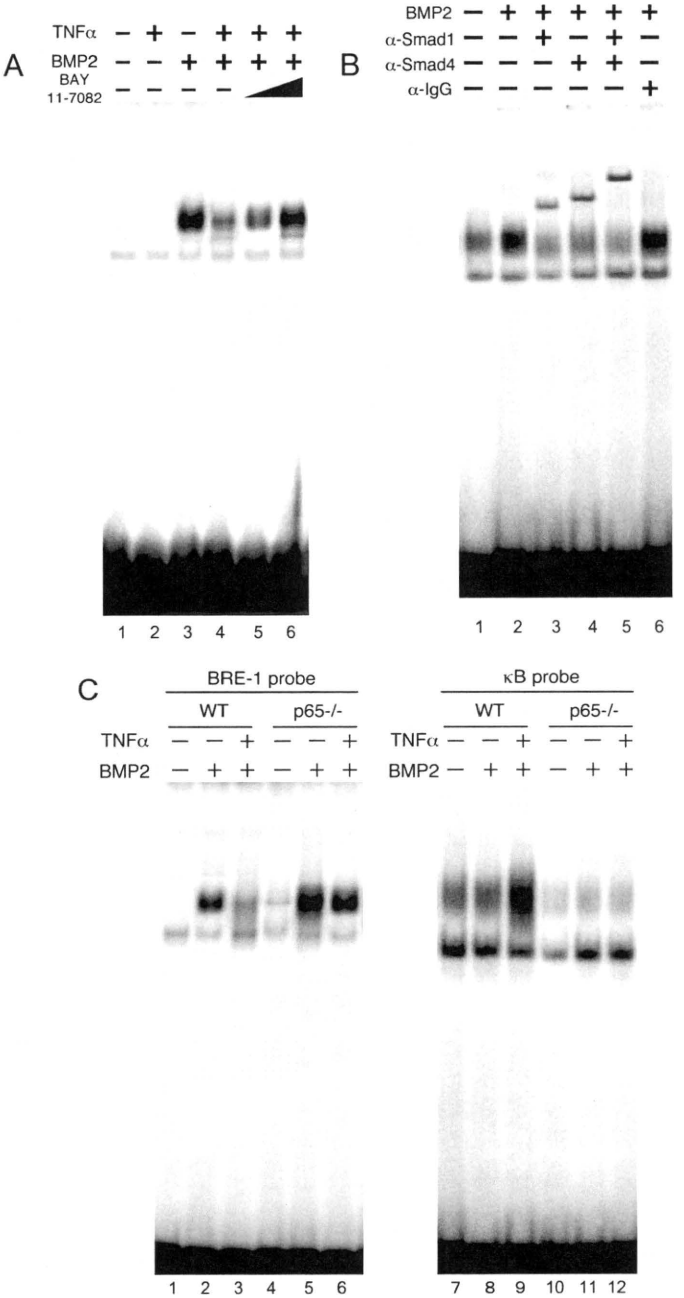

FIGURE 7. **Activation of p65 by TNFα inhibited the DNA-binding activities of Smad proteins induced by BMP2.** *A*, MC3T3-E1 cells were pretreated with or without increasing amounts of BAY11-7082 and TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for 30 min. The BMP2-induced DNA binding activity in the nuclear fractions was measured by EMSA using a BRE probe. *B*, subunit composition of the BMP2-induced DNA-binding proteins in MC3T3-E1 cells. The nuclear extracts were pretreated at 4 °C for 60 min with vehicle (*lanes 1* and *2*) or with 1 μl of the following polyclonal antibodies: anti-Smad1 (*lane 3*), anti-Smad4 (*lane 4*), anti-Smad1 and anti-Smad4 (*lane 5*), and control IgG (*lane 6*) antibodies. EMSAs were performed on these pretreated nuclear extracts. *C*, wild-type or $p65^{-/-}$ MEFs were pretreated with or without TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for 30 min. The Smad and NF-κB DNA binding activities in the nuclear fractions were measured by EMSA using a BRE probe and a κB probe, respectively. Similar results were obtained in three independent experiments.

A Specific Inhibitor of NF-κB Signaling Restored the TNFα-induced Inhibition of the DNA Binding Activities of Smad Proteins and the ALP Activity Induced by BMP2—Because NF-κB signaling was found to antagonize Smad activation by BMP2,

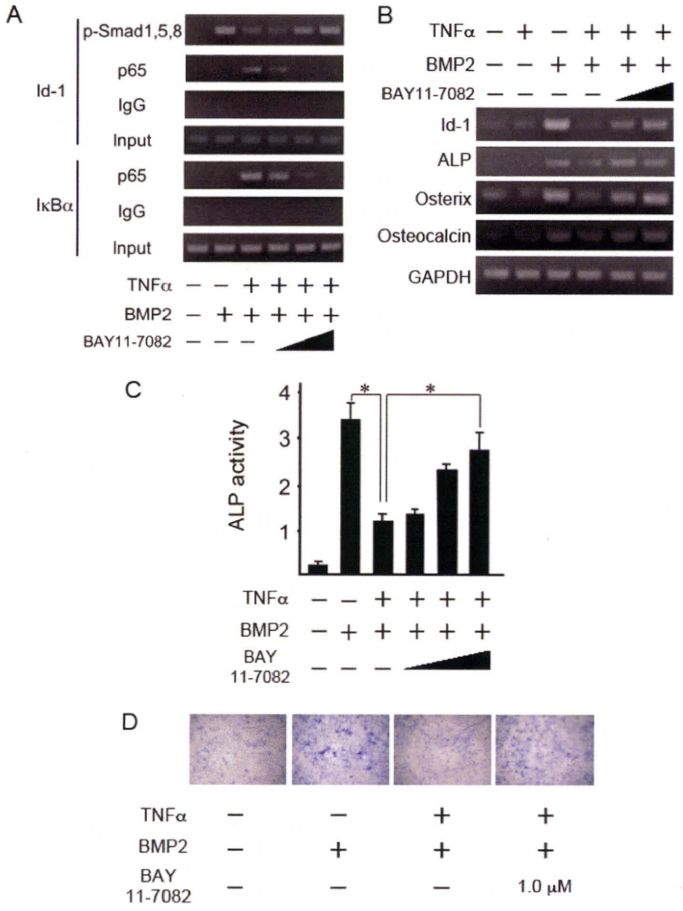

FIGURE 8. **A specific inhibitor of NF-κB signaling restored the TNFα-induced inhibition of the DNA binding activities of Smad proteins, expression of osteoblast marker genes, and ALP activity, which were all induced by BMP2.** *A*, MC3T3-E1 cells were pretreated with BAY11-7082 and TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for the indicated time. Chromatin from individual samples was precipitated using the indicated antibodies or control IgG. The Id1 or IκBα promoter was amplified by PCR from the precipitated DNA. *B*, MC3T3-E1 cells were pretreated with BAY11-7082 and TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for 12 h. Total RNA was extracted and subjected to reverse transcription with a random primer. The first strand cDNAs were submitted to PCR analysis for Id-1, ALP, osterix, osteocalcin, and GAPDH. *C*, MC3T3-E1 cells were pretreated with BAY11-7082 and TNFα (10 ng/ml) for 30 min and were subsequently treated with BMP2 (100 ng/ml) for 72 h. The cells were fixed with an acetone/ethanol mixture (50:50, v/v) and were incubated with a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$, 10 mg/ml *p*-nitrophenyl phosphate). ALP activity was then determined. Data are means + S.E. ($n = 3$). Similar results were obtained in three independent experiments. *, $p < 0.01$. *D*, The cells were stained for ALP activity.

we next examined the effect of BAY11-7082 on the TNFα-induced inhibition of the DNA binding activities of Smad proteins by BMP2. NF-κB suppression by BAY11-7082 led to a recovery of the binding of phosphorylated Smad1,5,8 to the Id1 promoter (Fig. 8*A*). By contrast, TNFα recruited p65 to the IκBα promoter, a target gene of NF-κB, but BAY11-7082 suppressed this recruitment in a dose-dependent manner (Fig. 8*A*). BAY11-7082 also rescued the inhibitory effect of TNFα on BMP2-induced Smad DNA binding in a dose-dependent manner (Fig. 7*A*, *lanes 4–6*). We next examined the expression of osteoblast differentiation marker genes in MC3T3-E1 cells. BMP2 induced Id1, ALP, osterix, and osteocalcin mRNA expression within 12 h, whereas TNFα pretreatment strongly suppressed the expression of these genes. BAY11-7082 restored TNFα-mediated inhibition of gene expression in a dose-dependent manner (Fig. 8*B*). In addition, the TNFα-mediated inhibition of ALP activity and the staining induced by BMP2 were also restored by pretreatment with BAY11-7082 (Fig. 8, *C* and *D*). These results strongly suggest that TNFα represses BMP signaling by interfering with the DNA binding of Smads through the activation of NF-κB.

DISCUSSION

TNFα is a potent inflammatory cytokine that contributes to local and systemic bone loss in inflammatory bone diseases such as rheumatoid arthritis and periodontitis, as well as in estrogen deficiency (28–31). In patients with rheumatoid arthritis, TNFα and other cytokines are overproduced in inflamed joints by various cells that infiltrate the synovial membrane (31). Anti-TNF drugs such as infliximab, etanercept, and adalimumab have been shown not only to diminish signs and symptoms of disease, but also to prevent joint damage (32). Furthermore, elevated production of TNFα in postmenopausal women and in animal models of postmenopausal osteoporosis augments bone destruction by stimulating osteoclastic bone resorption (29, 33). Under these conditions, osteoblast-mediated bone formation cannot compensate for accelerated osteoclastic bone resorption. These findings suggest a direct inhibitory effect of TNFα on osteoblasts. Further support for this inhibitory effect comes from research showing that although TNFα transgenic mice, a well-established animal model of rheumatoid arthritis, exhibit well-described features of rheumatoid arthritis, these animals also develop symptoms of general osteoporosis, such as a reduction in trabecular bone in the metaphysic, as compared with wild-type littermates (34). In addition, osteoblastic cells derived from TNFα transgenic mice form significantly fewer and smaller nodules under basal conditions and in the presence of BMP2, again indicating that TNFα reduces osteoblast function. Not only in pathological conditions, but also at the endogenous level, the level of TNFα present is of a magnitude sufficient to reduce bone formation, leading to a decrease in the maximum achievable peak bone mineral density (BMD) and bone mass. Both TNFα- and TNF type I receptor-deficient mice exhibit higher BMDs than wild-type littermates (35).

The inhibitory effects of TNF on bone formation *in vitro* were first reported in 1987 using a neonatal rat calvarial organ culture system (9). Subsequent studies demonstrated that TNFα blocks osteoblast differentiation in multiple models including fetal calvaria, bone marrow stromal cells, and MC3T3-E1 cells (10–12). TNFα treatment of fetal calvaria precursor cells, which spontaneously acquire the osteoblast phenotype over 21 days, inhibited differentiation, as shown by reduced formation of mineralizing nodules and decreased secretion of osteocalcin, a mature osteoblast marker. TNFα inhibited the expression of insulin-like growth factor I (IGF-I) but not the expression of BMP2, BMP4 or BMP6. Also, the addition of IGF-I or BMP6 could not rescue this TNF inhibition, suggesting that TNF acted downstream of these proteins in the differentiation pathway (11).

TNFα inhibited not only spontaneous osteoblast differentiation, but also BMP-induced osteoblast differentiation via

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Smad or via Smad-independent mechanisms (23). TNFα inhibited the BMP2-induced expression of Runx and osteocalcin and BMP2-induced ALP activity in C2C12 cells, a mouse myoblastic cell line, in a dose-dependent manner. TNFα inhibited phosphorylation of Smad1,5,8 by inducing Smad6 expression, an inhibitory Smad (23). We did not observe an inhibitory effect of TNFα on phosphorylation nor on translocation into the nucleus of Smad1,5,8 induced by BMP2 in MC3T3-E1. We treated cells with TNFα for 30 min and did not observe any induction of Smad6 or Smad7 by the TNFα treatment (data not shown). Although we could not explain this discrepancy, the discrepancy might depend on the stage of osteoblast differentiation and in the type of cell. Pretreatment with TNFα did inhibit the Smad-dependent Id1-luciferase activity induced by BMP2. These results strongly suggest that TNFα inhibits BMP/Smad signaling in the nucleus by interfering with the DNA binding or transcriptional modification of Smad proteins.

TNF signals through multiple intracellular pathways. In osteoblasts, a TNF trimer binds two receptor forms, type I and type II, of which only type I mediates the inhibition of osteoblast differentiation (36). In a well-established paradigm, the bound receptor activates MAP kinase or NF-κB signals (13, 14). We focused on NF-κB signals, because BMP and NF-κB have opposite biological functions during inflammatory processes (15, 16, 18, 19). We clearly showed that NF-κB is involved in the inhibitory effect of TNFα on osteoblast differentiation that is induced by BMP2. First, the inhibition of BMP2-induced Id1-luciferase activity coincided with the activation of NF-κB in $p65^{-/-}$ MEFs in a p65 dose-dependent manner. The overexpression of p65 completely abrogated the Id1-luciferase activity induced by the constitutively active forms of Smad1 and Smad4. Second, although TNFα inhibited BMP-induced DNA binding of Smad proteins in wild-type MEFs, TNFα failed to inhibit the DNA binding in $p65^{-/-}$ MEFs. Finally, a pharmacological NF-κB inhibitor restored the inhibitory effects of TNFα on both the BMP-induced DNA binding of Smad proteins and ALP activity. Whereas others have shown that NF-κB antagonizes BMP/Smad signaling by enhancing Smad7 expression (37), this study suggests that NF-κB, particularly the p65 subunit, binds the Smad1 and Smad4 complex, directly or indirectly, and that this binding interferes with the DNA binding of Smad proteins induced by BMP2.

There are some reports that TNFα inhibits BMP signaling by signals other than NF-κB. TNFα promotes Runx2 degradation through the up-regulation of Smurf1 and Smurf2 in osteoblasts, leading to suppressed osteoblast differentiation (34). Inhibitors of MEK and ERK, but not inhibitors of JNK or p38 kinase, abrogate TNFα inhibition of osterix mRNA and promoter activity (12). Moreover, TNFα acts to inhibit BMP signaling, including Smad1,5,8 phosphorylation and Id1 transcription, through activating the JNK pathway (23). These results suggest that TNFα inhibits osteoblast differentiation by inhibiting multiple steps that are required for differentiation from osteoblast progenitors to mature osteoblasts by interfering with several signals downstream of the TNF type I receptor.

Two transcriptional factors, Runx2 (Cbfa1/AML3/Pebp2αA) and osterix (Osx/Sp7), are required for differentiation of the osteoblast lineage (38, 39). The *in vivo* significance of these two factors has been verified by the observations that targeting disruption of either gene in mice results in a complete lack of both endochondral and intramembranous ossification and that these mice are characterized by an absence of mature osteoblasts throughout the body (38, 39). TNFα inhibits bone formation by promoting Runx2 proteasomal degradation through the up-regulation of Smurf1 and Smurf2 expression (34, 40). Furthermore, TNF inhibits the expression of osterix mRNA by suppressing osterix promoter activity via the MAP kinase and NF-κB pathways (41). These results indicate that TNFα inhibits osteoblast differentiation by suppressing the master regulators of osteoblast differentiation, such as Runx2 and osterix.

Collectively, our present study reveals that the inhibitory effect of TNFα on BMP signaling in the process of osteoblast differentiation is closely linked to the NF-κB pathway. Smad1,5,8 signaling is now generally accepted as a very early downstream effector in the BMP signaling pathway and is thus fundamental for osteoblastic differentiation in response to BMP ligands. The impairment of this central activation step, which is evoked by TNFα, is critical for bone differentiation and cellular sensitivity to BMP ligands. TNFα ablates osteoblast differentiation, at least in part, through NF-κB activation and subsequent interference with the DNA binding of Smad proteins. An intracellular balance of the signal intensities between TNFα/NF-κB and BMP/Smad is therefore crucial for osteoblast differentiation and could also be useful for bone regeneration.

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Constitutively Activated ALK2 and Increased SMAD1/5 Cooperatively Induce Bone Morphogenetic Protein Signaling in Fibrodysplasia Ossificans Progressiva*[S]

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Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder characterized by congenital malformation of the great toes and by progressive heterotopic bone formation in muscle tissue. Recently, a mutation involving a single amino acid substitution in a bone morphogenetic protein (BMP) type I receptor, ALK2, was identified in patients with FOP. We report here that the identical mutation, R206H, was observed in

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. S1 and S2.

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19 Japanese patients with sporadic FOP. This mutant receptor, ALK2(R206H), activates BMP signaling without ligand binding. Moreover, expression of Smad1 and Smad5 was up-regulated in response to muscular injury. ALK2(R206H) with Smad1 or Smad5 induced osteoblastic differentiation that could be inhibited by Smad7 or dorsomorphin. Taken together, these findings suggest that the heterotopic bone formation in FOP may be induced by a constitutively activated BMP receptor signaling through Smad1 or Smad5. Gene transfer of Smad7 or inhibition of type I receptors with dorsomorphin may represent strategies for blocking the activity induced by ALK2(R206H) in FOP.

Fibrodysplasia ossificans progressiva (FOP[2]; OMIM135100) is a rare autosomal dominant genetic disorder with ectopic bone formation in skeletal muscle tissue (1–4). At birth, most patients with FOP have malformations of the great toes, with hallux valgus, but do not have significant ectopic ossification. Heterotopic bone formation in the muscles and other soft tissues begins in early childhood and is further exacerbated by trauma, surgical treatment, lesional biopsies, and intramuscular injection (4, 5).

Ectopic bone formation similar to that observed in FOP is induced by implantation of bone morphogenetic proteins (BMPs) into muscle tissue (6–8). BMPs are members of the transforming growth factor-β (TGF-β) superfamily that were originally isolated from demineralized bone matrix and identified as factors responsible for induction of bone formation (6, 7). BMP signaling is transduced by two different types of serine/threonine kinase receptors, termed type I and type II receptors (9, 10). The ligand-bound type II receptor activates type I receptor kinase through phosphorylation of the glycine-serine (GS) domain, which is highly conserved among type I BMP and TGF-β receptors. ACVR1/ALK2, BMPR-IA/ALK3, BMPR-IB/ALK6, and ALK1 function as BMP type I receptors. Activated BMP type I receptor kinase activity in turn phosphorylates receptor regulated Smads, including Smad1, Smad5, and Smad8. Phosphorylated regulated Smads form heteromeric complexes with Smad4 and translocate into the nucleus to regulate transcription of various target genes, including *ID1*, which encodes an inhibitor of myogenesis (10–13). Inhibitory Smads (I-Smads), Smad6 and Smad7, are also induced by BMPs. I-Smads inhibit the BMP signaling pathways and thus form a negative feedback loop that down-regulates BMP signaling (14, 15). Altered BMP signaling in FOP cells

[2] The abbreviations used are: FOP, fibrodysplasia ossificans progressiva; BMP, bone morphogenetic protein; ACVR1, activin A type I receptor; ALK, activin receptor-like kinase; ALP, alkaline phosphatase; MHC, myosin heavy chain; RT, reverse transcription; TGF-β, transforming growth factor-β; I-Smad, inhibitory Smad.

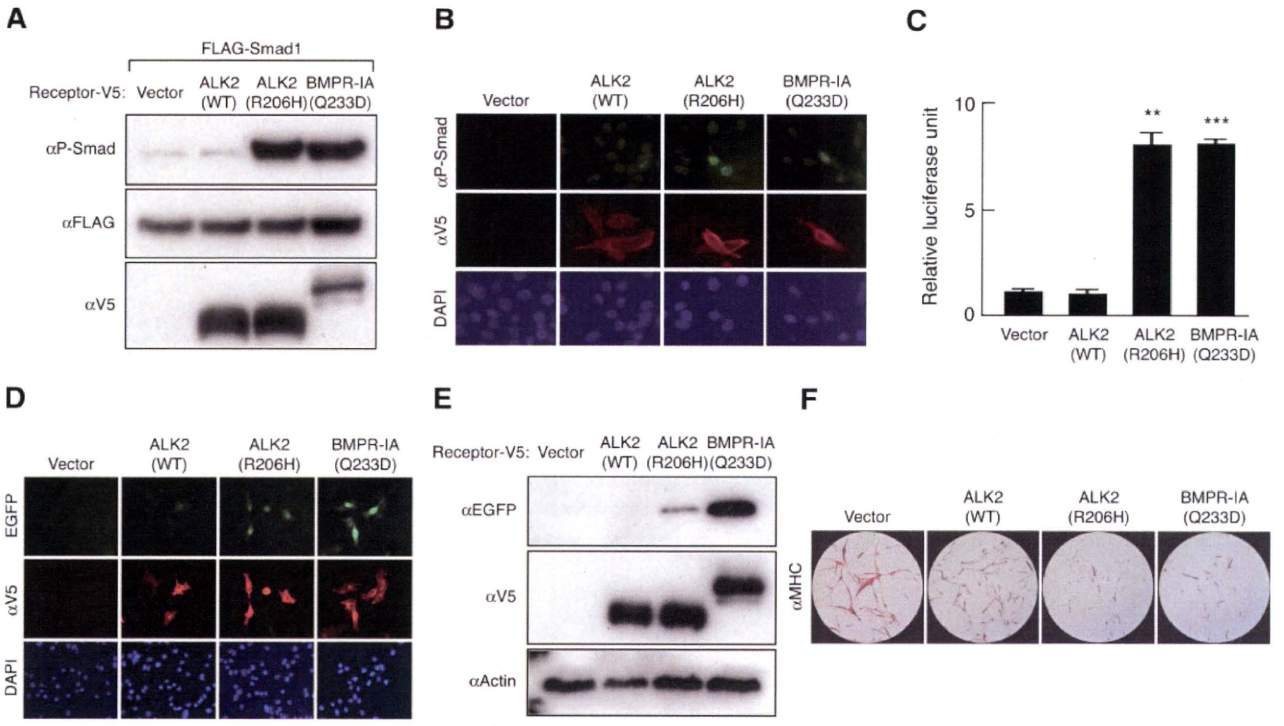

FIGURE 1. **ALK2(R206H) acts as a constitutively activated BMP receptor.** *A*, C2C12 cells were co-transfected with FLAG-tagged Smad1 and a V5-tagged wild-type ALK2 (*WT*), ALK2(R206H), or BMPR-IA(Q233D). Cell lysates were immunoblotted with anti-phospho-Smad1/5/8, anti-FLAG, or anti-V5 antibody. Constitutively active BMPR-IA(Q233D) was used as a positive control. *B*, C2C12 cells transfected with wild-type ALK2 or ALK2(R206H) were immunostained with anti-phospho-Smad1/5/8 or anti-V5 antibody and 4′,6-diamidino-2-phenylindole (*DAPI*). *C*, C2C12 cells were co-transfected with IdWT4F-luc reporter plasmid and wild-type ALK2, ALK2(R206H), or BMPR-IA(Q233D). Results are the means ± S.D. ($n = 3$). **, $p < 0.01$; ***, $p < 0.001$ compared with vector transfection. *D* and *E*, C2C12 cells were co-transfected with Id-EGFPd2 reporter plasmid and wild-type ALK2, ALK2(R206H), or BMPR-IA(Q233D). Levels of enhanced green fluorescent protein were determined by fluorescence microscopy (*D*) and immunoblotting (*E*). *F*, C3H10T1/2 cells co-transfected with a MyoD expression construct (24) and empty vector, wild-type ALK2, ALK2(R206H), or BMPR-IA(Q233D) were stained with anti-MHC antibody.

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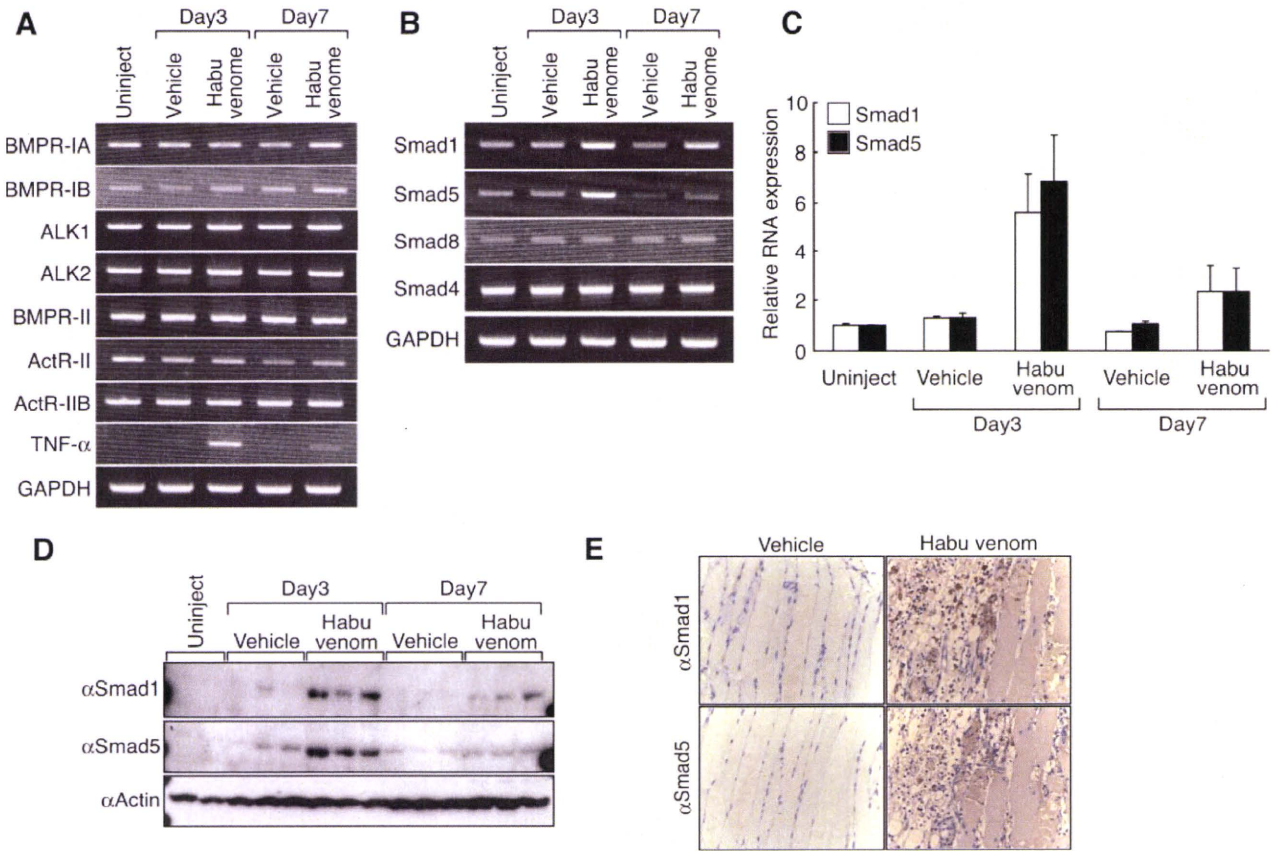

FIGURE 2. **Increased Smad1 and Smad5 mRNA during muscular injury *in vivo*.** *A–C*, mice were injected with vehicle (saline) or habu venom in femoral muscle, and total RNA was prepared after 3 or 7 days. Messenger RNA levels of BMP receptors (*A*) and Smads (*B* and *C*) were determined by RT-PCR (*A* and *B*) or real time quantitative PCR (*C*). Tumor necrosis factor-α (*TNF-α*) expression was examined to confirm inflammatory reaction. *D*, levels of Smad1 and Smad5 proteins in injured muscle *in vivo* were detected by immunoblotting at 3 and 7 days after injury. Two and three independent mice were analyzed in the control (uninjected) and vehicle and Habu venom-injected groups, respectively. *E*, localization of Smad1 and Smad5 in muscle tissues was determined by immunohistochemistry using specific antibodies to Smad1 and Smad5, respectively, on day 3 after injection. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

suggests that molecules involved in BMP signaling are responsible for FOP (16–20).

Recently, a recurrent heterozygous mutation in the *ACVR1/ALK2* gene was identified at 617G→A in both familial and sporadic patients with FOP (21, 22). This mutation causes an amino acid substitution of Arg to His at codon 206 (R206H) within the GS domain of the ALK2 receptor (21). Although a conformational change in the GS domain leading to activation of the receptor has been suggested to occur, the functional changes of the mutant receptor are still unclear.

In this study, we report that the common ALK2(R206H) mutation was identified in 19 of 19 Japanese patients with sporadic FOP and determined that ALK2(R206H) constitutively activates BMP signaling in *in vitro* assays. Expression of ALK2(R206H) in C2C12 myoblasts induced osteoblastic differentiation that was mediated through Smad1 and Smad5, and BMP signaling through ALK2(R206H) could be suppressed by Smad7 or dorsomorphin, two BMP type I receptor inhibitors. We further determined that mRNA levels of Smad1 and Smad5, but not Smad8 or ACVR1/ALK2, are increased in response to muscle injury *in vivo*. Because heterotopic bone formation in FOP commonly occurs following soft tissue injury, these data support the notion that the Smad1 and Smad5 increase following injury further enhances BMP signaling that has been pre-stimulated by a constitutively active ALK2 receptor mutation and leads to heterotopic bone formation. Smad7 and dorsomorphin may represent therapeutic approaches for inhibition of the BMP signaling induced by ALK2(R206H) in FOP.

MATERIALS AND METHODS

Genomic Sequence—Peripheral blood samples were obtained following informed consent from patients and their relatives in accordance with a protocol approved by the Ethics Committee of Saitama Medical University. Genomic DNA was extracted using a QIAamp DNA blood kit (Qiagen, Hilden, Germany), and exon 4 in the *ALK2* gene amplified by PCR was directly sequenced using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). The following oligonucleotides were used as primers: 5′-CCAGTCCTTCTTCCTTCTTCC-3′ and 5′-AGCAGATTTTCCAAGTTCCATC-3′.

Cell Culture, Transfection, and Reporter Assay—Mouse C2C12 myoblasts and C3H10T1/2 fibroblasts were maintained as described (23, 24). HEK293 cells were maintained in Dulbec-

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co's modified Eagle's medium containing 10% fetal bovine serum. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions (12). Stable ALK2(R206H)-expressing C2C12 cell lines were established by transfecting an expression vector, pcDEF3-ALK2(R206H), and selecting G418 at 700 μg/ml. BMP signaling was monitored using IdWT4F-luc or Id985-EGFPd2 reporter plasmids, which express a luciferase and a destabilized enhanced green fluorescent protein, respectively, under the control of a BMP-responsive element in the human *ID1* gene as described previously (12).

Alkaline Phosphatase Activity—Alkaline phosphatase (ALP) activity was measured as a marker of osteoblastic differentiation as described (23, 25). In brief, cells were incubated with a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$, and 10 mg/ml *p*-nitrophenyl phosphate). After appropriate incubation, reactions were terminated by adding 3 M NaOH, and absorbance was measured at 405 nm.

Immunoprecipitation and Immunoblotting—Cells and tissues were lysed in TNE buffer (10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and 1% Nonidet P-40). Immunoblotting was performed using anti-FLAG antibody (clone M2, Sigma), anti-phosphorylated Smad1/5/8 antibody (Cell Signaling, Beverly, MA), anti-V5 antibody (Invitrogen), anti-green fluorescent protein antibody (GF090R, Nakalai Tesque, Kyoto, Japan), anti-Smad1 antibody (sc-6201, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Smad5 antibody (sc-7443, Santa Cruz Biotechnology). Myogenic cells were detected using anti-myosin heavy chain (MHC) antibody (clone MF-20, Developmental Studies Hybridoma Bank, Iowa City, IA) (24).

Induction of Muscular Injury in Vivo—To induce muscular injury, 50 μl of habu (*Trimeresurus flavoviridis*) snake venom at 100 μg/kg or vehicle was injected into femoral muscles of 3-week-old C57BL/6 mice. After 3 and 7 days, RT-PCR, real time quantitative RT-PCR, immunoblotting, and immunohistochemistry were performed. The primers used were as described in supplemental Table S1. Real time RT-PCR for Smad1 and Smad5 was performed on Mx3000p (Stratagene, Tokyo, Japan) using QuantiTect Primer Assay (Qiagen). Muscle tissues were fixed with formalin and embedded in paraffin for immunohistochemical analysis.

Statistical Analysis—Comparisons were made by using Student's *t* test. Data were expressed as mean ± S.D.

RESULTS

Identification of a 617G→A Mutation in ALK2 in 19 Japanese Patients with FOP—To determine whether FOP in Japanese patients is caused by the same recurrent mutation in ALK2 that was recently reported in familial and sporadic patients with FOP (21), we examined the genomic DNA of 19 Japanese FOP patients. Through DNA sequencing, we confirmed the identical 617G→A (R206H) mutation in the *ACVR1/ALK2* gene in all 19 Japanese patients with FOP; however, none of the relatives that were examined carried the mutation, indicating that each of the 19 patients are sporadic cases (supplemental Fig. S1).

ALK2(R206H) Is a Constitutively Activated BMP Receptor—To examine functional changes of the mutant ALK2 identified in FOP, we examined its intracellular signaling *in vitro*. Trans-

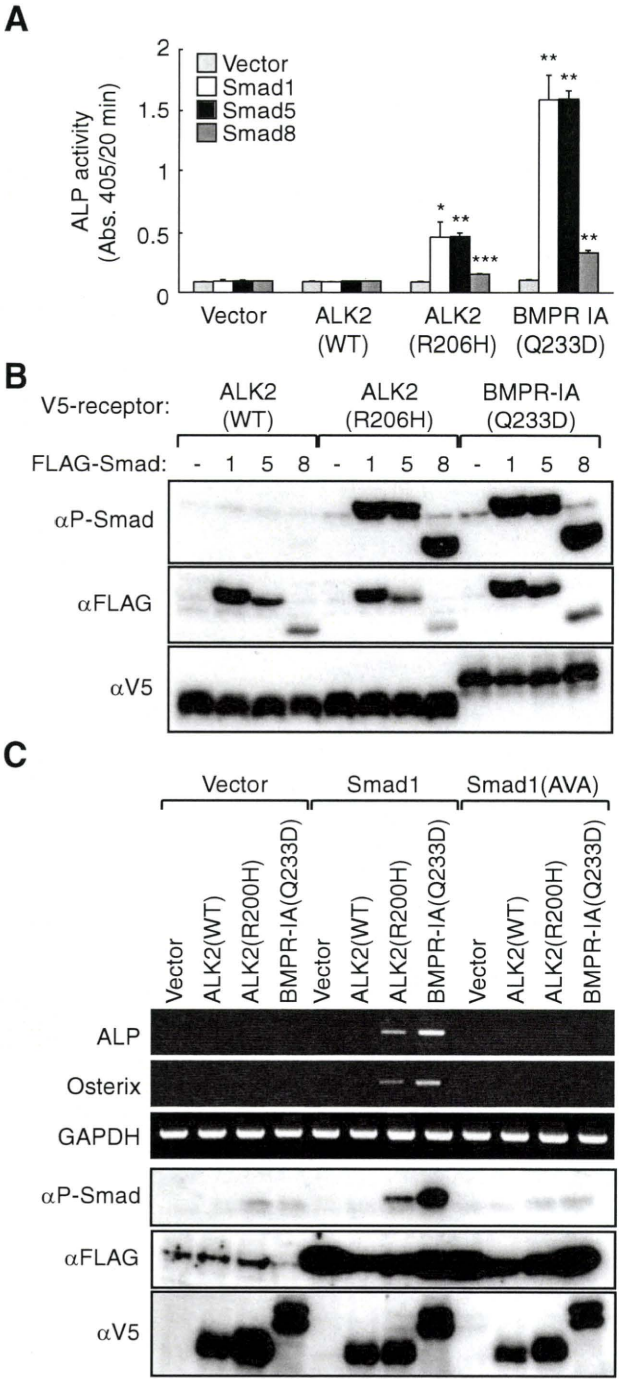

FIGURE 3. **Cooperative effect of ALK2(R206H) with Smad1/5 in induction of osteoblastic differentiation.** *A* and *B*, C2C12 cells were co-transfected with FLAG-tagged Smad1, Smad5, or Smad8 with V5-tagged wild-type ALK2(WT), ALK2(R206H), or BMPR-IA(Q233D). ALP activity (*A*) and levels of phosphorylation of Smads (*B*) were determined on day 3. Results are the means ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with vector transfection in each group. *C*, C2C12 cells were co-transfected with FLAG-tagged Smad1 or Smad1(AVA) and V5-tagged wild-type ALK2, ALK2(R206H), or BMPR-IA(Q233D). RT-PCR was performed to determine levels of expression of ALP and osterix mRNAs after 3 days. Levels of phosphorylated Smads and receptors were determined by immunoblotting using anti-phospho-Smad1/5/8, anti-FLAG, or anti-V5 antibody (*lower panels*). *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

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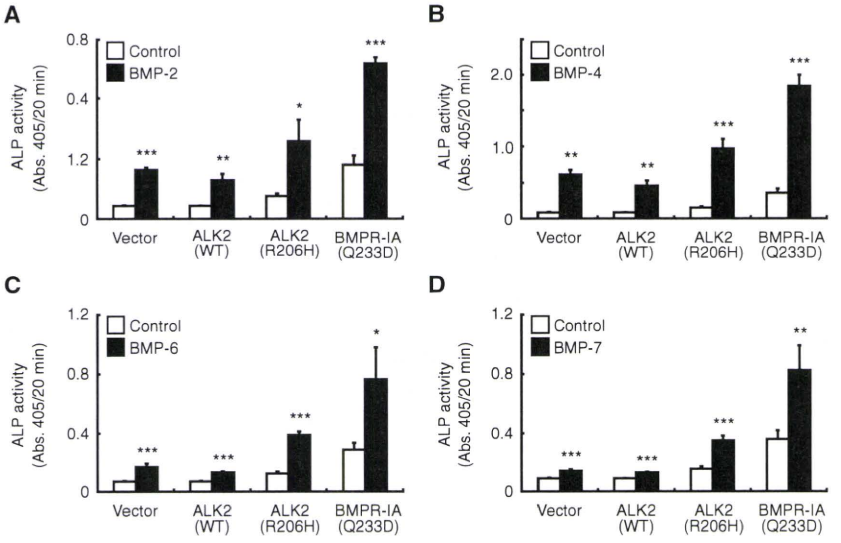

FIGURE 4. **BMPs further stimulate ALP activity induced by ALK2(R206H) and Smad1 in C2C12 myoblasts.** C2C12 cells co-transfected with Smad1 and wild-type (*WT*) ALK2, ALK2(R206H), or BMPR-IA(Q233D) were treated for 3 days with 300 ng/ml of BMP-2 (*A*) or 100 ng/ml of BMP-4 (*B*), BMP-6 (*C*), or BMP-7 (*D*), and ALP activity was then determined. Results are the mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with controls.

fection of Smad1, a signaling protein specific for the BMP pathway, with ALK2(R206H), but not wild-type ALK2, induced phosphorylation of Smad1 in the absence of BMPs (Fig. 1*A*). Immunodetection assays showed that endogenous Smad1/5/8 were phosphorylated and accumulated in nuclei in response to ALK2(R206H) as well as BMPR-IA(Q233D), a form of this BMP type I receptor previously shown to be constitutively active (Fig. 1*B*) (26). Promoter activity of the *Id1* gene, one of the transcriptional targets of the BMP-Smad axis, was induced by ALK2(R206H) and by BMPR-IA(Q233D) but not wild-type ALK2 in a luciferase assay (Fig. 1*C*). Induction of the *Id1* promoter by ALK2(R206H) was further confirmed using another construct, Id-EGFPd2 (12) (Fig. 1, *D* and *E*). We also examined the effects of ALK2(R206H) on myogenic differentiation and found that ALK2(R206H) as well as BMPR-IA(Q233D) markedly suppressed myogenesis in C3H10T1/2 cells transfected with a MyoD expression construct (Fig. 1*F*). Similar suppression of myogenesis by ALK2(R206H) was also observed in C2C12 myoblasts (data not shown). These findings indicate that ALK2(R206H) constitutively activates an intracellular signaling pathway specific for BMPs.

Expression of Smad1 and Smad5 Are Up-regulated during Muscular Regeneration—Because injuries of muscle tissue induce heterotopic bone formation in FOP patients, we hypothesized that receptors or transcription factors that cooperate with ALK2(R206H) in stimulating bone formation are induced in response to muscular injury. To test this hypothesis, we quantified mRNA levels of BMP type I and type II receptors, and of Smads as downstream BMP signaling molecules, during muscular regeneration induced by an intramuscular injection of habu venom in mice. No BMP receptor mRNA levels were changed during muscular regeneration (Fig. 2*A*). Levels of Smad8, a BMP receptor-regulated Smad, and Smad4, a Co-Smad common to BMP and TGF-β signaling, were not changed during muscular regeneration (Fig. 2*B*). In contrast, expression levels of two BMP pathway-specific Smads, Smad1 and Smad5, were up-regulated as detected by RT-PCR (Fig. 2*B*), quantitative RT-PCR (Fig. 2*C*), and immunoblot analysis (Fig. 2*D*). Levels of Smad1 and Smad5 mRNAs were increased up to ~6–7-fold by day 3 (Fig. 2, *B* and *C*). Smad1 and Smad5 proteins were mainly detected in cells within the regenerating muscle tissues rather than myofibers (Fig. 2*E*).

To examine the functional interaction of ALK2- and BMP-specific Smads, we co-transfected Smad1, Smad5, or Smad8 expression constructs with wild-type ALK2 or ALK2(R206H) into C2C12 myoblasts. Co-transfection and overexpression of Smad1 or Smad5 with ALK2(R206H) increased ALP activity, although enzyme activities were less than those induced by constitutively active BMPR-IA(Q233D) with Smad1 or Smad5 (Fig. 3*A*). In contrast, co-transfection of Smad8 with ALK2(R206H) induced lower levels of ALP activity than with co-transfection of Smad1 or Smad5, although levels of phosphorylation were not distinguishable among Smad1, Smad5, and Smad8 (Fig. 3, *A* and *B*). Moreover, co-transfection of Smad1 with ALK2(R206H) induced mRNAs related to osteoblastic differentiation such as ALP and osterix, although the levels of mRNAs were lower than that of BMPR-IA(Q233D) (Fig. 3*C*). However, these mRNAs were not induced by co-transfection of ALK2(R206H) or BMPR-IA(Q233D) with an inactive Smad1 mutant, Smad1(AVA), in which the carboxyl-terminal serine residues phosphorylated by BMP receptors were substituted with alanine residues. These findings suggest that the stimulatory effects of ALK2(R206H) and BMPR-IA(Q233D) on osteoblastic differentiation are mediated through phosphorylation of BMP-specific Smads. The ALP activity induced by ALK2(R206H) and Smad1 was further increased by treatment with BMP-2, BMP-4, BMP-6, or BMP-7 (Fig. 4). Co-transfection of ALK2(R206H) and Smad1 with one of the BMP type II receptors (BMPR-II, ActR-II, or ActR-IIB) further increased ALP activity in the presence and absence of BMPs (data not shown).

Smad7 and Dorsomorphin Inhibit ALK2(R206H) Activity—Addition of a BMP antagonist, Noggin that binds to BMPs and blocks their binding to specific receptors in the extracellular space, failed to suppress the ALP activity induced by ALK2(R206H) or BMPR-IA(Q233D) (Fig. 5*A*). We compared the effects of I-Smads on the intracellular signaling induced by ALK2(R206H) and BMPR-IA(Q233D). Both Smad6 and Smad7 at low amounts markedly inhibited the ALP activity induced by BMPR-IA(Q233D); however, only Smad7 markedly inhibited signaling by ALK2(R206H), confirming a recent report by Goto *et al.* (27) (Fig. 5, *B* and *C*).

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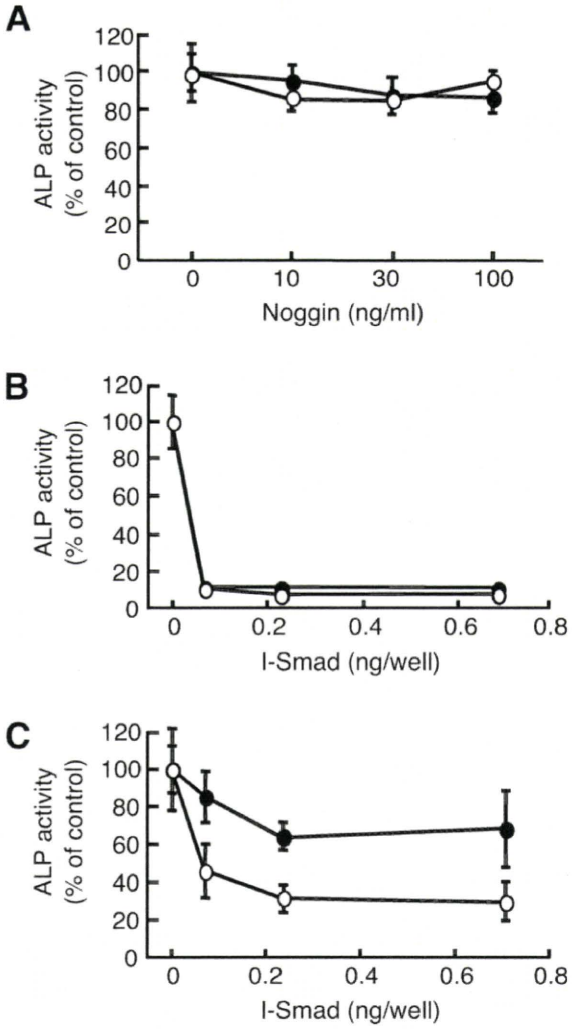

FIGURE 5. **Inhibition of biological activities of ALK2(R206H) by Noggin and I-Smads.** *A*, C2C12 cells co-transfected with Smad1 and ALK2(R206H) (*closed circles*) or BMPR-IA(Q233D) (*open circles*) were treated with Noggin. ALP activity was determined on day 3. *B* and *C*, C2C12 cells were co-transfected with BMPR-IA(Q233D) (*B*) or ALK2(R206H) (*C*) and Smad1, with increasing amounts of Smad6 (*closed circles*) or Smad7 (*open circles*). Total amounts of DNA transfected were adjusted equally with an empty vector. ALP activity was determined on day 3. Results are the mean ± S.D. ($n = 3$).

Recently, the small molecule dorsomorphin was identified as a specific inhibitor of Smad-dependent signaling induced by BMP type I receptors ALK2, BMPR-IA, and BMPR-IB (28). Dorsomorphin almost completely inhibited the phosphorylation of FLAG-Smad1 induced by ALK2(R206H) (Fig. 6*A*). Moreover, dorsomorphin dose-dependently suppressed the ALP activity induced by ALK2(R206H) in C2C12 cells in conditions of both transient and stable overexpression (Fig. 6*B* and supplemental Fig. S2). ALK2(R206H) was less sensitive to dorsomorphin than BMPR-IA(Q233D) in suppression of ALP activity (Fig. 6*B*). We established subclonal cell lines of C2C12 myoblasts that stably expressed wild-type ALK2 or ALK2(R206H). Myogenesis of ALK2(R206H)-expressing C2C12 cells was suppressed in the absence of dorsomorphin (Fig. 6*C*). However, dorsomorphin dose-dependently increased the numbers of MHC-positive myotubes in ALK2(R206H)-expressing C2C12 cells (Fig. 6*C*).

DISCUSSION

Recently, a recurrent mutation of 617G→A in the *ACVR1/ALK2* gene was identified as the mutation responsible for FOP (21), a rare skeletal disorder associated with heterotopic bone formation in muscle and other soft connective tissues (1–5). In this study, we identified the same mutation in 19 of 19 Japanese FOP patients. These findings strongly support a causal role of the 617G→A mutation in the pathogenesis of disease for FOP patients with classic FOP.

ALK2 is one of the type I receptors for BMPs, the most potent bone-inducing factors in vertebrates (6, 7). The common mutation identified in FOP patients causes a single amino acid substitution in ALK2, Arg to His in codon 206 within the GS domain. The GS domain is phosphorylated by BMP and TGF-β type I receptors following activation by ligand-bound type II receptors (9). Substitutions of codon Gln-207 to aspartic acid in ALK2 and homologous positions in other type I receptors in the TGF-β superfamily result in constitutive activation of the serine/threonine kinases of these receptors without binding of ligands (26, 29–31). These findings led us to examine whether ALK2(R206H) is activated in FOP as a BMP receptor. As shown here, we found that ALK2(R206H) induces BMP-specific signaling via phosphorylation of Smad1/5/8 even in the absence of BMPs or type II receptors, although the osteoblastic differentiation-inducing activity of ALK2(R206H) was weaker than those of BMPR-IA(Q233D) and ALK2(Q207D) (Fig. 3*A* and data not shown). Ours is thus the first study to elucidate biochemically that ALK2(R206H), the mutant receptor commonly identified in FOP, acts as a mild constitutively activated BMP type I receptor. The ALK2(R206H) mutation found in FOP is the first case of a natural gain-of-function mutation among the TGF-β superfamily receptors.

Injury of muscle tissue induces local heterotopic bone formation in patients with FOP (33–35). We speculated that additional signals may be altered in response to muscular injury. This hypothesis was confirmed by our finding that levels of Smad1 and Smad5, two downstream signal transducers for the BMP receptors, were increased during muscle regeneration. Moreover, co-expression of ALK2(R206H) with Smad1 or Smad5 synergistically induced myoblasts to show increased phenotypic expression related to osteoblastic differentiation. These findings suggest that the heterotopic bone formation in patients with FOP may, in part, be caused by cooperative activity of the constitutively activated BMP receptor (ALK2(R206H)) with trauma-induced up-regulation of Smad1 and Smad5. Although up-regulation of Smad1 and Smad5 in patients with FOP should be examined, tissue samples from patients with FOP are not available because biopsy and surgery must be avoided in such patients to prevent induction of heterotopic bone formation.

Moreover, treatments with BMPs further stimulated the osteoblastic differentiation of C2C12 myoblasts expressing ALK2(R206H) (Fig. 4). We and others have identified BMP-4 and other osteogenic BMPs in serum in vertebrates (25, 36, 37), and BMP-4 has been found to be overexpressed in lymphocytes

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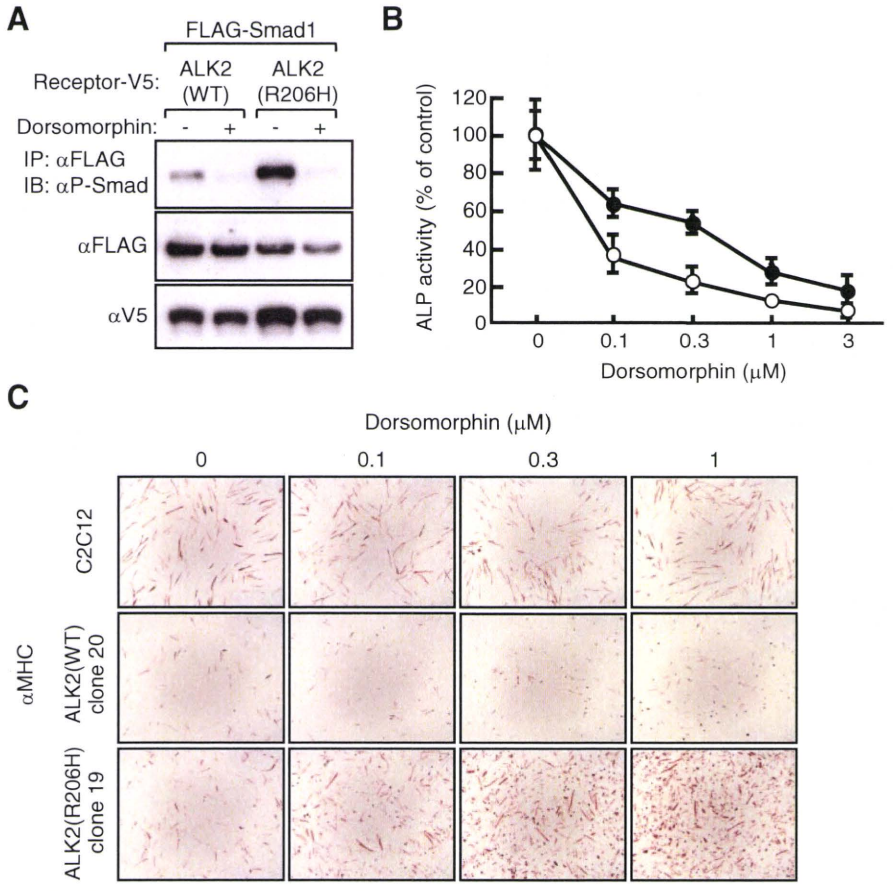

FIGURE 6. **Dorsomorphin inhibits ALK2(R206H) activity.** *A*, HEK293 cells were co-transfected with FLAG-tagged Smad1 and V5-tagged ALK2(R206H) or V5-BMPR-IA(Q233D) and then treated for 3 days with dorsomorphin at 3 μM. Levels of phosphorylated Smad1 were determined by immunoblotting (*IB*). *IP*, immunoprecipitation. *B*, C2C12 cells were co-transfected with Smad1 and ALK2(R206H) (*closed circles*) or BMPR-IA(Q233D) (*open circles*) and treated with graded concentrations of dorsomorphin. ALP activity was determined on day 3. *C*, parental C2C12 cells, C2C12-ALK2 (*WT*) clone 20, and C2C12-ALK2(R206H) clone 19 were treated with increasing concentrations of dorsomorphin in low serum medium and stained with anti-MHC antibody on day 3.

in FOP (20). It might thus be possible that myoblasts in FOP patients are exposed to BMP ligands through bleeding after muscle injury, and that these events cooperatively stimulate the process of heterotopic bone formation further in muscle tissue.

Interestingly, overexpression of wild-type ALK2 moderately inhibited myogenesis in the absence of BMPs and suppressed the ALP activity induced by BMPs. Because wild-type ALK2 did not induce detectable levels of the BMP-specific Smad pathway in the absence of BMPs (Fig. 1*F*), a non-Smad pathway might also play a role in this receptor inhibition (32). Alternatively, wild-type ALK2 may bind to other ligand(s) rather than BMPs in our culture conditions. Activin appears to be a possible ligand of ALK2, because it has been shown to be present in the circulation (38) and to suppress both myogenesis and osteoblastic differentiation *in vitro* (39, 40). Moreover, ALK2 was shown originally to bind to activin in the presence of appropriate type II receptors (41, 42). Further studies will be required to test this hypothesis.

At present, no treatments are available to prevent heterotopic bone formation in FOP. Recently, the unique small molecule dorsomorphin was identified as a specific inhibitor of Smad-dependent signaling induced by BMP type I receptors (28). Because dorsomorphin was found to inhibit the BMP-specific signaling induced by the ALK2(R206H) mutant receptor, this compound provides proof of concept for intracellular signal transduction inhibition in the design of novel drugs for the treatment of FOP. As Smad7 is an intrinsic intracellular molecule, drugs that induce Smad7 expression might be useful as well. The findings presented here suggest that not only ALK2(R206H) but also a novel type of signaling that induces Smad1/5 are potential targets of treatment in patients with FOP. Unfortunately, however, we were unable to test these possibilities *in vivo* because there is no suitable *in vivo* model system reflecting the phenotypes of FOP. We are currently attempting to establish new mouse models of FOP using ALK2(R206H). We will, in the near future, be able to examine the inhibitory effects of dorsomorphin and other compounds *in vivo* using these models.

In conclusion, we identified the ALK2(R206H) mutation in Japanese FOP patients. ALK2(R206H) is the first case of identification of a naturally activated BMP type I receptor in vertebrates. We found that Smad1 and Smad5 were induced in response to muscular injury and may play important roles in heterotopic bone formation after injury of muscle tissue in FOP. Molecules, including dorsomorphin and Smad7, will aid in the establishment of novel methods of treatment of FOP.

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The fibrodysplasia ossificans progressiva R206H ACVR1 mutation activates BMP-independent chondrogenesis and zebrafish embryo ventralization

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Patients with classic fibrodysplasia ossificans progressiva, a disorder characterized by extensive extraskeletal endochondral bone formation, share a recurrent mutation (R206H) within the glycine/serine-rich domain of ACVR1/ALK2, a bone morphogenetic protein type I receptor. Through a series of in vitro assays using several mammalian cell lines and chick limb bud micromass cultures, we determined that mutant R206H ACVR1 activated BMP signaling in the absence of BMP ligand and mediated BMP-independent chondrogenesis that was enhanced by BMP. We further investigated the interaction of mutant R206H ACVR1 with FKBP1A, a glycine/serine domain–binding protein that prevents leaky BMP type I receptor activation in the absence of ligand. The mutant protein exhibited reduced binding to FKBP1A in COS-7 simian kidney cell line assays, suggesting that increased BMP pathway activity in COS-7 cells with R206H ACVR1 is due, at least in part, to decreased binding of this inhibitory factor. Consistent with these findings, in vivo analyses of zebrafish embryos showed BMP-independent hyperactivation of BMP signaling in response to the R206H mutant, resulting in increased embryonic ventralization. These data support the conclusion that the mutant R206H ACVR1 receptor in FOP patients is an activating mutation that induces BMP signaling in a BMP-independent and BMP-responsive manner to promote chondrogenesis, consistent with the ectopic endochondral bone formation in these patients.

Introduction

Fibrodysplasia ossificans progressiva (FOP; MIM 135100), a rare genetic disorder of progressive extraskeletal (heterotopic) ossification, is the most severe form of human heterotopic ossification known and results in profoundly decreased mobility of affected individuals (1). Patients with classic FOP have congenital malformation of the great toes and develop progressive heterotopic ossification within soft connective tissues in characteristic anatomic patterns (2, 3). Ectopic bone formation in FOP occurs through an endochondral pathway in which cartilage forms initially at the lesional site and is subsequently replaced by bone (4, 5). The genetic mutation in FOP is therefore a likely key regulator of cartilage and bone formation.

The gene mutation for patients with the classic FOP clinical phenotype was mapped to chromosome 2q23–24, and mutations were identified in activin A receptor, type I (*ACVR1*; also known as *ALK2*), which encodes a bone morphogenetic protein (BMP) type I receptor (6). ACVR1 is expressed in several tissues, including cartilage and skeletal muscle, consistent with both the congenital skeletal malformations and the sites of postnatal endochondral heterotopic ossification in FOP. Previous reports demonstrated that constitutive activation of ACVR1 induces alkaline phosphatase (ALP) activity, expands cartilage elements, and induces joint fusions, all of which are associated with enhanced chondrogenesis and/or dysregulation of the BMP signaling pathway (7–9).

BMPs, members of the TGF-β superfamily, were identified initially as a family of proteins that could induce ectopic bone formation through an endochondral process (10–12). BMPs are critical signaling proteins, not only for bone and cartilage formation, but also in many stages of vertebrate development (13). During in vivo vertebrate development, BMP signaling is first required in early embryogenesis and is involved in the patterning of the tissues along the dorsal-ventral axis (14).

BMPs are extracellular ligands that exert their effects by binding to complexes of type I and type II serine/threonine kinase BMP receptors that are located at the cell membrane (15–19). Four type I receptors (ACVR1 [ALK2], BMPR1A [ALK3], BMPR1B [ALK6], and ACVRL1 [ALK1]) can mediate BMP signal transduction. Three type II receptors have been identified: BMPR2 (BMPRII), ACVR2A (ActRII), and ACVR2B (ActR-IIB). In the absence of BMP binding, the FK506-binding protein 1A (FKBP1A protein, also called FKBP12) binds the glycine-serine (GS) domain of BMP type I receptors to inhibit internalization of the receptor and down-

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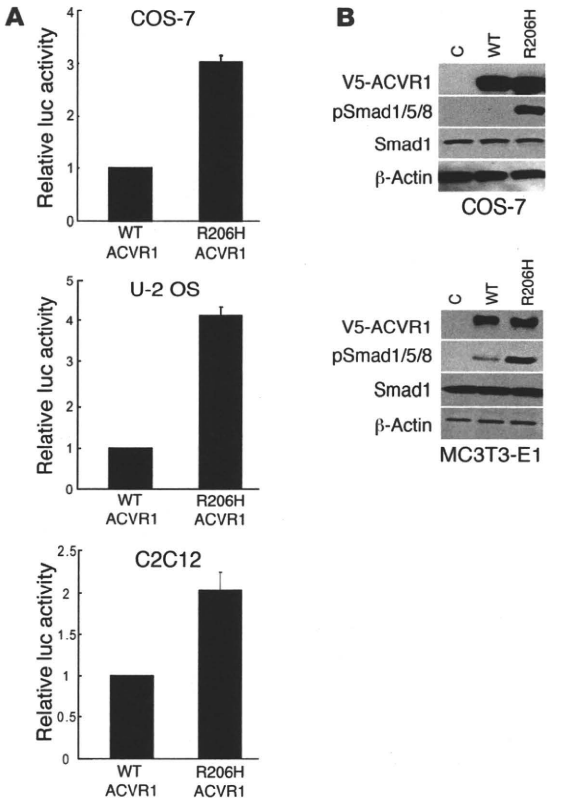

Figure 1
Mutant ACVR1 (c617A; R206H) activates the BMP signaling pathway. (**A**) Activation of ID1 transcription. COS-7 cells were cotransfected with an ID1 promoter luciferase reporter construct (–985/+94) and either human wild-type ACVR1 or mutant ACVR1 (R206H) expression vectors. Similar assays used U-2 OS and C2C12 cells. Data represent mean ± SEM (n > 3). (**B**) Induction of Smad1/5/8 phosphorylation. COS-7 (top) or MC3T3-E1 cells (bottom) were transfected with V5-tagged wild-type *ACVR1* or c617A mutant *ACVR1* (R206H) constructs or with the empty vector control (C). Immunoblot analysis for phosphorylated Smad1/5/8 showed induction of Smad1/5/8 phosphorylation without BMP treatment in cells expressing the mutant but not wild-type *ACVR1* or empty vector constructs. Total Smad1 protein is shown. V5 antibody was used to detect the expression of the V5-tagged ACVR1 proteins. β-Actin was detected as a loading control. Data are representative of 3 individual experiments.

stream signal activation (20–23). Upon ligand binding, BMP receptors form tetramers of 2 BMP type I receptors with 2 BMP type II receptors. The type II receptor phosphorylates the type I receptor within its GS domain, and the phosphorylated GS domain activates downstream signal transduction proteins (15, 24). BMP-specific R-Smads (Smad1, Smad5, and Smad8) are canonical mediators of the intracellular signals activated by BMP type I receptors. Following R-Smad phosphorylation of serine residues in the carboxyterminal SSXS motif, the common partner Smad4 (co-Smad) forms a complex with the phosphorylated R-Smads, translocates into the nucleus, and is recruited to target gene promoters to regulate transcription (25, 26).

The zebrafish (*Danio rerio*) is a valuable genetic model system for studying BMP signaling activity during development and has been used to show that BMPs induce the formation of ventral tissues in the early embryo. Activin receptor–like kinase 8 (Alk8), the zebrafish functional ortholog of human ACVR1, acts as a BMP2/4/7 receptor, upstream of Smad5. The zebrafish Alk8 mutant model (*lost-a-fin*; *laf*) develops a dorsalization of the embryonic axis, indicating insufficient BMP signaling (27, 28). By contrast, overactivation of BMP signaling induces embryonic ventralization (14).

In this study we investigated the effect of the FOP ACVR1 (c.617G→A; R206H) mutation on the BMP signaling pathway. A series of in vitro assays consistently demonstrated that the ACVR1 mutant activates ligand-independent BMP signaling and support the hypothesis that this GS domain mutation results in impaired ACVR1-FKBP1A binding, leading to leaky receptor activation in the absence of ligand. We used zebrafish *alk8* embryos injected with wild-type and mutant *ACVR1* mRNAs to study the function of the FOP ACVR1 mutant in BMP signaling in vivo. Assays for zebrafish embryonic development confirm ligand-independent hyperactivation of BMP activation in vivo. Furthermore, in cell differentiation assays, the ACVR1 mutant receptor induces enhanced chondrogenesis that is both BMP independent and BMP responsive. These data provide strong evidence suggesting that the mutant R206H ACVR1 receptor induces dysregulation of BMP signaling to promote heterotopic endochondral ossification in FOP patients.

Results

The FOP mutant ACVR1 receptor (c.617A; R206H) activates BMP-independent signaling of the BMP pathway. To examine the functional effects of the ACVR1 c.617G→A; R206H mutation that occurs in classic FOP patients, we compared the activity of wild-type (c.617G; R206) and mutant (c.617A; R206H) ACVR1 expression constructs on expression of the BMP pathway transcriptional target gene inhibitors of differentiation 1 (*ID1*) (29, 30). The wild-type construct contains arginine at codon 206 (R206), while the mutant construct replaces codon 206 with histidine (R206H). Cells were transfected with the human *ID1* promoter (–985/+94) luciferase reporter construct (31) together with the wild-type or mutant ACVR1 expression construct. In COS-7 cells expressing the R206H ACVR1 mutant, the *ID1* promoter activity was enhanced 3.0 ± 0.2–fold compared with cells expressing wild-type ACVR1 (Figure 1A). Similar results were observed in C2C12 and U-2 OS cells (Figure 1A). Both wild-type ACVR1 and mutant ACVR1 showed increased *ID1* promoter activity in response to BMP treatment; however, the relative activity levels between the mutant and wild-type responses were not significantly different in these assays (data not shown). These data indicate that although the ACVR1 R206H mutant remains responsive to BMP ligand, this mutation does not require BMP ligand in order to activate BMP pathway signaling and *ID1* gene transcription.

To determine whether BMP-independent expression of BMP target genes by mutant ACVR1 is mediated through specific BMP signaling pathways, we examined activation of Smad1, -5, and -8, which transduce intracellular signals upon phosphorylation by BMP receptors. Following transfection of COS-7 cells with wild-type ACVR1 or mutant R206H ACVR1 expression constructs, total proteins were assayed by immunoblotting for phosphorylated Smad1/5/8. Mutant ACVR1 induced Smad1/5/8 phosphorylation in the absence of exogenous BMP ligand (Figure 1B), consistent with BMP-independent stimulation of *ID1* promoter activity. No Smad phosphorylation was observed in protein extracts from cells expressing equal levels of the wild-type ACVR1 in the absence of

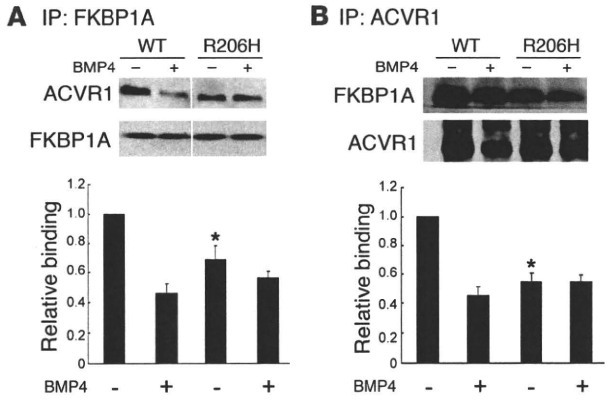

Figure 2
Reduced binding of FKBP12 to mutant ACVR1. COS-7 cells were cotransfected with FKBP1A expression vectors and wild-type ACVR1 or mutant ACVR1 (R206H) constructs. Following no treatment (–) or treatment (+) with 150 ng/ml BMP4, proteins were (**A**) immunoprecipitated with anti-FKBP1A/FKBP12 antibody, then immunoblotted with V5 antibody to detect V5-tagged ACVR1 or (**B**) immunoprecipitated with anti-ACVR1 antibody and immunoblotted to detect FKBP1A (top panels). The relative quantitative interactions between ACVR1 and FKBP12 are shown in the lower panels. Data represent mean ± SEM. $^*P < 0.05$ versus wild-type without BMP treatment. The blots in lanes in **A** were run on the same gel but were noncontiguous (white line).

BMP ligand. Phosphorylation of Smad2, a Smad specifically activated by TGF-β signaling, was not changed in response to mutant ACVR1, indicating specificity for the BMP signaling pathway (data not shown). Similar results were obtained using MC3T3-E1 (preosteoblast) cells (Figure 1B).

Independently of Smads, BMP signaling also activates the p38 MAPK pathway (32–34); however, additional assays in COS-7 cells showed no increase in p38 MAPK phosphorylation in response to ACVR1 (R206H) (data not shown). These data support the hypothesis that the BMP-independent activation of BMP signaling by the mutant R206H ACVR1 receptor is mediated, at least in part, through the canonical BMP Smad signaling pathway.

Mutant R206H ACVR1 shows impaired binding of FKBP1A in the absence of BMP ligand. To explore the mechanism for BMP-independent activation of the BMP pathway by the ACVR1 GS domain R206H mutation, we examined the interaction of this receptor with the inhibitory protein FKBP1A/FKBP12, which binds to the GS domain of type I receptors and prevents leaky signal transduction in the absence of ligand binding (20–23). COS-7 cells were cotransfected with an FKBP1A expression construct and with wild-type or R206H mutant ACVR1 expression constructs. Immunoprecipitation of FKBP1A and subsequent immunoblotting to detect associated ACVR1 showed decreased interaction of FKBP1A with the ACVR1 mutant, compared with wild-type, in the absence of BMP (Figure 2A). Furthermore, while wild-type ACVR1 responded to exogenous BMP as expected by releasing ACVR1-FKBP1A binding, little change was observed in the level of interaction between FKBP1A and the mutant ACVR1 receptor following BMP treatment (Figure 2A). Reciprocal immunoprecipitation experiments (IP for ACVR1, immunoblot for FKBP1A) showed consistent results (Figure 2B). These data suggest that altered GS domain conformation and/or GS domain phosphorylation that may affect either direct or indirect binding of FKBP1A to mutant ACVR1 are possible mechanisms for the BMP-independent activation of the downstream signals by the FOP mutant receptor.

The R206H ACVR1 mutation induces BMP-independent hyperactivity of BMP signaling in vivo. Gradients of BMP signaling in zebrafish embryos direct dorsal-ventral cell fate specification during embryogenesis. Low levels of BMPs allow dorsal cell fate specification (e.g., neural tissue; formation of head structures), whereas higher levels of BMPs direct ventral cell fate specification (e.g., epidermis, blood, formation of tail structures). In zebrafish, Alk8, the ortholog of human ACVR1/ALK2, is a BMP type I receptor that mediates dorsal-ventral axial patterning in the embryo. In the zygotic *alk8* mutant *lost-a-fin* (*laf*), BMP signaling is strongly reduced or absent in post-gastrula stages, causing an expansion of dorsal cell fates at the expense of ventral cell fates that results in a dorsalized phenotype with defects restricted to the tail (Figure 3, A and B, and refs. 27, 28, 35). Microinjection of wild-type human ACVR1 RNA into *alk8*$^{-/-}$ zebrafish embryos rescued approximately 80% of the injected embryos completely or partially (Figure 3, C and F), showing that human ACVR1 can function as a BMP type I receptor in this zebrafish model and substitute for Alk8.

By contrast, microinjection of mutant ACVR1 RNA (encoding R206H) into *alk8*$^{-/-}$ zebrafish embryos induced a strongly ventralized phenotype at 1 day after fertilization (Figure 3, D–F), indicating that the FOP ACVR1 mutation induces hyperactivity of BMP signaling in the zebrafish embryo. Compared with the *alk8* mutants rescued with wild-type ACVR1, R206H ACVR1–injected embryos displayed defects in both head and tail tissues (Figure 3), evidence that development was affected during gastrulation (35). This is further supported by the greatly expanded expression of the ventral markers *eve1* and *gata2* and the more restricted expression of the dorsal marker *foxb1* during gastrulation (Figure 3, G–L).

As further evidence of BMP signaling activation, proteins isolated from zebrafish embryos were examined by immunoblotting for phosphorylated Smad1/5. (There is no known Smad8 in zebrafish.) Embryos that expressed the mutant R206H ACVR1 showed increased levels of Smad1/5 phosphorylation relative to embryos expressing the control ACVR1 (Figure 3M).

Mutant ACVR1 signaling during zebrafish embryogenesis is mediated through Smads. Injection of mutant R206H *ACVR1* mRNA into wild-type embryos induced a range of ventralized phenotypes (Figure 4, A–D). To examine dependence of mutant ACVR1-induced ventralization on downstream signal transduction by Smad5, *smad5* morpholinos, which cause strongly dorsalized (C4 and C5) phenotypes in wild-type embryos (36), were used to inhibit Smad5 expression. Similar to the effect on wild-type embryos (Figure 4, E and F), inhibition of Smad5 induced a dorsalized phenotype in 4-somite-stage embryos (Figure 4, G and H) showing a complete block of the ventralizing effect of the R206H ACVR1 mutant and indicating that Smad5 is required downstream of the mutant ACVR1 receptor to activate BMP signaling in zebrafish. These experiments demonstrate that robust activation of BMP signaling by the mutant R206H ACVR1 receptor requires the Smad pathway in vivo.

Additional evidence that the R206H ACVR1 mutant receptor activates BMP signaling through the canonical Smad pathway was provided by treatment with dorsomorphin (DM), a small mol-

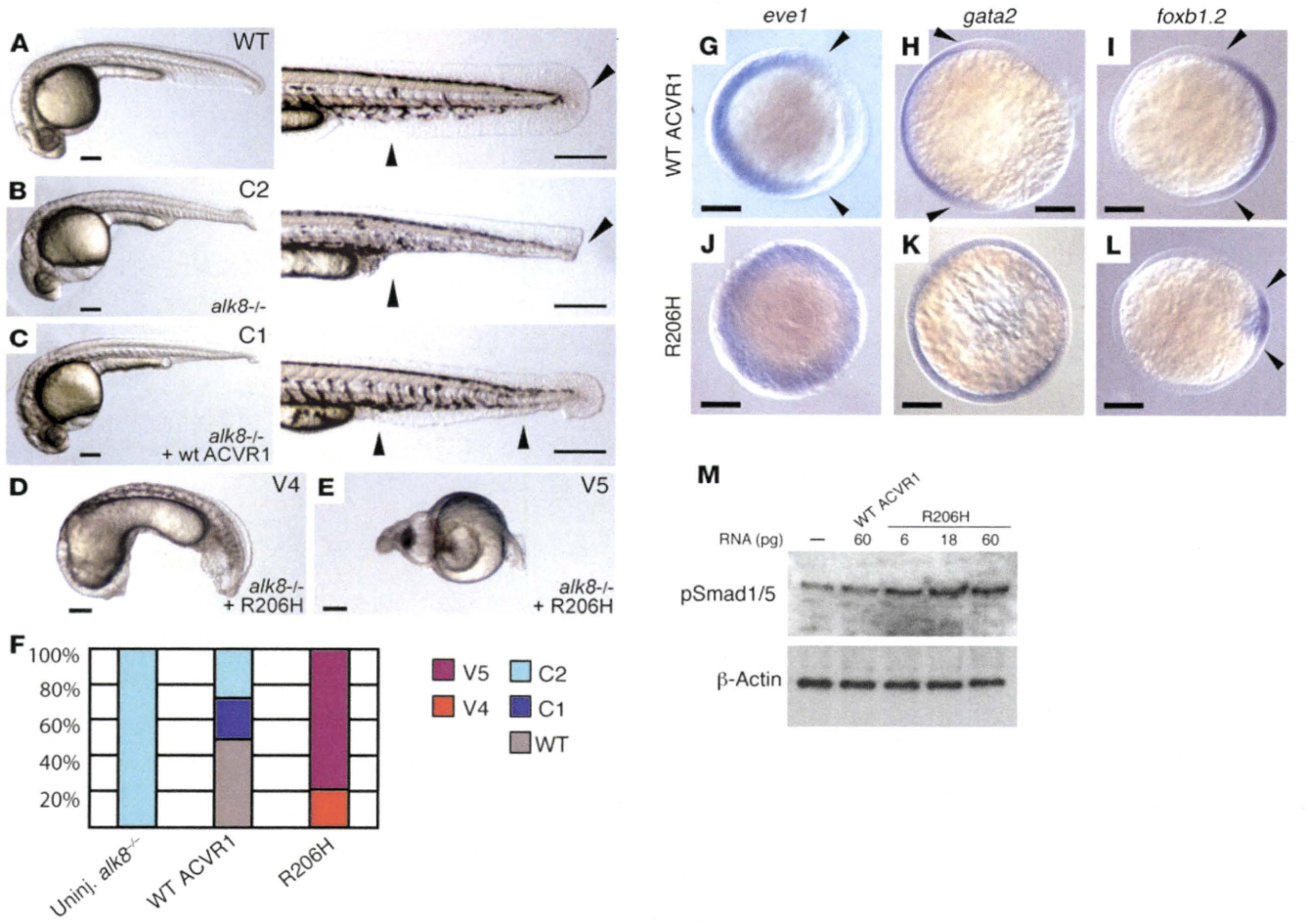

Figure 3

Overexpression of R206H ACVR1 in zebrafish embryos causes strong ventralization by enhancing BMP signaling. Embryos from crosses between *alk8*$^{+/-}$ heterozygotes were injected with wild-type or mutant (R206H) *ACVR1* mRNA, grouped by phenotype, then genotyped for the *alk8* mutation. Compared with wild-type embryos (**A**), uninjected *alk8*$^{-/-}$ embryos are weakly (class 2; C2) dorsalized (**B**), with loss of the ventral fin fold (arrowheads). Wild-type *ACVR1* mRNA (6–60 pg) at the 1-cell stage fully (as in **A**) or partially rescued the mutant phenotype (C1 dorsalized; **C**). (**A**–**C**) Embryos at 1 day after fertilization. Right panels show posterior of same embryos at 2 days after fertilization. (**D** and **E**) Injection of 50 pg mutant *ACVR1* mRNA strongly (**D**) or severely (**E**) ventralized *alk8*$^{-/-}$ embryos. Scale bars: 0.2 mm. (**F**) Phenotype quantification of injected *alk8*$^{-/-}$ embryos (WT ACVR1, $n = 43$; R206H, $n = 15$). (**G**–**L**) In situ hybridization of wild-type embryos injected with wild-type (**G**–**I**) or mutant *ACVR1* (**J**–**L**) to detect ventral markers *eve1* (**G**, $n = 12/12$; **J**, $n = 10/13$; onset of gastrulation) and *gata2* (**H**, $n = 14/14$; **K**, $n = 15/19$; mid-gastrulation) or dorsal marker *foxb1.2* (**I**, $n = 12/12$; **L**, $n = 6/13$, 7/13 showed no expression; mid-gastrulation). Animal pole views with dorsal at right. Arrowheads delineate the dorsal-ventral expression domains. Scale bars: 0.2 mm. (**M**) Total mid-gastrulation stage protein from wild-type zebrafish embryos injected with wild-type or mutant *ACVR1* mRNA was immunoblotted to detect phospho-Smad1/5. Increased Smad1/5 phosphorylation was observed even at a low dose (6 pg) of mutant *ACVR1*. β-Actin was detected as a loading control.

ecule that inhibits BMP type I receptor signaling (ACVR1/ALK2, BMPR1A/ALK3, BMPR1B/ALK6) by specifically blocking Smad pathway activation (37). The reduced BMP signaling caused by DM induces severely dorsalized embryonic phenotypes in the zebrafish (Figure 4I) (37). To determine whether DM can disrupt the ventralizing activity of the R206H ACVR1 mutation, we injected wild-type embryos with mutant *ACVR1* mRNA and treated them with DM or carrier solvent only (DMSO). As with the response of uninjected embryos, the majority of R206H-ACVR1–injected embryos that were treated with DM developed a class 5 severely dorsalized phenotype (Figure 4I). Inhibition of the mutant R206H ACVR1 receptor by DM is consistent with Smad-dependent signaling in the embryo and indicates that the mutant receptor requires the Smad signaling pathway to activate BMP signaling and influence zebrafish development.

Mutant R206H ACVR1 effects on dorsal-ventral patterning are BMP independent. To complement our in vitro studies showing BMP-independent activation of BMP signaling, we investigated the function of the R206H ACVR1 mutant receptor in vivo by using zebrafish embryos lacking bmp2b and/or bmp7, the BMP ligands responsible for zebrafish dorsal-ventral patterning. Both *bmp7* and *bmp2b* are required independently and nonredundantly for BMP signaling in the early zebrafish embryo due to the function of these ligands in a Bmp2b-Bmp7 heterodimer (38, 39). Thus, loss of either of these ligands causes loss of all BMP signaling in the early embryo and a C5 dorsalized phenotype that is identical to the