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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.

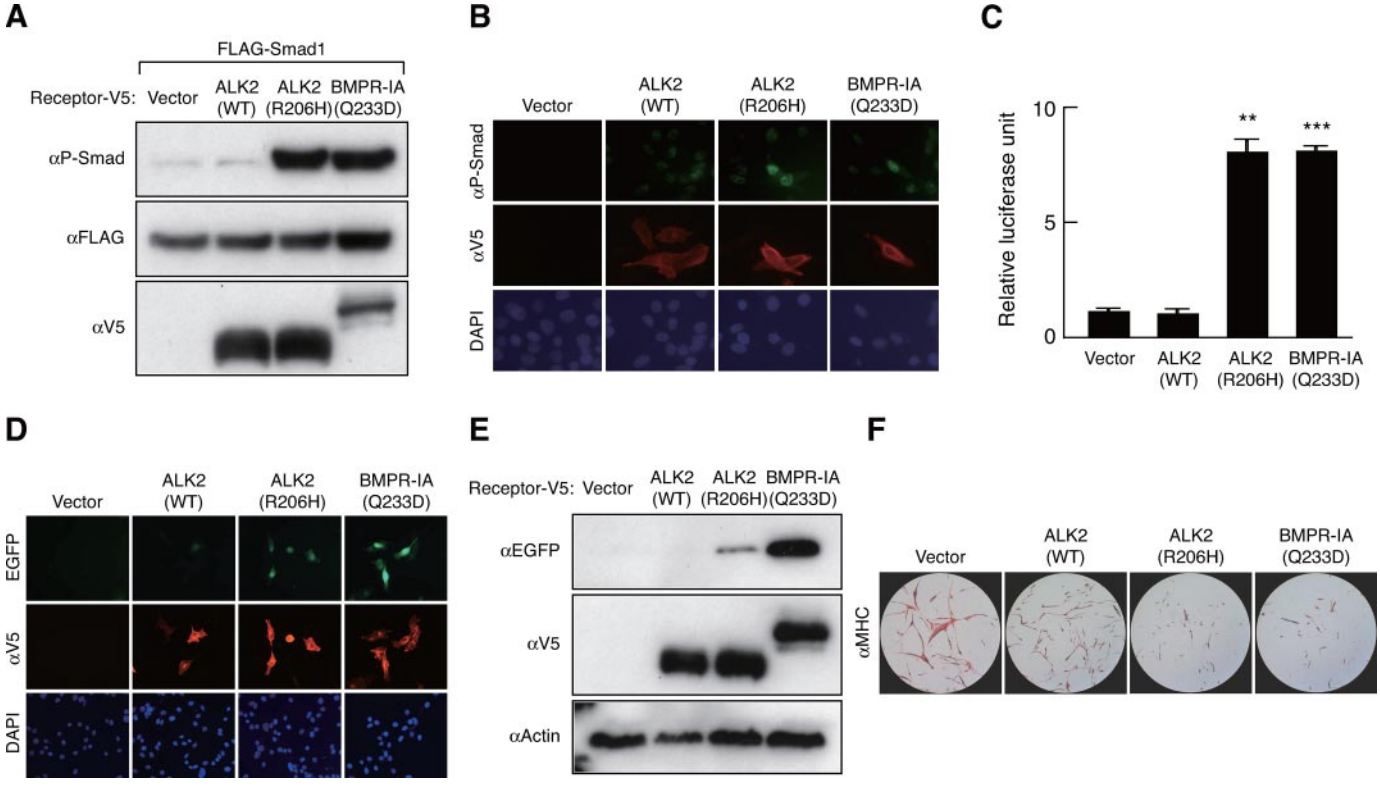

FIGURE 1. **ALK2(R206H) acts as a constitutively activated BMP receptor.** *A,* C2C12 cells were co-transfected with FLAG-tagged Smad1 and a V5-tagged wild-type ALK2 (*WT*), ALK2(R206H), or BMPR-IA(Q233D). Cell lysates were immunoblotted with anti-phospho-Smad1/5/8, anti-FLAG, or anti-V5 antibody. Constitutively 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.

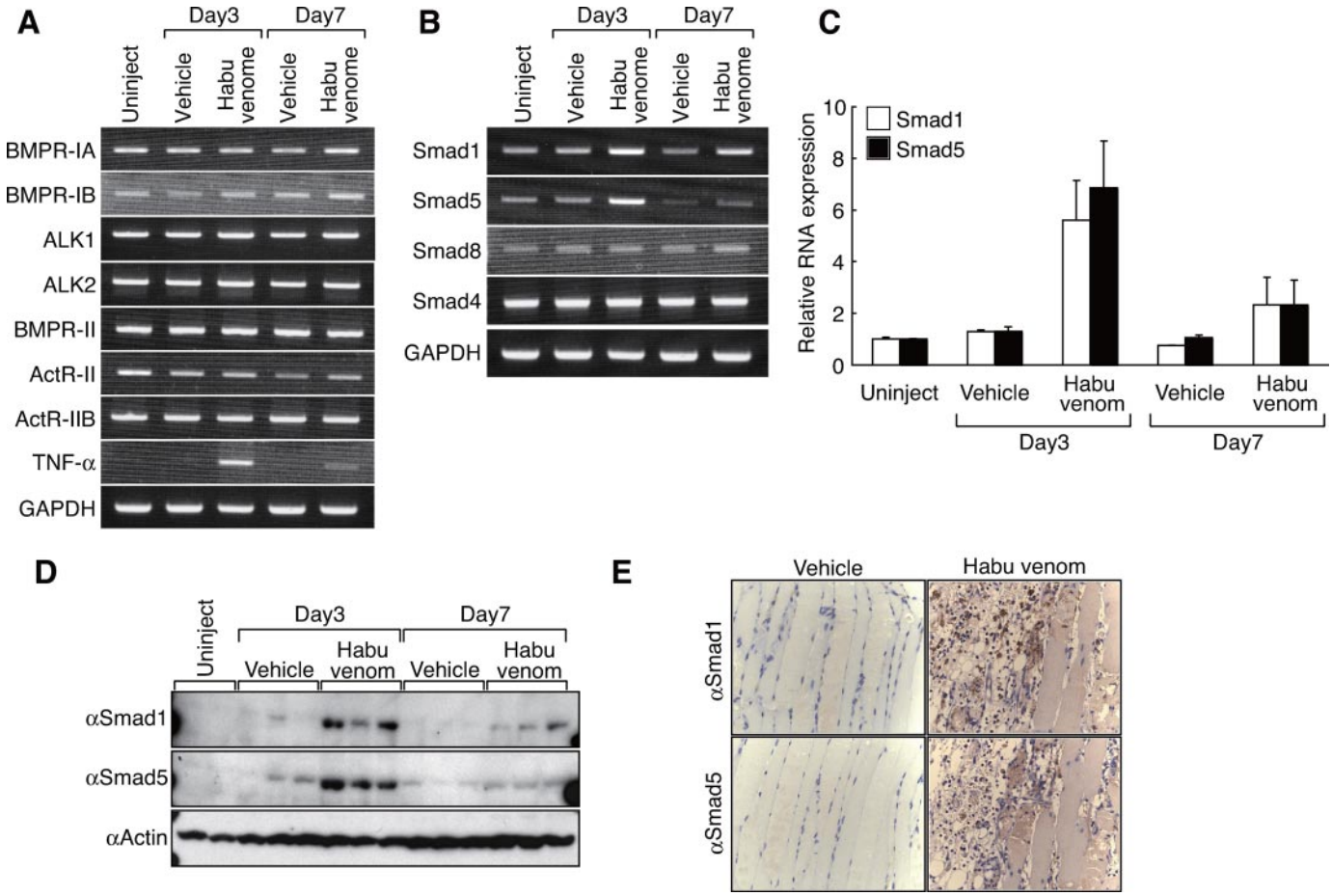

FIGURE 2. **Increased Smad1 and Smad5 mRNA during muscular injury *in vivo*.** *A–C,* mice were injected with vehicle (saline) or habu venom in femoral muscle, and total RNA was prepared after 3 or 7 days. Messenger RNA levels of BMP receptors (*A*) and Smads (*B* and *C*) were determined by RT-PCR (*A* 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-

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-

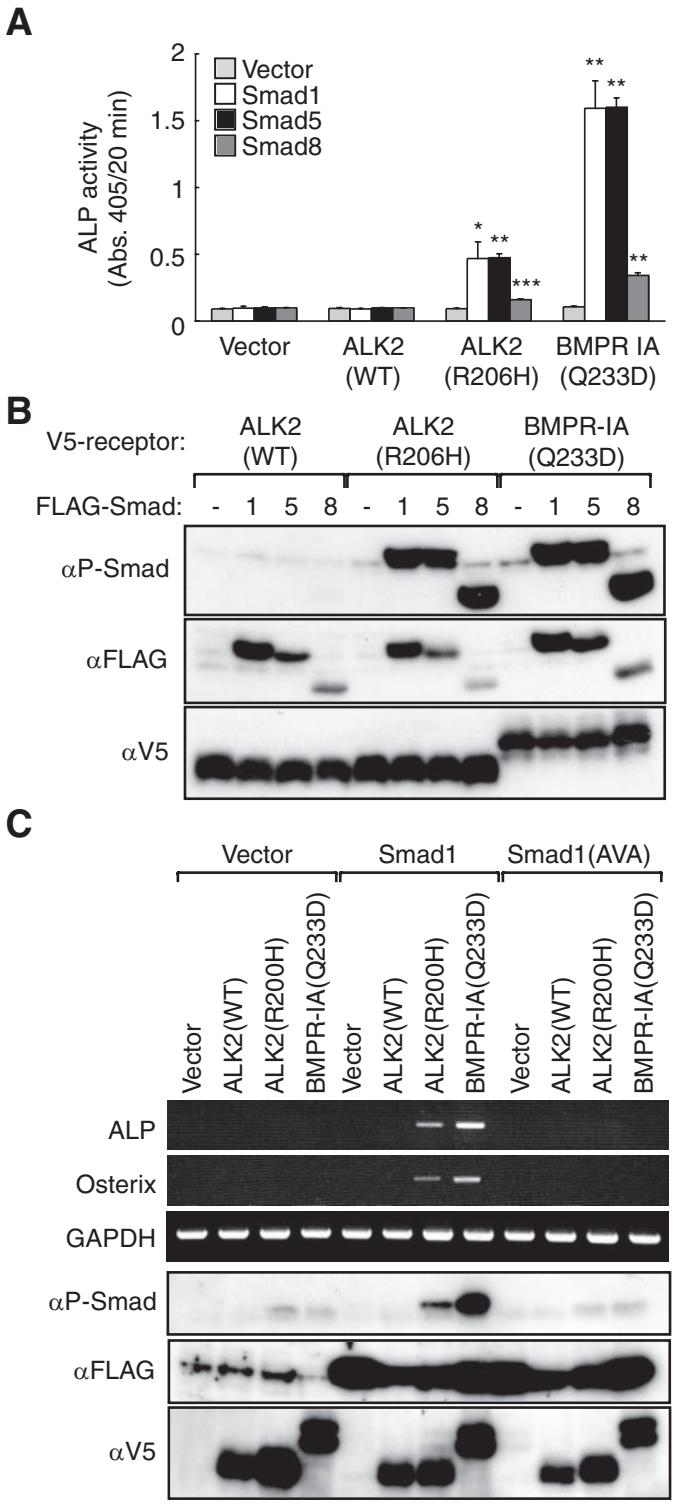

FIGURE 3. **Cooperative effect of ALK2(R206H) with Smad1/5 in induction of osteoblastic differentiation.** *A* and *B*, C2C12 cells were co-transfected with FLAG-tagged Smad1, Smad5, or Smad8 with V5-tagged wild-type ALK2(WT), ALK2(R206H), or BMPR-IA(Q233D). ALP activity (*A*) and levels of phosphorylation 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.

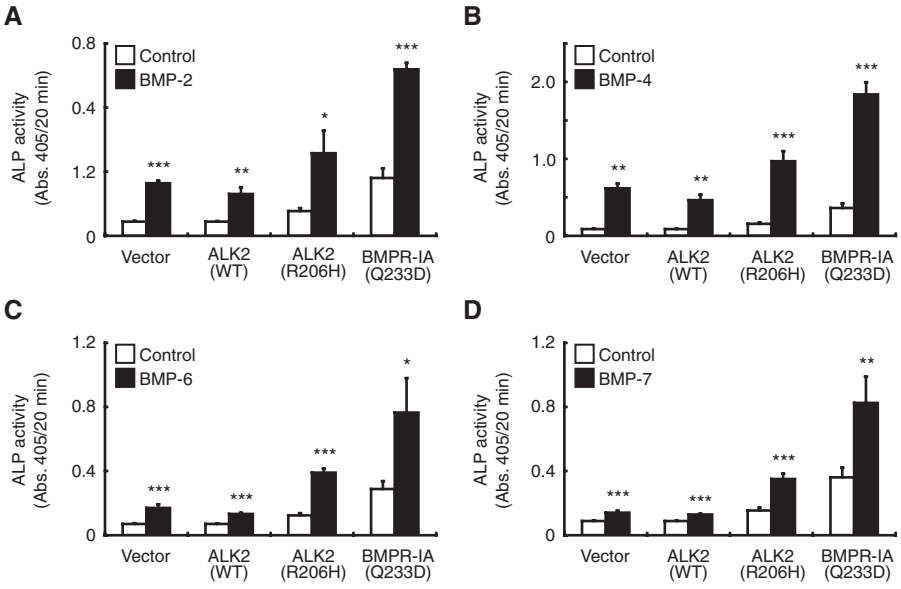

FIGURE 4. **BMPs further stimulate ALP activity induced by ALK2(R206H) and Smad1 in C2C12 myoblasts.** C2C12 cells co-transfected with Smad1 and wild-type (*WT*) ALK2, ALK2(R206H), or BMPR-IA(Q233D) were treated for 3 days with 300 ng/ml of BMP-2 (*A*) or 100 ng/ml of BMP-4 (*B*), BMP-6 (*C*), or BMP-7 (*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*).

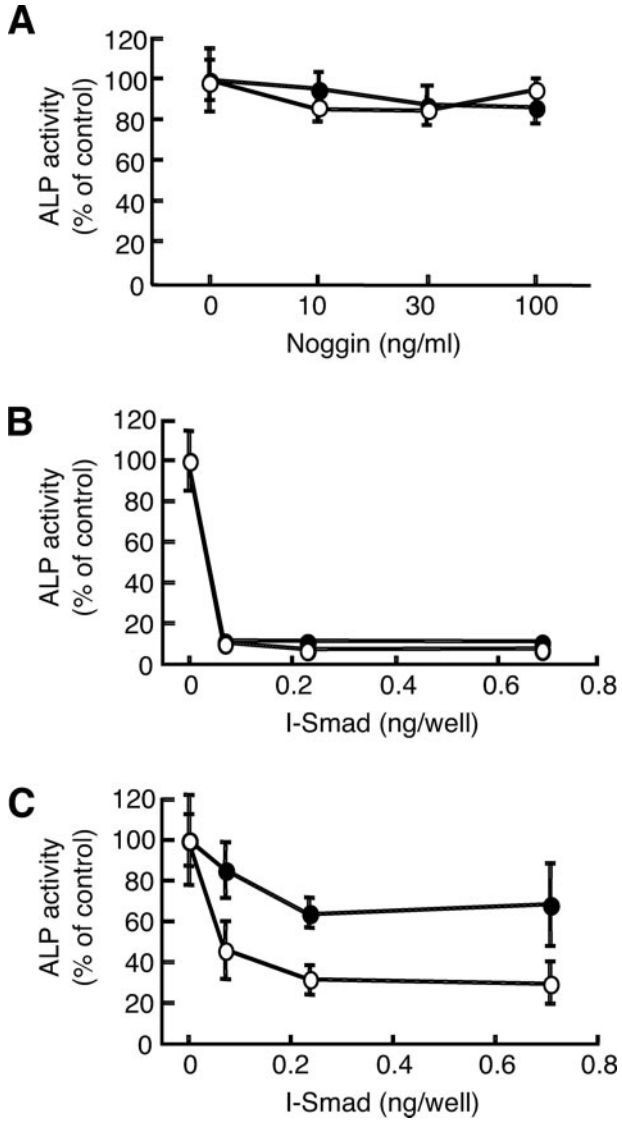

FIGURE 5. **Inhibition of biological activities of ALK2(R206H) by Noggin and I-Smads.** *A,* C2C12 cells co-transfected with Smad1 and ALK2(R206H) (*closed circles*) or BMPR-IA(Q233D) (*open circles*) were treated with Noggin. ALP activity was determined on day 3. *B* and *C,* C2C12 cells were co-transfected 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

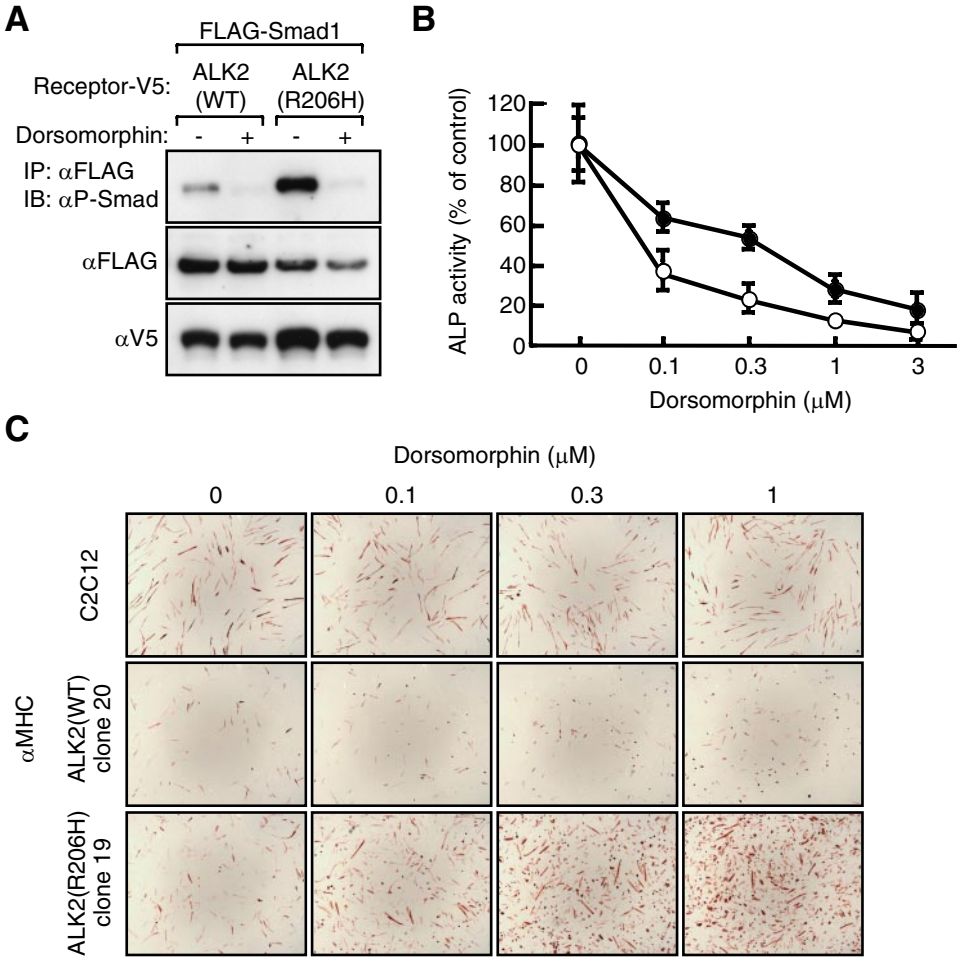

FIGURE 6. **Dorsomorphin inhibits ALK2(R206H) activity.** *A,* HEK293 cells were co-transfected with FLAG-tagged Smad1 and V5-tagged ALK2(R206H) or V5-BMPR-IA(Q233D) and then treated for 3 days with dorsomorphin at 3 μM. Levels of phosphorylated Smad1 were determined by immunoblotting (*IB*). *IP,* immunoprecipitation. *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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