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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.

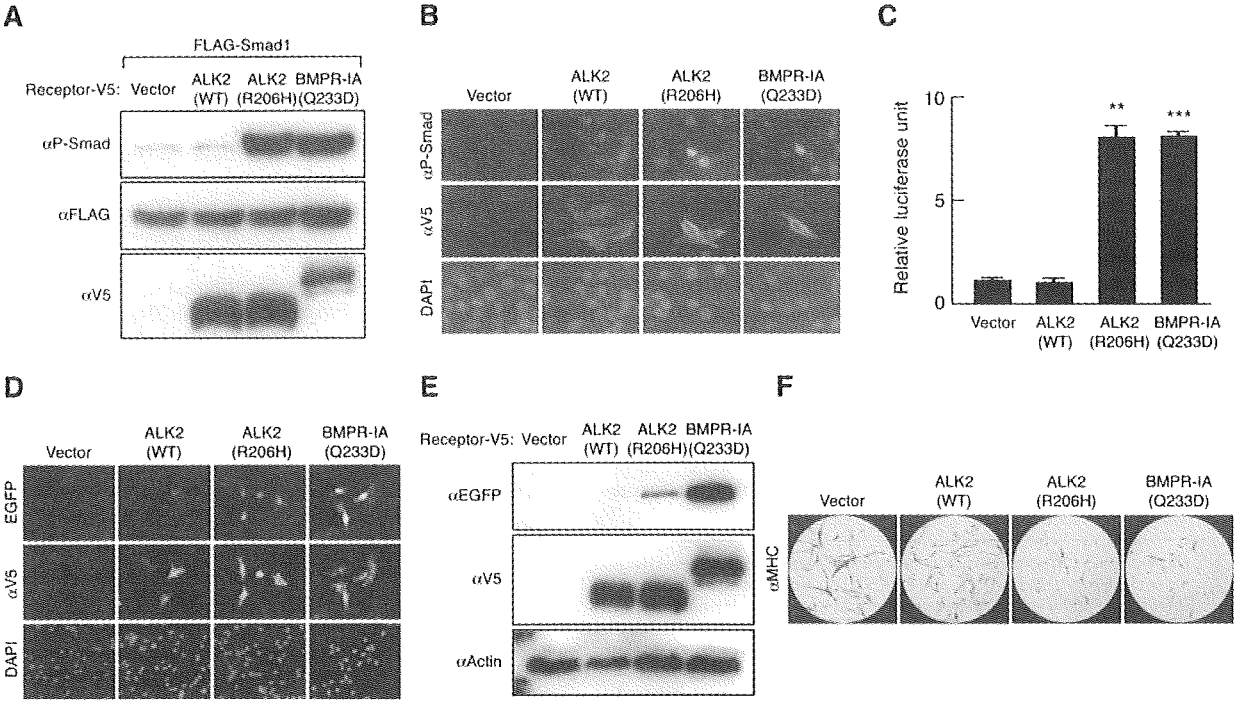

FIGURE 1. **ALK2(R206H) acts as a constitutively activated BMP receptor.** *A*, C2C12 cells were co-transfected with FLAG-tagged Smad1 and a V5-tagged wild-type ALK2 (*WT*), ALK2(R206H), or BMPR-IA(Q233D). Cell lysates were immunoblotted with anti-phospho-Smad1/5/8, anti-FLAG, or anti-V5 antibody. Constitutively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

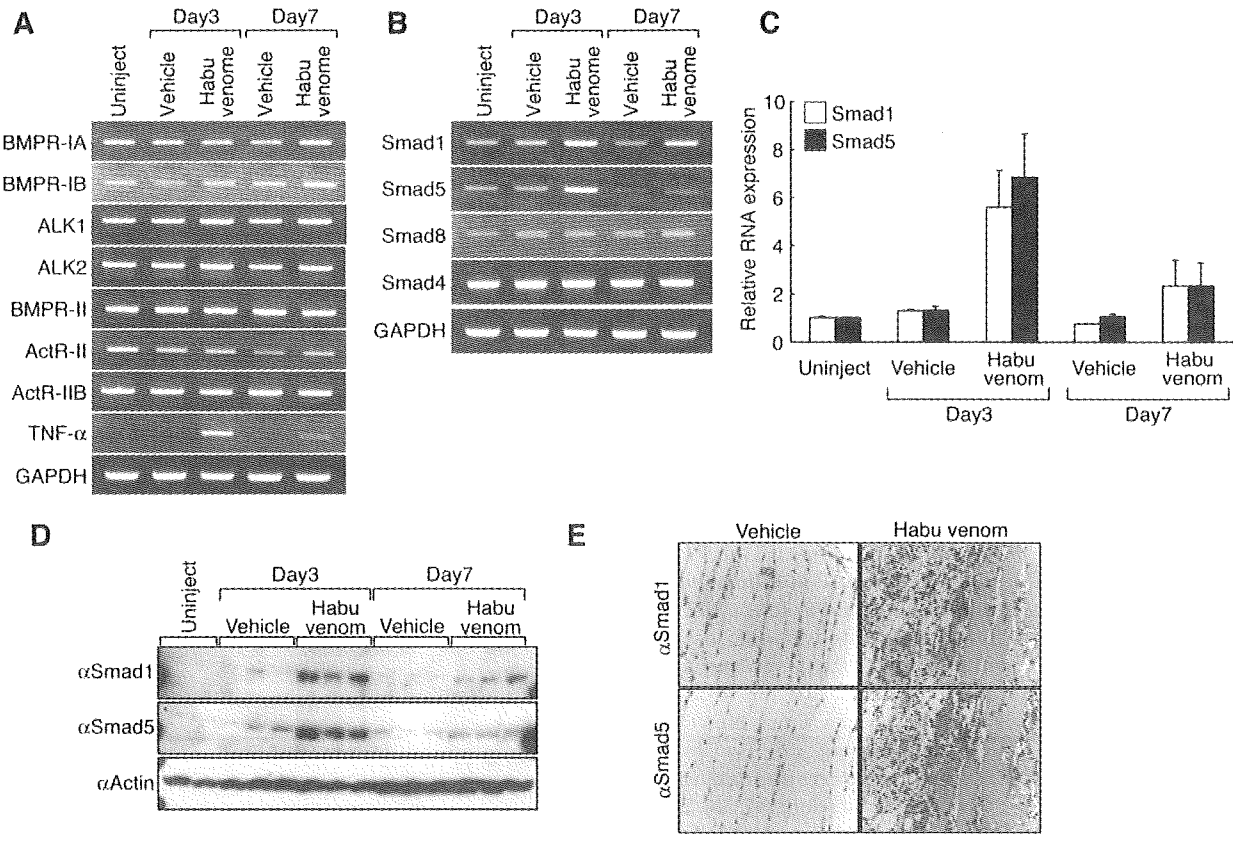

FIGURE 2. **Increased Smad1 and Smad5 mRNA during muscular injury *in vivo*.** *A–C*, mice were injected with vehicle (saline) or habu venom in femoral muscle, and total RNA was prepared after 3 or 7 days. Messenger RNA levels of BMP receptors (*A*) and Smads (*B* and *C*) were determined by RT-PCR (*A*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

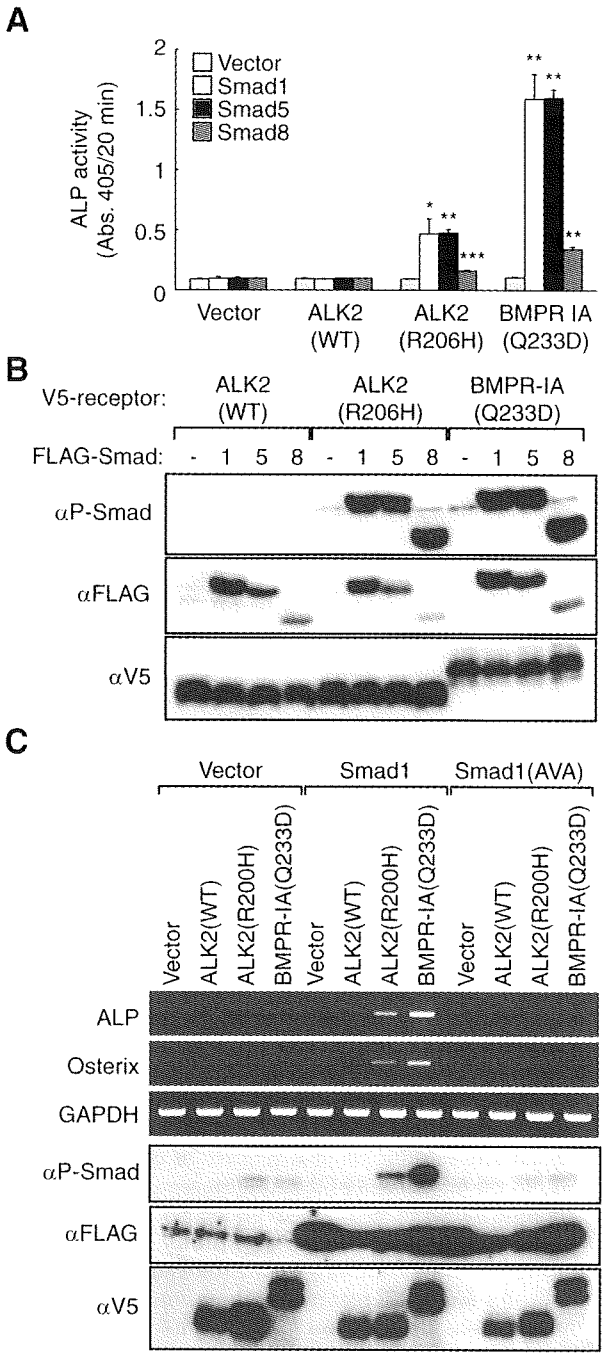

FIGURE 3. **Cooperative effect of ALK2(R206H) with Smad1/5 in induction of osteoblastic differentiation.** *A* and *B*, C2C12 cells were co-transfected with FLAG-tagged Smad1, Smad5, or Smad8 with V5-tagged wild-type ALK2(WT), ALK2(R206H), or BMPR-IA(Q233D). ALP activity (*A*) and levels of phosphorylation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

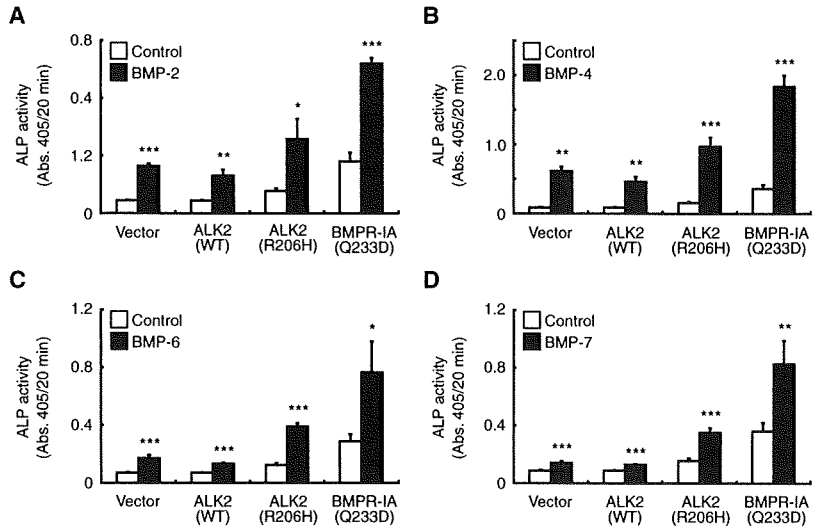

FIGURE 4. **BMPs further stimulate ALP activity induced by ALK2(R206H) and Smad1 in C2C12 myoblasts.** C2C12 cells co-transfected with Smad1 and wild-type (*WT*) ALK2, ALK2(R206H), or BMPR-IA(Q233D) were treated for 3 days with 300 ng/ml of BMP-2 (*A*) or 100 ng/ml of BMP-4 (*B*), BMP-6 (*C*), or BMP-7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

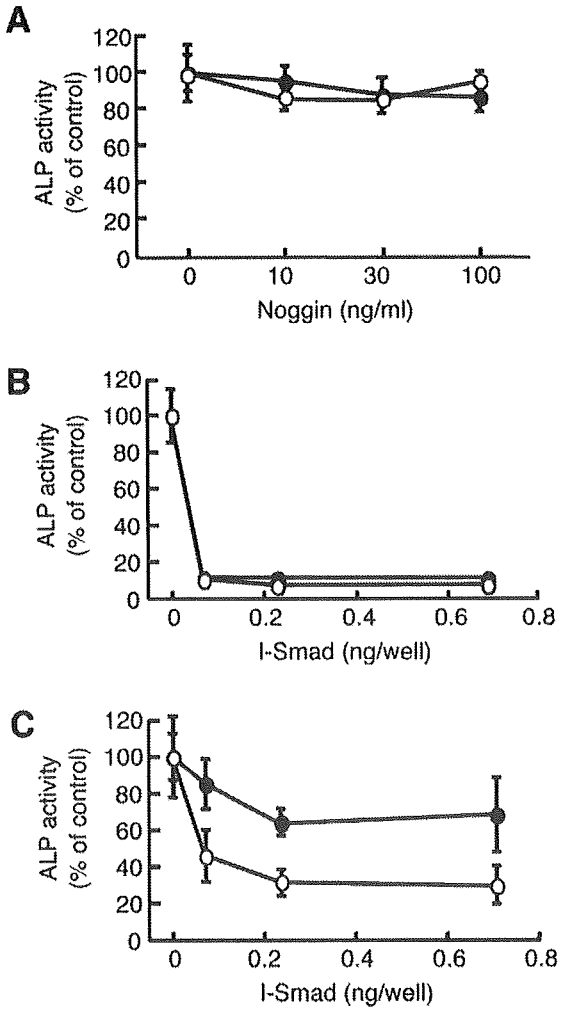

FIGURE 5. **Inhibition of biological activities of ALK2(R206H) by Noggin and I-Smads.** *A*, C2C12 cells co-transfected with Smad1 and ALK2(R206H) (*closed circles*) or BMPR-IA(Q233D) (*open circles*) were treated with Noggin. ALP activity was determined on day 3. *B* and *C*, C2C12 cells were co-transfected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

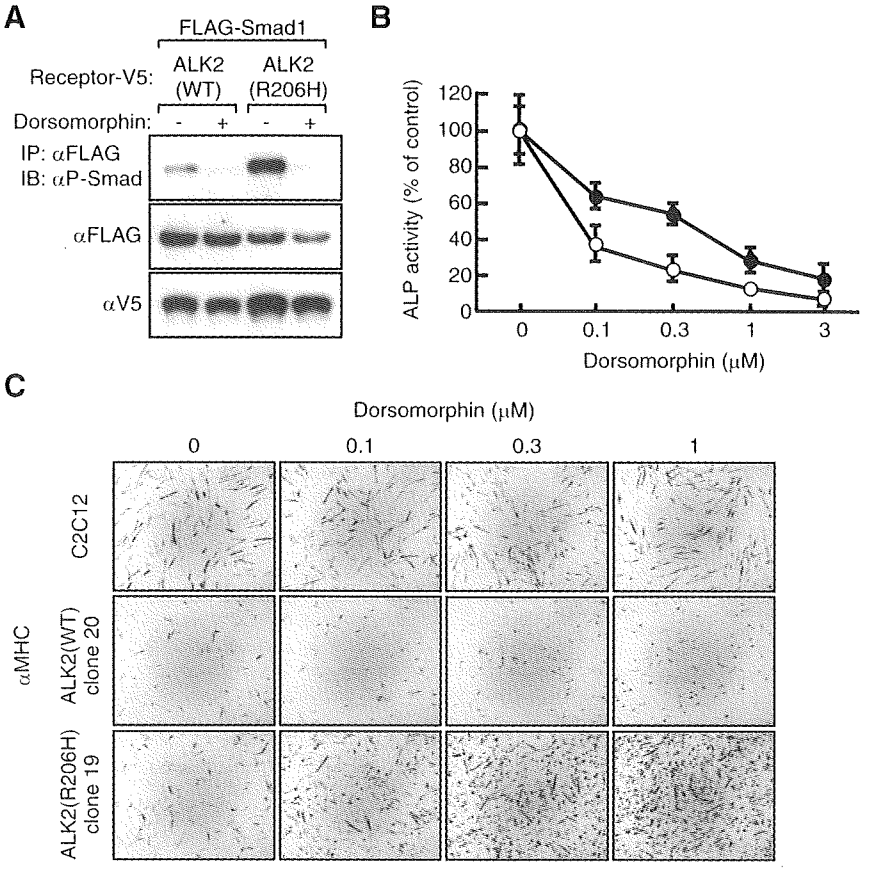

FIGURE 6. **Dorsomorphin inhibits ALK2(R206H) activity.** *A*, HEK293 cells were co-transfected with FLAG-tagged Smad1 and V5-tagged ALK2(R206H) or V5-BMPR-IA(Q233D) and then treated for 3 days with dorsomorphin at 3 μM. Levels of phosphorylated Smad1 were determined by immunoblotting (*IB*). *IP*, immunoprecipitation.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

1. Cohen, R. B., Hahn, G. V., Tabas, J. A., Peeper, J., Levitz, C. L., Sando, A., Sando, N., Zasloff, M., and Kaplan, F. S. (1993) *J. Bone Jt. Surg. Am.* 75, 215–219

2. Kaplan, F. S., McCluskey, W., Hahn, G., Tabas, J. A., Muenke, M., and Zasloff, M. A. (1993) *J. Bone Jt. Surg. Am.* **75,** 1214–1220
3. Kaplan, F. S., Tabas, J. A., Gannon, F. H., Finkel, G., Hahn, G. V., and Zasloff, M. A. (1993) *J. Bone Jt. Surg. Am.* **75,** 220–230
4. Kaplan, F. S., Shen, Q., Lounev, V., Seeman, P., Groppe, J., Katagiri, T., Pignolo, R. J., and Shore, E. M. (2008) *J. Bone Miner. Metab.* **26,** 521–530
5. Kaplan, F. S., Glaser, D. L., Shore, E. M., Pignolo, R. J., Xu, M., Zhang, Y., Senitzer, D., Forman, S. J., and Emerson, S. G. (2007) *J. Bone Jt. Surg. Am.* **89,** 347–357
6. Urist, M. R. (1965) *Science* **150,** 893–899
7. Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988) *Science* **242,** 1528–1534
8. Glaser, D. L., Economides, A. N., Wang, L., Liu, X., Kimble, R. D., Fandl, J. P., Wilson, J. M., Stahl, N., Kaplan, F. S., and Shore, E. M. (2003) *J. Bone Jt. Surg. Am.* **85,** 2332–2342
9. Miyazono, K., Maeda, S., and Imamura, T. (2005) *Cytokine Growth Factor Rev.* **16,** 251–263
10. Katagiri, T., Suda, T., and Miyazono, K. (2008) in *The TGF-β Family* (Miyazono, K., and Derynck, R., eds) pp. 121–149, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
11. Afrakhte, M., Moren, A., Jossan, S., Itoh, S., Sampath, K., Westermark, B., Heldin, C. H., Heldin, N. E., and ten Dijke, P. (1998) *Biochem. Biophys. Res. Commun.* **249,** 505–511
12. Katagiri, T., Imada, M., Yanai, T., Suda, T., Takahashi, N., and Kamijo, R. (2002) *Genes Cells* **7,** 949–960
13. Takase, M., Imamura, T., Sampath, T. K., Takeda, K., Ichijo, H., Miyazono, K., and Kawabata, M. (1998) *Biochem. Biophys. Res. Commun.* **244,** 26–29
14. Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., and Miyazono, K. (1997) *Nature* **389,** 622–626
15. Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H., and ten Dijke, P. (1997) *Nature* **389,** 631–635
16. Fiori, J. L., Billings, P. C., de la Pena, L. S., Kaplan, F. S., and Shore, E. M. (2006) *J. Bone Miner. Res.* **21,** 902–909
17. Gannon, F. H., Kaplan, F. S., Olmsted, E., Finkel, G. C., Zasloff, M. A., and Shore, E. (1997) *Hum. Pathol.* **28,** 339–343
18. Kaplan, F. S., Fiori, J., De La Pena, L. S., Ahn, J., Billings, P. C., and Shore, E. M. (2006) *Ann. N. Y. Acad. Sci.* **1068,** 54–65
19. Kaplan, F. S., Tabas, J. A., and Zasloff, M. A. (1990) *Calcif. Tissue Int.* **47,** 117–125
20. Shafritz, A. B., Shore, E. M., Gannon, F. H., Zasloff, M. A., Taub, R., Muenke, M., and Kaplan, F. S. (1996) *N. Engl. J. Med.* **335,** 555–561
21. Shore, E. M., Xu, M., Feldman, G. J., Fenstermacher, D. A., Cho, T. J., Choi, I. H., Connor, J. M., Delai, P., Glaser, D. L., LeMerrer, M., Morhart, R., Rogers, J. G., Smith, R., Triffitt, J. T., Urtizberea, J. A., Zasloff, M., Brown, M. A., and Kaplan, F. S. (2006) *Nat. Genet.* **38,** 525–527
22. Nakajima, M., Haga, N., Takikawa, K., Manabe, N., Nishimura, G., and Ikegawa, S. (2007) *J. Hum. Genet.* **52,** 473–475
23. Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda, T. (1994) *J. Cell Biol.* **127,** 1755–1766
24. Katagiri, T., Akiyama, S., Namiki, M., Komaki, M., Yamaguchi, A., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda, T. (1997) *Exp. Cell Res.* **230,** 342–351
25. Kodaira, K., Imada, M., Goto, M., Tomoyasu, A., Fukuda, T., Kamijo, R., Suda, T., Higashio, K., and Katagiri, T. (2006) *Biochem. Biophys. Res. Commun.* **345,** 1224–1231
26. Akiyama, S., Katagiri, T., Namiki, M., Yamaji, N., Yamamoto, N., Miyama, K., Shibuya, H., Ueno, N., Wozney, J. M., and Suda, T. (1997) *Exp. Cell Res.* **235,** 362–369
27. Goto, K., Kamiya, Y., Imamura, T., Miyazono, K., and Miyazawa, K. (2007) *J. Biol. Chem.* **282,** 20603–20611
28. Yu, P. B., Hong, C. C., Sachidanandan, C., Babitt, J. L., Deng, D. Y., Hoyng, S. A., Lin, H. Y., Bloch, K. D., and Peterson, R. T. (2008) *Nat. Chem. Biol.* **4,** 33–41
29. Souchelnytskyi, S., ten Dijke, P., Miyazono, K., and Heldin, C. H. (1996) *EMBO J.* **15,** 6231–6240
30. Aoki, H., Fujii, M., Imamura, T., Yagi, K., Takehara, K., Kato, M., and Miyazono, K. (2001) *J. Cell Sci.* **114,** 1483–1489
31. Wieser, R., Wrana, J. L., and Massague, J. (1995) *EMBO J.* **14,** 2199–2208
32. Perry, R. L., Parker, M. H., and Rudnicki, M. A. (2001) *Mol. Cell* **8,** 291–301
33. Connor, J. M., and Evans, D. A. (1982) *J. Bone Jt. Surg. Br.* **64,** 76–83
34. Lanchoney, T. F., Cohen, R. B., Rocke, D. M., Zasloff, M. A., and Kaplan, F. S. (1995) *J. Pediatr.* **126,** 762–764
35. Luchetti, W., Cohen, R. B., Hahn, G. V., Rocke, D. M., Helpin, M., Zasloff, M., and Kaplan, F. S. (1996) *Oral Surg. Oral Med. Oral Pathol. Oral. Radiol. Endod.* **81,** 21–25
36. Davies, M. R., Lund, R. J., Mathew, S., and Hruska, K. A. (2005) *J. Am. Soc. Nephrol.* **16,** 917–928
37. David, L., Mallet, C., Keramidas, M., Lamande, N., Gasc, J. M., Dupuis-Girod, S., Plauchu, H., Feige, J. J., and Bailly, S. (2008) *Circ. Res.* **102,** 914–922
38. Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T. K., Andries, M., Smith, J. C., Heldin, C. H., and Miyazono, K. (1995) *J. Cell Biol.* **130,** 217–226
39. Ikenoue, T., Jingushi, S., Urabe, K., Okazaki, K., and Iwamoto, Y. (1999) *J. Cell. Biochem.* **75,** 206–214
40. Link, B. A., and Nishi, R. (1997) *Exp. Cell Res.* **233,** 350–362
41. Attisano, L., Carcamo, J., Ventura, F., Weis, F. M., Massague, J., and Wrana, J. L. (1993) *Cell* **75,** 671–680
42. ten Dijke, P., Ichijo, H., Franzen, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C. H., and Miyazono, K. (1993) *Oncogene* **8,** 2879–2887

DRAGON, a GPI-anchored membrane protein, inhibits BMP signaling in C2C12 myoblasts

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Bone morphogenetic proteins (BMPs) induce osteoblastic differentiation of myoblasts via binding to cell surface receptors. Repulsive guidance molecules (RGMs) have been identified as BMP co-receptors. We report here that DRAGON/RGMb, a member of the RGM family, suppressed BMP signaling in C2C12 myoblasts via a novel mechanism. All RGMs were expressed in C2C12 cells that were differentiated into myocytes and osteoblastic cells, but RGMc was not detected in immature cells. In C2C12 cells, only DRAGON suppressed ALP and Id1 promoter activities induced by BMP-4 or by constitutively activated BMP type I receptors. This inhibition by DRAGON was dependent on the secretory form of the von Willbrand factor type D domain. DRAGON even suppressed BMP signaling induced by constitutively activated Smad1. Over-expression of neogenin did not alter the inhibitory capacity of DRAGON. Taken together, these findings indicate that DRAGON may be an inhibitor of BMP signaling in C2C12 myoblasts. We also suggest that a novel molecule(s) expressed on the cell membrane may mediate the signal transduction of DRAGON in order to suppress BMP signaling in C2C12 myoblasts.

Introduction

Bone morphogenetic proteins (BMPs) were first isolated from demineralized bone matrix and identified as factors responsible for inducing ectopic bone formation in muscular tissues (Urist 1965; Wozney et al. 1988). BMPs are responsible not only for artificial ectopic bone formation but also for physiological skeletal development (Thomas et al. 1997). BMP signaling is initiated when a ligand binds to complexes of type I and type II serine/threonine kinase receptors (Miyazono et al. 2005; Katagiri et al. 2008). The BMP-bound type II receptor phosphorylates the intracellular "GS" domain of the type I receptor. The activated type I receptor then phosphorylates Smad1/5/8 transcription factors in the cytoplasm. The phosphorylated Smad1/5/8 form transcriptionally active complexes with Smad4, translocate into the nucleus and bind to the regulatory elements of target genes (including Id1, which encodes an inhibitor of myogenesis) (Afrakhte et al. 1998; Katagiri et al. 2008). Among the type I receptors, substitution mutations of the specific conserved residues in the GS domains activate intracellular signaling without the binding of ligands or type II receptors (Wieser et al. 1995; Akiyama et al. 1997; Fujii et al. 1999; Aoki et al. 2001). Recently, we found that substitution of the phosphorylation site at the carboxyl terminus in Smad1 also activated downstream signaling events, including Id1 expression and osteoblastic differentiation, without phosphorylation by BMP type I receptors (Nojima & Katagiri, unpublished observation).

The repulsive guidance molecule (RGM) family members RGMa, DRAGON/RGMb and RGMc/hemojuvelin/HEF2 are secretory glycosylphosphatidylinositol (GPI)-anchored membrane proteins that have been implicated in axonal guidance and neural tube formation during embryogenesis (Monnier et al. 2002; Niederkofler et al. 2004; Papanikolaou et al. 2004; Samad et al. 2004). They have significant sequence homology to one another (50–60% amino acid identity) (Papanikolaou et al. 2004;

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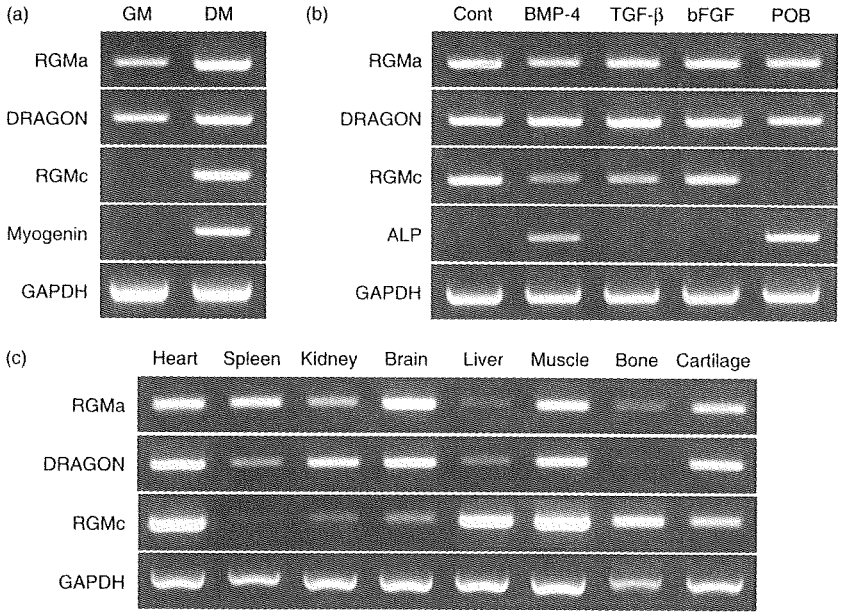

Figure 1 Expression levels of RGMs in C2C12 cells and various murine tissues. (a,b) RT-PCR analysis of RGM levels in C2C12 cells. C2C12 myoblasts were cultured with growth medium (GM) and differentiation medium (DM) for 7 days (a) or with DM in the presence of BMP-4 (100 ng/mL), TGF- 1 (5 ng/mL) or bFGF (20 ng/mL) for 3 days (b). Myogenin and ALP mRNA expression levels were used as markers of myogenic and osteoblastic differentiation, respectively. (c) RT-PCR analysis of RGMs in various murine tissues.

Babitt et al. 2005). All three proteins have a conserved von Willbrand factor (vWF) type D domain (Monnier et al. 2002), although its physiological function is still unknown. All of the RGM family members were found to act as co-receptors for the BMP subfamily. These proteins bind to BMP ligands as well as type I and type II BMP receptors, thereby enhancing BMP signaling (Babitt et al. 2005, 2006; Samad et al. 2005). A genetic mutation was found in the hemojuvelin gene in patients with juvenile hemochromatosis, a condition that is caused by the decreased expression of hepcidin, a key regulator of iron homeostasis in hepatocytes. Hemojuvelin enhanced the BMP-dependent up-regulation of hepcidin expression in hepatocytes (Papanikolaou et al. 2004; Babitt et al. 2006, 2007). Cells transfected with RGMa have been shown to utilize both BMPR-II and ActR-IIA for BMP-2 or BMP-4 signaling (Xia et al. 2007). However, to our knowledge, a physiological role for DRAGON, especially in bone metabolism, remains unestablished.

The ectopic bone-inducing activity of BMPs in muscle tissue is, at least in part, reflected in the in vitro culture of myoblasts, including mouse C2C12 cells. Treatment of these cells with BMPs inhibits the myogenic differentiation of myoblasts and converts their differentiation pathway into that of osteoblast lineage cells (Katagiri et al. 1994). Over-expression of a constitutively activated BMP type I receptor also induces similar differentiation conversions of myoblasts (Akiyama et al. 1997; Fujii et al. 1999; Aoki et al. 2001). We report here that DRAGON inhibits BMP signaling in C2C12 cells via a region that includes the secretory form of the vWF type D domain. DRAGON also inhibits the BMP signaling induced by a constitutively active form of Smad1. These findings indicate that a novel molecule(s) expressed on the cell membrane may mediate the signal transduction of DRAGON to suppress BMP signaling in C2C12 myoblasts.

Results

Expression levels of RGMs in C2C12 cells and mouse tissues

First, we examined the expression levels of RGM mRNAs in C2C12 myoblasts. RGMa and DRAGON were expressed in both growing and differentiated myocytes, but RGMc was detected only in myogenin-expressing differentiated cells (Fig. 1a). Although all DRAGON mRNAs were detected in C2C12 cells in the presence of BMP-4, TGF- 1 or bFGF, the expression levels of RGMc were decreased by BMP-4 (Fig. 1b). Primary osteoblasts expressed RGMa and DRAGON, but did not express RGMc (Fig. 1b), suggesting that osteoblastic differentiation induced by BMPs suppresses RGMc expression in C2C12 cells. RT-PCR analysis using RNAs prepared from various murine tissues indicated that RGMa and DRAGON were highly expressed in the heart, brain, muscle and cartilage (Fig. 1c). RGMc was strongly expressed in heart, liver, muscle, bone and cartilage (Fig. 1c). In bone tissue, RGMc may be expressed in osteocytes and/or bone marrow cells rather than

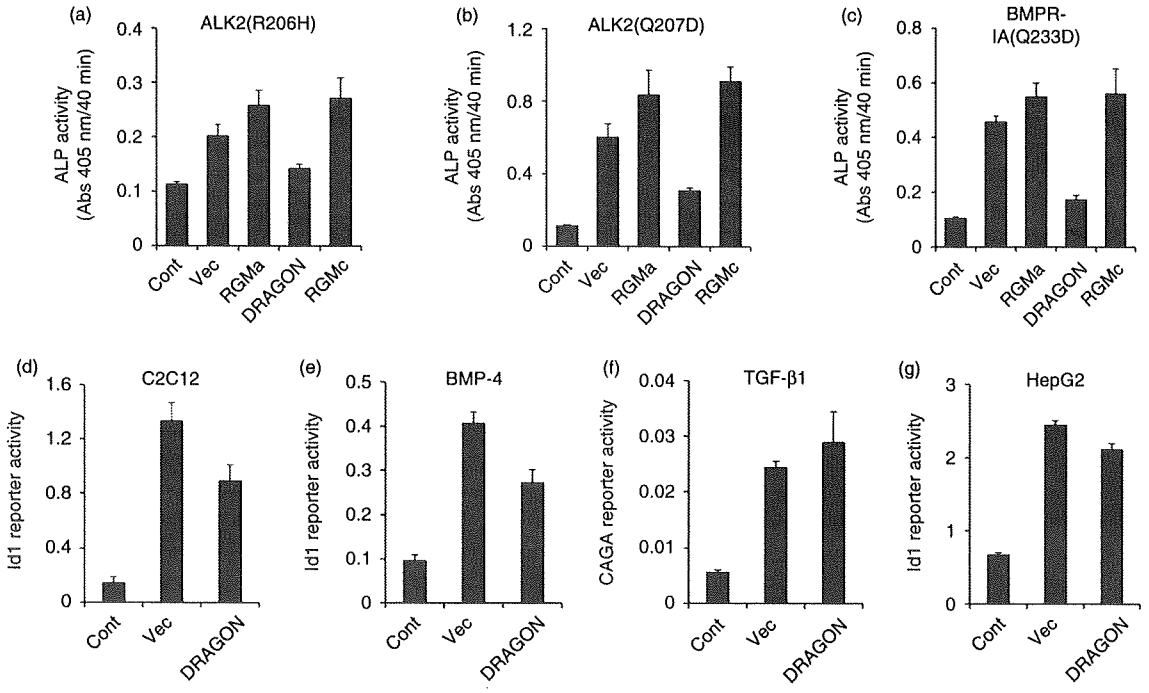

Figure 2 DRAGON inhibits BMP signaling in C2C12 cells. (a–c) Effects of the RGM family on BMP activity in C2C12 cells. C2C12 cells were co-transfected with ALK2(R206H) (a), ALK2(Q207D) (b) or BMPR-IA(Q233D) (c) and RGMa, DRAGON or RGMc in the presence of Smad1. ALP activity was determined on day 3. Data represent the mean ï SD (n ï 3). (d,g) IdWT4F-luc was co-transfected with DRAGON or an empty vector into C2C12 (d) and HepG2 (g) cells. Luciferase activity was determined on day 2. Data represent the mean ï SD (n ï 3). (e,f) C2C12 cells were co-transfected with DRAGON or an empty vector and the IdWT4F-luc (e) or CAGA-luc (f) reporter plasmid and then treated with 100 ng/mL of BMP-4 (e) or 5 ng/mL of TGF- 1 (f). Data represent the mean ï SD (n ï 3).

osteoblasts because it was not expressed in primary osteoblasts (Fig. 1b,c).

DRAGON inhibits BMP signaling in C2C12 cells

Next, we examined the effect of RGMs on BMP-induced osteoblastic differentiation. A constitutively activated BMP receptor—ALK2(R206H), ALK2(Q207D) or BMPR-IA(Q233D)—was co-transfected with one of the RGMs into C2C12 cells, and as a result of that process we identified alkaline phosphatase (ALP) activity as a typical marker of osteoblastic differentiation. Unexpectedly, DRAGON suppressed ALP activity in all of the cultures that were examined (Fig. 2a–c). The suppression of BMP signaling by DRAGON was also confirmed in a luciferase assay using BMP-specific IdWT4F-luc in C2C12 cells (Fig. 2d,e). In contrast to BMP signaling, TGF- signaling (as determined by CAGA-luc) was not suppressed by DRAGON in C2C12 cells (Fig. 2f). DRAGON showed a weaker inhibitory activity on IdWT4F-luc induced by ALK2(Q207D) in HepG2 cells compared with the inhibitory activity seen in C2C12 cells (Fig. 2d,g).

Deletion analysis of DRAGON to determine the inhibitory domain affecting BMP signaling

We generated Sol- and Cyt-DRAGON mutants, in which a GPI-anchor domain and a signal peptide, respectively, were deleted (Fig. 3a). The cellular localization of these DRAGON proteins was confirmed by immunohistochemical analysis in the presence and absence of detergent. Wild-type DRAGON, but not Sol- or Cyt-DRAGON, was detected on the cell membrane, even in the absence of detergent; Sol- and Cyt-DRAGON were detected only in the cytoplasm in the presence of detergent (Fig. 3b). ALP activity inhibition induced by ALK2(Q207D) was observed in the wild-type and Sol-DRAGON constructs, which may be secreted by a signal peptide. To investigate whether the signal peptide itself suppressed ALP activity, we further generated an additional mutant of DRAGON (Swap-DRAGON) in which the signal peptide was replaced with that of another protein (Fig. 3a). Swap-DRAGON was anchored on the cell membrane and suppressed ALP activity in a manner similar to that of wild-type DRAGON, indicating that the signal peptide of

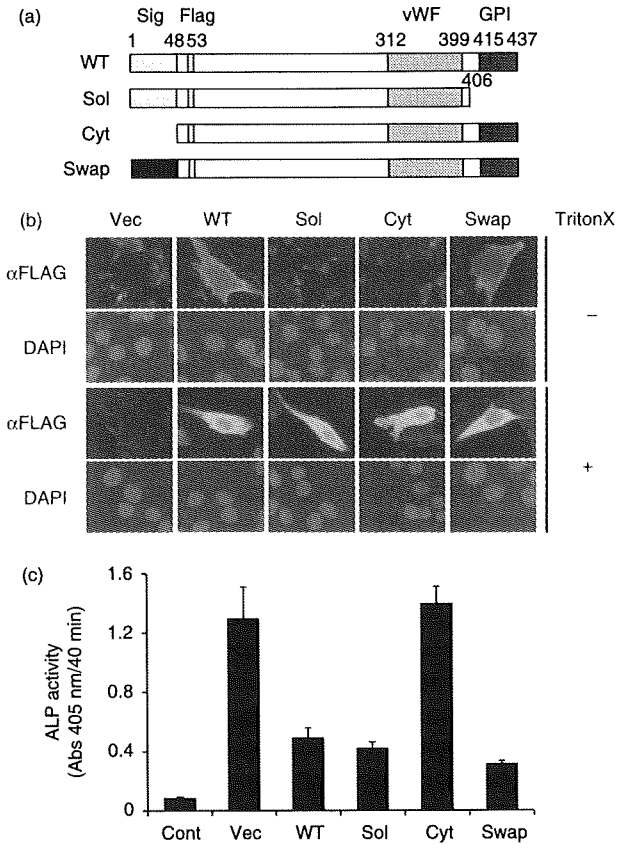

Figure 3 The secreted form of DRAGON inhibits BMP signaling. (a) Schematic diagram of DRAGON mutants. Location of a signal peptide (Sig), vWF type D domain (vWF), GPI-anchor domain (GPI) and FLAG-epitope tag (Flag) are indicated. (b) Cellular localization of DRAGON mutants in C2C12 cells. C2C12 cells were transfected with a wild-type (WT) form, a deleted form lacking the GPI-anchor domain (Sol), a deleted form lacking the signal peptide (Cyt) or a swapped signal peptide form (Swap) of the FLAG-tagged DRAGON construct. Cells were stained with an anti-FLAG antibody and DAPI in the absence and presence of Triton X-100. (c) C2C12 cells were co-transfected with one of the DRAGON mutants and ALK2(Q207D) in the presence of Smad1. ALP activity was determined on day 3. Data represent the mean ï SD (n ï 3).

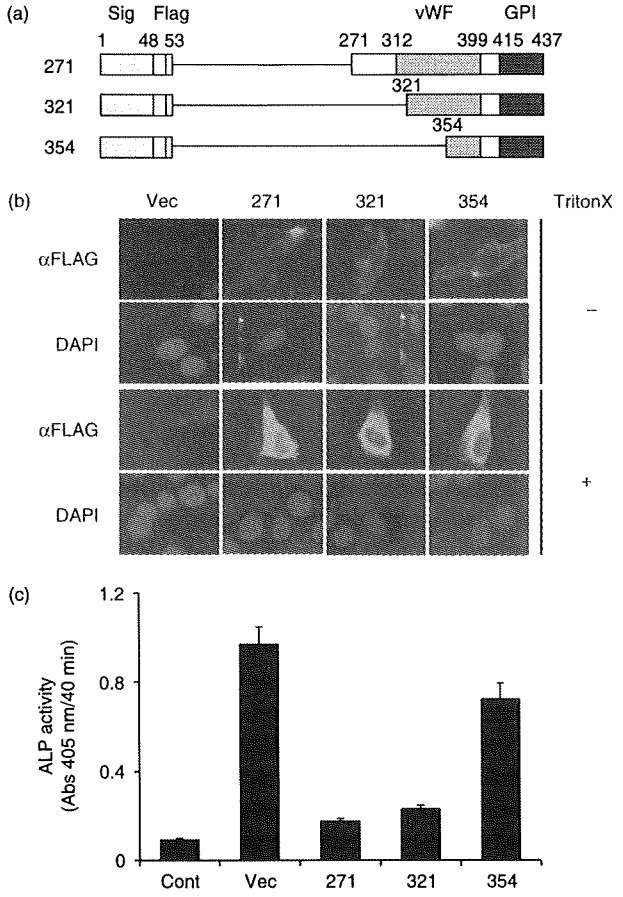

Figure 4 C-terminal region of DRAGON is important for BMP activity inhibition. (a) Schematic diagram of DRAGON deletion mutants. (b) Cellular localization of DRAGON mutants in C2C12 cells. C2C12 cells were transfected with one of the FLAG-tagged deletion DRAGON mutants and were stained with an anti-FLAG antibody and DAPI in the presence and absence of Triton X-100. (c) C2C12 cells were co-transfected with one of the DRAGON mutant constructs and ALK2(Q207D) in the presence of Smad1. ALP activity was determined on day 3. Data represent the mean ï SD (n ï 3).

DRAGON was not involved in the inhibition of BMP signaling (Fig. 3b,c).

We further examined the inhibitory domain of DRAGON using the three deletion mutants 271, 321 and 354 (Fig. 4a). All of these deletion mutant DRAGON constructs were detected on the cell membrane in the absence of detergent (Fig. 4b). Both the 271 and 321 mutants showed the capacity to inhibit ALP activity, but mutant 354 had little suppressive effect on enzyme activity (Fig. 4c). Together, these findings indicate that a region including the vWF type D-like domain may play an important role in the inhibition of BMP signaling by DRAGON.

DRAGON inhibits BMP signaling independent of neogenin

Because the secretory form of DRAGON inhibited BMP signaling without a GPI-anchor domain (Figs 3, 4), we hypothesized that a cell surface molecule(s) may be involved in the inhibitory activity of DRAGON. Neogenin has been identified as a cell surface receptor for RGM family members (Matsunaga et al. 2004; Rajagopalan et al. 2004). Because neogenin was only weakly expressed in C2C12 cells, we cloned its cDNA and transiently over-expressed it in C2C12 cells (Fig. 5a, unpublished observation). Co-transfection of neogenin showed no effect on the ability

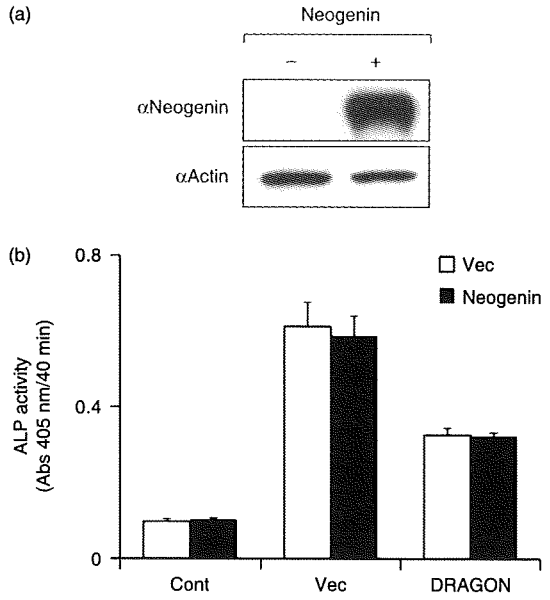

Figure 5 Inhibitory activity of DRAGON on BMP signaling is independent of neogenin. (a) Over-expression of neogenin in C2C12 cells. C2C12 cells were transfected with a neogenin expression vector, and protein levels were determined by immunoblots with anti-neogenin and anti-actin antibodies. (b) Neogenin did not affect the inhibitory activity of DRAGON on BMP signaling. C2C12 cells were co-transfected with ALK2(Q207D), DRAGON and Smad1 with or without neogenin. ALP activity was determined on day 3. Data represent the mean ± SD (n = 3).

of DRAGON to inhibit the ALP activity induced by ALK2(Q207D) (Fig. 5b).

DRAGON inhibits the transcriptional activity of Smads in C2C12 cells

Both wild-type and Sol-DRAGON decreased the expression levels of ALP and osterix mRNAs induced by ALK2(Q207D) in C2C12 cells, suggesting that DRAGON inhibited BMP signaling-induced osteoblastic differentiation (Fig. 6a). We examined the effect of DRAGON on phosphorylation levels of Myc-Smad1 and found that DRAGON did not significantly decrease phospho-Smad1 levels (Fig. 6b). Next, we asked whether a direct interaction between DRAGON and BMP receptor is involved in the inhibition process. Immunoprecipitation with immunoblotting analysis indicated that wild-type DRAGON, but not Sol-DRAGON, interacted with ALK2(Q207D) (Fig. 6c). Finally, we examined the effect of DRAGON on constitutively activated Smad1, which is able to induce downstream signal transduction cooperatively with Smad4 without phosphorylation by BMP receptors. Wild-type DRAGON, Sol-DRAGON and mutant 321 markedly inhibited the ALP activity and IdWT4F-luc activity induced by constitutively activated Smad1 and Smad4 (Fig. 6d,e). These results indicate that the inhibitory effect of DRAGON represses the transcriptional activity of Smad. Because both wild-type DRAGON and Sol-DRAGON suppressed ALK2 activity to a similar degree, we hypothesized that the interaction between DRAGON and BMP receptors may not be involved in this inhibition.

Discussion

The present study demonstrates that DRAGON, a member of the RGM family, inhibits BMP signaling in C2C12 myoblasts. The inhibitory domain of DRAGON was mapped around the vWF type D-like (but not GPI-anchor) domain. In our preliminary experiments, mouse vWF did not have any effect on BMP signaling in C2C12 cells, suggesting that a DRAGON-specific sequence in this domain may be involved in this inhibition. Although RGMs have been reported to interact with both BMP ligands and BMP receptors, DRAGON suppressed signaling induced by constitutively activated BMP receptors in the absence of BMP ligands. Moreover, secretory forms of DRAGON suppressed BMP signaling induced by constitutively activated Smad1. Taken together, our results suggest that DRAGON may bind to a novel molecule(s) expressed on the cell membrane to transduce inhibitory signaling on the transcriptional activity of Smads (Fig. 6f).

Neogenin, a homologue of deleted colorectal cancer (DCC) and the netrin-1 receptor, was a potential candidate for the DRAGON binding protein on the cell membrane because it has been shown to act as a high-affinity receptor for RGMs (Vielmetter et al. 1994; Keino-Masu et al. 1996; Stein et al. 2001). The interactions between RGMa and neogenin were involved in the regulation of neuronal survival (Wilson & Key 2006). Interestingly, the interaction with neogenin was required for RGMc release from muscle cells, and soluble RGMc played a critical role in the negative regulation of hepatic hepcidin expression through BMP signaling in hepatocytes (Zhang et al. 2007). However, neogenin over-expression did not affect the DRAGON activity in C2C12 cells. We noticed that the degree of BMP signaling inhibition by DRAGON is dependent on cell type; for example, HepG2 and other types of cells showed weaker activities than C2C12 cells. It is possible that the amount of novel molecule(s) expressed on the cell membrane may regulate the cell type-specific activity of DRAGON. In addition, DRAGON was expressed in bone, cartilage and primary osteoblasts. However, the expression of DRAGON was unaltered during osteoblastic differentiation in C2C12 cells. Thus, it is possible that the suppressive function of DRAGON depended

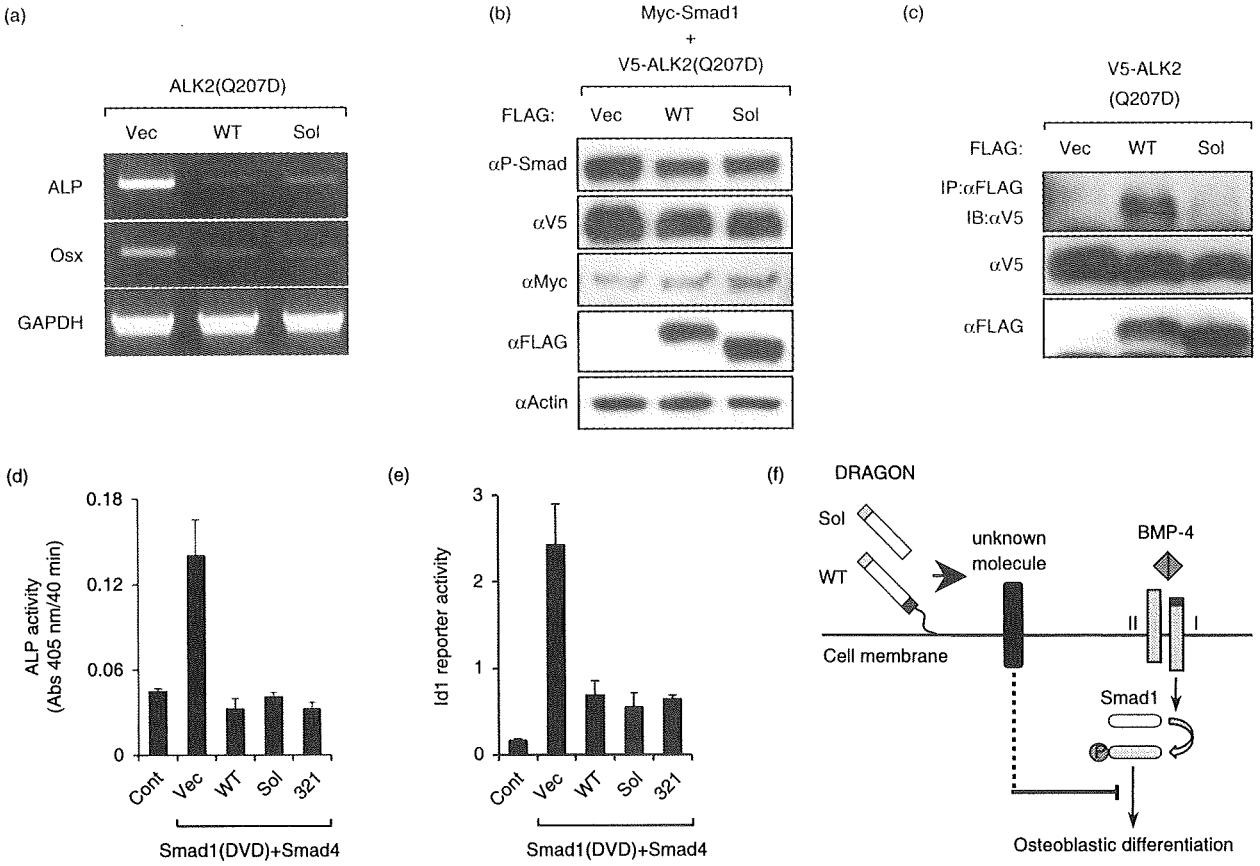

Figure 6 DRAGON inhibits the transcriptional activity of Smads independent of binding to the BMP receptor. (a) RT-PCR analysis of ALP and osterix in C2C12 cells. C2C12 cells were co-transfected with ALK2(Q207D) and an empty vector, wild-type or Sol-DRAGON in the presence of Smad1. ALP and osterix mRNA levels were determined by RT-PCR analysis on day 3. (b) Effect of DRAGON on the phosphorylation of Smad1. C2C12 cells were co-transfected with V5-ALK2(Q207D), Myc-Smad1 and FLAG-tagged wild-type, Sol-DRAGON or an empty vector. Whole cell lysates were immunoblotted with anti-phospho-Smad1/5/8, anti-V5, anti-Myc, anti-FLAG and anti-actin antibodies. (c) Interaction between DRAGON and ALK2(Q207D). C2C12 cells were co-transfected with V5-ALK2(Q207D) and FLAG-tagged wild-type, Sol-DRAGON or an empty vector. Whole cell lysates were immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-V5 antibody. (d,e) DRAGON inhibits BMP activity directly induced by Smads. (d) C2C12 cells were co-transfected with constitutively activated Smad1 and Smad4 with wild-type, Sol- or mutant 321 DRAGON. ALP activity was determined on day 3. Data represent the mean ± SD (n = 3). (e) C2C12 cells were co-transfected with IdWT4F-luc, constitutively activated Smad1 and Smad4 with wild-type, Sol- or mutant 321 DRAGON. Luciferase activity was determined on day 2. Data represent the mean ± SD (n = 3). (f) A schematic diagram of the inhibitory mechanism of DRAGON in C2C12 cells. Secreted DRAGON with and without a GPI-anchor domain may interact with an unknown molecule(s) expressed on the cell membrane. This interaction may activate an intracellular signaling pathway to suppress the transcriptional activity of Smads and the osteoblastic differentiation of C2C12 myoblasts.

on the alteration of expression levels of a novel molecule(s). The identification of the DRAGON-binding molecule(s) on the cell membrane will shed light on the novel regulatory mechanisms associated with BMP signaling.

Recently, heterozygous mutations in the ACVR1 gene, which encodes the BMP type I receptor ALK2, were identified in familial and sporadic patients with FOP (Shore et al. 2006; Nakajima et al. 2007; Fukuda et al. 2009). These mutations cause an amino acid substitution in the intracellular ALK2 domain. We found that these ALK2 mutants were constitutively activated BMP receptors and that they cooperatively induced BMP signaling and osteoblastic differentiation with Smad1 and Smad5 (Fukuda et al. 2009). At the present time, no treatments are available to prevent heterotopic bone formation in FOP. We found in this study that DRAGON clearly inhibited the activity of ALK2(R206H), a typical mutation in FOP patients. Therefore, DRAGON may provide a novel concept for intracellular signal transduction inhibition in the design of novel drugs for the treatment of FOP.

In conclusion, DRAGON inhibited the intracellular signaling of activated BMP receptors by suppressing the transcriptional activity of Smad in C2C12 cells. DRAGON may bind to a novel molecule(s) expressed on the cell surface to transduce the inhibitory signals of BMPs.

Experimental procedures

Cell culture, transfection, reporter assay and ALP assay

Mouse C2C12 myoblasts and human HepG2 hepatocytes were maintained as described (Katagiri et al. 1994; Babitt et al. 2006). Myogenic differentiation of C2C12 cells was induced by culturing the cells with differentiation medium (Dulbecco's modified Eagle's medium containing 2.5% fetal bovine serum) for 7 days. Cells were transfected with plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. BMP and TGF- signaling were monitored using IdWT4F-luc and CAGA-luc reporter plasmids, respectively (Dennler et al. 1998; Katagiri et al. 2002). ALP activity was measured as described (Kodaira et al. 2006). In brief, cells were incubated with a substrate solution (0.1 m diethanolamine, 1 mm $MgCl_2$ and 10 mg/mL of p-nitrophenylphosphate). After appropriate incubation, reactions were terminated by adding 3 m NaOH; absorbance was measured at 405 nm.

Reverse transcription-PCR analysis and plasmid construction

Total RNAs were prepared using TRIzol Reagent (Invitrogen) and then reverse transcribed by SuperScript III reverse transcriptase (Invitrogen) in accordance with the manufacturer's instructions. PCR reactions were performed using Go-Taq (Promega, Madison, WI). The primer sets used were previously described (Zhao et al. 2006) as follows: 5 -CACGCTGGCTGCCTACTATGCTTTG-3 (RGMa-U), 5 -TGGGCTGCATCCTGAGGCTGACTTG-3 (RGMa-L), 5 -ACTCATAGACAACAATTACCTTTCG-3 (DRAGON-U), 5 -GGGAAGAGTCTCTCACACACACATG (DRAGON-L), 5 -CTCCGGTGACCCCAACTTTACTGTG-3 (RGMc-U) and 5 -CTGTCCCCGCTGTTTCCTTTGGCAC (RGMc-L). Each cDNA of the RGM family was cloned into a pcDEF3 expression vector and confirmed by DNA sequencing. A series of mutant DRAGON constructs was generated using a standard PCR technique.

Immunoblotting and immunohistochemistry

Cells were lysed in TNE buffer [10 mm Tris-HCl (pH 7.5), 0.15 m NaCl, 1 mm EDTA and 1% Nonidet P-40] and subjected to immunoprecipitation and immunoblotting as described previously (Fukuda et al. 2009). The following antibodies were used: anti-phosphorylated Smad1/5/8 antibody (Cell Signaling, Beverly, MA), anti-V5 antibody (Invitrogen), anti-FLAG antibody (Sigma, St Louis, MO), anti-Myc antibody and anti-actin antibody (SantaCruz, Santa Cruz, CA). The cells transfected with DRAGON mutants were fixed with formalin and stained with an anti-FLAG antibody and DAPI.

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References

Afrakhte, M., Moren, A., Jossan, S., Itoh, S., Sampath, K., Westermark, B., Heldin, C.H., Heldin, N.E. & ten Dijke, P. (1998) Induction of inhibitory Smad6 and Smad7 mRNA by TGF- family members. Biochem. Biophys. Res. Commun. **249**, 505–511.

Akiyama, S., Katagiri, T., Namiki, M., Yamaji, N., Yamamoto, N., Miyama, K., Shibuya, H., Ueno, N., Wozney, J.M. & Suda, T. (1997) Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. Exp. Cell Res. **235**, 362–369.

Aoki, H., Fujii, M., Imamura, T., Yagi, K., Takehara, K., Kato, M. & Miyazono, K. (2001) Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction. J. Cell Sci. **114**, 1483–1489.

Babitt, J.L., Zhang, Y., Samad, T.A., Xia, Y., Tang, J., Campagna, J.A., Schneyer, A.L., Woolf, C.J. & Lin, H.Y. (2005) Repulsive guidance molecule (RGMa), a DRAGON homologue, is a bone morphogenetic protein co-receptor. J. Biol. Chem. **280**, 29820–29827.

Babitt, J.L., Huang, F.W., Wrighting, D.M., Xia, Y., Sidis, Y., Samad, T.A., Campagna, J.A., Chung, R.T., Schneyer, A.L., Woolf, C.J., Andrews, N.C. & Lin, H.Y. (2006) Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat. Genet. **38**, 531–539.

Babitt, J.L., Huang, F.W., Xia, Y., Sidis, Y., Andrews, N.C. & Lin, H.Y. (2007) Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. J. Clin. Invest. **117**, 1933–1939.

Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S. & Gauthier, J.M. (1998) Direct binding of Smad3 and Smad4 to critical TGF b-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J. **17**, 3091–3100.

Fujii, M., Takeda, K., Imamura, T., Aoki, H., Sampath, T.K., Enomoto, S., Kawabata, M., Kato, M., Ichijo, H. & Miyazono, K.

(1999) Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. Mol. Biol. Cell **10**, 3801–3813.

Fukuda, T., Kohda, M., Kanomata, K. et al. (2009) Constitutively activated ALK2 and increased smad1/5 cooperatively induce BMP signaling in fibrodysplasia ossificans progressiva. J. Biol. Chem. **284**, 7149–7156.

Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J.M., Fujisawa-Sehara, A. & Suda, T. (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J. Cell Biol. **127**, 1755–1766.

Katagiri, T., Imada, M., Yanai, T., Suda, T., Takahashi, N. & Kamijo, R. (2002) Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. Genes Cell **7**, 949–960.

Katagiri, T., Suda, T. & Miyazono, K. (2008) The Bone Morphogenetic Proteins. New York: Cold Spring Harbor Laboratory Press.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S., Culotti, J.G. & Tessier-Lavigne, M. (1996) Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. Cell **87**, 175–185.

Kodaira, K., Imada, M., Goto, M., Tomoyasu, A., Fukuda, T., Kamijo, R., Suda, T., Higashio, K. & Katagiri, T. (2006) Purification and identification of a BMP-like factor from bovine serum. Biochem. Biophys. Res. Commun. **345**, 1224–1231.

Matsunaga, E., Tauszig-Delamasure, S., Monnier, P.P., Mueller, B.K., Strittmatter, S.M., Mehlen, P. & Chedotal, A. (2004) RGM and its receptor neogenin regulate neuronal survival. Nat. Cell Biol. **6**, 749–755.

Miyazono, K., Maeda, S. & Imamura, T. (2005) BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev. **16**, 251–263.

Monnier, P.P., Sierra, A., Macchi, P., Deitinghoff, L., Andersen, J.S., Mann, M., Flad, M., Hornberger, M.R., Stahl, B., Bonhoeffer, F. & Mueller, B.K. (2002) RGM is a repulsive guidance molecule for retinal axons. Nature **419**, 392–395.

Nakajima, M., Haga, N., Takikawa, K., Manabe, N., Nishimura, G. & Ikegawa, S. (2007) The ACVR1 617G;A mutation is also recurrent in three Japanese patients with fibrodysplasia ossificans progressiva. J. Hum. Genet. **52**, 473–475.

Niederkofler, V., Salie, R., Sigrist, M. & Arber, S. (2004) Repulsive guidance molecule (RGM) gene function is required for neural tube closure but not retinal topography in the mouse visual system. J. Neurosci. **24**, 808–818.

Papanikolaou, G., Samuels, M.E., Ludwig, E.H. et al. (2004) Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat. Genet. **36**, 77–82.

Rajagopalan, S., Deitinghoff, L., Davis, D., Conrad, S., Skutella, T., Chedotal, A., Mueller, B.K. & Strittmatter, S.M. (2004) Neogenin mediates the action of repulsive guidance molecule. Nat. Cell Biol. **6**, 756–762.

Samad, T.A., Srinivasan, A., Karchewski, L.A., Jeong, S.J., Campagna, J.A., Ji, R.R., Fabrizio, D.A., Zhang, Y., Lin, H.Y., Bell, E. & Woolf, C.J. (2004) DRAGON: a member of the repulsive guidance molecule-related family of neuronal- and muscle-expressed membrane proteins is regulated by DRG11 and has neuronal adhesive properties. J. Neurosci. **24**, 2027–2036.

Samad, T.A., Rebbapragada, A., Bell, E., Zhang, Y., Sidis, Y., Jeong, S.J., Campagna, J.A., Perusini, S., Fabrizio, D.A., Schneyer, A.L., Lin, H.Y., Brivanlou, A.H., Attisano, L. & Woolf, C.J. (2005) DRAGON, a bone morphogenetic protein co-receptor. J. Biol. Chem. **280**, 14122–14129.

Shore, E.M., Xu, M., Feldman, G.J. et al. (2006) A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat. Genet. **38**, 525–527.

Stein, E., Zou, Y., Poo, M. & Tessier-Lavigne, M. (2001) Binding of DCC by netrin-1 to mediate axon guidance independent of adenosine A2B receptor activation. Science **291**, 1976–1982.

Thomas, J.T., Kilpatrick, M.W., Lin, K., Erlacher, L., Lembessis, P., Costa, T., Tsipouras, P. & Luyten, F.P. (1997) Disruption of human limb morphogenesis by a dominant negative mutation in CDMP1. Nat. Genet. **17**, 58–64.

Urist, M.R. (1965) Bone: formation by autoinduction. Science **150**, 893–899.

Vielmetter, J., Kayyem, J.F., Roman, J.M. & Dreyer, W.J. (1994) Neogenin, an avian cell surface protein expressed during terminal neuronal differentiation, is closely related to the human tumor suppressor molecule deleted in colorectal cancer. J. Cell Biol. **127**, 2009–2020.

Wieser, R., Wrana, J.L. & Massague, J. (1995) GS domain mutations that constitutively activate T R-I, the downstream signaling component in the TGF- receptor complex. EMBO J. **14**, 2199–2208.

Wilson, N.H. & Key, B. (2006) Neogenin interacts with RGMa and netrin-1 to guide axons within the embryonic vertebrate forebrain. Dev. Biol. **296**, 485–498.

Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M. & Wang, E.A. (1988) Novel regulators of bone formation: molecular clones and activities. Science **242**, 1528–1534.

Xia, Y., Yu, P.B., Sidis, Y., Beppu, H., Bloch, K.D., Schneyer, A.L. & Lin, H.Y. (2007) Repulsive guidance molecule RGMa alters utilization of bone morphogenetic protein (BMP) type II receptors by BMP2 and BMP4. J. Biol. Chem. **282**, 18129–18140.

Zhang, A.S., Anderson, S.A., Meyers, K.R., Hernandez, C., Eisenstein, R.S. & Enns, C.A. (2007) Evidence that inhibition of hemojuvelin shedding in response to iron is mediated through neogenin. J. Biol. Chem. **282**, 12547–12556.

Zhao, B., Katagiri, T., Toyoda, H., Takada, T., Yanai, T., Fukuda, T., Chung, U.I., Koike, T., Takaoka, K. & Kamijo, R. (2006) Heparin potentiates the in vivo ectopic bone formation induced by bone morphogenetic protein-2. J. Biol. Chem. **281**, 23246–23253.

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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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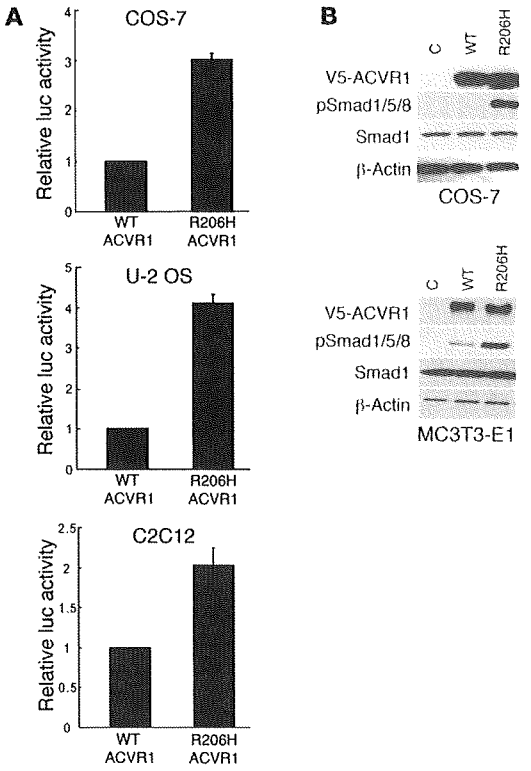

Figure 1

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

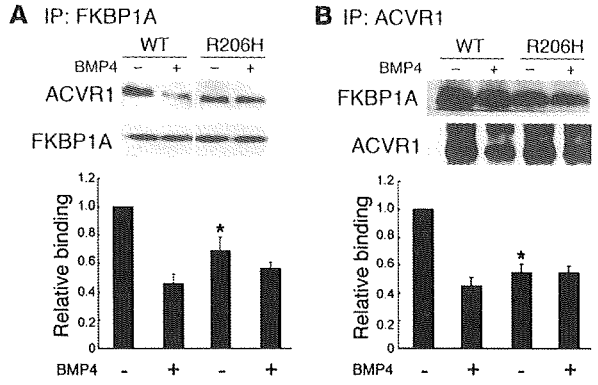

Figure 2
Reduced binding of FKBP12 to mutant ACVR1. COS-7 cells were cotransfected with FKBP1A expression vectors and wild-type ACVR1 or mutant ACVR1 (R206H) constructs. Following no treatment (–) or treatment (+) with 150 ng/ml BMP4, proteins were (**A**) immunoprecipitated with anti-FKBP1A/FKBP12 antibody, 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-

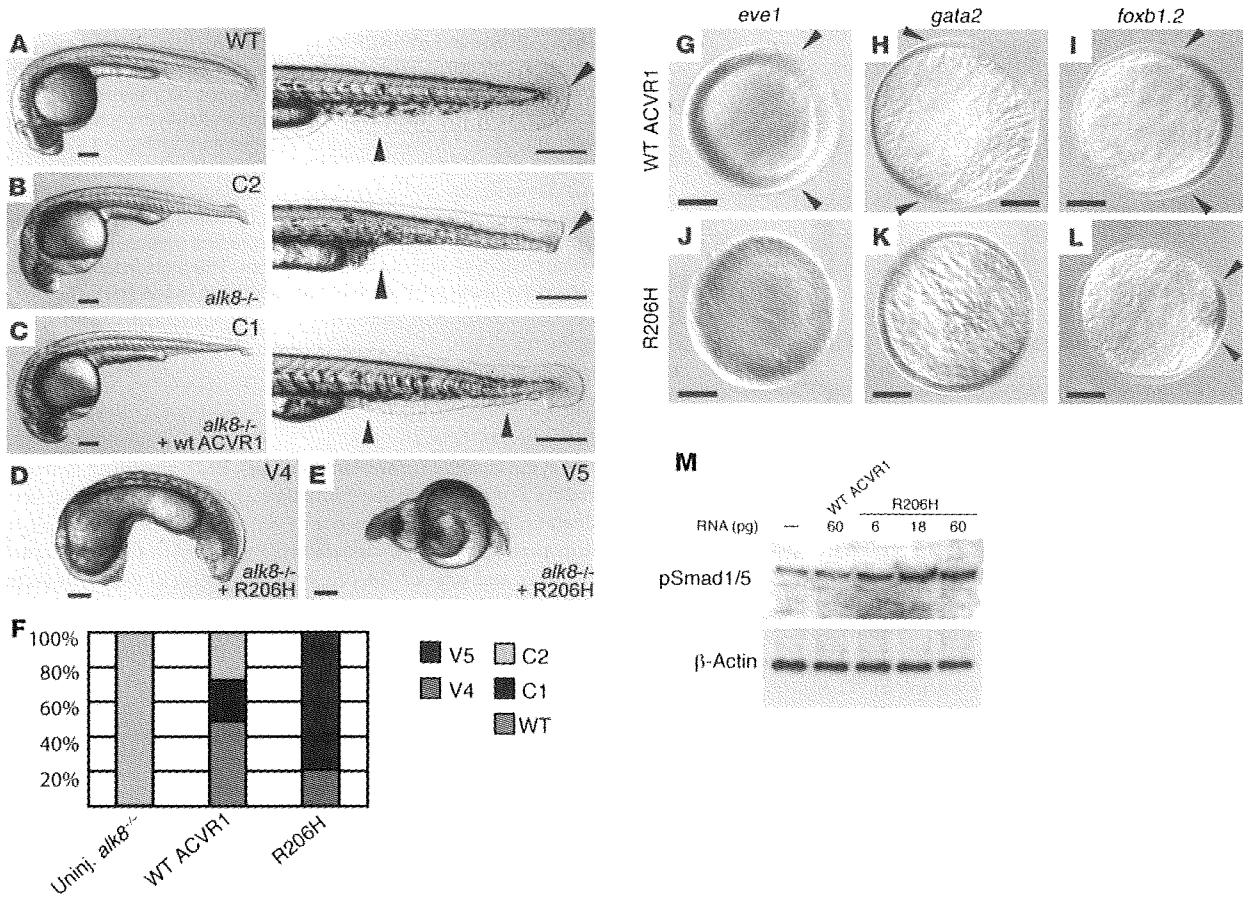

Figure 3

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