

# Molecular Consequences of the ACVR1<sup>R206H</sup> Mutation of Fibrodysplasia Ossificans Progressiva<sup>\*[S]</sup>

Received for publication, December 13, 2009, and in revised form, April 9, 2010. Published, JBC Papers in Press, May 12, 2010, DOI 10.1074/jbc.M109.094557

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Fibrodysplasia ossificans progressiva (FOP), a rare genetic and catastrophic disorder characterized by progressive heterotopic ossification, is caused by a point mutation, c.617G>A; p.R206H, in the activin A receptor type 1 (ACVR1) gene, one of the bone morphogenetic protein type I receptors (BMPR-Is). Although altered BMP signaling has been suggested to explain the pathogenesis, the molecular consequences of this mutation are still elusive. Here we studied the impact of ACVR1 R206H mutation on BMP signaling and its downstream signaling cascades in murine myogenic C2C12 cells and HEK 293 cells. We found that ACVR1 was the most abundant of the BMPR-Is expressed in mesenchymal cells but its contribution to osteogenic BMP signal transduction was minor. The R206H mutant caused weak activation of the BMP signaling pathway, unlike the Q207D mutant, a strong and constitutively active form. The R206H mutant showed a decreased binding affinity for FKBP1A/FKBP12, a known safeguard molecule against the leakage of transforming growth factor (TGF)- $\beta$  or BMP signaling. The decreased binding affinity of FKBP1A to the mutant R206H ACVR1 resulted in leaky activation of the BMP signal, and moreover, it decreased steady-state R206H ACVR1 protein levels. Interestingly, while WT ACVR1 and FKBP1A were broadly distributed in plasma membrane and cytoplasm without BMP-2 stimulation and then localized in plasma membrane on BMP-2 stimulation, R206H ACVR1 and FKBP1A were mainly distributed in plasma membrane regardless of BMP-2 stimulation. The impaired binding to FKBP1A and an altered subcellular distribution by R206H ACVR1 mutation may result in mild activation of osteogenic BMP-signaling in extraskeletal sites such as muscle, which eventually lead to delayed and progressive ectopic bone formation in FOP patients.

Fibrodysplasia ossificans progressiva (FOP)<sup>2</sup> is one of the most catastrophic disorders in humans because of extensive

\* This work was supported by a Korea Research Foundation grant funded by the Korean Government (KRF-2008-313-E00562) and by the Korea Health 21 R&D Project, Ministry of Health and Welfare (Project A010252).

[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

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<sup>2</sup> The abbreviations used are: FOP, fibrodysplasia ossificans progressiva; WT, wild type; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; ER, endoplasmic reticulum;

heterotopic ossification throughout the body. Until recently, it has been one of the most elusive and mysterious diseases, with ~2500 cases reported since the 1800s and a prevalence of about one in two million (1–2). At the time of birth no specific disability or skeletal deformity is apparent, except for a malformed big toe; however, once heterotopic ossification begins in the first decade of life, it progresses with aggravation, leading to ankylosis of the major joints or fusion of the rib cage with subsequent severe disability and fatal respiratory failure (1–4). Moreover, surgical attempts to remove heterotopic bone or incisional biopsies commonly lead to episodes of explosive and painful new bone growth (5–6). Thus, the difficulties in obtaining human tissues for study have limited the molecular and biochemical understanding of the disease. Presently, the underlying molecular mechanisms of FOP are still unclear, and there is no known treatment regimen.

Mutations in the activin A receptor, type I (ACVR1), also known as activin receptor-like kinase 2, (ALK2), one of the bone morphogenetic protein (BMP) type I receptors (BMPR-I), have been reported in FOP patients at several sites. These heterozygous missense point mutations are categorized into classic FOP, FOP-plus, and FOP variant (7–8). The recurrent c.617G>A; p.R206H mutation, which is considered as a major mutation, involves the substitution of arginine with histidine at codon 206, located right at the end of the glycine-serine-rich (GS) activation domain of ACVR1. This mutation is found in all cases of classic FOP and most cases of FOP-plus. Another point mutation in ACVR1, found in the FOP variant, is reported to be a site of interaction with arginine 206 (Arg-206) according to protein structure homology modeling (8). In BMP/BMP receptor (BMPR) signaling, the GS domain of the receptor is a very important site for transferring phosphorylation from a type II receptor to a type I receptor (9). In this process, FKBP1A/FKBP12 (a 12-kDa FK506-binding protein) plays a role as a safeguard molecule for signal leakage by binding to the unphosphorylated GS domain (10–12). During ligand binding to the receptors, the serine moiety of the GS domain is phosphorylated and a conformational change occurs around the domain, which subsequently causes dissociation of bound FKBP1A from the GS domain. R-Smad (Smad1, Smad5, and Smad8) then binds to the phosphorylated GS domain (12). The phosphorylation level of Smad1 or Smad5 was well known to be elevated

ALP, alkaline phosphatase; BMPR, bone morphogenetic protein receptors; ACVR1, activin A receptor, type I; DAPI, 4',6-diamidino-2-phenylindole.

with ACVR1 R206H mutation in C2C12 cells (13–14). Several roles for FKBP1A have been reported so far (15–19); however, the biological function of FKBP1A in TGF- $\beta$  or BMP signaling remains unresolved. Recently, the hypothesis that the R206H mutation of ACVR1 might be associated with an impaired FKBP1A interaction and the induction of abnormal BMP signaling was suggested, but without clear supporting evidence (20–21).

As shown in the hierarchy of osteogenic master transcription factors described in our previous reports, *Dlx5* is known for the most proximal target of BMP signaling and plays a pivotal role in stimulating downstream *Runx2*, *Osterix*, and *Osteocalcin* during osteoblast differentiation in the BMP signaling pathway (22–26). Also, *Alp* is known as an early representative marker of osteoblast differentiation and a direct target of *Dlx5*, which is stimulated by the activation of BMP signaling. *Msx2* antagonizes *Dlx5* action in the BMP-2 signaling pathway by competing with it for binding to common response elements in the promoters of bone marker genes such as *Osteocalcin*, *Alp*, and *Runx2* (23, 27–29). At present, there are no data on osteogenic transcription factor expression with respect to the R206H ACVR1 mutation in muscle cells to aid our understanding of the genotype-phenotype correlation.

To date, although the causative genetic mutation of FOP has been successfully identified, there are few ongoing studies on the molecular consequences of the mutation or its underlying mechanism. Mouse myogenic C2C12 cells are suitable for a functional study because they represent the target tissue of FOP pathogenesis. Here, we show that the recurrent R206H mutation in ACVR1 is a weak activating mutation, which results in leaky signaling through a decreased affinity for FKBP1A. In addition, we report, for the first time, that the ACVR1 R206H mutation has reduced ACVR1 protein levels and a different subcellular distribution from the wild-type protein, with molecular consequences for the pathogenesis of the disease.

## EXPERIMENTAL PROCEDURES

**Antibodies**—Anti-V5 (R960-25) antibody was purchased from Invitrogen (Carlsbad, CA). Anti-Myc (9E10) anti-mouse antibody was purchased from Covance (Princeton, NJ). Anti-Myc (2272) anti-rabbit antibody was purchased from Cell Signaling Technology (Denver, MA). Anti- $\beta$ -actin antibody was from Abcam (Cambridge, MA), and horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Pierce. Alexa Fluor 488-conjugated anti-mouse secondary antibody and a Qdot 655-conjugated anti-rabbit secondary antibody were purchased from Molecular Probes (Eugene, OR). Bioactive recombinant human BMP-2 protein was purchased from R&D systems (Minneapolis, MN).

**Plasmid Construction and Site-directed Mutagenesis**—Constructs encoding full-length human ACVR1 (GenBank<sup>TM</sup> accession no. NM\_001105.4) wild type (WT), and its mutants, K235R and Q207D, were purchased from Addgene Inc. (Cambridge, MA). For the WT construct, PstI-BamHI WT fragments were used for subcloning into new pCMV5 vectors. For the K235R and Q207D constructs, BsmBI-PpuMI fragments were substituted with the same restriction enzyme site fragments of the purchased K235R and Q207D constructs

from new pCMV5-ACVR1 WT. For subsequent cloning into pcDNA6/v5-HisA, the open reading frame corresponding to ACVR1 was amplified by PCR using the above constructs as templates, with DNA primers (IDT, Coralville, IA) containing an appropriate restriction site. For the R206H mutant construct, site-directed mutagenesis using PCR was performed to induce a point mutation at nucleotide 617 using the following primer pair with mutated nucleotides underlined: BsmBI-forward, 5'-TATGTCTTTT-AGCCTGCCTGCTGGGAG-TTG-3' and BsmBI-reverse, 5'-CCAACAGTGTAATCT-GGTGAGCCACTGTTCTTT-GT-3' and PpuMI-forward, 001105.4) wild type (WT), and its mutants 5'-ACAAAGAA-CAGTGGCT-CACCAGATTACACTGTTGG-3' and PpuMI-reverse, 5'-CCCAAATCTGCTATGCAACACTGTCCATTC-3'. All constructs were verified by restriction fragment length polymorphisms (BglII, NruI, and Cac8I for K235R, Q207D, and R206H, respectively) and full ORF sequencing. Full-length of human FKBP1A (GenBank<sup>TM</sup> accession no. NM\_054014.2) cDNA was amplified by PCR using cDNA from HEK293 cells as a template using the following primer pair: forward, 5'-TTTG-GATCCGCCACCATGGGAGTGC-AGGT-3', and reverse, 5'-TCTCGAGTCATTCCAGTTTT-AGAAGCTCCACA-3', and was then subcloned into pcDNA4/myc-HisA vectors. The product was also confirmed by sequencing. The Smad5 (pcDNA3-Smad5) construct, used previously, was a gift from K. Miyazono (Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan) (24–25).

**Cell Cultures**—Mouse myogenic C2C12 cells and human embryonic kidney 293 (HEK293) cells were grown in Dulbecco's modified Eagle's medium (Logan, UT) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C, in a 5% CO<sub>2</sub> atmosphere.

**Transient Transfection and Stable Cell Line Establishment**—C2C12 cells were plated in 100-mm plates and cultured to 90% confluency. After harvesting the cells, transfection by electroporation was performed using a Microporator (NanoEnTek, MA) as previously used (30–31). Stable C2C12 cell lines were generated by transfection with pcDNA6/v5-HisA constructs, followed by selection using 2–20  $\mu$ g/ml Blasticidin S-containing selection medium. We used early passage stable cells (P2–P4) after selection. HEK293 cells were seeded in a 6-well plate, and after overnight culture, the cells were transfected with Hilymax (Dojindo, Japan) according to the manufacturer's instructions. All plasmid DNAs were prepared using a DNA Maxi-prep kit (GENOMED, Loehne, Germany). Expression in cells and the transfection efficiency was confirmed by EGFP-vector transfection and immunoblotting. Reverse transcription PCR (RT-PCR) in Fig. 4D was performed by using the following primer pair: forward, 5'-TCCGCAAGACTCACAGCA-3' and reverse (for ACVR1-V5), 5'-AGAATCGAGAC-CGAGGAGAG-3' and reverse (for ACVR1-BGH rev): 5'-ACTAGAAGGCACAGTTCGA-GG-3'.

**Real-time Quantitative PCR**—Total RNA was extracted from C2C12 cells using the RNeasy Mini kit (Qiagen) or TRIzol® (Invitrogen). The concentration and purity of the RNA preparations were determined by measuring the absorbance of RNA at 230, 260, and 280 nm. The RNA integrity was analyzed on a 1% native agarose gel for checking 18 S and 28 S band, and

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**TABLE 1**  
Primer sequences for real-time quantitative PCR

Symbol	Name	GenBank™ No.	Primer
Dlx5	Distal-less homeobox 5	NM_010056	5'-TCTCTAGGACTGACGCAAACA-3' 5'-GTTACACGCCATAGGGTCGC-3'
Alp	Alkaline phosphatase, liver/bone/kidney	NM_007431	5'-GGCTACATTGGTCTTGAGCTTTT-3' 5'-CCAACCTCTTTGTGCCAGAGA-3'
Msx2	Homeobox, msh-like 2	NM_013601	5'-TTCACCACATCCCAGCTTCTA-3' 5'-TTGCAGTCTTTTCGCCCTTAGC-3'
Bmpr1a	BMP receptor, type 1A	NM_009758	5'-TTTCCAGCCCTACATCATGGC-3' 5'-GCTCCAACCTTACTTCATCGCT-3'
Bmpr1b	BMP receptor, type 1B	NM_007560	5'-CCCTCGGCCCAAGATCCTA-3' 5'-CAACAGGCATTCAGAGTCATC-3'
Bmpr2	BMP receptor, type II	NM_007561	5'-TTGGGATAGGTGAGAGTCGAAT-3' 5'-TGTTTCACAAGATTGATGTCCCC-3'
Acvr1	Activin A receptor, type I	NM_001110204	5'-GTGGAAGATTACAAGCCACCA-3' 5'-GGGCTGAGAACCATCTGTTAGG-3'
Acvr2a	Activin receptor IIA	NM_007396	5'-GCGTTCGCCGCTTTCTTATC-3' 5'-GTTGGTCTGTCTCTTTCCCAAT-3'
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	NM_008084	5'-CATGTTCCAGTATGACTCCACTC-3' 5'-GGCCTCACCCCATTTGATGT-3'

cDNAs were synthesized with Superscript II Reverse Transcriptase (Invitrogen). About 100 to 150 ng of total RNA was used for analysis. We performed real-time PCR using Takara SYBR premix Ex Taq (Takara, Japan) in an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA) according to the manufacturer's instructions. All primers were synthesized by Integrated DNA Technologies, Inc. (IDT; Coralville, IA). The primer sets for the analysis are listed in Table 1. The relative levels of target gene mRNAs were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). We calculated the  $\Delta\Delta C_t$  value, which represents the cycle threshold difference between the target gene primer pair and the *Gapdh* primer pair in each sample. Each experiment was repeated at least three times in duplicate. Data were expressed as means  $\pm$  S.D. of 2 ( $-\Delta\Delta C_t$ ).

**Knock-down Assay with siRNA**—To knock-down the BMP receptors or Smads, siRNAs against *Bmpr1a*, *Bmpr1b*, *Acvr1*, *Smad1*, and *Smad5* were purchased from Dharmacon (Lafayette, CO), a siGENOME SMART pool. The siGENOME Non-Targeting siRNA 2 was used as a control (scramble siRNA) as previously used (31). In  $1 \times 10^5$  C2C12 cells, 40 pmol of siRNA were transfected by electroporation. After transfection, the cells were cultured for 24–36 h to 90% confluency and then treated with, or without, rhBMP-2 (30 to 50 ng/ml) for 24 h.

**Immunoblotting and Immunoprecipitation**—Twenty-four hours after transfection, lysates were harvested in HEPES or TNE lysis buffer (25 mM HEPES or Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 3 mM EDTA, protease inhibitor mixture) or CHAPS-TNE lysis buffer (20 mM CHAPS, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3 mM EDTA, protease inhibitor mixture) (32). About 20  $\mu$ g of whole cell lysate were separated by 10% to 12% SDS-PAGE and blotted onto polyvinylidene fluoride membranes. Nonspecific sites were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween-20 (TBS-T). Blots were incubated with anti-V5 antibody (R960–25, Invitrogen) or anti-Myc antibody (9E10, Covance) followed by horseradish peroxidase-conjugated anti-mouse IgG. The enhanced chemiluminescence blot detection kit was used as described by the manufacturer to visualize reactive products. Band intensity was quantified using the Fujifilm LAS-2000 system and Multi-gauge software (Fujifilm). For immunoprecipitation analysis, 800  $\mu$ g

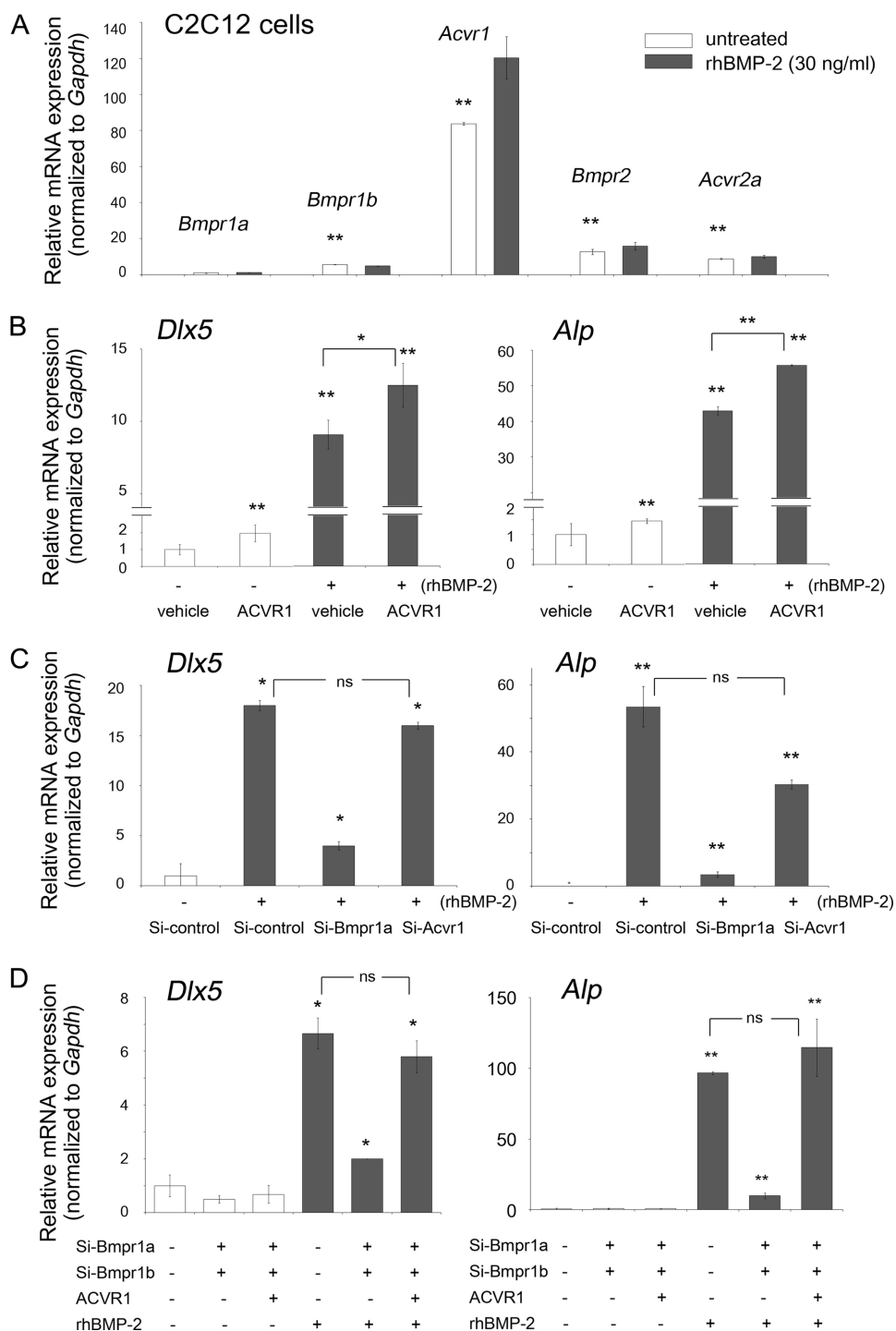
of whole cell lysate were incubated with Myc antibody (9E10, Covance) for 6 h at 4 °C under constant rotation. After washing and resuspension in Laemmli sample buffer, protein G-agarose beads were boiled at 80 °C for 5 min. Bound proteins were resolved by 10% to 12% SDS-PAGE and analyzed by immunoblotting with anti-V5 or anti-Myc antibodies as described above.

**Alkaline Phosphatase (ALP) Staining**—Twenty-four hours after C2C12 cell seeding, cells were treated with rhBMP-2 (30 to 50 ng/ml) for further 24 h, and the growth medium was changed after 1 or 2 days. After another 3–4 days of culture following rhBMP-2 treatment, cells were washed twice with PBS, fixed with 2% paraformaldehyde, and stained for alkaline phosphatase according to the manufacturer's instructions (Sigma).

**Immunocytochemistry**—Stable C2C12 cells, transfected with WT ACVR1 or the R206H mutant, were grown in 6-well plates with coverslips after transfection with the FKBP1A construct using an electroporator. After 24 h, cells were fixed with 4% (w/v) paraformaldehyde in PBS at 4 °C for 20 min, permeabilized with 0.1% Triton X-100 in PBS at 4 °C for 30 min and washed with PBS. Nonspecific sites were blocked for 30 min in PBS containing 0.2% bovine serum albumin. Coverslips were incubated with the mouse anti-V5 antibody (R960–25, Invitrogen, 1:100) and rabbit anti-Myc antibody (2272, Cell Signaling, 1:100) for 2 h at room temperature. Slides were washed and then incubated with a green-fluorescent Alexa Fluor 488-conjugated anti-mouse secondary antibody and a Qdot 655-conjugated anti-rabbit secondary antibody for 1 h. Confocal images were captured with an Olympus FV-300 microscope in Sequence scan mode using the HeNe laser at 543 nm and the Ar laser at 488 nm with the pinhole adjusted to three Airy units for both excitation and wavelengths. Images were collected and saved using the Software FV300 (Olympus Software) version 4.3 and exported to Adobe PhotoShop for digital processing.

## RESULTS

*ACVR1 Is an Abundant BMP Type I Receptor, but Plays a Minor Role in BMP Signal Transduction in Normal C2C12 Cells*—Despite its name, ACVR1 forms heteromeric complexes with BMPR-II, but not with ACVR2, binds to BMP-2, -4, -6, -7, but

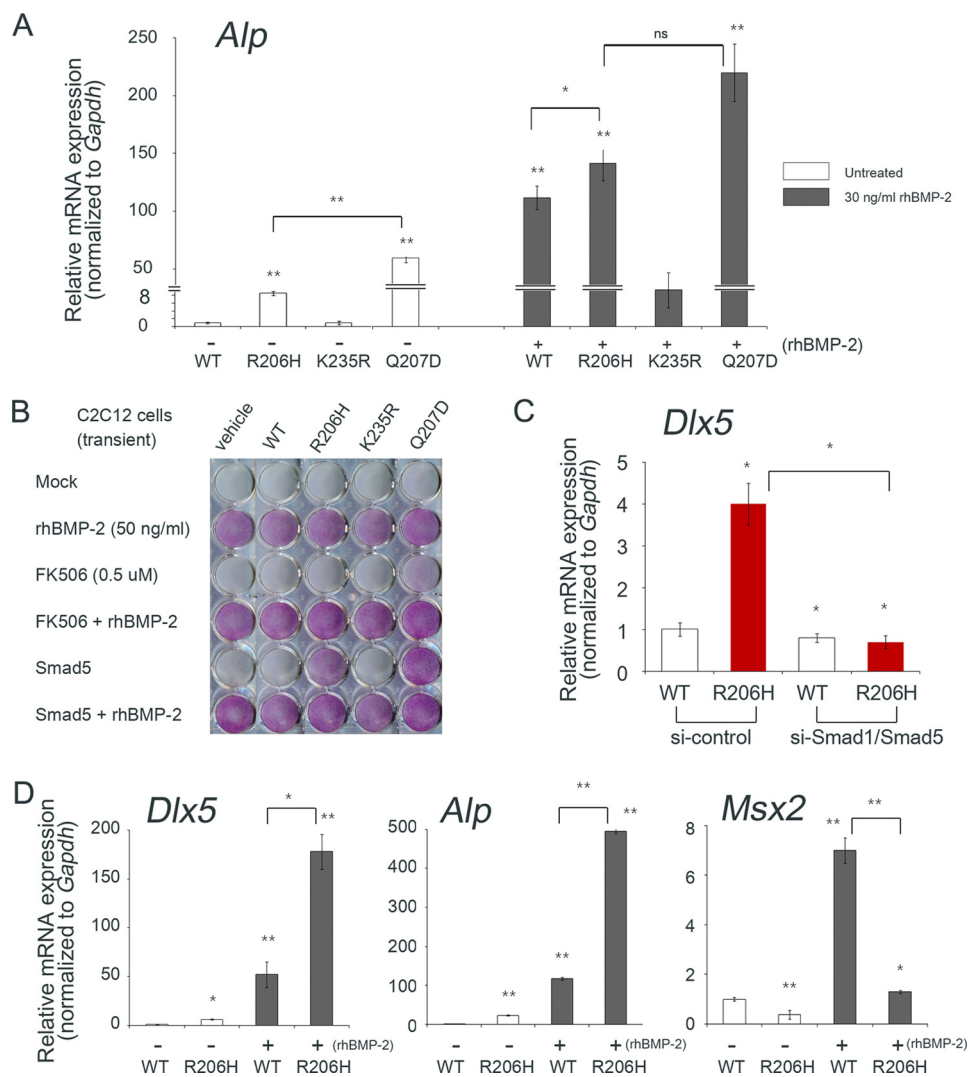


**FIGURE 1. ACVR1 is a strongly expressed BMP type I receptor but plays a minor role in BMP signal transduction in myogenic C2C12 cells.** *A*, after visual confluence of seeded C2C12 cells, the cells were cultured for an additional 24 h with, or without, rhBMP-2 (30 ng/ml). Total RNA was harvested, and mRNA levels of BMP receptors were analyzed by RT-qPCR. (\*\*,  $p < 0.001$  versus *Bmpr1a* expression in the untreated condition.) *B*, C2C12 cells were transiently transfected with ACVR1 or empty vector, and the transfected cells were cultured for an additional 24 h with, or without, rhBMP-2 (30 ng/ml). The mRNA levels of the BMP downstream genes, *Dlx5* and *Alp*, were determined by RT-qPCR. (\*\*,  $p < 0.001$ , \* $p < 0.01$  versus untreated vehicle.) *C*, C2C12 cells were transfected with a control siRNA (*si-control*), *Bmpr1a* (*si-Bmpr1a*), and ACVR1 (*si-ACVR1*). Cells were cultured for 24–36 h and treated with, or without, rhBMP-2 (30 ng/ml) for an additional 24 h. (\*\*,  $p < 0.001$ ; \*,  $p < 0.01$  versus untreated Si-control.) *D*, C2C12 cells were transfected with the siRNAs with, or without, ACVR1 overexpression. After reaching visual confluence, cells were cultured for an additional 24 h with, or without, rhBMP-2 (30 ng/ml). (Data represent mean  $\pm$  S.D. Statistical differences compared with the respective controls are depicted by \*\*,  $p < 0.001$ ; \*,  $p < 0.01$  by the two-tailed Student's *t* test. *ns*, not significant.)

not to activin (33–34), and transduces BMP and Mullerian-inhibiting substance (MIS) group signaling, but not activin-mediated signaling (35–36). However, the precise role of ACVR1 compared with other type I receptors in the BMP signaling pathway remains unclear. To characterize ACVR1, first we assessed the expression profiles of the BMP receptor family in murine myogenic C2C12 cells by real-time quantitative PCR (RT-qPCR) (Fig. 1*A*). Cycle threshold (Ct) values reflecting the levels of expressed mRNA encoding each receptor were analyzed and normalized to that of *Gapdh*. We found that the expression of *Bmpr1a* was consistently the lowest of the five BMP receptors, *Bmpr2* and *Acvr2a* were expressed at intermediate levels, and *Acvr1* was the most abundantly expressed type I receptor, at levels  $\sim 80$ -fold higher than *Bmpr1a*. There was a slight (less than 1.5-fold) increase in all receptor mRNA levels over a period of 24 h in response to BMP-2 treatment.

Next we evaluated the contribution of ACVR1, in myogenic C2C12 cells, to the acquisition of osteoblastic characteristics by analyzing the expression of the osteogenic marker genes, *Dlx5* and *Alp* (Fig. 1*B*). Overexpression of ACVR1 in C2C12 cells stimulated an increase in the expression of the BMP downstream genes, *Dlx5* and *Alp*, but the increase was less than 2-fold. Although the increase of those genes in ACVR1-overexpressed cells by the treatment of BMP-2 was still apparent and statistically significant, the fold increase of *Dlx5* and *Alp* mRNAs in response to BMP-2 treatment was much weaker in ACVR1 overexpressed C2C12 cells compared with that in vehicle-transfected cells. Similarly, ACVR1 knock-down did not alter the BMP-2-stimulated expression of *Dlx5*, *Alp* significantly (Fig. 1*C*). In contrast, blocking *Bmpr1a* expression with a siRNA resulted in a decrease of  $\sim 90\%$  in *Dlx5* and *Alp* expression levels. In addition, blocking the

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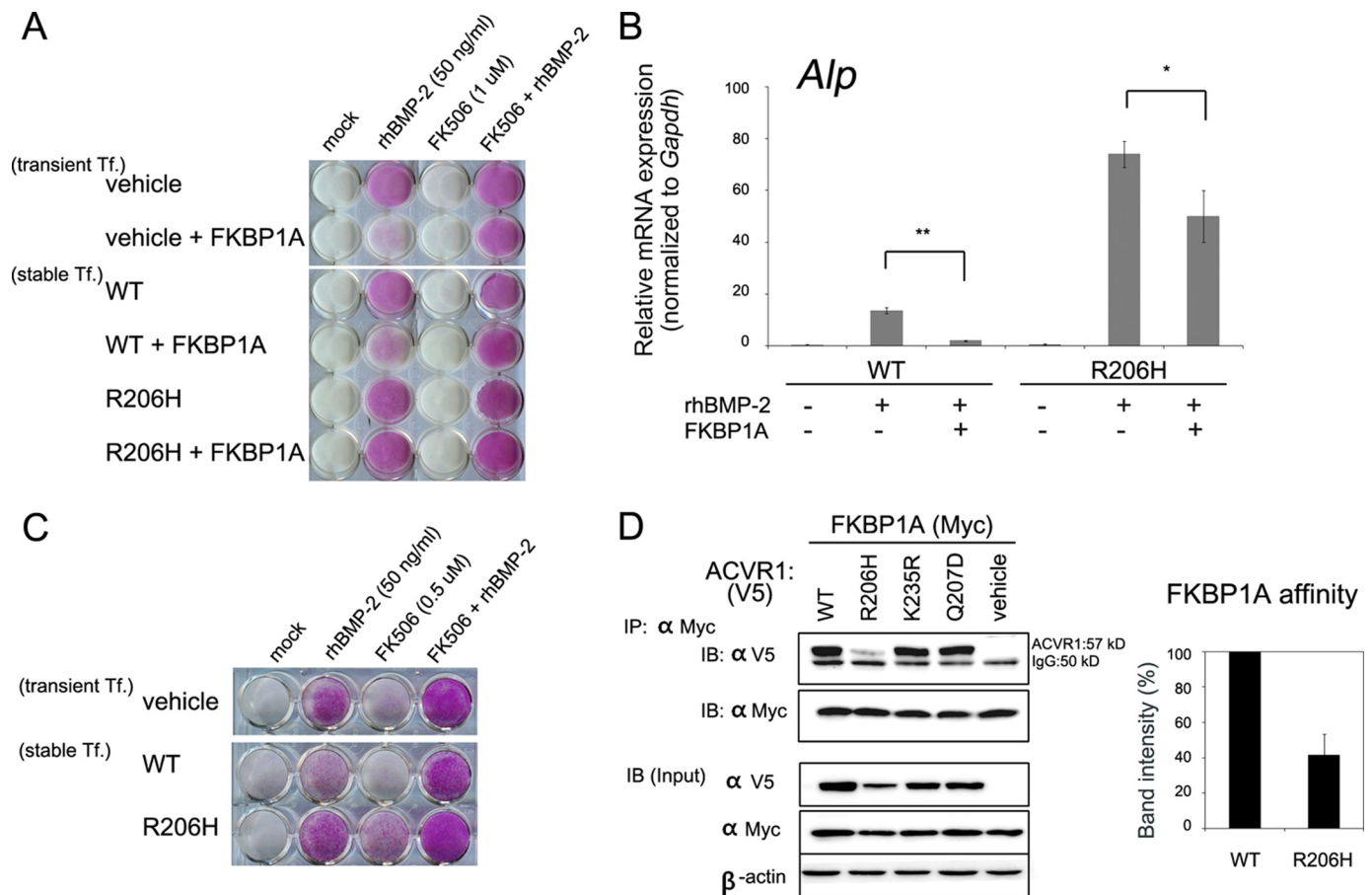
**FIGURE 2. ACVR1<sup>R206H</sup> is a weak activating mutant unlike ACVR1<sup>Q207D</sup>, a constitutively active mutant.** A, C2C12 cells were transiently transfected with ACVR1 WT, R206H mutant, dominant negative mutant (K235R), or constitutively active mutant (Q207D). After 24 h of transfection, cells were treated with, or without, rhBMP-2 (30 ng/ml) for an additional 24 h. The *Alp* mRNA level was determined by RT-qPCR. (\*\*,  $p < 0.001$ ; \*,  $p < 0.01$  versus untreated WT.) B, C2C12 cells were transiently transfected with four different expression constructs of ACVR1 or Smad5. After 24 h of transfection, cells were treated with 50 ng/ml rhBMP-2, 500 nM FK506, or a combination of both agents for additional 24 h. Then after a further 3 days of culture, cells were fixed, and ALP activity was cytochemically determined. C, C2C12 cells were co-transfected with a control siRNA (si-control) or si-Smad1 plus si-Smad5 with ACVR1 WT or R206H overexpression. Cells were cultured for 24–36 h, and the mRNA levels of the BMP downstream genes, *Dlx5*, were determined by RT-qPCR (\*,  $p < 0.01$  versus WT with si-control.) D, C2C12 cells, stably transfected with ACVR1 WT or R206H, were cultured. After reaching visual confluence, cells were cultured for an additional 24 h with, or without, rhBMP-2 (30 ng/ml). The mRNA levels of BMP downstream genes (*Dlx5*, *Alp*, and *Msx2*) were determined by RT-qPCR. (\*\*,  $p < 0.001$ ; \*,  $p < 0.01$  versus untreated WT by the two-tailed Student's *t* test. ns, not significant.)

expression of both *Bmpr1a* and *Bmpr1b* with their siRNAs also produced a marked reduction in BMP-2-induced *Dlx5* or *Alp* expression. Interestingly, ACVR1 played a significant role in BMP-2-induced *Dlx5* or *Alp* expression when both *Bmpr1a* and *Bmpr1b* were knocked down (Fig. 1D). We analyzed the knock-down efficiency of siRNAs against *Bmpr1a*, *Bmpr1b*, and *Acvr1* by measuring their mRNA levels using RT-qPCR, and found that the expression of BMP type I receptors was decreased by ~60% (data not shown).

**ACVR1 p.R206H Is a Gain-of-Function Mutation but Is Not Constitutively Active**—To investigate the genotype-phenotype correlation of the R206H mutation, we evaluated the expres-

sion levels of BMP-2-downstream osteogenic marker genes by RT-qPCR. The mRNA expression level of *Dlx5*, *Alp*, and *Msx2* in WT, R206H, dominant negative (K235R), and constitutively active (Q207D) ACVR1-transfected cells were examined. Transient transfection of R206H significantly stimulated *Alp* mRNA expression but the level of expression was not comparable with that following BMP-2 treatment or transfection with the constitutively active form, Q207D (Fig. 2A). In the absence of BMP-2 stimulation, the *Alp* mRNA expression level in cells overexpressing the R206H mutant was ~8-fold higher than that in ACVR1 WT cells ( $p < 0.001$ ). Interestingly, the *Alp* expression level in Q207D-transfected cells was significantly higher (about 8-fold) than that of the R206H-transfected cells ( $p < 0.001$ ). Treatment with BMP-2 for 24 h significantly stimulated *Alp* mRNA expression in all of the ACVR1 mutant-expressing cells; however, because of differences in basal *Alp* expression levels, the fold stimulation of *Alp* mRNA by BMP-2 treatment varied considerably: about 120-fold in WT, 18-fold in R206H, 6-fold in K235R, and 3-fold in Q207D compared with the respective BMP-2-untreated cells (Fig. 2A).

To characterize the functional changes of the R206H mutant compared with the Q207D constitutively active mutant further, we analyzed changes in *Alp* activity in the presence of Smad or FK506. FKBP1A, the FK506-binding protein 1A, binds around the GS domain of the transmembrane domain in the type I receptor and prohibits leakage of the signal in the absence of ligand binding. As FK506 is known to sequester FKBP1A from the GS domain (37), treatment with a sufficient amount of FK506 could cause the BMP signal to leak intentionally. In addition, overexpression of the Smad5 protein can also amplify the response of the BMP signaling pathway even if the original signal is very weak. Treatment with 0.5 μM FK506 is sufficient to increase *Alp* activity in Q207D-transfected cells but is not enough to stimulate *Alp* activity in R206H-transfected cells over the threshold level during a transient transfection. However, co-expression of Smad5 is sufficient to stimulate *Alp* activity in C2C12 cells transfected with



**FIGURE 3. Reduced and impaired FKBP1A binding for ACVR1<sup>R206H</sup> results in leakage of BMP signaling.** *A*, C2C12 cells, stably transfected with ACVR1 WT or R206H, were then transfected with FKBP1A. After 24 h of transfection, the cells were treated with 50 ng/ml rhBMP-2 or 1  $\mu$ M FK506 for an additional 24 h. ALP activity was cytochemically determined as for Fig. 2*B*. *B*, cells described in *A* were harvested after treatment with rhBMP-2 or FK506 for 24 h. The *Alp* mRNA level was analyzed by RT-qPCR. (\*\*,  $p < 0.001$ ; \*,  $p < 0.01$  versus untreated WT by the two-tailed Student's *t* test.) *C*, C2C12 cells, stably transfected with ACVR1 WT or R206H, were treated with 50 ng/ml of rhBMP-2 or 500 nM FK506 for 24 h and ALP activity was determined cytochemically. *D*, whole cell lysates of HEK293 cells transfected with the four ACVR1 expression constructs (WT, R206H, K235R, Q207D) were immunoprecipitated by  $\alpha$ -Myc (pcDNA4-FKBP1A-myc) and then immunoblotted with  $\alpha$ -V5 (pcDNA6-ACVR1-V5) or  $\alpha$ -Myc antibody. The intensities of the immunoprecipitated ACVR1 bands were normalized to respective FKBP1A band using the average intensity of the WT as 100% from two independent experiments. Data represent the means  $\pm$  S.D.

R206H or Q207D, even in the absence of BMP-2 treatment (Fig. 2*B*). Both gain-of-function mutants stimulated Alp activity over the threshold level; however, Q207D-overexpressing cells showed much stronger activity than cells overexpressing R206H (Fig. 2*B*).

In Fig. 2*B*, cotransfection of R206H mutant with *smad5* showed enhanced ALP staining compared with that of R206H alone. To examine whether the activation of the downstream signaling of ACVR1 by R206H is mediated by Smad-dependent or Smad-independent signaling pathways, we cotransfected the si-Smad1 and si-Smad5 with WT or R206H to C2C12 cells by electroporation and checked the effect of the blockade of Smad 1 and Smad 5 on *Dlx5* gene expression. R206H mutation-induced *Dlx5* gene expression was completely abrogated by the knockdown of both Smads (Fig. 2*C*), clearly indicating that R206H ACVR1 signaling was mediated by the Smad-dependent pathway.

We next examined the effect of the R206H mutation in stably transfected cells to confirm the molecular event shown in transiently transfected cells. C2C12 cells, stably transfected with ACVR1 WT or the R206H mutant, showed very similar *Alp* expression patterns when compared with transiently trans-

fected cells. *Dlx5* ( $p < 0.01$ ) and *Alp* ( $p < 0.001$ ) mRNA levels were increased significantly by R206H overexpression alone but the increase was much lower than that seen in response to BMP-2 treatment (Fig. 2*D*). *Runx2* and *Osterix*, an important transcription factors in regulation of osteoblast differentiation, showed similar expression pattern with that of *Dlx5* and *Alp* (supplemental Fig. S1). In contrast, the expression of *Msx2* was significantly ( $p < 0.001$ ) decreased by R206H overexpression (Fig. 2*D*). This up- and down-regulation of the expression patterns of BMP-2 downstream bone marker genes, as a result of R206H overexpression, was still maintained after the BMP-2 treatment.

*The ACVR1 p.R206H Mutation Causes Leakage in Osteogenic BMP Signaling Because of a Reduced Affinity for FKBP1A*—In our experience, 30–50 ng/ml of rhBMP-2 is the optimal concentration required to ensure a positive result in the ALP staining assay in C2C12 cells. BMP-2-induced Alp activation was suppressed by overexpression of FKBP1A in C2C12 (Fig. 3*A*). The involvement of FKBP1A was further supported by the result that treatment with FK506, a sequestering agent of FKBP1A, released FKBP1A-blocked BMPR signaling and allowed the cells fully to respond to BMP-2 treatment. These

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results were reproduced in WT ACVR1 stably-transfected C2C12 cells, whereas R206H ACVR1-transfected cells showed unaltered Alp activity, which was not successfully blocked by FKBP1A overexpression (Fig. 3A).

Next we examined the inhibitory function of FKBP1A on BMP-2-induced *Alp* mRNA expression by RT-qPCR (Fig. 3B). FKBP1A overexpression showed ~85% inhibition of *Alp* mRNA expression in mock-transfected (data not shown) or WT cells. In contrast, *Alp* mRNA levels in R206H mutant-transfected cells were not successfully inhibited by FKBP1A overexpression, consistent with the result of ALP staining. To confirm the signaling leakage in the R206H mutant, we treated the cells with 0.5  $\mu\text{M}$  of FK506 for the first 24 h, to dissociate FKBP1A from the GS domain of ACVR1, and then after three more days in culture, we performed ALP staining (Fig. 3C). FK506 alone was not sufficient to produce a positive ALP staining in mock and WT-transfected cells. In contrast, R206H-overexpressed cells showed stronger ALP staining than WT or mock cells even in the FK506-treated cells.

To date, protein modeling analysis has predicted that FKBP1A binds to ACVR1 in order to maintain the autoinhibited state unless receptor activation by the ligand is diminished or perturbed in the mutant. However, there are no molecular data for direct verification of the interaction of FKBP1A with wild type or R206H mutant of ACVR1. To confirm their interaction, we performed immunoprecipitation with an anti-Myc antibody for FKBP1A and then immunoblotting with an anti-V5 and anti-Myc antibody for ACVR1 and FKBP1A, respectively (Fig. 3D). The intensities of the immunoprecipitated ACVR1 bands were normalized to respective FKBP1A band using the average intensity of the WT as 100%. The binding affinity was reduced by over a half in R206H-transfected cells. This result was the first strong evidence to show the loose binding of FKBP1A to the R206H mutant of ACVR1. In addition, the decreased affinity of the R206H mutant for FKBP1A was accompanied by the reduced expression of the mutant protein (Fig. 3D).

**Decreased Expression of ACVR1 p.R206H Protein and Its Elevation by FKBP1A**—Western blot analysis of whole cell lysates obtained by using HEPES lysis buffer showed a discrepancy in the amount of protein expression between WT and R206H. The ACVR1 band intensities were firstly normalized to the actin bands and then intensity of R206H was compared with the WT as 100% from three independent experiments. The R206H protein level was consistently lower than that of the WT in HEK293 cells (Fig. 4A). Overexpression of FKBP1A strongly increased the protein levels of all of the ACVR1 including wild type or mutants, compared with those in FKBP1A non-transfected cells. However, the protein level of the R206H mutant was consistently lower than that of WT or the other mutants even in the forced expression of FKBP1A (Fig. 4B). To negate cell type-specific changes and variable transfection efficiencies, we examined these molecular phenomenons in C2C12 cells stably overexpressing ACVR1 WT or R206H. Similar to the result in HEK293 cells, the R206H protein level showed a decrease of ~40% in its protein expression compared with that of WT (Fig. 4C). In this experiment, FKBP1A co-transfection strongly elevated the ACVR1 protein level in both HEK293 and C2C12

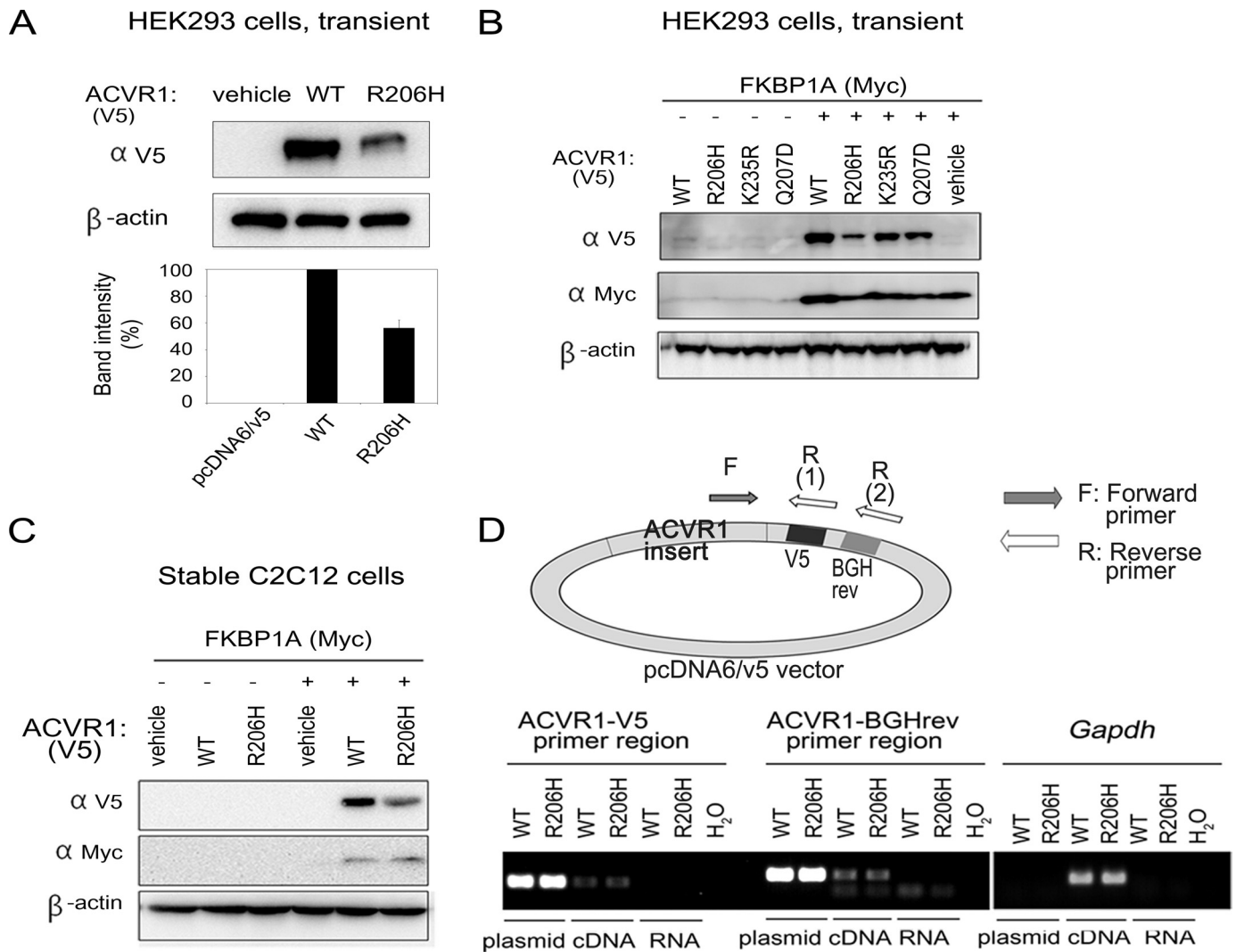
cells, although the R206H protein level was consistently and markedly lower than that of the WT, at about 60%.

To discover whether the reduced level of protein expression is caused by the decrease in the mRNA level or not, we performed reverse transcription PCR (RT-PCR) with cDNA from each stable C2C12 cell line. Our RT-PCR results showed the same levels of transcription when plasmids or reverse transcribed cDNAs were used as templates in reactions with low cycle numbers (25 cycles) (Fig. 4D). Thus, we assumed that the decreased protein level of the R206H mutant was due to a post-transcriptional event.

**Different Subcellular Distribution between ACVR1 WT and the p.R206H Mutant**—Because ACVR1 protein level was higher in FKBP1A-transfected cells, and the R206H mutant, with its weaker binding affinity for FKBP1A, consistently produced lower ACVR1 protein levels (Fig. 4B). We considered that the ACVR1-FKBP1A interaction might affect the amount of receptor protein. To investigate this hypothesis in detail, we co-transfected FKBP1A and ACVR1 WT or the R206H mutant into HEK293 cells. We then harvested the protein with CHAPS-TNE lysis buffer to solubilize the insoluble integrated proteins from the plasma membrane, or TNE lysis buffer to collect total protein, including the soluble proteins, from the cytosol (32). Different lysis buffers resulted in different amounts of protein in Western blot analysis. The proteins solubilized with CHAPS-TNE lysis buffer showed that both WT and R206H protein levels gradually increased in response to FKBP1A overexpression in a dose-dependent manner. The expressed R206H protein level was ~80% compared with WT after actin normalization (Fig. 5A). On the other hand, the proteins solubilized with TNE lysis buffer showed that R206H levels were maintained at consistently low level of about 40% to 50% of WT. Interestingly, the R206H protein levels were little affected by the FKBP1A co-transfection, in contrast to WT, which showed a sensitive response to the increase in FKBP1A (Fig. 5B).

To investigate the different subcellular locations of ACVR1 WT and its R206H mutant further, C2C12 cells, stably overexpressed with the different forms of ACVR1, were immunostained after FKBP1A co-transfection and analyzed under a confocal laser microscope. WT ACVR1 was observed in the nucleus, cytoplasm and plasma membrane. We also detected WT ACVR1 in the endoplasmic reticulum (ER) and the Golgi apparatus, which were easily distinguished phenotypically (38–39). Although the R206H mutants were also detected in the nucleus, cytoplasm, plasma membrane, ER and Golgi, they were mainly concentrated in the plasma membrane (Fig. 6A). There was no specific difference in cell morphology between WT and R206H. FKBP1A overexpression did not alter distribution of both ACVR1 WT and R206H. However, while FKBP1A, a generally abundant and ubiquitous cytoplasmic protein, was detected with a broadly dispersed pattern in the cytoplasm of WT ACVR1 cells, FKBP1A in the R206H mutant cells were observed mainly in the area of the plasma membrane.

Next, to explore the effect of BMP stimulation on receptor distribution, we performed immunostaining after 30 min treatment with 50 ng/ml rhBMP-2. In the absence of rhBMP-2 treatment, ACVR1 and FKBP1A were detected broadly throughout



**FIGURE 4. ACVR1-FKBP1A interaction stabilizes ACVR1 protein, ACVR1<sup>R206H</sup> mutation causes reduced amount of protein because of reduced affinity for FKBP1A.** *A*, lysates of HEK293 cells, transfected with ACVR1 WT, or R206H, were analyzed by SDS/PAGE and Western blotting with the  $\alpha$ -V5 (ACVR1) antibody. The intensities of the ACVR1 bands were normalized to the actin bands using the average intensity of the WT as 100% from three independent experiments. *B*, HEK293 cells were transfected with the four ACVR1 constructs with, or without FKBP1A, and then analyzed by Western blotting with the  $\alpha$ -V5 or  $\alpha$ -Myc antibodies. *C*, stable C2C12 cells carrying WT or R206H were analyzed by Western blotting. *D*, RT-PCR analysis of the mRNA levels of overexpressed *Acvr1* WT and R206H. Forward and reverse primers were designed to include the V5 and BGH reverse region, which are distal sequences from ACVR1 ORF insert in pcDNA6-ACVR1-v5 plasmids. Plasmid and RNA without reverse transcription, as a template, was included for a positive and negative control reaction, respectively.

the cytoplasm in WT ACVR1 cells. However, after rhBMP-2 treatment, ACVR1 and FKBP1A were identified prominently around the plasma membrane, and the merging of ACVR1 and FKBP1A signals also increased only in the area of the subplasma membrane (Fig. 6*B*). Regardless of rhBMP-2 stimulation, the R206H mutant and FKBP1A were observed mainly in the area of the plasma membrane. The mutant receptor and FKBP1A signals colocalized around the plasma membrane in all serial confocal slices from R206H ACVR1 cells (Fig. 6*C*).

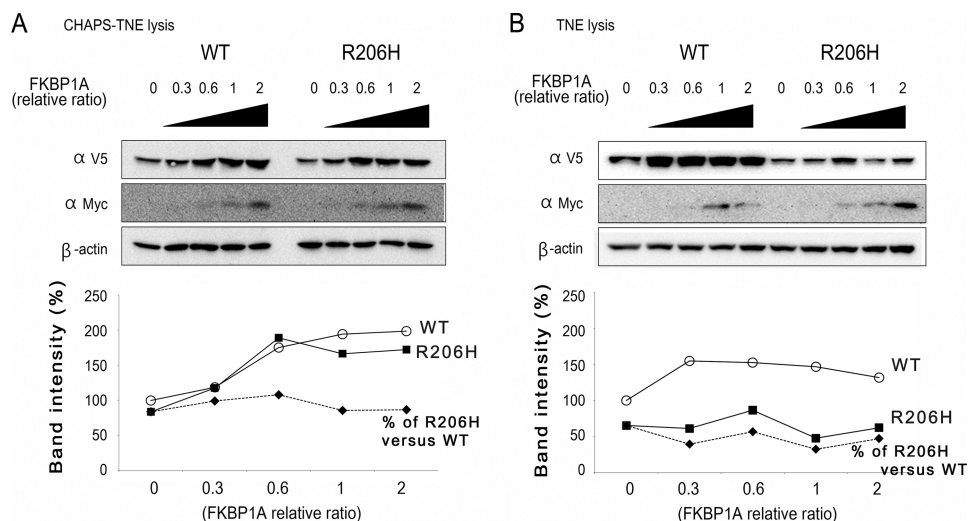
## DISCUSSION

BMPs regulate a myriad of homeostatic events during embryonic development and throughout adult life through their heterotetrameric type I and type II receptor complexes (40). However, which BMP receptor complexes are operating in various BMP signaling pathways is complex and unclear (41).

The activin A receptor, type 1 (ACVR1), also known as ALK2, is known to contribute to left-right pattern formation during embryogenesis and to the formation of primordial germ cells (PGCs) in the visceral endoderm (42–43). In addition, disrupted ACVR1 results in gastrulation defects or congenital heart defects (44–47). However, the contribution of ACVR1 to BMP signaling is ill defined. A previous report revealed that ACVR1/ALK2 is the most predominantly expressed type I receptor and that the BMP receptor, type 1A (BMPRI1A) also shows strong activity, although it has the lowest expression among the BMPRI-I receptors in primary human mesenchymal stem cells (hMSCs) (48). ACVR1-ACVR1 complexation has the weaker activity than BMPRI-IA complexation in transduction of BMP signaling (48). Our data showed that ACVR1 was the most abundantly expressed of the type I BMP receptors in murine myogenic C2C12 cells (Fig. 1*A*). Our RT-qPCR analysis showed



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**FIGURE 5. The ACVR1<sup>R206H</sup> mutation shows a different subcellular distribution to WT by quantitative analysis.** A and B, FKBP1A with WT or R206H were transiently transfected in HEK293 cells with an increasing ratio of FKBP1A plasmid to ACVR1 plasmid as indicated (FKBP1A relative ratio). After 24 h, whole cell lysates were harvested using TNE-CHAPS lysis buffer to solubilize transmembrane receptors or TNE lysis buffer to collect total soluble cytosolic proteins. The graphs in the lower panel represent the densitometric analysis of the Western blots of the upper panel. Each experiment was repeated at least twice.

that *Acvr1* mRNA was also the most abundantly expressed of the *Bmpr1* receptors in mouse skeletal muscle, ST2 cells and mouse primary bone marrow stromal cells (data not shown). Expression levels of the osteogenic master genes, *Dlx5* and *Alp*, were not significantly altered by ACVR1 overexpression or by knock-down with si-RNA of ACVR1. However, ACVR1 could replace the function of the other BMPR-I because ACVR1 was able to transduce the osteogenic BMP-signal efficiently when the other BMPR-Is were knocked-down by their respective siRNAs suggesting that ACVR1 may not be the main pathway for osteogenic BMP-signal transduction in mesenchymal cells despite their high expression level. Therefore, although ACVR1 may be the most common BMP type I receptor in mesenchymal cells, it appears to play a minor role in BMP signal transduction.

In addition, this characteristic of ACVR1 may explain the clinically unexpressed features of FOP at birth and then slow progression of the disease. Accumulation or positive feedback of BMP signaling in the ACVR1 R206H mutant may be involved in generating the slow progressive character of FOP. Put simply, a low concentration of BMPs may satisfy the environment of heterotopic ossification in the presence of the R206H mutation. The impact or necessity of environmental factors, such as hypoxia or inflammation, on the pathogenesis of FOP should also be investigated carefully (49–51).

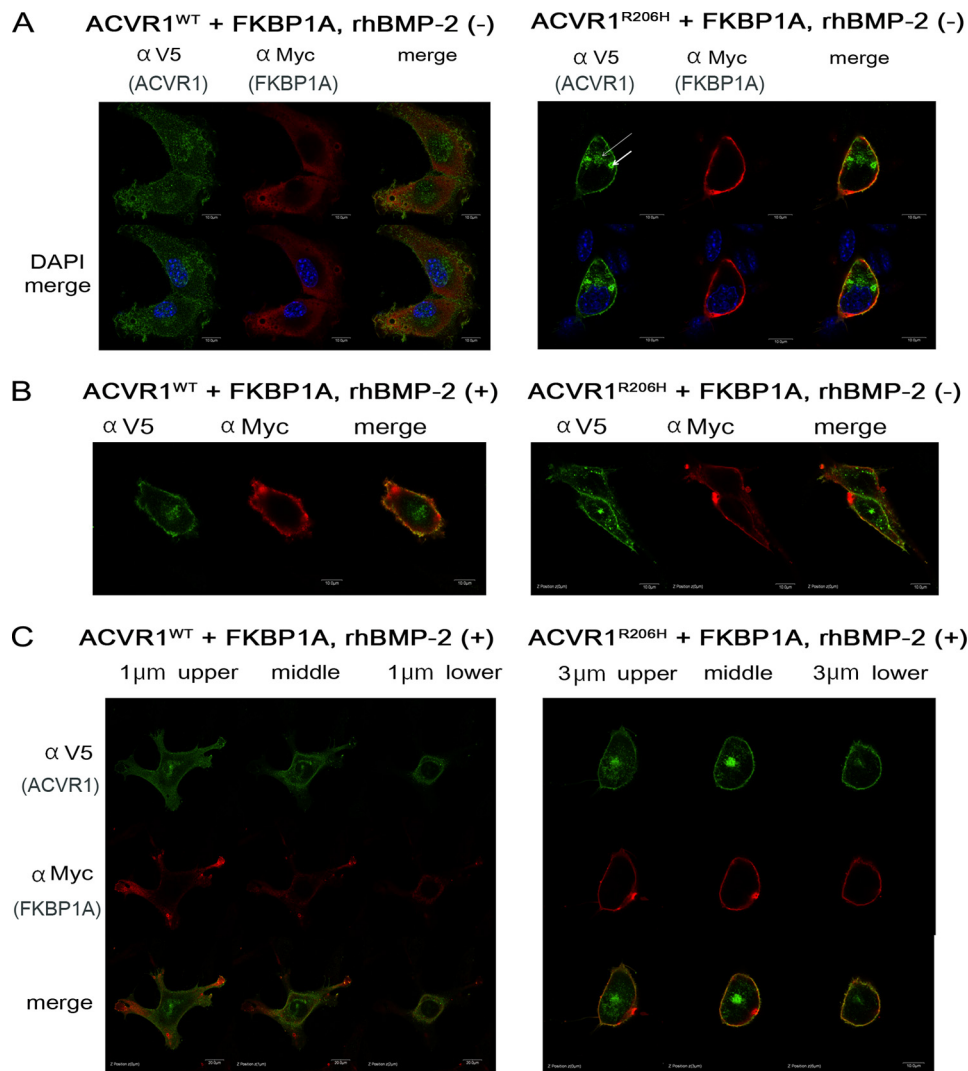
The weak ACVR1-FKBP1A interaction in the R206H mutant causes a leaky BMP signal, which is quite different from the ligand-independent mutant, Q207D. The Q207D mutant can be compared with R206H based on their nearby locations and dramatic *in vivo* impacts in mouse (52). Q207D overexpressed cells show stronger osteogenic activity than R206H-overexpressed cells both in our data and previous reports (13, 21). Different ranges of the BMP response suggest that R206H can have discrete mechanism with Q207D, despite the fact that they involve adjacent mutations in the GS domain, because Q207D shows a normal binding affinity for FKBP1A compared with wild type (Fig. 3D).

FKBP1A is a cis-trans peptidyl-prolyl isomerase as a member of the immunophilin protein family, which plays a role in immunoregulation and basic cellular processes involving protein folding and trafficking (53–55). It interacts with multiple intracellular calcium release channels including the tetrameric skeletal muscle ryanodine receptor, and the immunosuppressants FK506 and rapamycin, as well as type I receptors of the TGF- $\beta$  superfamily (56). A reduction in the ACVR1-FKBP1A interaction by the R206H mutation may lead to a decrease in the steady-state ACVR1 protein level and to a different subcellular distribution. Interestingly, most mutations identified in FOP patients are single nucleotide substitutions that produce missense

mutations, not frameshifts or nonsense mutations, suggesting that in each case the mutant receptor protein has an altered function (8). The impaired binding with FKBP1A is a result of protein conformational changes (8, 12), but it is not clear that the R206H mutant protein is misfolded. Moreover, no direct evidence has been found to show that the consequences of functional activation were due to activation of the receptor itself rather than activation through other compensatory mechanisms.

Post-translational modifications of many proteins in the secretory pathway are intrinsically linked to protein folding and quality control (57–58). Incorrectly folded proteins in the ER are targeted by a process known as endoplasmic reticulum-associated degradation (ERAD), which ensures that misfolded proteins are removed from the cell by protein lysis, thereby leading to reduced protein levels (59–61). Decreased levels of R206H protein can be presumed to be the result of ERAD because the missense mutation alters the conformational structure of the GS domain, leading to an alteration in the overall structure of ACVR1. However, detection of the protein in the plasma membrane by Western blot analysis and confocal microscopy means that normal transport of the mutant protein from the ER to the Golgi, and finally to the plasma membrane does occur safely. Therefore, FOP is different from several other genetic disorders, which show defective protein folding or ER retention (62–63). Our data provide clear evidence that the mutant R206H is in a functionally active state without defective folding despite its low levels of expression and minor activity.

The alteration in the subcellular distribution of ACVR1 due to the R206H mutation may be a biologically important clue to the molecular consequences of the mutation. Altered affinity with FKBP1A, a cis-trans peptidyl-prolyl isomerase, may change the trafficking of ACVR1. Figs. 5 and 6 shows that WT ACVR1 was distributed broadly in cytoplasm and plasma membrane whereas the R206H mutant was found



**FIGURE 6. Different subcellular distribution between WT and ACVR1<sup>R206H</sup>.** A, C2C12 stable cells carrying WT ACVR1 or R206H were transiently transfected with FKBP1A and then immunostained with  $\alpha$ -V5 (green: ACVR1) and  $\alpha$ -Myc (RED: FKBP1A) antibody followed by Alexa F488-conjugated anti-mouse and Qdot 655-conjugated anti-rabbit secondary antibodies. The view of a confocal slice midway through the thickness of the cells was taken. WT shows a broadly dispersed cytosolic pattern with FKBP1A. (Long thin white arrow, ER; short thick white arrow, Golgi apparatus.) B, after 30 min of treatment with 50 ng/ml rhBMP-2, the cells were fixed and immunostained. The view of a confocal slice was taken midway through the height of the cells. The subcellular distribution of WT with rhBMP-2 treatment, and the R206H mutant without rhBMP-2 treatment was compared. C, serial confocal slices were taken in the upper and lower 1 to 3  $\mu$ m from the midway through the height of the cells. (Blue, DAPI; yellow, merged area; scale bars: 10  $\mu$ m or 20  $\mu$ m.)

mainly in plasma membrane. It suggests that an altered trafficking or endocytosis can be related to the R206H mutant because accelerated endocytosis or rapid recycling is necessary for reduced steady-state of protein level to bring the higher osteogenic signal. Moreover, FKBP1A is reported as a negative regulator of TGF- $\beta$  receptor internalization (16). A few previous studies on the TGF- $\beta$  receptor or BMP receptors have demonstrated that BMP receptors are constitutively endocytosed with rates closely resembling those of the TGF- $\beta$  receptors (32). BMPR-I and BMPR-II are continuously internalized mainly via the clathrin-mediated pathway mainly, while BMPR-II is also additionally endocytosed via lipid rafts (32, 64–65). Ligand activation to preformed receptor complexes (PFCs) can induce Smad phosphorylation at the plasma membrane even before clathrin-coated pit

(CCP) formation or internalization, while receptor endocytosis is required for the continuation of signal transduction and target gene expression (32, 64). Internalized cell surface receptors are either recycled to the plasma membrane or transported to lysosomes for degradation (66). In several pathogenesis, endocytosis is indicated as one of the key processes controlling the level of receptors at the cell surface (57, 67). We suggest that the reduced levels of the R206H receptor, accompanied with altered subcellular distribution, possibly occur in FOP through an accelerated endocytosis, or rapid turnover, or rapid recycling.

Our results explain why FOP has delayed and progressive characteristics in ectopic bone formation. First, ACVR1 is an abundant BMP type I receptor, but plays a minor role in BMP signal transduction and second, ACVR1 R206H is a gain-of-function mutation, but unlike Q207D, it is not strong or constitutively active. Rather, ACVR1 R206H mutation causes the leakage in BMP signaling through the loose binding to FKBP1A. Finally, as compared with WT ACVR1 there is a decreased expression of ACVR1 R206H protein due to reduced affinity to FKBP1A, which contribute to post-transcriptional regulatory events. Although the ACVR1 R206H mutant shows gain-of-function, its steady-state protein level is low, so it may not cause an immediate and abrupt phenotype expression of ectopic osteogenesis like Q207D.

Taken together, our results have provided interesting insights for understanding the molecular consequences of FOP and its biology. The R206H mutation of ACVR1 induced subcellular translocation of mutant ACVR1 and FKBP1A to the plasma membrane regardless of rhBMP-2 stimulation. The impaired binding to FKBP1A and an altered subcellular distribution suggests a molecular mechanism by which the R206H mutation may lead to progressive heterotopic ossification.

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