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Variable signaling activity by FOP ACVR1 mutations

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Abstract

Most patients with fibrodysplasia ossificans progressiva (FOP), a rare genetic disorder of heterotopic ossification, have the same causative mutation in ACVR1, R206H. However, additional mutations within the ACVR1 BMP type I receptor have been identified in a small number of FOP cases, often in patients with disease of lesser or greater severity than occurs with R206H mutations. Genotype-phenotype correlations have been suggested in patients, resulting in classification of FOP mutations based on location within different receptor domains and structural modeling. However while each of the mutations induces increased signaling through the BMP-pSmad1/5/8 pathway, the molecular mechanisms underlying functional differences of these FOP variant receptors remained undetermined. We now demonstrate that FOP mutations within the ACVR1 receptor kinase domain are more sensitive to low levels of BMP than mutations in the ACVR1 GS domain. Our data additionally confirm responsiveness of cells with FOP ACVR1 mutations to both BMP and Activin A ligands. We also have determined that constructs with FOP ACVR1 mutations that are engineered without the ligand-binding domain retain increased BMP-pSmad1/5/8 pathway activation relative to wild-type ACVR1, supporting that the mutant receptors can function through ligand-independent mechanisms either directly through mutant ACVR1 or through indirect mechanisms.

Keywords

Fibrodysplasia ossificans progressiva; heterotopic ossification; BMP signaling activity; protein kinase mutation; ACVR1; classic/variant mutation

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Declaration of interest

All authors declare no conflicts of interests.

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1. Introduction

Fibrodysplasia ossificans progressiva (FOP; OMIM: #135100) can be clinically diagnosed based on the post-natal formation of extra-skeletal bone in soft connective tissues, such as skeletal muscle, beginning in early childhood. In most cases, this heterotopic ossification (HO) was preceded by prenatal skeletal malformations, most characteristically noted in the great toes. FOP is caused by heterozygous mutations in ACVR1, a BMP type I receptor (also known as ALK2, ACTRIA, ACVRLK2); most are *de novo* mutations in the affected individual [1]. All patients clinically diagnosed with FOP who have been examined for mutations in ACVR1 have been found to have a mutation in this gene. Those who have a 'classic' clinical presentation (heterotopic endochondral ossification and great toe malformations) nearly always have the same recurrent mutation – c.617G>A; R206H. However, a small number show less or greater severity of disease with regard to extent of skeletal malformations and/or timing of onset of HO [2]. Most of these clinically variable patients have been determined to carry heterozygous ACVR1 mutations other than R206H.

ACVR1 is a transmembrane protein composed of an extracellular ligand-binding domain (LBD), an intracellular glycine-serine (GS) rich domain, and a protein kinase (PK) domain. Initially, ACVR1 was identified as an Activin binding receptor but was later shown to also bind BMP ligands. Both classes of ligands belong to the BMP/TGF-beta superfamily of growth and differentiation factors and have been associated with the HO formation processes in FOP [3–8].

ACVR1 is one of 7 known BMP/TGF-beta type 1 receptors that mediate transduction of ligand-initiated extracellular signaling into the cell nucleus [9–11]. Ligands of the BMP/TGF-beta family form dimers that bind to or initiate formation of heteromeric receptor complexes composed of at least two type 1 and two type 2 receptors. Upon ligand-binding, type 2 receptors activate the GS domain of the type 1 receptors by transphosphorylation resulting in the activation of its kinase domain. The type 1 receptor subsequently phosphorylates and activates intracellular transducers of the signaling cascade such as Smad1, Smad5 and Smad8 (for BMP) or Smad2 and Smad3 (for TGF-beta) [12]. Structural modeling of ACVR1 mutant proteins predicted that FOP ACVR1 variant mutations alter protein folding and interactions that are likely to lead to an activated receptor state [2,13,14]. Previous reports supported that each of the FOP ACVR1 mutations examined confers increased BMP-pSmad1/5/8 pathway signaling in cells that express these mutations [13–16].

However, whether the mechanisms of aberrant receptor activation are the same for each of the FOP ACVR1 mutant proteins or if alternate mechanisms to prompt receptor activation are used by various mutants remained uninvestigated. In this study, we focused on the response of ACVR1 mutants to ligands, including whether ligand activation of these receptors is required for enhanced pSmad1/5/8 signaling in cells with mutant ACVR1 receptors.

2. Materials and Methods

2.1 Plasmid constructs

A human wild-type (WT) ACVR1 expression plasmid was generated by cloning the protein-coding sequence of human ACVR1 into pcDNA 3.1 D V5-His-TOPO vector (Invitrogen) and the ACVR1 R206H plasmid generated by site-directed mutagenesis of the WT ACVR1 [15]. This same approach was used with the indicated oligonucleotides (with mutant nucleotide underlined) for ACVR1 constitutively active (CA) (forward 5': GTACAAAGAACAGTGGCTCGCGATATTACTG-3', reverse 5': GCGAGCCACTGTTCTTTGTACCAGAAAAGGAAG-3'); ACVR1 G328E (forward 5': GATATTTGGGACCCAAGAGAAACCAGCCATTGC-3'; reverse 5': CTTGGGTCCCAAATATCTCTATGTGCAAATGTGC-3'); ACVR1 G328W (forward 5': GATATTTGGGACCCAATGGAAACCAGCCATTGC-3'; reverse 5': TTGGGTCCCAAATATCTCTATGTGCAAATGTGCT-3'); ACVR1 G356D (forward 5': TTGCATAGCAGATTTGGACCTGGCAGTCATGC-3'; reverse 5': CCAAATCTGCTATGCAACACTGTCCATTCTTCT-3'). The pSmad1/5/8-responsive BRE luciferase reporter (BRE-Luc; [17]) was used in the luciferase reporter gene assay with the pRL-TK renilla luciferase (Renilla-Luc) expression construct (Promega) as a control.

To generate ACVR1 ligand-binding domain deletion (LBD) expression plasmids, amino acids 35–99, encoding the ligand-binding domain of the ACVR1 receptor, were deleted from full-length expression plasmids by site-directed mutagenesis PCR; an HA-tag was inserted at the N-terminal end of the protein [14].

2.2 Immortalized mouse embryonic fibroblasts (iMEFs) and cell culture

MEFs were isolated from mice [18] at embryonic day 13.5 (E13.5) as previously described [19]. Cells were immortalized with recombinant lentivirus expressing SV40 large T antigen (Capital Biosciences, Rockville, MD, USA). Expression of SV40 T antigen was confirmed via qRT-PCR on mRNA using specific primer SV40 (forward 5'-GACTCAGGGCATGAAACAGG-3' and reverse 5'-ACTGAGGGGCCTGAAATGA-3'). Immortalized cell line was genotyped for ACVR1 by DNA sequencing. Cells were cultured in Dulbecco's modified Eagle's medium, high glucose (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37°C and routinely passaged prior to confluency using TrypLE (Invitrogen). C2C12 or C2C12-BR [20] cells were cultured in DMEM (Gibco) supplemented with 5% FBS (Invitrogen) at 37°C and routinely passaged prior to confluency using TrypLE (Invitrogen).

2.3 Cell transfection

C2C12, C2C12-BR cells or iMEFs were plated in cell culture dishes in DMEM and incubated at 37°C for 18–20 hours. Cells were transfected with indicated expression plasmids using FugeneHD (Promega) following manufacturer's instruction. Transfection complexes were removed after 5 hours by substituting media. Cells were then incubated overnight at 37°C. To remove endogenous ligands, medium was replaced with serum-reduced media (0.5% FBS in DMEM) for 2 hours prior stimulation with growth factors.

Cells were then treated with indicated concentrations of BMP4 (R&D Systems) or Activin A (Peprotech) followed by analysis.

2.4 Luciferase reporter gene assay

C2C12-BR cells or iMEFs were plated in 48-well plates at 2×10^4 cells/well. C2C12-BR cells were co-transfected with renilla luciferase (Renilla-Luc) normalization reporter (pRL-TK; Promega; 0.2 μ g) and the indicated ACVR1 receptor expression plasmid (0.4 μ g). Additionally, iMEFs, which lack an endogenous BRE-Luc, were co-transfected with a BRE-Luc reporter [17]. Cells were treated with indicated amounts of BMP4 or Activin A for 16 hours and then washed with 1xD-PBS prior cell lysis. Cell lysis and detection of firefly and renilla activities used Dual-Luciferase® Reporter Assay System (Promega; catalog #E1960) following manufacturer's instruction and using the Synergy HT instrument system from BioTek. Since transfected receptors show variable expression in cells (Suppl. Figure 1) relative firefly activity was corrected to renilla activity before normalization to cells expressing the WT ACVR1 receptor.

To segregate BMP pathway activation by other BMP receptors in the LBD assays, we calculated the basal activity level of the WT by subtracting LBD values from full-length. This step is based on the assumption that WT LBD receptor is functionally inactive and thus the calculation eliminates background noise emanating from remaining type I receptors in the cell system. Activity levels from mutant receptors (both full-length and LBD) were then calculated by normalization to WT full-length followed by a correction against basal WT activity level.

2.5 Smad1 phosphorylation assay

To indirectly measure kinase activity of ACVR1 receptors, we used the AlphaScreenSureFire Smad1 p-Ser463/p465 assay (PerkinElmer, catalog # TGRSM1S500). The assay is based on the formation of sandwich antibody complex (comprised of a donor bead recognizing Smad1 and an acceptor bead, recognizing Smad1, when phosphorylated at Ser463/465) that only forms, when both beads are in close proximity to allow energy transfer after excitation with light at 680nm. Emission was captured at 520–620 nm using an EnSpire Alpha Plate Reader (PerkinElmer). Immortalized MEFs were plated in 24-well plates (4.5×10^4 cells/well) and incubated at 37°C overnight. Cells were transfected as described above (cell transfection) with indicated ACVR1 receptor expression plasmid and renilla luciferase normalization reporter. The next day cells were starved for 2 hours in serum-reduced media (0.5% FBS in DMEM) followed by stimulation with ligand for 1 hour. Cells were then lysed as described above. Detection of phosphorylated Smad1 was done following manufacturer's instruction. Renilla activity was detected as described in previous section (Luciferase reporter gene assay). Relative pSmad1 level was calculated by normalization to renilla activity and to value of cells expressing the WT ACVR1 receptor under untreated condition.

2.6 Immunoblotting

C2C12 cells were plated into 6-well plates (2.5×10^5 cells/well) and cultured overnight at 37°C. Cells were transfected with V5- or HA-tagged ACVR1 constructs as described above.

For stimulation with BMP4, cells were serum-starved (0.5% FBS in DMEM) for 2 hours followed by treatment with 30ng/ml BMP4 (R&D Systems) for 1 hour. Cells were lysed in RIPA buffer (Sigma-Aldrich) supplemented with Halt Protease and Halt Phosphatase Inhibitor Cocktail (Pierce), and cleared by centrifugation. Protein concentrations of lysates were quantified by BCA Protein Kit (Thermo Scientific). Proteins were electrophoresed through 10% Tris-Glycine gels (Invitrogen), and transferred to nitrocellulose (Invitrogen). Membranes were blocked in 5% milk in 1xTBS (BioRad; #1706435) and incubated overnight at 4°C with primary antibodies against phospho-Smad1/5/8 (Cell Signaling Technology, #9511), V5 (Invitrogen R960-25), HA (Sigma Aldrich, #H6908) or β -actin (Cell Signaling Technology, #4967) followed by detection using an anti-rabbit (Cell Signaling Technology, #7074) or anti-mouse (Santa Cruz, #H2208) HRP-conjugated secondary antibody. Membranes were incubated with HRP substrate (LI-COR) and chemiluminescence was detected and quantified with a C-DiGit Blot Scanner (LI-COR).

2.7 Statistical analysis

Unless otherwise indicated, results are presented as the mean $\pm$ SEM of at least 3 independent experiments each performed in duplicate or triplicate (technical replicates). Statistical significance was calculated in GraphPad Prism 6 (GraphPad Software, Inc.) by one-way or two-way ANOVA with Tukey's multiple comparison test or Bonferroni post-hoc test, respectively. Data were additionally analyzed by two-tailed t-test to compare each variant to wild-type ACVR1. Differences were considered significant at $p < 0.05$ (*); $p < 0.01$ (**) or $p < 0.001$ (***).

3. Results

3.1 Basal BMP-pSMAD1/5/8 signaling activity by FOP ACVR1 variants is elevated

The most prevalent FOP ACVR1 mutation, referred to as the FOP 'classic' mutation, is R206H (c.617G>A) and is located within the GS domain (Figure 1) named for the abundance of glycine and serine residues. In the absence of ligand-receptor interaction through the extra-cellular ligand-binding domain (LBD), the GS domain controls activity of the receptor, acting as a repressor of the kinase domain by acquiring an inhibitory conformation and also by acting as a docking site for inhibitory molecules (such as FKBP12).

In silico modeling of the R206H mutant receptor indicated that in the absence of ligand the inhibitory conformation is lost, and biochemical investigations confirmed significantly reduced binding of FKBP12 to the receptor; these data support enhanced receptor activity as has been demonstrated by Smad1/5/8 phosphorylation assays [1,2,13,15,21–23]. BMP-pSmad1/5/8 signaling by the ACVR1 R206H mutation has been extensively studied and shown to be elevated in a partially ligand-dependent manner [14,15].

Additional FOP mutations in the GS domain occur in amino acid 207 (Q207E), adjacent to the arginine at position 206, and as L196P, P197-F198 del ins L, and R202I. All other known FOP-associated mutations are in the protein kinase (PK) domain of ACVR1, including R258G/S, G328R/W/E, G356D, and R375P. The effects of these FOP variant mutations on

the BMP signaling pathway are less established. However, similarly to the R206H mutation, *in silico* modeling predicts that each of these mutations could lead to increased receptor signaling [2,13].

In this study, we examined the signaling activity of a subset of FOP ACVR1 variant mutations (G356D, G328W, and G328E) that occur within the PK domain and have been associated with severe clinical phenotypes. They were compared to the common FOP mutation in the GS domain, R206H. As controls, we also included wild-type (WT) ACVR1 and the strongly constitutively active (CA) Q207D mutation, which does not occur in patients and is likely incompatible with normal development and function.

In the absence of exogenous ligands, C2C12 cells (a myoblast cell line) transfected with V5-tagged constructs containing the FOP ACVR1 receptor GS domain mutation R206H showed elevated phosphorylated Smad1/5/8 (pSmad1/5/8) levels by immunoblotting, indicating increased receptor activity under basal conditions. As expected, the CA Q207D mutation showed much higher levels of activation than the FOP ACVR1 mutations (Figure 2A–D). Among the PK domain ACVR1 FOP mutations, only the variant G356D KD mutant receptor showed significantly increased activation under unstimulated conditions (Figure 2A,B).

Stimulation with BMP4 ligand increased phosphorylation of Smad1/5/8, as detected by immunoblotting, with increases in pSmad1/5/8 relative to WT similar to the relative differences under basal conditions for each of the FOP variant mutations and showing no statistically significant differences (Figure 2C,D).

The C2C12 cell line has been previously used in FOP research due to the ability to undergo osteogenic differentiation, a cell fate relevant to the formation of ectopic endochondral bone. However, the low transfection efficiency that is characteristic of these cells led us to use C2C12-BR cells, which differ only by additionally carrying a pSmad1/5/8-responsive luciferase (Luc) reporter, for the following luciferase reporter assays. To evaluate downstream transcriptional activation of the pSmad1/5/8 pathway, C2C12-BR cells were quantified for luciferase reporter activity. Under basal conditions (no added ligand), results from the luciferase reporter gene assay (Figure 3A, white bars) were consistent with pSmad1/5/8 immunoblot data (Figure 2A–B) showing slightly increased (~1.5–1.9-fold) basal activation of the pathway, although these differences were not statistically significant. When using a two-tailed t-test all FOP variant receptors as well as the engineered CA receptor showed statistically significant differences under basal conditions ($p < 0.001$) compared to the WT receptor.

We additionally investigated BMP-Smad pathway activation in mouse embryonic fibroblasts (MEFs), a population of mesenchymal progenitor cells widely used for cell differentiation studies including for FOP [24–27]. In contrast to results in C2C12-BR cells, expression of the ACVR1 FOP receptor mutations in an immortalized MEF line (iMEFs) showed significantly up-regulated pSmad1/5/8-Luc reporter signaling under unstimulated conditions for all of the ACVR1 FOP variants relative to WT, indicating that the basal signaling level is increased in this cell line compared to C2C12-BR cells (Figure 3B, white bars). These data

suggest cell type-specific responses for ACVR1 receptor signaling activity. In the iMEF cell assays, among FOP mutations, we found that increased pSmad1/5/8-luc activity was most pronounced with the classic R206H mutation and that the increased activity by all PK mutants was lower, suggesting that the mechanisms that increase pSmad1/5/8 signaling pathway activity by GS and PK domain FOP ACVR1 mutations may differ.

3.2 BMP signaling activity by FOP variants in response to BMP4

The ACVR1 receptor has previously been shown to respond to BMP ligands and FOP ACVR1 mutant receptors are responsive to BMPs, including BMP4 [19,28,29]. BMP4 is highly expressed in injured muscles [30–32] as well as during the inflammatory and fibroproliferative early stages of FOP HO lesions supporting a role for this BMP ligand in the pathology of FOP [5–8].

BMP4 treatment (30ng/ml) of transfected C2C12-BR cells revealed that all mutant receptors significantly increase pSmad1/5/8 signaling activity relative to the WT ACVR1 receptor (Figure 3A, black bars), although the differences among the mutants are small, perhaps because the activation is approaching maximal levels under the experimental conditions. In response to a lower BMP4 dose (10ng/ml), iMEFs transfected with mutant receptors showed significant increases in BMP pathway activation (Figure 3B, black bars). In addition, we noted that the magnitude of the increase in response to BMP4 is less in FOP mutant receptors compared to WT, consistent with mutant receptors that are already activated at their basal state.

3.3 Activin A activates BMP signaling by ACVR1 PK and GS domain mutants

Recent studies have implicated Activin A as having an important role in the pathology of R206H ACVR1-induced HO formation [3,16,33]. Activin A is a member of the BMP/TGF-beta family of ligands that usually signals through the pSmad2/3 pathway, but also has been reported to bind the ACVR1 receptor to antagonize BMP-pSmad1/5/8 signaling by competing with BMP ligands for receptor binding [3,33–36]. Immortalized MEFs were transfected with ACVR1 receptor constructs, and treated with 100ng/ml Activin A or left untreated (Figure 3C). In contrast to previous findings in HEK cells [3], we detected increased BMP-pSmad1/5/8 signaling in iMEF cells with the WT receptor in response to Activin A (~20% increase compared to untreated cells; $p < 0.003$ by two-tailed t-test). Increased pSmad1/5/8 pathway activation by GS domain mutations (CA and R206H mutant receptors) were at a similar ~20% increase as WT ACVR1. The responses to Activin A by the PK domain variants G328E and G356D were highest (~40% in comparison to untreated).

When comparing receptor response to both tested ligands we found that WT luciferase activity levels with BMP4 treatment are about 1.43-fold greater than with Activin A treatment. Of note, almost no differences were found between Activin A and BMP4 treatment in each of the tested ACVR1 mutants.

3.4 GS domain and PK variant mutations show different dose-response sensitivity to BMP4

To investigate whether ACVR1 FOP mutant receptors show varied sensitivity and signaling response to levels of ligands, transfected iMEFs were treated with increasing concentrations

of BMP4 followed by a Smad1 phosphorylation assay (Figure 4). The sensitivity of this assay is much greater than the luciferase reporter assay, as it directly measures phosphorylation of the receptor substrate Smad1 rather than quantifying a transcriptional target reporter, and therefore indirectly assays the receptor kinase activity.

Basal receptor activity (0 ng/ml BMP4) is significantly increased in the GS domain mutants R206H and Q207E as well as in the G356D PK domain mutant (Figure 4A, B) compared to WT in untreated, serum-starved cells; pSmad1/5/8 pathway activation by KD mutant variants at position G328W and G328E showed increased activity but did not significantly differ from WT by two-way ANOVA. However, comparison of WT to each of the variant FOP receptors revealed a ~1.6 to 1.8-fold higher induction of BMP signaling that were statistically significant when using a two-tailed t-test ($p < 0.001$).

For GS domain mutations, doses of BMP4 up to 10 ng/ml induced Smad1/5/8 phosphorylation to levels that were not significantly different from WT receptor activity (Figure 4C). However, at 20 ng/ml BMP4, both R206H and Q207E receptors induced a significantly increased response compared to WT ACVR1 by two-way ANOVA (Figure 4A). The constitutively active Q207D mutation response was much greater and significantly different from WT at all doses.

ACVR1 PK domain mutations showed a more sensitive response to lower doses of ligand, with significantly increased activity of G328W, G328E, and G356D receptors at 10 ng/ml BMP4 (Figure 4C). In addition, G328W and G328E appear to be reaching a plateau of activity at lower doses (10–20 ng/ml) than the GS domain and G356D mutant receptors which continued to increase at the highest dose tested (20 ng/ml) (Figure 4A, C). In other words, at 10–20 ng/ml, the rate of increased activity by the GS domain and G356D receptors appears to be accelerating relative to the WT receptor, while the rate of increased activity by G328E and G328W is decreasing.

3.5 Ligand-independent activation of FOP mutant ACVR1 receptors

To test whether ACVR1 mutants require the extracellular ligand-binding domain of the receptor in order to activate the pSmad1/5/8 signaling pathway, we engineered HA-tagged ligand-binding-domain deletion constructs (LBD) (Figure 5A) corresponding to each of the GS and PK domain mutants. Expression of the truncated receptor proteins at the predicted sizes was confirmed by immunoblotting and HA-tag detection. (Suppl. Figure 1). Full-length and deletion constructs were transfected into iMEFs in the absence of added ligands and pSmad1/5/8 activity assayed by luciferase reporter assays.

Under these basal conditions, all mutant ACVR1 receptors activate signaling to higher levels relative to WT full-length ACVR1 when the ligand-binding domain is absent (Figure 5B).

The LBD CA Q207D receptor retains most (~72% of full-length) under basal conditions (Figure 5B), while FOP ACVR1 mutant receptors retain less activity. The PK domain mutations show the greatest dependence on the ligand-binding domain, with G328E and G356D mutants retaining only 43% and 37% of full-length receptor activity, respectively.

The G328W LBD receptor maintains ~49% activity compared to its full-length construct while the LBD R206H receptor retained ~53% activity.

Levels of pSmad1/5/8 signaling activity by LBD receptors were similar under basal conditions or with BMP4 or Activin A treatment (Figure 5C,D).

4. Discussion

Most FOP patients with a ‘classic’ clinical presentation of FOP share a recurrent *de novo* mutation in ACVR1, R206H. However, FOP ‘variant’ patients, often with more severe skeletal malformations than occur in classic FOP, have also been identified. These FOP variants have non-R206H mutations in ACVR1. Phenotypic variations observed in patients have been suggested to correlate with the structural domain that is mutated in the ACVR1 receptor [2], possibly by inducing higher levels of ligand dependent or ligand-independent pathway activation or through perturbation of different signaling mechanisms.

In silico studies investigating the conformational changes induced by FOP mutations support that R206H and Q207E mutations prevent an inactive conformation of the GS domain by disruption/prevention of FKBP12 binding, resulting in permanent or leaky activation of the ACVR1 signaling pathway. Binding by FKBP12 to the ACVR1 receptor is essential for keeping the receptor inactive in the absence of ligands [2,37–41]. In contrast, *in silico* modeling of the ACVR1 G328E, G328W, or G356D mutations, all within the receptor PK domain and all associated with more severe skeletal malformations than in patients carrying an ACVR1 R206H mutation, identified no obvious structural consequences of these mutations. It is hypothesized that such mutations might induce higher or lower binding efficiency of substrates (R-Smads), enhance overall kinase activity, or show altered sensitivity to BMP/TGF-beta ligands.

In this study we examined the signaling activity of three FOP ACVR1 mutations in the protein kinase (PK) domain of ACVR1 that were associated with severe skeletal malformation phenotypes. As shown for the ACVR1 R206H mutation, we determined that these PK domain mutant receptors over-activate the BMP-pSmad1/5/8 signaling pathway, although to a lesser degree. We additionally determined that the PK domain mutations that we examined (G328E, G328W, G356D) are more sensitive to low levels of BMP ligands than mutations in the ACVR1 GS domain (R206H and Q207E).

Our data also raise the question of cell type specific differences that affect the signaling activity of ACVR1. Using the myoblast cell line C2C12-BR, we did not detect significant differences between WT and mutant receptors in downstream BMP-pSmad1/5/8 pathway activation under basal conditions. However, in iMEFs, a mesenchymal progenitor cell system, all of the tested mutant receptors elevated basal BMP-pSmad1/5/8 signaling, with R206H being the most active. These data are consistent with previous findings in C2C12 or U2OS cells [13,16]. The differences found among cell systems suggest a requirement for accessory receptors or other components that are not present or available for binding in the C2C12-BR cell line. Previous reports indicate that the type 2 BMPR2 receptor is mandatory for BMP pathway overactivation in FOP cells [42–44]. Studies in zebrafish support the

dependency of ACVR1-mediated BMP pathway activation by other receptors, with full activation of the BMP signaling pathway depending on the formation of Bmpr1a-Acvr1 and Bmpr1b-Acvr1 heteromeric receptor complexes [45]. It is also important to keep in mind that forced overexpression of a single component in the fine-tuned signal transduction cascade will alter stoichiometric distribution of signaling components in the cell system and impact BMP pathway activity. The endogenous repertoire and expression levels of signaling components also vary among different cell types and modify BMP pathway activity. Further investigations would be required to fully resolve mechanistic differences of ACVR1 function in C2C12-BR and iMEF cells and other cell types.

Under usual circumstances, BMP/TGF-beta receptor signaling is tightly-regulated through ligand activation. Inflammatory and fibroproliferative pre-osteogenic stages of heterotopic ossification in FOP highly express BMP4 protein, implicating that ligand-induced activation of the mutant receptor has a crucial role [5–8]. Damaged skeletal muscles also overexpress BMP4 [30–32]. Activin A, another ligand in the BMP/TGF-beta superfamily, has recently drawn considerable attention in FOP research as inhibition of this ligand prevents HO formation by administration of a humanized antibody to mice expressing the Acvr1 R206H mutation [3]. This study suggested that the WT ACVR1 receptor forms inactive receptor complexes with ACVR2A and -B in the presence of Activin A, efficiently blocking BMP pathway activation, but that the R206H receptor responds to Activin A by aberrantly activating the pSmad1/5/8 signaling pathway. The underlying molecular mechanism however, remains unresolved.

Our data showed that either BMP4 or Activin A induced increased BMP pathway activation in iMEFs by GS and PK domain mutant ACVR1. In contrast to previous reports, we did not observe an inhibitory effect of Activin A on the pSmad1/5/8 pathway in cells with the WT receptor [3,16]. This discrepancy could be due to differences in the cell systems used in these studies. Alternatively, the overexpression systems used in each of these studies would have changed stoichiometric ratios of ACVR1 to co-receptors or regulatory/adaptor proteins, and could therefore account for the observed differences in signaling response. Ligand-binding specificity of ACVR1 receptors towards Activin versus TGF-beta/BMPs was reported to depend on complex formation with type 2 receptor [33,44,46,47]. The expression of type 2 receptors may therefore be expected to determine which signaling cascade will be activated.

We also found that Activin A stimulation of FOP mutant receptors resulted in similar activation levels as by BMP4, while the WT receptor activated the pSmad1/5/8 signaling pathway >1.4fold stronger with BMP4 compared to Activin A. This result could indicate that all tested ACVR1 receptors interpret Activin A as a ‘BMP-like’ ligand. The functional consequence (as well as the molecular mechanism) of this finding however remains unresolved.

Additionally, we investigated ligand independent activation of the ACVR1 PK domain mutants by designing receptors lacking the ligand-binding domain (LBD). We demonstrated that truncated GS and PK domain mutant ACVR1 receptors retain increased BMP-pSmad1/5/8 pathway activation relative to wild-type ACVR1, supporting that the mutant

receptors can function through ligand-independent mechanisms. Receptor signaling activity by all truncated FOP receptors was significantly reduced compared to the corresponding full-length receptors. However, we found differences in the relative ligand dependency between GS and PK mutants: while the ‘classic’ R206H mutation retains more than 50% activity in the absence of a functional LBD confirming previous reports [14], PK mutations are less active indicating a higher/lower degree of ligand-dependency/-independency.

ACVR1 mutations in the PK domain are associated with severe skeletal effects, such as malformations of multiple digits of hands and feet. To further investigate the molecular mechanisms that could lead to more severe developmental alterations, we examined the dose response of PK domain mutations to ligands in comparison to the GS domain mutations. We found that ACVR1 receptors with mutant PK domains have increased Smad1/5/8 phosphorylation compared to the WT receptor at low BMP4 doses while R206H and Q207E respond similarly as WT ACVR1. However, PK domain mutants reach maximal signaling levels more quickly, at the same doses that are beginning to stimulate GS domain mutants to highly activate the BMP pathway. We suggest that the PK domain mutations result in receptors that are more sensitive and responsive to ligand, while GS domain mutations more strongly enhance receptor activity once a threshold of ligand is present/reached. Given that the physiological ligand levels that are required to regulate embryonic development of highly complex structures, such as the limb, are potentially low, increased sensitivity to low levels of BMP ligand by PK mutant receptors could explain the more severe digit phenotypes in many of the patients carrying a FOP variant.

5. Conclusions

Taken together, our results indicate that all FOP variant mutations investigated increased BMP pathway activation upon BMP4 and/or Activin A treatment. Furthermore we find that ACVR1 receptor-pSmad1/5/8 signaling activity shows cell type-specific responses. Mutations in the ACVR1 protein kinase (PK) domain, G328E, G328W and G356D, sensitize the receptor to low levels of BMP ligand resulting in mild activation of the receptor when normally unresponsive. In contrast, mutations located in the GS domain strongly over-activate the BMP-pSmad1/5/8 pathway at higher BMP4 doses but are indistinguishable from WT receptor activity at lower concentrations. We additionally found that all FOP mutant as well as the WT receptor respond similarly to Activin A and BMP4 stimuli indicating that ACVR1 interprets Activin A as a ‘BMP-like’ ligand. While each of the FOP ACVR1 variant mutations induces increased signaling through the BMP-pSmad1/5/8 pathway, there appears to be more than a single molecular mechanism that leads to enhanced activation. Currently, we can only speculate about the *in vivo* consequences of our findings and how it impacts prenatal skeletal malformation as well as postnatal HO formation in FOP patients (or explain differences in FOP-classic and -variant phenotypes) but further investigations clarifying the molecular consequences of FOP mutations are required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACVR1	activin A receptor type 1
BMP	bone morphogenetic protein
HO	heterotopic ossification
FOP	fibrodysplasia ossificans progressiva
LBD	extracellular ligand-binding domain
GS	glycine-serine rich domain
PK	protein kinase domain
TGF-β	transforming growth and differentiation factor beta

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Highlights

- While each of the FOP ACVR1 variant mutations induces increased signaling through the BMP-pSmad1/5/8 pathway, there appears to be more than a single molecular mechanism that leads to enhanced activation.
- ACVR1 PK domain mutations are more sensitive to low levels of BMP ligands while GS domain mutations more strongly enhance receptor activity once a threshold of ligand is present.
- BMP4 or Activin A induced increased BMP pathway activation in PK and GS domain mutant ACVR; WT and mutant ACVR1 receptors respond similarly to Activin A and BMP4 indicating that ACVR1 interprets Activin A as a ‘BMP-like’ ligand.
- Ligand-binding domain deletions show ligand-independent pSmad1/5/8 pathway activation by FOP mutant ACVR1 receptors.
- ACVR1 receptor-pSmad1/5/8 signaling activity shows cell type-specific responses

Figure 1. Schematic of human ACVR1 protein with functional domains

Functional domains are indicated: signaling peptide (SP); ligand-binding domain (LBD); transmembrane domain (TM); glycine-serine rich (GS) domain; protein kinase (PK) domain. Amino acid positions are indicated below and mutations identified in FOP patients are shown above the protein structure. Q207D is an engineered constitutively active (CA) mutation that does not occur in FOP patients.

Figure 2. BMP-pSMAD1/5/8 pathway activity by FOP ACVR1 receptors

C2C12 cells were transiently transfected with V5-tagged ACVR1 receptor expression constructs and stimulated with 30ng/ml BMP4 or left untreated. Total proteins were isolated, electrophoresed through SDS-PAGE, and immunoblotted to detect pSmad1/5/8 (~60 kDa); the same blot was re-probed to detect V5-tagged ACVR1 (~57 kDa) and beta-actin (~45 kDa). A representative immunoblot from three independent repeats under unstimulated conditions (A) and BMP4 treatment (C) is shown. (B and D) Band intensity of phosphorylated Smad1/5/8 was quantified and then normalized to V5-tag, which served as an internal control for receptor variant expression. Beta-actin detection served as a control for equal protein loading. Graphs represent mean $\pm$ SD of three independent repeats. One-way ANOVA was performed and significant differences compared to WT (*) or R206H (#) indicated ($p < 0.05$ */#; $p < 0.01$ **/##; or $p < 0.001$ ***/###). Note that in B and D, data are relative to WT within each condition.

Figure 3. Comparison of BMP pathway activation by FOP receptors in transfected C2C12-BR cells and immortalized MEFs (iMEFs)

(A) C2C12-BR cells, co-transfected with Renilla-Luc and either empty vector, WT, CA or indicated FOP ACVR1 mutation, were treated with 30ng/ml BMP4. Relative luciferase activities were corrected for transcription efficiency (Renilla-Luc) before normalizing to untreated WT samples. Graph represents means $\pm$ SEM of two independent experiments. (B, C) Immortalized MEFs were co-transfected with Renilla-Luc, BRE-Luc reporter and either empty vector, WT, CA or indicated FOP mutation and treated with 10ng/ml BMP4 (B) or 100ng/ml Activin A (C) or left untreated (control; ctr) prior detection of luciferase activities. Relative luciferase activities were normalized to untreated WT. Graph represents means $\pm$ SEM of five (in B) and three (in C) independent experiments. Two-way ANOVA with a Bonferroni post-test was performed and significant differences compared to WT (*) or R206H (#) indicated ($p < 0.05$ */#; $p < 0.01$ **/##; or $p < 0.001$ ***/###). Note that in B and C, data are relative to WT under basal conditions.

Figure 4. BMP4 dose response by FOP mutants

Immortalized MEFs were transfected with the indicated receptor constructs and treated with a range of concentrations of BMP4 (0; 0.1; 1; 5; 10; 20 ng/ml) for 1 hour followed by a Smad1 phosphorylation assay. Cells were co-transfected with Renilla-Luc as a transfection efficiency control. (A) Normalization to renilla adjusted for transfection efficiency and was followed by normalization to untreated WT samples. Graphs represent means $\pm$ SEM of three independent experiments. (B,C) Magnification of boxed area in (A) depicting basal activity level of mutant receptors and response to very low (B) and medium (C) doses of BMP4. Significant differences (two-way ANOVA with a Bonferroni post-test) compared to untreated WT (0 ng/ml BMP4) are: R206H*, Q207E*, G356D*; compared to WT-10ng/ml BMP4 are: G356D***, G328E*, G328W**; and compared to WT-20ng/ml BMP4 are: R206H***, Q207E***, G356D***, G328W*; ns=not significant. CA*** compared to WT at all tested concentrations.

Figure 5. BMP-pSMAD signaling activity by ACVR1 ligand-binding domain deleted (LBD) receptor proteins

(A) To investigate ligand independent activity of FOP mutant receptors the LBD domain was deleted from the HA-tagged (HA), full-length ACVR1 WT, CA, and FOP mutant receptors.

(B–D) Immortalized MEFs were co-transfected with Renilla-, BRE-Luc and either full-length or ΔLBD ACVR1 receptors and treated with 10ng/ml BMP4 (C), 100ng/ml Activin A (D) or left untreated (B). Luciferase activities from BRE-Luc were first corrected for transcription efficiency by normalizing to Renilla-Luc. Relative receptor activity levels were then normalized to WT (full-length) followed by correction against WT basal level. Graph represents means ± SEM of five (B), two (C) or three (D) independent experiments. Significant differences (two-way ANOVA with a Bonferroni post-test) compared to WT (*) or R206H (#) are indicated ($p < 0.05$ */#; $p < 0.01$ **/##; or $p < 0.001$ ***/###). Note that a moderate dose of BMP4 was used for the experiments in (C) while a relatively high dose of Activin A was used in (D).