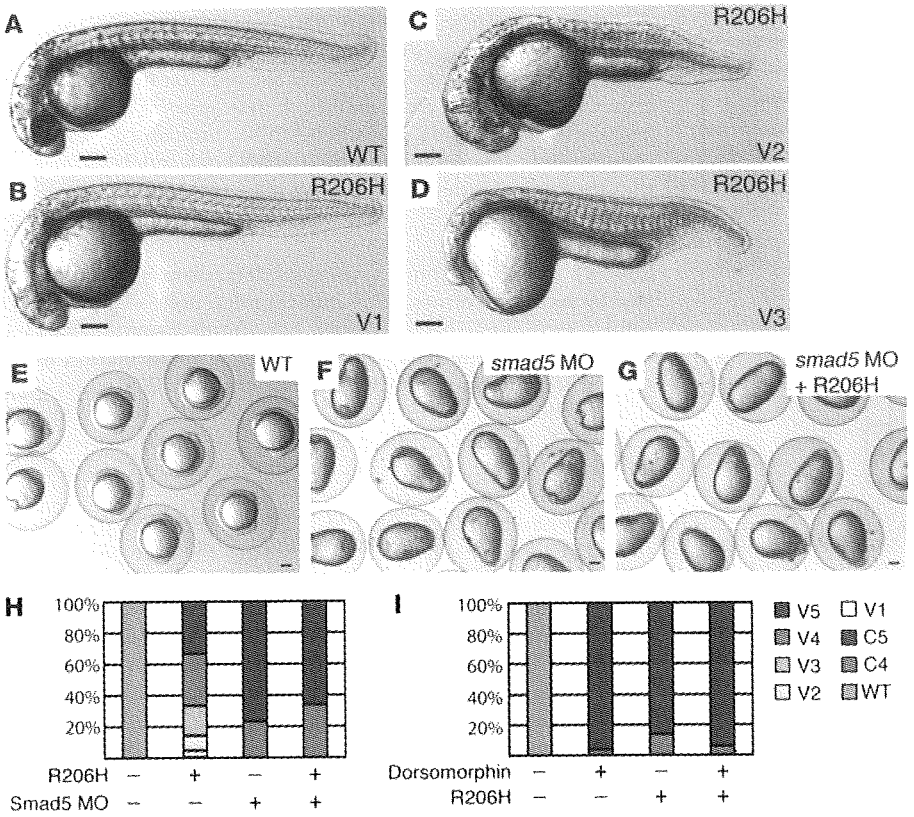

Figure 4
ACVR1 mutation signaling requires the BMP/Smad signaling pathway. (**A–D**) Compared with uninjected embryos (**A**), wild-type embryos injected with 10 pg mutant R206H *ACVR1* mRNA showed a range of ventralized phenotypes at 1 day after fertilization: weak (V1, **B**; and V2, **C**), moderate (V3, **D**), and strong (V4 and V5, examples shown in Figure 3, D and E). Scale bars: 0.2 mm. (**E–H**) Compared with uninjected embryos (**E**), wild-type embryos injected with translation-blocking morpholinos (MO) against smad5 (**F**) showed strongly dorsalized phenotypes, evident by the characteristic elongated morphology at the 4-somite stage. When coinjected with smad5 MO, mutant *ACVR1* (10 pg) does not induce ventralization (**G**). Scale bars: 0.2 mm. (**H**) Quantification of embryo injection results (uninjected, $n = 82$; mutant ACVR1 only, $n = 289$; smad5 MO only, $n = 169$; mutant *ACVR1* plus smad5 MO, $n = 202$). (**I**) DM inhibits mutant *ACVR1*–induced ventralization. Wild-type embryos treated with DM (40 μM) become severely dorsalized ($n = 54$), whereas solvent alone has no effect ($n = 42$). Ventralization induced by 20 pg mutant *ACVR1* mRNA ($n = 79$) is completely blocked by DM ($n = 75$).

double *bmp2b/bmp7* mutant phenotype (39). Homozygous mutant *bmp2b* or *bmp7* embryos exhibited a strongly dorsalized phenotype (25% of all progeny were C5), whereas sibling embryos (wild-type and heterozygotes; 75% of all progeny) showed a wild-type phenotype (Figure 5A). Microinjection of mutant R206H ACVR1 RNA induced embryonic ventralization, indicating BMP signaling overactivation, in the presence of BMP (wild-type or heterozygotes, Figure 5A), as well as in *bmp7*- or *bmp2b*-null embryos (Figure 5A). To completely knock down all BMP ligand expression in the embryos, we injected a *bmp2b* morpholino into *bmp7*$^{-/-}$ embryos. The single and double BMP ligand–deficient embryos (Figure 5B) showed strongly dorsalized phenotypes due to loss of BMP signaling. By contrast, single and double BMP-deficient embryos responded to injected mutant R206H ACVR1 by exhibiting a strongly ventralized phenotype (Figure 5B). Importantly, the wild-type ACVR1 showed no rescuing ability when injected into *bmp7*-null mutant embryos ($n = 290$, 60 pg *ACVR1* mRNA). Thus, these results show that the mutant ACVR1 acts downstream of BMP ligand and activates BMP signaling in vivo in a BMP-independent manner.

Mutant R206H ACVR1 enhances chondrogenesis. Heterotopic bone formation in FOP patients develops through an endochondral ossification pathway in which a fibroproliferative vascular stage is followed by chondrogenesis and then replacement of cartilage by bone (4, 40). To investigate whether mutant R206H ACVR1 can influence chondrogenesis, we examined effects on chondrocyte differentiation by the ACVR1 mutation in chick limb bud micromass cultures that were infected with retroviral constructs containing either wild-type or mutant ACVR1. Overexpression of wild-type ACVR1 had no effect on extracellular matrix accumulation, which was monitored by Alcian blue staining, or on ALP activity when compared with cultures infected with empty viral vector (Figure 6). This is consistent with effects of other BMP receptors, such as BMPR1B (41). By contrast, overexpression of the R206H ACVR1 mutant induced a steady increase in matrix accumulation and ALP activity that were clearly visible after 1 week in culture. This effect was milder than that caused by a previously described constitutively active ACVR1 (caACVR1; Q207D), which was designed based on studies using the TGF-β receptor 1 (42). The dramatic increase in chondrogenic differentiation in response to the caACVR1 Q207D was already visible after 2 days in culture and saturated after 1 week in culture (Figure 6).

To determine whether the effect of R206H ACVR1 is BMP independent, we coexpressed the receptor constructs with a construct for the BMP antagonist Noggin that directly binds BMP ligands, blocking the ability of BMP to bind receptors (43). Noggin had a dominant effect on wild-type ACVR1, as shown by complete inhibition of chondrogenic differentiation, while Noggin had no effect on caACVR1 Q207D activity. However, Noggin-treated cultures expressing mutant R206H ACVR1 had reduced chondrogenic nodule formation and ALP activity (Figure 6), showing that the FOP R206H ACVR1 mutation has a milder self-activating effect than the Q207D variant but, like this constitutively active variant, seems to be, at least in part, BMP independent. Significantly, by contrast to caACVR1 Q207D, R206H ACVR1 is BMP responsive. These data support the hypothesis that while the FOP ACVR1 mutation is an activating mutation that promotes BMP-independent chondrogenesis, this activity may be additionally responsive to BMP ligands.

To investigate chondrogenic differentiation on a molecular level, we analyzed the relative expression levels of marker genes that are characteristic for distinct stages of chondrogenesis (Figure 6E).

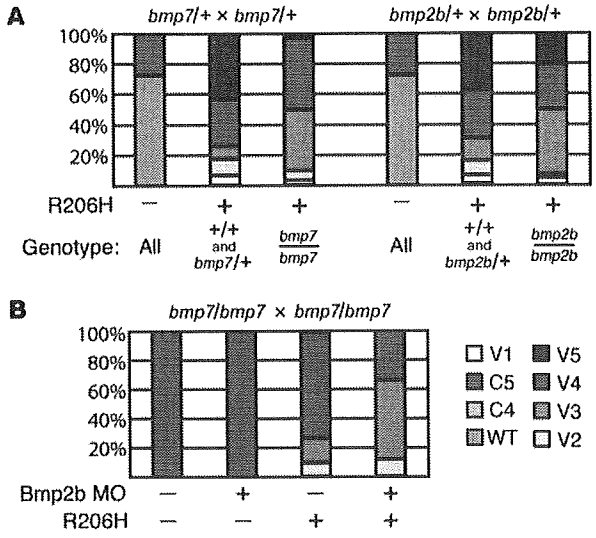

Figure 5

Mutant R206H ACVR1 induces ventralization in a BMP7 and BMP2b ligand–independent manner. (**A**) Embryos from crosses of bmp7 heterozygous adults (bmp7/+ × bmp7/+) or bmp2b heterozygous adults (bmp2b/+ × bmp2b/+) were injected with 10 pg mutant *ACVR1* mRNA (indicated as R206H +), examined for extent of ventralization, then identified by genotyping (bmp7/bmp7, homozygous null for bmp7; bmp7/+, heterozygous; +/+, wild-type). Genotyping showed that bmp7 homozygotes (n = 32) and bmp2b homozygotes (n = 44) are ventralized by mutant *ACVR1*, as are wild-type and heterozygous siblings (bmp7/+ crosses, n = 94; bmp2b/+ crosses, n = 144). Of the uninjected (indicated as R206H –) control embryos examined (for bmp7/+ × bmp7/+ crosses, n = 146; for bmp2b/+ × bmp2b/+ crosses, n = 29), approximately one-quarter displayed a strongly dorsalized C5 phenotype. (**B**) To examine whether mutant *ACVR1* could activate BMP signaling in embryos lacking both Bmp7 and Bmp2b, embryos from crosses of bmp7 homozygous adults were injected with 10 pg mutant *ACVR1* mRNA with or without injection of a translation-blocking MO targeting bmp2b. Embryos exhibited a strongly dorsalized C5 phenotype in the absence of BMP ligands (uninjected, n = 34; bmp2b MO, n = 34), whereas mutant *ACVR1* caused ventralization of embryos deficient in bmp7 (n = 126) or both bmp7 and bmp2b (n = 103).

The condensation phase of mesenchymal prechondrogenic cells is the first event in chondrogenesis, characterized by the expression of collagen type II (44). Aggrecan is a major component of cartilage matrix (45, 46) and is expressed in chondrocytes that are not terminally maturated (47). Within the condensation, increasing expression of Indian hedgehog (Ihh) and collagen type X occurs as chondrocytes differentiated into prehypertrophic and hypertrophic chondrocytes, respectively. Overexpression of wild-type ACVR1 in chick micromass cultures resulted in expression levels of marker genes that are comparable to those of the empty viral vector, while cells overexpressing R206H ACVR1 showed upregulation of collagen type II and aggrecan. The later-stage differentiation markers Ihh and collagen type X showed expression levels comparable to those in control cultures. By contrast, overexpression of caACVR1 Q207D resulted in a dramatic increase in Ihh and collagen type X expression. Aggrecan expression was only slightly enhanced, whereas collagen type II was markedly downregulated. Remarkably, the relative expression levels of all marker genes were only slightly decreased when R206H ACVR1 and Q207D caACVR1 cultures were cotransfected with Noggin, whereas cells expressing the wild-type ACVR1 or empty viral vector showed clear downregulation of all analyzed marker genes for chondrogenesis. These data support the hypothesis that while caACVR1 stimulates advanced chondrogenic differentiation, R206H ACVR1 only mildly induces the expression of early chondrogenesis markers.

Discussion

FOP is a severely disabling musculoskeletal disease characterized by extensive formation of endochondral bone within soft connective tissues. This heterotopic bone formation is the most clinically relevant feature of FOP. However, malformation of skeletal bones during embryogenesis also occurs in FOP patients and illustrates that the underlying genetic mutation occurs in a gene with functional importance in regulating chondro-osseous differentiation during embryonic skeletal development as well as in adult musculoskeletal tissues.

A series of recent investigations in FOP cells (48–50) demonstrated altered signal transduction through the BMP pathway, with increased phosphorylation of the BMP-specific Smads and increased expression of BMP transcriptional targets in the absence of exogenous BMP ligand. Our recent discovery (6) of mutations in the gene encoding ACVR1, a BMP type I receptor, in all examined FOP patients confirms that changes in the BMP signaling pathway cause FOP.

In this study, we conducted functional analyses of the *ACVR1* c.617G→A (R206H) mutation that is found in all FOP patients with classic features of the disease (malformed great toes and progressive heterotopic ossification). Consistent with our studies in FOP patient cells (48–50), in vitro and in vivo assays determined that the R206H ACVR1 mutation is an activating mutation that stimulates BMP signaling without requiring BMP to initiate the signaling cascade. Our in vitro assays showed that BMP-independent BMP signaling by R206H ACVR1 specifically activates the Smad pathway, as was also found in recent studies using C2C12 cells (51). We further demonstrated that Smad signaling is required for R206H ACVR1 signaling in vivo through zebrafish embryo assays using DM, a small molecule that inhibits BMP type I receptor phosphorylation of BMP pathway–specific Smads (37).

The FOP *ACVR1* c.617A mutation causes an amino acid substitution of histidine for arginine in codon 206 (R206H), a highly conserved amino acid among vertebrates and among human ACVR1 family members (6). Codon 206 is within the GS activation domain adjacent to the protein kinase domain of ACVR1. Protein homology modeling of the ACVR1 receptor predicts that the protein conformation of the R206H ACVR1 mutant is altered and could lead to changes in the ability of the receptor to interact with proteins that bind the receptor GS domain (6, 52). The GS domain of all type I TGF-β/BMP superfamily receptors is a critical site for binding and activation of the pathway-specific Smad signaling proteins and is a specific binding site for FKBP1A (also known as FKBP12), a highly conserved inhibitory protein that prevents leaky activation of type I receptors in the absence of ligand (21–23).

Our data demonstrate that the R206H ACVR1 protein has reduced interaction with FKBP1A in the absence of BMP and suggest that this impaired FKBP1A-ACVR1 interaction contributes to BMP-independent BMP pathway signaling. Although our data cannot distinguish between direct and indirect FKBP1A-ACVR1 binding, the data are consistent with the hypothesis that altered ACVR1 GS domain

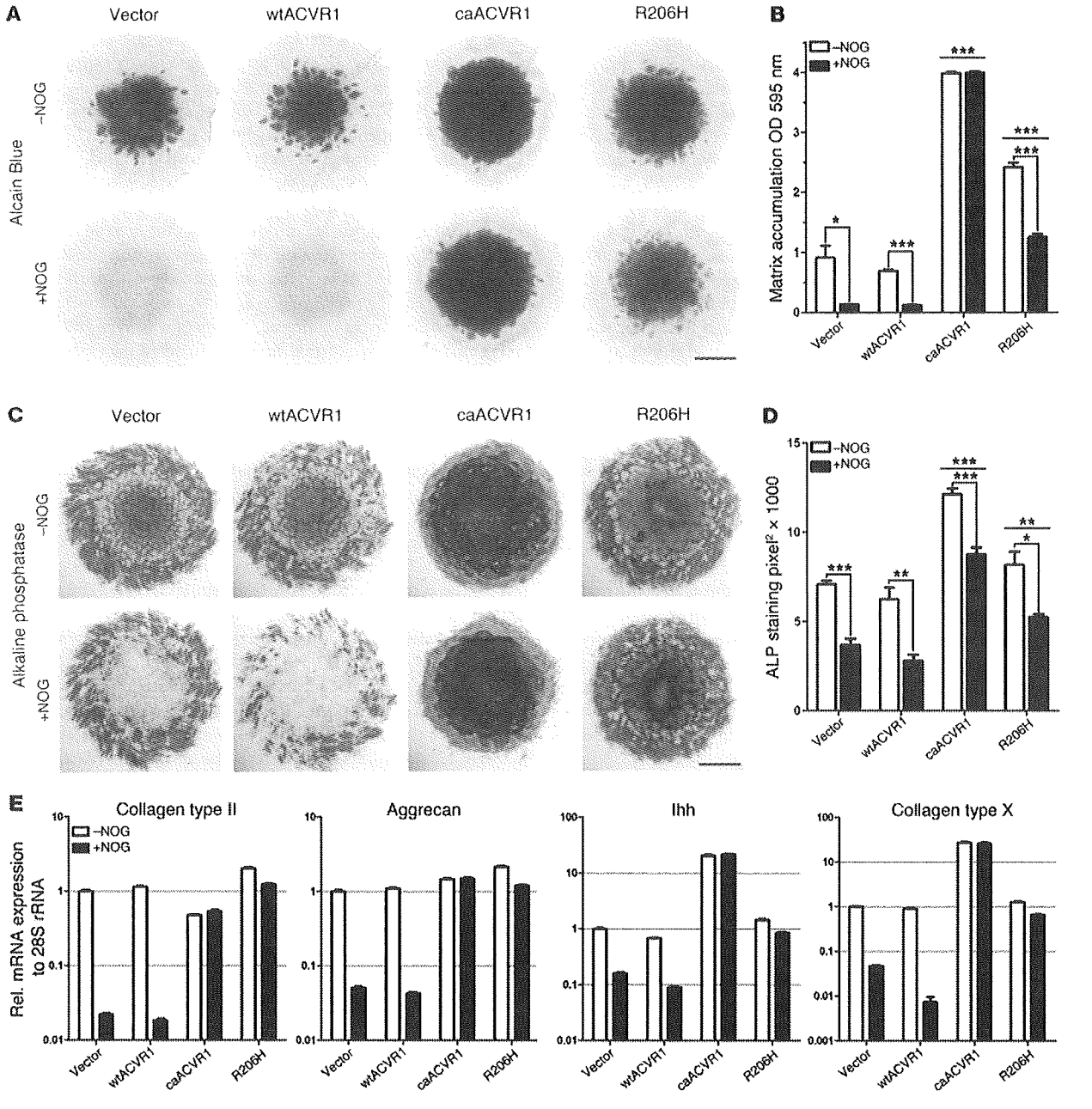

Figure 6

Mutant R206H ACVR1 enhances chondrogenesis in a BMP-independent and BMP-responsive manner. Micromass cultures were infected with RCASBP(A) alone (control) or containing either wild-type *ACVR1*, a constitutively active *caACVR1* Q207D construct, or the R206H *ACVR1* mutation either alone or together with RCASBP(B) expressing Noggin (NOG). (**A**) Micromass cultures were stained with Alcian blue to visualize early chondrogenic differentiation by extracellular matrix accumulation after 7 days. (**B**) Quantification of Alcian blue incorporation into the extracellular matrix is shown. Significant differences: wtACVR1 vs. caACVR1***; wtACVR1+NOG vs. caACVR1+NOG***; wtACVR1 vs. R206H***; wtACVR1+NOG vs. R206H+NOG***. (**C**) ALP-stained micromass cultures visualize prehypertrophic differentiation after 9 days in culture. Scale bars in **A** and **C**: 2 mm. (**D**) Quantification of ALP staining by histomorphometric analysis (ALP activity quantified as pixel² x 10,000) is shown. Significant differences: wtACVR1 vs. caACVR1***; wtACVR1+NOG vs. caACVR1+NOG***; wtACVR1 vs. R206H (NS); wtACVR1+NOG vs. R206H+NOG**. (**E**) Relative expression of marker genes for chondrogenic differentiation by qRT-PCR of cDNA from 8-day micromass cultures. Amplification was normalized to the expression of 28S rRNA. For quantification in **B** and **D**, significant differences in comparison to wtACVR1 or wtACVR1+NOG are indicated. Graphs in **B** and **D** show mean ± SEM of quadruplicate samples from 1 of 3 representative experiments. Horizontal lines represent significant differences at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Data in **E**, shown as mean ± SEM, are representative results from 2 independent cDNA syntheses and qRT-PCR.

structure changes the affinity or ability of GS domain–interacting proteins, such as FKBP1A, to properly regulate BMP pathway activity. Additionally, our data show that the R206H ACVR1 mutation does not fully prevent FKBP1A interaction, suggesting that FKBP1A–mutant ACVR1 interactions are less stable and/or of shorter duration. The mutant GS domain may interact with FKBP1A in a manner that allows activation of Smads and downstream signaling in the presence of aberrant FKBP1A binding.

The BMP pathway is an important signaling pathway in many tissues and during many stages of development. Disruption of this pathway has severe consequences, as illustrated by knockout mouse models (53, 54) and by overactivation or by mutation of Alk8, the functional ACVR1 homolog in zebrafish (7, 27, 28), and recent data demonstrated that Alk8 is required for zebrafish dorsal-ventral embryonic patterning (38). It was therefore surprising that the mutation that causes FOP overactivates the BMP pathway yet allows human embryonic development to occur relatively unimpaired, with only mild skeletal effects. One explanation may be that the FOP mutation is only moderately activating. By comparing FOP R206H ACVR1 with caACVR1 Q207D in micromass chondrogenesis assays, we found that the R206H ACVR1 receptor has a milder stimulation of cell differentiation compared with caACVR1 Q207D. The effects of the FOP ACVR1 mutant receptor during embryonic development may therefore induce only relatively minor malformations as observed in FOP skeletal formation (1, 6). Furthermore, the data are consistent with the idea that, in postnatal connective tissues, increased BMP signaling from the mutant receptor may be only moderately "on" under basal in vivo conditions, allowing for the quiescent periods that are observed in patients between active episodes of heterotopic bone formation, but priming the cells to respond to changes in the local tissue environment by forming extraskeletal bone.

This possibility is supported by micromass cultures expressing wild-type ACVR1, caACVR1 Q207D, or R206H ACVR1 that showed significant differences in expression of specific marker genes for chondrogenic differentiation. While expression levels of all marker genes remained almost unchanged in wild-type ACVR1 cultures compared with the empty viral vector, the expression profile induced by caACVR1 Q207D displayed evidence of advanced chondrogenic differentiation as shown by decreased levels of the early differentiation stage marker collagen type II together with enhanced expression of the later-stage markers Ihh and collagen type X. Indian hedgehog has been shown previously to be induced by BMP signaling transmitted via the ACVR1 receptor (9). By contrast, the R206H ACVR1 mutant only slightly enhances the expression of initial and early chondrogenesis markers (collagen type II and aggrecan), consistent with a milder stimulatory effect of the FOP ACVR1 mutation on chondrocyte differentiation. Remarkably, the BMP antagonist Noggin resulted in dramatically reduced expression of chondrogenic markers in wild-type ACVR1 and empty viral vector cultures, but not in those expressing caACVR1 or R206H ACVR1, suggesting that the enhanced chondrogenesis induced by the mutant receptors is mediated predominantly through their BMP-independent activation.

The extraskeletal bone that develops within soft tissues in FOP patients occurs through an endochondral ossification pathway, forming ectopic cartilage that is subsequently replaced by ectopic bone (4, 40). In vitro chondrogenesis assays demonstrated that the R206H ACVR1 mutation can stimulate progenitor cells to differentiate to cartilage in concert with the appropriate cell environment and differentiation factors and that this mutant receptor activates BMP signaling constitutively without BMP stimulation. However, chondrogenesis assays in chick micromass cultures showed that enhanced signaling by the mutant R206H ACVR1 can be partially inhibited by the BMP antagonist Noggin, suggesting that in addition to BMP-independent activity by the R206H ACVR1 receptor, this mutant receptor is also responsive to BMP, consistent with previous BMP signaling pathway studies that used cells derived from FOP patients (48–50).

While our studies provide important insight into the molecular pathophysiology of the mutant ACVR1 receptor on the BMP signaling pathway in FOP, interpretations of these data are limited to those allowed by the experimental systems used. Most notably, the studies are based on transient transfection of wild-type and mutant gene constructs into cultured cells or into zebrafish embryos and are unlikely to reproduce the specific mutant gene dose that is present in heterozygous mutant cells in FOP patients. The balance of expression of mutant and wild-type ACVR1 protein is likely to affect ligand binding dynamics, receptor oligomerization, internalization, and/or activation of downstream signaling pathways, as well as receptor response to environmental triggers that are clinical features of the natural history of FOP. Further refinement of our understanding of BMP signaling in FOP will follow with development of an *ACVR1* mutant knock-in mouse that will more precisely mimic the naturally occurring gene dose effect of the mutant R206H ACVR1 allele. Such a model is under development.

In conclusion, we show here that the recurrent single nucleotide missense mutation (c.617G→A) in the gene encoding ACVR1, a BMP type I receptor that causes FOP in all classically affected individuals, induces enhanced chondro-osseous differentiation and can activate the BMP signaling pathway in a BMP-independent manner. However, this mutant receptor also shows the ability to respond to BMP to enhance receptor activity. Additionally, our study provides mechanistic insight into the constitutive activity of this mutant ACVR1 receptor. These findings have practical implications in developing model systems for testing new pharmacologic approaches for blocking this renegade receptor. Finally, this study provides insight into a signaling pathway that regulates skeletal morphogenesis and also that, when dysregulated in a specific manner, orchestrates the promiscuous and ectopic chondro-osseous differentiation of soft connective tissue into a disabling second skeleton of heterotopic bone.

Methods

Plasmid constructs. A human ACVR1 expression vector was generated by insertion of the hACVR1 protein-coding sequence (GenBank accession number NM_001105.4) into the pcDNA 3.1 D V5-His-TOPO vector (Invitrogen). Nucleotide numbering reflects the ACVR1 cDNA sequence, with +1 corresponding to the A of the ATG translation initiation codon in the GenBank reference sequence, according to nomenclature guidelines (http://www.hgvs.org/mutnomen). The protein initiation codon is numbered +1. The FOP mutant ACVR1 expression vector was generated by site-directed mutagenesis of the wild-type ACVR1 sequence at cDNA position 617 (from G to A) using the GeneTailor Site-Directed Mutagenesis System (Invitrogen). The oligonucleotides used to generate the mutant construct were: forward 5′-GTACAAAGAACAGTGGCTCaCCAGATTACACTG-3′; reverse 5′-GTGAGCCACTGTTCTTTGTACCAGAAAAGGAAG-3′. The wild-type sequence is represented by "c.617G" and the FOP mutation by "c.617A." The FKBP1A/FKBP12 expression vector was from Origene. The human *ID1* promoter (-985/+94) luciferase reporter construct was previ-

ously described (31). The *ID1* gene is a direct target of BMP signaling and encodes a dominant-negative inhibitor of basic helix-loop-helix transcription factors, including members of the MyoD family that are important in myoblast differentiation (55, 56).

Cell culture. COS-7 African green monkey kidney cells, C2C12 mouse myoblastic cells, MC3T3-E1 human osteoblastic cells, and U-2 OS human osteosarcoma cells were obtained from ATCC. Cells were cultured in DMEM (COS-7 and C2C12), α-MEM (MC3T3-E1), or McCoy's 5A medium (U-2 OS) plus 10% FBS (all from Invitrogen). All cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

Transfection and luciferase assay. COS-7 cells were seeded into 24-well plates at 7×10^4 cells per well in culture medium without antibiotics. After 24 hours, expression vectors were transfected into the cells using FuGene 6 (Roche) according to the manufacturer's protocol. Efficiency of transfection, as assessed by cotransfection with GFP constructs and subsequent GFP detection, was estimated at 60%–70%. At 48 hours, cells were washed twice with PBS and lysed in 1× passive lysis buffer (Dual-Luciferase Reporter Assay, Promega). Luciferase activity was assayed following the recommended protocol and normalized to pRL-TK-*Renilla* luciferase signals.

Immunoblot analysis. COS-7 cells, plated at 70% confluence in 100-mm tissue culture dishes, were transfected with vector alone or pcDNA3-ACVR1 (c.617G) or pcDNA3-ACVR1 (c.617A). Total proteins were harvested in lysis buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, phosphatase inhibitors [Pierce], protease inhibitors [C complete protease inhibitor cocktail, Roche], and 1% Triton X-100). For immunoblot analysis, 50 μg protein from each total cell lysate was electrophoresed through 10% SDS-polyacrylamide gels, then transferred to PVDF membranes (Bio-Rad). Membranes were incubated overnight at 4°C with antibodies specific for phospho-Smad1/5/8 and Smad1 (Cell Signaling Technology), V5 (Invitrogen), or β-actin (Santa Cruz Biotechnology Inc.) in PBS containing 5% nonfat milk and 0.5% BSA. Membranes were washed with PBS and incubated for 1 hour with the corresponding secondary antibody conjugated with horseradish peroxidase. The enhanced chemiluminescent Western blotting detection system (Pierce) was used to detect the antigen-antibody complex. Similar protocols were used for immunoblot analysis of cell protein extracts from MC3T3-E1 (mouse preosteoblasts), U-2 OS (human osteosarcoma), and C2C12 (mouse myoblasts with osteogenic potential). Western blotting of phospho-Smad1/5/8 from zebrafish embryo extracts was previously described (35).

Immunoprecipitation. To examine the interaction between FKBP1A and ACVR1 in the absence or presence of BMPs, COS-7 cells were cotransfected with normal (c.617G) or mutant (c.617A) ACVR1 expression vector and the FKBP1A expression vector. After 48 hours of transfection, cells were starved for 2 hours in serum-free medium then treated for 1.5 hours with 150 ng/ml BMP4 or BMP7 (R&D Systems). Total proteins were isolated, and protein concentration was detected by the Bradford assay. Immunoprecipitation assays used 500 μg protein from each experimental sample and 2 μg FKBP1A or ACVR1 antibody (both from Santa Cruz Biotechnology Inc.) at 4°C overnight, followed by treatment with 30 μl of Protein A/G agarose beads (Pierce) at 4°C for 1 hour and centrifugation at 800 *g* for 5 minutes. The immunoprecipitated complex was dissociated by 12% SDS-PAGE and detected with V5 monoclonal antibody (Invitrogen) or FKBP1A antibody (N19; Santa Cruz Biotechnology Inc.).

Micromass cultures. Viral constructs were designed and prepared for infection of chick limb bud mesenchymal stem cells in micromass culture. The coding sequence of chicken Acvr1 was PCR amplified from chicken embryo cDNA using the following primer pair: chAcvr1-NcoI-fwd, 5′-ACCATGGCTCTCCCCGTGCTGCTG-3′ and chAcvr1-BamHI-rev, 5′-AGGATCCTCAACAGTCAGCCTTCAGTTT-3′. The PCR product was digested with NcoI and BamHI and subcloned into the pSLAX-13 shuttle vector. This construct was used to introduce the corresponding human FOP mutation R206H and the constitutive active variant of the receptor Q207D by Site-Directed Mutagenesis (QuikChange, Stratagene) using the following primer pairs (with lower-case letters indicating the nucleotides changed relative to wild-type Acvr1 sequence): R206H-chAcvr1-fwd, 5′-GCAAAGAACAGTGGCTCaCCAGATCACGCTTGTGG-3′ and R206H-chAcvr1-rev, 5′-CCACAAGCGTGATCTGGtGAGCCACTGTTCTTTGC-3′; chAcvr1-ca-Q207D-fwd, 5′-GCAAAGAACAGTGGCTCGCgAcATCACGCTTGTGGAGTG-3′ and chAcvr1-ca-Q207D-rev, 5′-CACTCCACAAGCGTGATgTcGCGAGCCACTGTTCTTTGC-3′). Inserts were subcloned by ClaI into the avian-specific viral vector RCASBP(A). chNog in RCASBP(B) was a gift from A. Vortkamp (Universität Duisburg-Essen, Duisburg, Germany). To produce the virus, RCASBP plasmids were transfected into DF1 cells, culture medium was harvested, and viral particles were concentrated by ultracentrifugation as described previously (57). Titers of all receptor-expressing RCASBP(A) viruses were determined, and concentrations of greater than 1×10^8 were used throughout the experiments. The titer of Nog-expressing RCASBP(B) was 1×10^7.

Micromass cultures were prepared from dissected limb buds from chicken embryos at Hamburger-Hamilton stage HH24 as described previously (58). Cells (2×10^5) were plated in a drop containing 10 μl growth medium (DMEM:F12, 10% FCS, 0.2% chicken serum, 2 mM L-Gln, penicillin/streptomycin), and 1 μl of each indicated viral concentrate was applied in a 24-well plate. Growth media were replaced 3 times a week. Alcian blue staining was performed after fixation of the cells in Kahle's fixative (28.9% [v/v] EtOH; 0.37% formaldehyde; 3.9% [v/v] acetic acid) with 1% Alcian blue in 0.1N HCl overnight. Excess dye was removed by washing with water, and cultures were dried before photographs were taken. For quantification of incorporated Alcian blue into the proteoglycan-rich extracellular matrix, cultures were incubated with 6 M guanidine hydrochloride overnight, followed by photometric measurement at OD_{595}. ALP staining was performed after fixation of the cells with 4% PFA and followed by incubation with NBT/BCIP. The reaction was stopped with TE buffer, and photographs were taken. For quantification, histomorphometric analyses were performed using Autmess AxioVision 4.6 software (Zeiss).

RNA isolation and relative quantitative RT-PCR. RNA was extracted from chicken micromass cultures at day 8 using peqGold Trifast (peqLab Biotechnologie GmbH) following the manufacturer's instructions, then was digested with DNase (RNase-Free DNase kit, QIAGEN). cDNA synthesis was performed using the TaqMan Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) (ABIPrism 7900HT cycler, Applied Biosystems) was performed by using Power SYBR Green PCR Master Mix (Applied Biosystems) and the following primer pairs: ch-collagen type II-fwd, 5′-GAGGGCAACAGCAGGTTCAC-3′ and ch-collagen type II-rev, 5′-TGCCCCATTTGCCAGTGT-3′; ch-collagen type X-fwd, 5′-CACTCTACTGCCTTGCATTGGA-3′ and ch-collagen type X-rev, 5′-AACAGCAGCAGTAACGATATTTGTAAA-3′; ch-Ihh-fwd, 5′-GGCTTTGACTGGGTCTACTACGA-3′ and ch-Ihh-rev, 5′-CAGCCGAGTGCTCTGACTTG-3′; ch-aggrecan-fwd, 5′-TGTTTGTGTGTTTGCAAGCCA-3′ and ch-aggrecan-rev, 5′-AGGCCATCGCTACTGTCTGAG-3′. To normalize gene expression, we used ch-28S rRNA-fwd, 5′-GGTATGGGCCCGACGCT-3′ and ch-28S rRNA-rev, 5′-CCGATGCCGACGCTCAT-3′ (59) as a housekeeping gene. Relative expression levels normalized to 28S rRNA expression were calculated using qBase software (60).

Zebrafish embryo injection, plasmids, strains, and genotyping. PCR-amplified cDNA encoding control (c.617G) or mutant (c.617A) hACVRI was inserted into a derivative of pCS2+ vector (61) containing an in-frame C-terminal FLAG epitope (provided by M. Deardorff and P. Klein, University of Pennsylvania). mRNA was in vitro transcribed using the SP6 mMessage mMachine kit (Ambion) from plasmids linearized with NotI. Bmp7 and Bmp2b (ortholog of BMP2) are the BMPs that pattern the embryonic dorsal-ventral axis in zebrafish (14). A previously described morpholino against *bmp2b* (62)

was injected at 1 ng per embryo. A morpholino mixture of 2 ng smad5MO1 (5′-ATGGAGGTCATAGTGCTGGGCTGC-3′) and 2.5 ng smad5MO3 (5′-GCAGTGTGCCAGGAAGATGATTATG-3′) per embryo was used to knock down *smad5* translation. All injections were performed at the 1-cell stage as described previously (63). Genotyping of the *lost-a-fin*^{tm110b} allele was performed as described (28). Homozygous null *bmp7* or *bmp2b* mutant embryos were obtained from crosses between *bmp7* or *bmp2b* heterozygous zebrafish. PCR genotyping for *swirl*^{tdc24} (*bmp2b*) (64) used primers DC245P (5′-CGCTTGCTCAATATGTTCGGATTGAAT-3′) and DC243P (5′-CGTGATGAAAACTTCGTATCGTGTTTG-3′), which create a recognition site for the restriction enzyme TaqI only when amplifying the wild-type allele. Homozygous *snb*^{tb1aub} embryos (*bmp7*) (39) were identified by failure to PCR-amplify a closely linked simple sequence-length polymorphism (SSLP) using primers BMP7s42F (5′-CTCAGACACATAAGCCTAAGTGC-3′) and BMP7s42R (5′-TCTATCCCAACTCAAATGCACCAG-3′). For treatment with DM, a BMP signaling inhibitor that dorsalizes zebrafish embryos (37), embryos were placed in E3 embryo medium containing DMSO either alone or with 40 μM DM prior to first cleavage. All images of embryos were from an MZ12.5 stereomicroscope (Leica) with a ColorSNAP-cf digital camera (Photometrics) and processed using Adobe Photoshop. In situ hybridization and subsequent imaging were carried out as described previously (65) with probes against *eve1* (66), *gata2* (67), and *foxb1.2* (68).

Statistics. Results are expressed as mean ± SEM. A 2-tailed Student's *t* test was used for statistical comparison of 2 groups. *P* values of less than 0.05 were considered significant.

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REVIEW (Molecular Regulation of Mineralization and Its Failure)

Heterotopic Bone Formation Induced by Bone Morphogenetic Protein Signaling : Fibrodysplasia Ossificans Progressiva

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Abstract : Bone morphogenetic proteins (BMPs) were originally identified *via* their unique activity in demineralized bone matrix—they induce heterotopic bone formation in skeletal muscle. BMP activity is transduced by two types of BMP-specific transmembrane serine/threonine kinase receptors and downstream transcription factors, known as Smads. Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder characterized by progressive heterotopic bone formation in skeletal muscle tissue. In patients with FOP, heterozygous mutations involving single amino-acid substitutions have been identified in the BMP type I receptor, ALK2. These mutations in ALK2 are scattered in an intracellular domain containing the kinase and GS domains. The mutant receptors constitutively activate downstream intracellular signaling, even in the absence of BMPs. In FOP, muscle injury induces acute heterotopic bone formation. Smad1 and Smad5 are up-regulated during muscle regeneration and induce osteoblastic differentiation of myoblasts in cooperation with mutant ALK2. Some types of BMP receptor inhibitors block the signal transduction induced by mutant ALK2 receptors *in vitro* and *in vivo*. Taken together, this work demonstrates that BMP signaling induces heterotopic bone formation in skeletal muscle. Inhibitors of BMP receptors may aid in the establishment of novel treatments to prevent heterotopic bone formation in FOP.

Introduction

Skeletal tissues are formed *via* two related but distinct processes during development in vertebrates : endochondral ossification and intramembranous ossification[1,2]. In endochondral ossification, undifferentiated mesenchymal cells condensed in skeletal muscle tissue are first committed to differentiate into chondrocytes. They form a cartilaginous model of bone tissue and gradually differentiate into proliferating and hypertrophic chondrocytes. The calcified cartilage formed by terminally differentiated hypertrophic chondrocytes is destroyed by chondroclasts, which derive from macrophages invading blood vessels, and is replaced by bone matrix secreted from osteoblasts. In contrast, cells undergoing mesenchymal condensation differentiate into osteoblasts and directly form bone tissue during intramembranous ossification. These developmental processes are orchestrated by various regulators, such as systemic hormones and local cytokines.

Bone morphogenetic proteins (BMPs) play critical roles in skeletal development by regulating the proliferation, differentiation, and apoptosis of chondrocytes,

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osteoblasts, and osteoclasts[3,4]. Although BMPs are multifunctional factors in vertebrates and invertebrates, they were originally discovered due to their bone-inducing activity in bone matrix in 1965. Urist[5] first prepared demineralized bone by treating bone matrix with hydrochloric acid and then implanted it in skeletal muscular tissues to induce re-calcification of the matrix. A few weeks after transplantation, new cartilage and bone tissues with bone marrow, rather than simple re-calcification, were ectopically formed in the demineralized bone matrix (Fig. 1). These findings suggested that the demineralized bone matrix contained unknown bioactive molecule(s) capable of inducing the differentiation of chondrocytes and osteoblasts from mesenchymal cells in muscular tissues. This ectopic bone-inducing activity was subsequently named "bone morphogenetic protein" because it was sensitive to trypsin digestion[6].

Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder characterized by congenital malformation of the big toes and progressive heterotopic bone formation in skeletal muscle tissue[7,8]. The frequency of FOP is estimated at one per 2 million, regardless of race, sex, and area of the world[8]. Genes encoding BMP signaling molecules have been suggested as possible candidates responsible for FOP because the process of heterotopic bone formation in FOP is similar to that induced by BMP implantation in muscle tissue[7]. In 2006, a concurrent heterozygous mutation in the *ACVR1* gene was identified from both familial and sporadic FOP patients[9]. Their *ACVR1* genes had a substitution mutation of guanine to adenine in position 617 ; this changes histidine to arginine in position 206 of the type I BMP receptor ALK2[9]. The mutant receptor, ALK2 (R206H), activates intracellular signaling specific to BMPs, even in the absence of ligands[10] ; therefore, it is considered a constitutively active BMP receptor. Moreover, the process of muscle regeneration seems to enhance BMP signaling by increasing downstream transcription factors[10]. Taken together, these findings shed light on the molecular mechanisms underlying pathological bone formation in skeletal muscle tissue by BMP signaling and the development of new treatments for FOP.

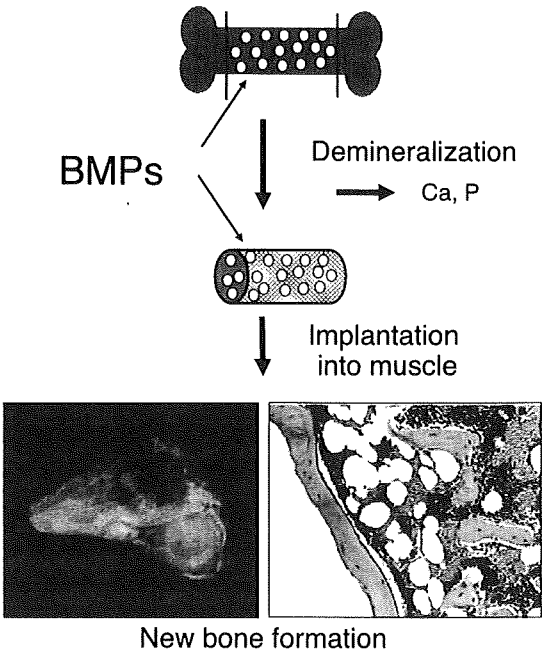

Fig. 1 Ectopic bone formation induced by BMPs

BMPs are stored in bone matrix and retained after demineralization by HCl. Implantation of the demineralized bone matrix containing BMPs into skeletal muscle induces heterotopic bone formation after 1—2 weeks.

Signal Transduction of BMPs

BMPs, except BMP-1, are members of the transforming growth factor-β (TGF-β) family[3]. Heterotopic bone-inducing activity in skeletal muscle is conserved between several BMP family members, including BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-9, and GDF-5 but not BMP-3, TGF-β, or activin[11]. The difference in bone-inducing activity *in vivo* can be reproduced in C2C12 myoblast cultures *in vitro* ; BMPs inhibit myogenesis and induce osteoblastic differentiation, but TGF-β1 fails to induce osteoblastic differentiation even though it inhibits myogenesis[12]. C2C12 myoblasts are widely used today for studying the molecular mechanisms of heterotopic bone formation by BMP signaling *in vitro*.

Similar to other members of the TGF-β family, the

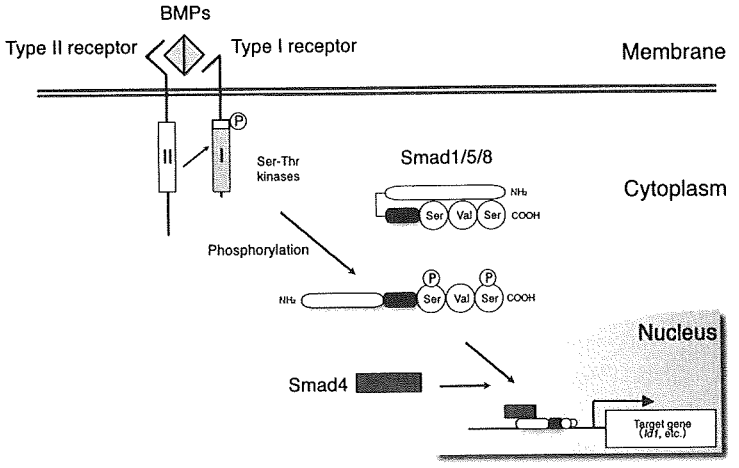

Fig. 2 Signal transduction of BMPs

BMPs bind to type I and type II receptors, which are transmembrane serine/threonine kinases expressed on the cell membrane. The type II receptor phosphorylates the type I receptor at a "GS domain", then the activated type I receptor phosphorylates Smad1/5/8 in the cytoplasm. Phospho-Smad1/5/8 form complexes with Smad4, move into the nucleus, and regulate gene expression.

intracellular signal transduction of BMPs is activated by two types of transmembrane serine/threonine kinase receptors : type I and type II receptors (Fig. 2)[3,13]. Combinations among three types of type II (BMPR-II, ActR-II, and ActR-IIB) and four types of type I (ALK1, ALK2, BMPR-IA/ALK3, and BMPR-IB/ALK6) receptors bind to BMPs and activate BMP-specific biological activities[3,13]. In contrast to TGF-β/activin receptors, BMP type I receptors do not seem to require type II receptors for ligand binding. In the ternary complex containing the BMP-type II receptor and type I receptor, the type II receptor phosphorylates the type I receptor at the "GS domain", which consists of a glycine and serine residue-rich domain located intracellularly between the transmembrane and kinase domains of type I receptors. The phosphorylated type I receptor at the GS domain activates downstream signaling pathways, including Smads, p38 MAP kinase, and PI3 kinase, *via* phosphorylation.

The GS domain of the type I receptor serves as a "molecular switch" for kinase activity. Substitution of a conserved asparagine residue in the GS domain of the type I receptors (which is present not only among BMP receptors but also TGF-β and activin receptors) activates kinase activity without stimulation by ligands or type II receptors[14]. A kinase domain-truncated type I receptor, which still retains a ligand binding domain, blocks the biological activities of BMPs as a dominant negative receptor[15,16]. These findings suggest that type I receptors play a central role in the signal transduction of BMPs due to phosphorylation-dependent regulation at the GS domain.

Among the intracellular signaling pathways activated by BMP type I receptors, the Smad-dependent pathway seems to be critical for bone formation induced by BMPs[13]. Smads are the mammalian counterparts of Sma and Mad in *C. elegans* and *Drosophila*, respectively, that transduce intracellular signaling of the TGF-β family of ligands. Smads are divided into three subgroups based on their structures and functions : receptor-regulated Smads (R-Smads), common Smads (Co-Smads), and inhibitory Smads (I-Smads)[3,13]. R-Smads are phosphorylated at the carboxyl termini by type I receptors, form ternary complexes with Co-Smad and Smad4, and move into nuclei to regulate the transcription of the target genes (Fig. 2). Specificity of ligand activity depends at least in part on receptor-activated Smads ; BMP receptors

mainly phosphorylate Smad1, Smad5 and Smad8, but TGF-β/activin receptors phosphorylate Smad2 and Smad3.

When activated by BMP type I receptors, Smad1 is introduced negative charges at both the Ser463 and Ser465 positions *via* phosphorylation. Substitution of these serine residues by aspartic acid, which is an acidic amino acid with an additional negative charge, seems to reproduce a state similar to phosphorylation[17]. This mutant Smad1, called Smad1 (2 SD) or Smad1(DVD), is a constitutively activated Smad1 because it activates target gene expression (such as that of *Id1*) and induces osteoblastic differentiation of C2C12 myoblasts in cooperation with Smad4 in the absence of ligands or type II or type I receptors[17] (unpublished observation). A specific chemical inhibitor of BMP-Smad phosphorylation, but not MAP kinase inhibitors, inhibits the osteoblastic differentiation of C2C12 cells induced by BMPs. Taken together, these findings suggest that the BMP receptor-Smad axis plays an important role in heterotopic bone formation induced by BMPs.

Clinical Features of FOP

FOP is an autosomal dominant disorder characterized by congenital malformation of the big toes and progressive heterotopic bone formation in skeletal muscle tissues that causes joint fusion with resultant permanent immobility[8]. The diaphragm, tongue, extra-ocular muscles, cardiac muscle and smooth muscles are not involved in the bone formation in FOP[8]. Almost all patients with FOP have malformation of the big toes at birth, suggesting that a protein encoded by the gene responsible for FOP regulates the patterning of the big toes during development[7,8]. A conductive hearing impairment is commonly observed in patients with FOP.

Prior to heterotopic bone formation in skeletal muscle, FOP patients develop painful flare-ups composed of inflammatory soft tissue swellings that contain a large number of mast cells[8]. The heterotopic bones are usually formed at flare-up sites *via* the process of endochondral bone formation. Minor trauma, such as intramuscular immunizations, mandibular blocks for dental work, overstretching of the jaw, muscle fatigue, blunt muscle trauma from bumps, bruises, falls, or influenza-like illnesses, is known to trigger new flare-ups of FOP and lead to heterotopic bone formation. Surgical operation and biopsy are prohibited in FOP because they also lead to new flare-ups and heterotopic bone formation. Thus, the heterotopic bone formation in FOP is an event inducible by muscle injury and muscle regeneration.

In addition to typical FOP features, some additional and/or different clinical features have been identified in atypical FOP[18]. These include severe but variable reduction deficits of the digits, absent finger/toenails of digits with severe reduction deficits, sparse and/or thin scalp hair, minimal change to the big toe, and slow progression of heterotopic bone formation in muscle. Recently, it has been reported that these differences are caused by different mutations in the *ACVR1* gene (see below).

Mutations in ALK2 Identified from FOP Patients

Several proteins and genes related to BMP signaling have been suggested to be responsible for FOP. The overexpression of BMP-4 was observed in lymphocytes prepared from FOP patients. Deletion of *NOGGIN* at 17q21-22, which encodes a BMP antagonist, was identified in a FOP patient ; however, this mutation was not detected by others and was excluded by linkage analysis[19]. Additional point mutations in the *NOGGIN* gene have been reported in FOP patients[20]. Genome-wide linkage analysis identified the 4q27-31 region as responsible for FOP, and this region contains *SMAD1*[21].

In 2006, the FOP mutation was mapped to chromosome 2q23-24, which contains the *ACVR1* gene encoding the BMP type I receptor ALK2[9]. A recurrent mutation (617G>A) was identified in the *ACVR1* gene in all examined individuals with FOP, including 5 familial and 32 sporadic cases (Fig. 3). This mutation was also confirmed in a patient who has been reported to carry a *NOGGIN* mutation[9]. In addition, this mutation was also identified in at least 22 Japanese FOP patients[10,22].

This *ACVR1* 617G>A mutation causes an amino

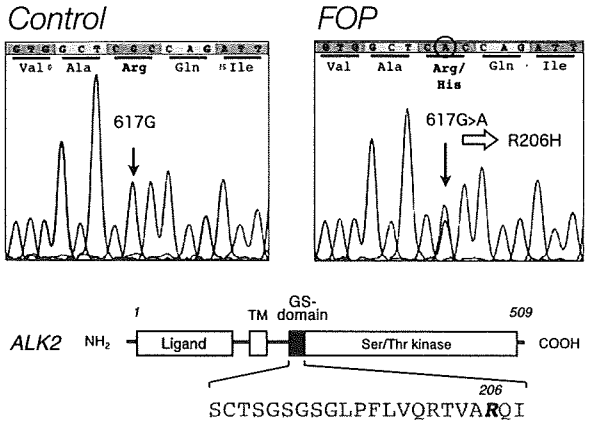

Fig. 3 R206H mutation in ALK2 identified in patients with FOP

The *ACVR1* gene encodes the BMP type I receptor ALK2. The control *ACVR1* gene has a "G" at position 617, but alleles from patients with FOP show heterozygosity for "G" and "A" ; this results in a change of arginine to histidine residue at position 206 in the ALK2 protein. Arg206 is localized in the GS domain, which is a "molecular switch" for the serine/threonine kinase activity of ALK2.

acid change from arginine to histidine residues in codon 206 (Fig. 3)[9]. Arg206 is highly conserved among vertebrates and a proximal amino acid of Gln207 ; a substitution mutation in Gln207 to glutamic acid has been reported to activate constitutive kinase activity[14]. Indeed, overexpression of ALK2 (R206H) in C2C12 myoblasts inhibits myogenesis and induces the nuclear accumulation of phospho-Smad1/5/8, *Id1* promoter/enhancer activity, and alkaline phosphatase activity[10] . Up-regulation of endogenous *ID1* expression and activation of p38 MAP kinase are also observed in lymphocytes immortalized from patients with FOP[23]. These findings clearly indicate that ALK2(R206H) is a genetically activated BMP type I receptor. This is the first case of a gain-of-function mutation among BMP type I receptors.

In addition to ALK2(R206H), several mutations in the same *ACVR1* gene at different positions have been identified in patients with atypical FOP. These novel mutations are scattered between the GS domain and kinase domain, and include delP197-F198 insL, R202I, Q207E, R258S, G328E, G328R, G328W, G356D, and R375P[18,24—26]. The structural changes of mutant ALK2 (including R206H) have been analyzed in silico, and this analysis suggests that they exhibit reduced ability to bind to FKBP12 (a binding repressor of the BMP/TGF-β type I receptors)[27]. It has also been suggested that a substitution of Arg206 to histidine creates a pH-sensitive switch in the GS domain that leads to ligand-independent activation of ALK2[27].

Muscle Regeneration and Ectopic Bone Formation

In FOP patients, muscle injury induces acute heterotopic bone formation, suggesting that the process of muscle regeneration may stimulate BMP signaling. A snake venom injection into skeletal muscle tissue in mice induces the whole process of muscle regeneration within a couple of weeks. In this model, expression of Smad1 and Smad5, but not Smad8, is up-regulated within 3 days of injection[10]. Smad1 and Smad5 are expressed in mononuclear cells appearing in the regenerating muscle. Although both Smad1 and Smad5 are not phosphorylated in wild-type mice *in vivo*, they become phosphorylated and activated for osteoblastic differentiation after co-transfection with ALK2(R206H) in C2C12 myoblasts[10]. Thus, cooperation of genetically activated mutant ALK2 and increased Smad1 and Smad5 may induce downstream signaling to induce heterotopic bone formation in skeletal muscle in FOP (Fig. 4). Injection of snake venom into skeletal muscle also stimulates heterotopic bone formation in BMP-4-expressing transgenic mice.

Clinical features of FOP suggest that myogenic cells are potential progenitors of the chondrocytes and osteoblasts forming heterotopic skeletal tissue in patients with FOP. In addition, the activity of ALK2 (R206H) in C2C12 cells further confirms this hypothesis[10]. These cells are believed to be derived from satellite cells, which are immature progenitor cells of myoblasts in skeletal muscle tissue. Recently, Tie2-expressing cells were identified as progenitor cells that contribute heterotopic cartilage and bone

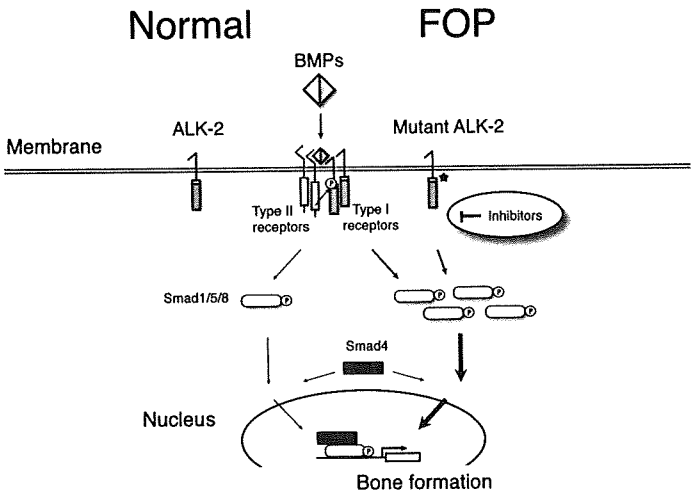

Fig. 4 A schematic model of signal transduction in FOP
Under normal conditions, ALK2 induces bone formation *via* Smad1/5/8 only after binding to BMP ligands. In contrast, in FOP, mutant ALK2 phosphorylates Smad1/5/8 and induces signal transduction without binding to BMPs. Moreover, levels of expression of Smad1 and Smad5 increase during muscle regeneration and mediate the mutant ALK2-stimulated bone formation. Inhibitors of ALK2, such as Dorsomorphin and LDN-193189, may aid in the establishment of novel treatments for FOP.

formation induced by BMP-2 using a Cre/LoxP tracing system *in vivo*[28]; however, MyoD-expressing cells did not differentiate into either chondrocytes or osteoblasts in this model. These findings suggest that endothelial progenitors, but not myoblasts, contribute to heterotopic bone formation in FOP.

Establishment of New Treatments for FOP

Today, there is no effective treatment for preventing heterotopic bone formation in FOP ; however, the establishment of new treatments for FOP has begun based on the recent findings regarding ALK2. Since the genetic activation of ALK2 activity seems to play a central role in heterotopic bone formation in FOP, specific inhibitors of the BMP receptors have been developed to block intracellular signaling from mutant ALK2. Dorsomorphin is a specific small chemical inhibitor of BMP type I receptors, such as ALK2, BMPR-IA and BMPR-IB[29]. Interestingly, Dorsomorphin inhibits the phosphorylation of Smads, but not p38, induced by BMPs[29]. Treatment of C2C12 cells expressing ALK2(R206H) or ALK2(G356D) with Dorsomorphin blocks the induction of osteoblastic differentiation *in vitro*[10]. In addition, overexpression of Smad7 also blocks the ALK2(R206H)-induced osteoblastic differentiation of C2C12 cells. Smad6 shows only minimal activity in this model.

LDN-193189 is a potent derivative of Dorsomorphin and inhibits the BMP-4-induced phosphorylation of Smad1/5/8 at an approximately 80-fold lower concentration than Dorsomorphin *in vitro*[30]. Yu *et al.* [30] established a new *in vivo* model system of heterotopic bone formation in skeletal muscle using mice carrying a floxed ALK2(Q207D) mutation ; however, it should be noted that this mutation has not been found in patients with FOP. An injection of Cre-expressing adenovirus into skeletal muscle of floxed ALK2(Q207D) mice induces local heterotopic bone formation, joint fusion and functional impairment similar to that in FOP. Treatment of these mice with LDN-19318 reduces heterotopic bone formation and functional impairment, suggesting that this type of inhibitor may be useful for curing FOP (Fig. 4)[30].

In the early stages of FOP research, it was suggested that bone marrow transplantation would be

effective in preventing heterotopic bone formation in FOP since increased BMP-4 expression in lymphocytes seemed to be responsible for this disorder[31]. In 2007, a report described a FOP patient with the ALK2 (R206H) mutation who had received bone marrow transplantation at age 10 and thereby avoided heterotopic bone formation for 14 years while undergoing immunosuppressive treatments[32]. However, the patient experienced heterotopic bone formation after cessation of the immunosuppressive treatments, suggesting that the immune system may also contribute to heterotopic bone formation in FOP[32].

Conclusion

BMP signaling is a potent inducer of heterotopic bone formation in skeletal muscle tissue. Activation of the type I receptor-Smad axis plays a central role in the bone formation induced by BMPs. Patients with FOP have mutations in ALK2 that exist as constitutively activated forms of the BMP type I receptor. Muscle regeneration in FOP may cooperatively stimulate heterotopic bone formation by increasing Smad1/5 expression. Development of specific inhibitors for ALK2 would be useful to prevent heterotopic bone formation in FOP.

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Ortho visual

FOP
―進行性骨化性線維異形成症―

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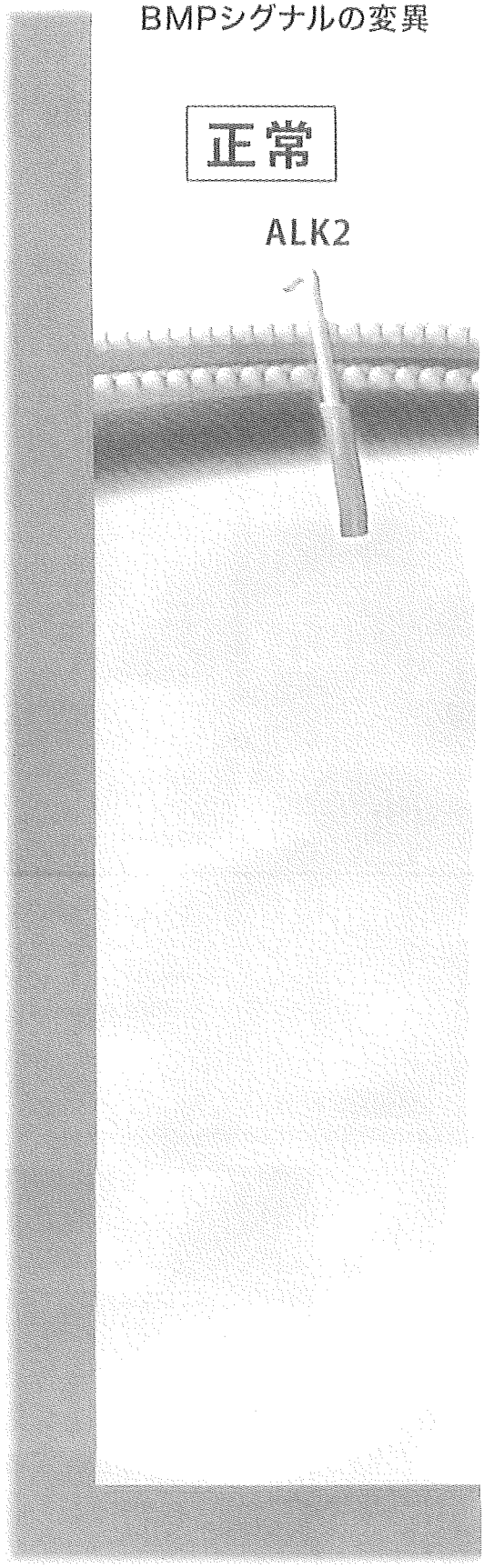

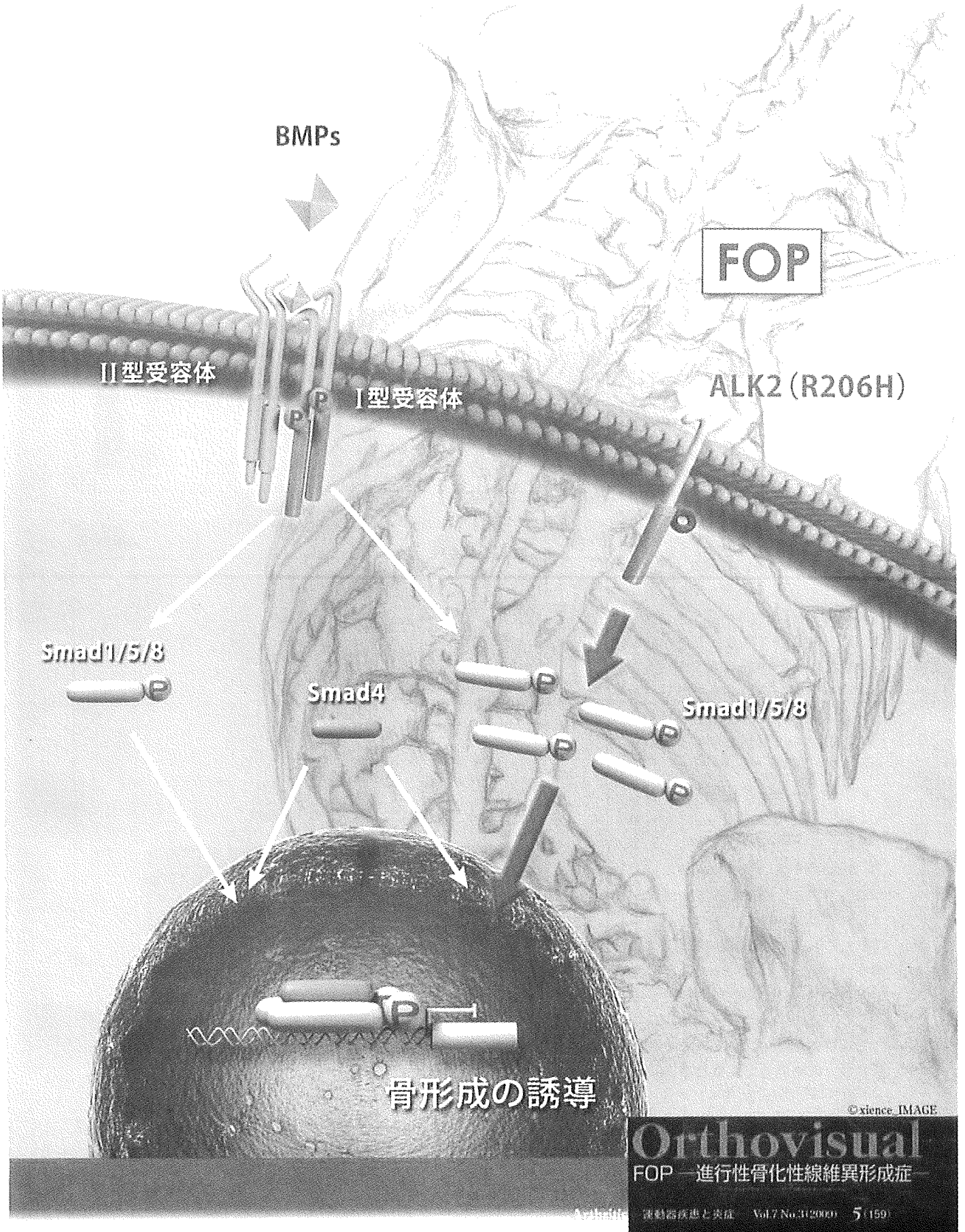

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運動器疾患と疾症　Vol.7 No.3 2009　5（159）

1 緒言

進行性骨化性線維異形成症(fibrodysplasia ossificans progressiva；FOP)は，成長に伴う全身の骨格筋や靱，靱帯などの異所性骨形成を特徴とする遺伝性疾患である[1](図1)．FOPは，2007年3月にわが国の難治性疾患克服研究事業対象疾患(いわゆる難病)のひとつに認定されている．FOPの進行が直接生命を脅かすことはないが，異所性骨形成により多くの関節が癒合するため，顔面を含む運動が困難となる．

これまで，FOPの診断・治療・研究には複数の大きな障害があった．現時点でも異所性骨化を防ぐ有効な治療法がないことは，その代表的一例である．その一方，これまで分子レベルでの発症機序がまったく不明であったのに対し，FOPの責任遺伝子が判明し発症機序の一端が明らかになり，それに基づいた新しい治療薬の開発が始まっている．また，異所性骨化発症前でもFOPの確定診断遺伝子診断が可能となった．

FOPは，筋の損傷が異所性骨形成を惹起するため，生検や手術など侵襲的医療行為は禁忌とされている．しかし，FOPの症例はきわめて少なく，医療従事者にもFOPに関する情報が十分に周知されていない．また，形成された骨組織は正常骨組織と同様に骨髄を伴いリモデリングされるため，異所性骨組織のみを薬剤などで除去することも困難である．したがって，現時点では可能な限り早期にFOPを確定診断し，FOP患者と医療従事者らが最新の情報を共有しながら，異所性骨化を防ぐよう努めることが重要と思われる．本稿では，最新の研究成果を交えてFOPを概説する．

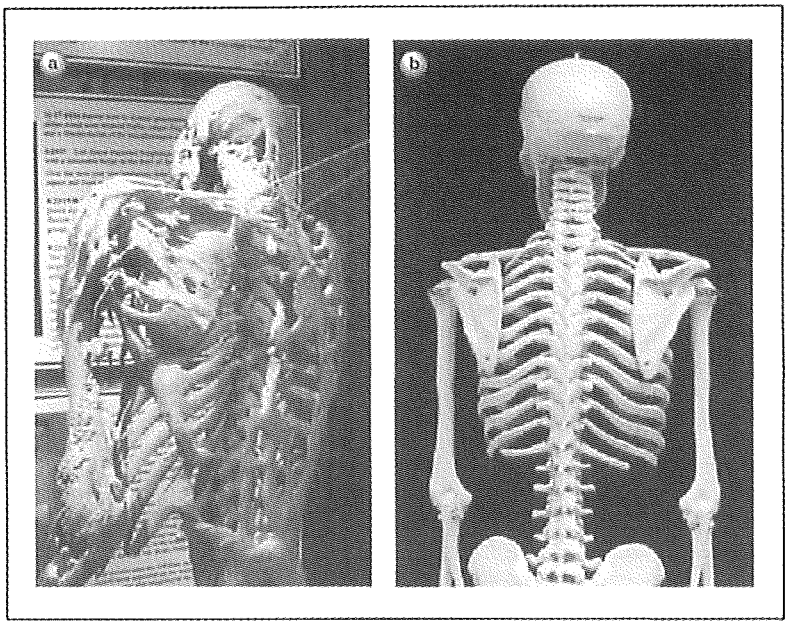

図1　FOPにおける異所性骨形成
米国ペンシルバニア州フィラデルフィアのMutter Museumに展示されているFOPの骨格標本(a)と正常骨格モデル(b)の比較．
(筆者作成)

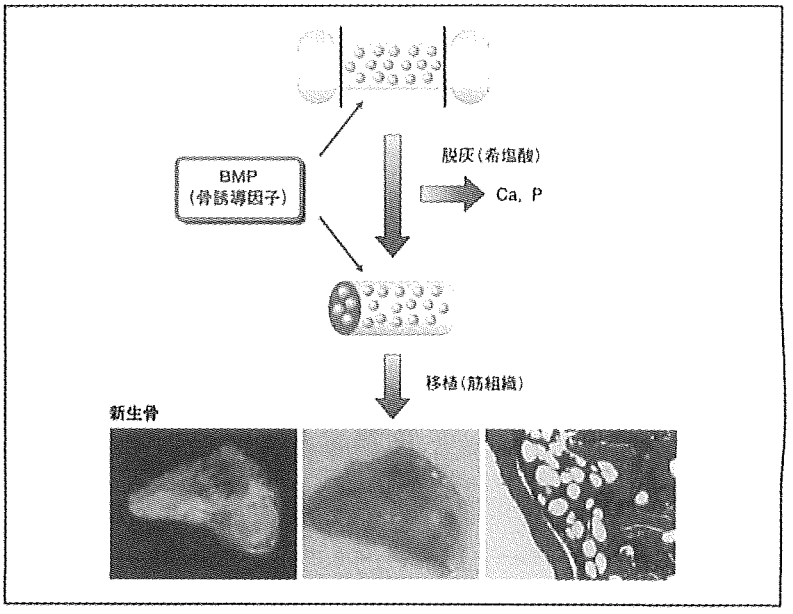

図2　BMPによる異所性骨誘導実験
酸で脱灰した骨基質を筋組織内に移植すると，数週間後に骨髄を伴った骨組織が誘導される．これは，骨基質に含まれるBMPの活性による．
(筆者作成)

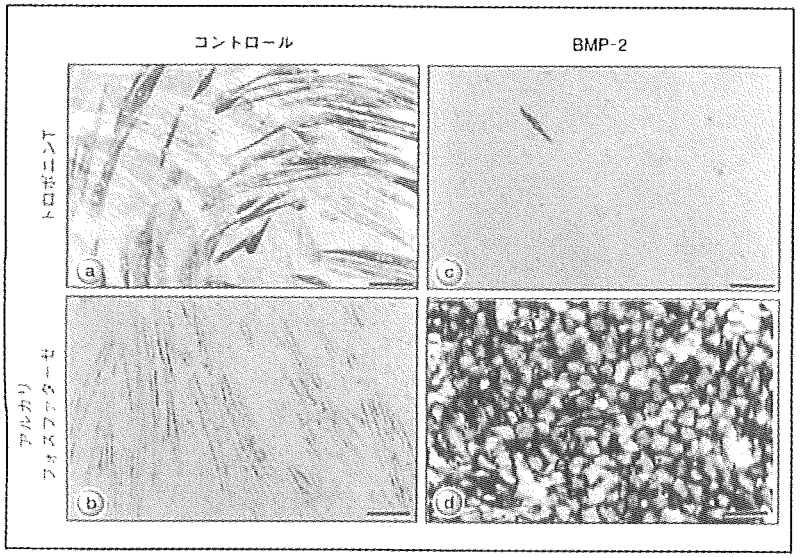

図3　BMPによる筋芽細胞から骨芽細胞への分化誘導実験

マウスの筋芽細胞C2C12をBMP-2存在下で培養すると，トロポニンTを発現する成熟筋細胞への分化が阻害され，代わりにアルカリフォスファターゼを発現する骨芽細胞様細胞に分化する．

（文献3より改変）

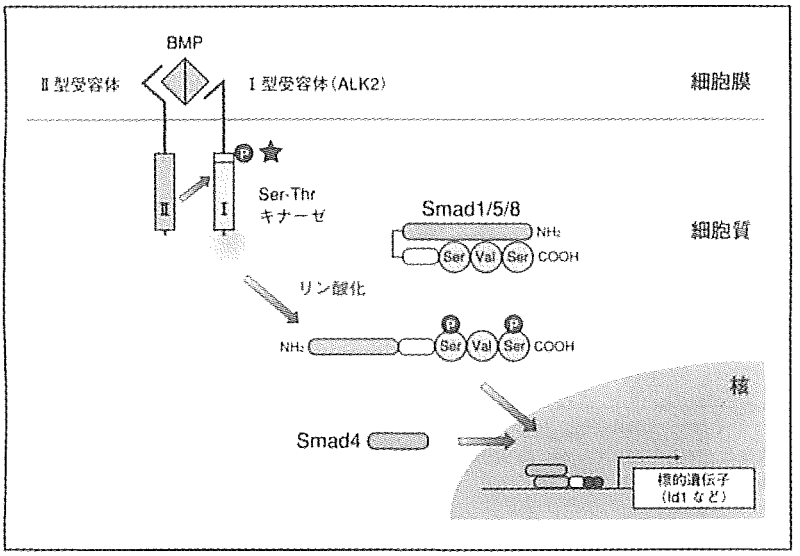

図4　BMP受容体による細胞内情報伝達

BMPは，標的細胞表面の2種類の膜貫通型セリン・スレオニン受容体（Ⅰ型とⅡ型）に結合する．Ⅱ型受容体がⅠ型受容体をリン酸化によって活性化し，Ⅰ型受容体がさらに下流の転写因子Smad1/5/8をリン酸化反応によって活性化する．Smad1/5/8は，Smad4と複合体を形成して核へ移行し，標的遺伝子の転写を制御する．

（筆者作成）

骨誘導因子BMPとFOP

以前から，酸で脱灰した骨基質を筋組織や皮下に移植すると，そこに異所性骨形成が誘導されることが知られていた[2]（図2）．この活性本体は，骨形成蛋白質（bone morphogenetic protein；BMP）と呼ばれる成長因子で，BMPは筋芽細胞の培養系でも筋分化を阻害するとともに骨芽細胞様細胞への分化を誘導する“骨誘導因子”である[3]（図3）．

BMP活性は，細胞表面の2種類の膜貫通型受容体と，細胞質でⅠ型受容体によってリン酸化される転写因子Smadによって伝達される（図4），最近，FOPの責任遺伝子として同定された第2染色体23-24の遺伝子は，BMPのⅠ型受容体ALK2をコードする遺伝子*ACVR1*であった[4]（図4，5）．

FOPはBMP受容体遺伝子の変異による

わが国の21例の孤発性FOPを含め，世界的なFOP症例の大部分は*ACVR1*遺伝子の617番目の塩基がグアニンとアデニンのヘテロ接合体である[4-6]（図5），この617G>A変異は，ALK2受容体の206番目のアルギニン残基をヒスチジンに置換させる（図5）．

このFOPで見出された変異ALK2（R206H）を培養筋芽細胞に発現させると，BMP非存在下でも，Smadのリン酸化や標的遺伝子の転写が誘導されたことから，ALK2（R206H）は構成的に活性化されたBMP受容体であることが判明した[6]（図6）．

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