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Investigations of Activated ACVR1/ALK2, a Bone Morphogenetic Protein Type I Receptor, That Causes Fibrodysplasia Ossificans Progressiva

Frederick S. Kaplan^{*,†,‡}, Petra Seemann[§], Julia Haupt[§], Meiqi Xu^{*,‡}, Vitali Y. Lounev^{*,‡}, Mary Mullins^{||}, and Eileen M. Shore^{*,||,‡}

^{*}Department of Orthopaedic Surgery, The University of Pennsylvania School of Medicine, Philadelphia, USA

[†]Department of Medicine, The University of Pennsylvania School of Medicine, Philadelphia, USA

[‡]The Center for Research in FOP and Related Disorders, The University of Pennsylvania School of Medicine, Philadelphia, USA

[§]Berlin Brandenburg Center for Regenerative Therapies (BCRT), Charité-Universitätsmedizin Berlin, Berlin, Germany

^{||}Department of Cell and Developmental Biology, The University of Pennsylvania School of Medicine, Philadelphia, USA

^{||}Department of Genetics, The University of Pennsylvania School of Medicine, Philadelphia, USA

Abstract

Bone morphogenetic protein (BMP) type I receptors are serine-threonine kinase transmembrane signal transduction proteins that regulate a vast array of ligand-dependent cell-fate decisions with temporal and spatial fidelity during development and postnatal life. A recent discovery identified a recurrent activating heterozygous missense mutation in a BMP type I receptor [Activin receptor IA/activin-like kinase 2 (ACVR1; also known as ALK2)] in patients with the disabling genetic disorder fibrodysplasia ossificans progressiva (FOP). Individuals with FOP experience episodes of tissue metamorphosis that convert soft connective tissue such as skeletal muscle into a highly ramified and disabling second skeleton of heterotopic bone. The single nucleotide ACVR1/ALK2 mutation that causes FOP is one of the most specific disease-causing mutations in the human genome and to date the only known inherited activating mutation of a BMP receptor that causes a human disease. Thus, the study of FOP provides the basis for understanding the clinically relevant effects of activating mutations in the BMP signaling pathway. Here we briefly review methodologies that we have applied to studying activated BMP signaling in FOP.

1. Introduction

Fibrodysplasia ossificans progressiva (FOP), a rare and illustrative genetic disorder of skeletal malformations and progressive heterotopic ossification, is the most disabling condition of ectopic skeletogenesis in humans (Kaplan *et al.*, 2005). Typically, during the first decade of life, episodes of inflammatory soft tissue swellings (flare-ups) seize the body's skeletal muscles and connective tissue, and transform them through an endochondral

process into an immobilizing second skeleton of heterotopic bone (Kaplan *et al.*, 2005). Most cases are caused by spontaneous new mutations. Inheritance, when observed, is autosomal dominant with variable expression (Shore *et al.*, 2005). At present, there is no definitive treatment (Kaplan *et al.*, 2008a).

A large body of work has supported dysregulated bone morphogenetic protein (BMP) signaling in the pathogenesis of FOP (Ahn *et al.*, 2003; Billings *et al.*, 2008; Fiori *et al.*, 2006; Glaser *et al.*, 2003; Kaplan *et al.*, 2007a; Lounev *et al.*, 2009; Serrano de la Peña *et al.*, 2005; Shafritz *et al.*, 1996; Shen *et al.*, 2009). Heterozygous missense mutations were identified in the glycine-serine activation domain of Activin receptor IA/Activin-like kinase 2 (ACVR1; also known as ALK2), a BMP type I receptor, in all affected individuals of several multigenerational families and in all sporadically affected individuals with features of classic FOP (Shore *et al.*, 2006). Remarkably the same single nucleotide mutation and substituted amino acid residue (c.617G>A; R206H) are altered in all classically affected FOP patients, providing the basis for elucidating the dysregulated BMP signaling that underlies the catastrophic phenotype of FOP (Groppe *et al.*, 2007; Shen *et al.*, 2009; Shore *et al.*, 2006). Conversely, FOP provides an important clinical and scientific window through which to understand the complexities of dysregulated BMP signaling relevant to human pathology and regenerative medicine (Kaplan *et al.*, 2007b, 2009a; Shore *et al.*, 2006). To date, FOP is the only human condition caused by inherited activating mutations in a BMP receptor. Recent detailed articles on FOP (Kaplan *et al.*, 2008a) and the BMP signaling pathway (Sieber *et al.*, 2009) provide an in-depth review of these critical background topics for readers.

Here, we briefly review methodologies that have been useful in understanding activated BMP signaling in FOP. While it may be possible in some instances to indicate optimal techniques, there are other instances, such as in the development of relevant animal models, where methodologies are rapidly evolving.

2. Patient Methodologies

2.1. Precise definition of FOP phenotype

In all laboratory studies in which FOP patient samples are used, it is critical that the clinical FOP phenotype be clearly and unequivocally established before proceeding with molecular and biochemical analyses (Kaplan *et al.*, 2005, 2009b; Shore *et al.*, 2006). The diagnosis of FOP is made on a clinical basis, however, mutational analysis can be confirmative, aid in early diagnosis, and be extremely valuable in cases of suspected variants (Kaplan *et al.*, 2008b, 2009b).

Patients with classic FOP have two defining clinical features: characteristic congenital malformations of the great toes and progressive heterotopic ossification in characteristic anatomic patterns (Kaplan *et al.*, 2009b; Shore *et al.*, 2006). In addition, common but variable features are seen in most individuals with FOP including proximal medial tibial osteochondromas, cervical spine malformations, short, broad femoral necks, conductive hearing impairment, and malformations of the thumbs (Kaplan *et al.*, 2009b).

Some patients with FOP-type heterotopic ossification have more or less severe clinical presentations. Individuals with FOP-plus have classic features of FOP plus one or more atypical features (Kaplan *et al.*, 2009b). Individuals with FOP variants have major variations in one or both of the classic defining features of FOP (Kaplan *et al.*, 2009b).

2.2. FOP mutational analysis

Mutational analysis of ACVR1/ALK2 in affected members of the few identified multigenerational families and in all sporadically affected individuals worldwide identified a recurrent missense activating mutation in codon 206 of the cytoplasmic glycine-serine domain of the receptor (Shore *et al.*, 2006). Follow-up studies identified alternate mutations in ACVR1/ALK2 in patients with highly variable and extremely rare clinical variants of the condition (Kaplan *et al.*, 2009b). While the recurrent ACVR1/ALK2 (R206H) mutation was found in all cases of classic FOP and most cases of FOP-plus, mutations at different positions occur in ACVR1/ALK2 in all FOP variants and in rare cases of FOP-plus (Kaplan *et al.*, 2009b).

2.3. ACVR1/ALK2 mutational analysis

In order to determine the genotype of individuals suspected of having classic FOP, genomic DNA is commonly screened from buccal swabs, blood, or lymphoblastoid cell lines for the canonical R206H mutation in ACVR1/ALK2. For individuals with classic FOP, genomic DNA is screened for mutations in ACVR1 by PCR amplification using primers that flank the recurrent c.617G>A mutation. For individuals with a suspected FOP variant phenotype, mutations in ACVR1/ALK2 can be screened by PCR amplification of the nine exons containing protein-coding sequences (ACVR1/ALK2 transcript report ensemble v35, accessions number ENST00000263640, GenBank RefSeq NM_001105.4 and NP_001096.1) using exon-flanking primers (Shore *et al.*, 2006). When available, DNA samples from parents are also examined. To date, no mutations have been found in unaffected parents.

PCR amplification is performed using Amplitaq Gold enzyme on PE2700 automated thermocyclers (Perkin Elmer, ABI, Waltham, MA). The PCR volume is 25 μ l, with 60 ng of genomic DNA, 10 pmol each of forward and reverse primer (Table 18.1), 200 μ M of deoxyribonucleotide triphosphate, 1.5 mM of MgCl₂, and 10 $\times$ PCR buffer. The general cycling conditions are 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, then 1 cycle of 72°C for 5 min. The PCR product amplification is verified by agarose gel electrophoresis. The amplicons are subsequently treated with Shrimp Alkaline Phosphatase and Exonuclease (USB, Cleveland, OH) to eliminate unincorporated nucleotides and primers before DNA sequence analysis. The products are purified on a 96-well purification plate (Edge Biosystems, Gaithersburg, MD), dissolved in water, and analyzed by dye terminator cycle sequencing with an ABI 3700XL sequencer (ABI) using either the reverse or forward PCR primer (Kaplan *et al.*, 2009b; Shore *et al.*, 2006).

Sequence data are analyzed using 4Peaks software v.1.6 (available online: www.mekentosj.com/4peaks). Mutations are identified by nucleotide numbering that reflects the cDNA sequence, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to gene nomenclature guidelines (www.hgvs.org/

mutnomen). The protein initiation codon is codon 1 (Kaplan *et al.*, 2009b; Shore *et al.*, 2006).

As an alternative to DNA sequence analysis, the c.617G>A; R206H ACVR1 mutation can be readily identified through differences in restriction endonuclease recognition (Shore *et al.*, 2006). Genomic DNA (0.1 μ g) is amplified using primers for protein-coding exon 4 (Table 18.1). Following agarose gel electrophoresis, PCR products (350 bp) are recovered from agarose using QIAquick Gel Extraction reagents (Qiagen). Purified PCR products are digested with either *Hph*I (5 U/ μ l) or *Cac*8I (4 U/ μ l) (New England Biolabs) at 37°C for 2 h and fragments resolved on 3% NuSieve 3:1 agarose (FMC BioProducts) gels with 100 bp ladder (New England Biolabs) as size markers.

Similarly, some FOP variant *ACVR1* mutations can be detected by differential restriction endonuclease digestion (Kaplan *et al.*, 2009b). New cleavage sites are created by the c. 619C>G (*Nru*I) and c.1067G>A (*Drd*I) nucleotide substitutions. A *Sty*I digestion site is eliminated by each of the single nucleotide substitutions identified in codon 328.

3. Cellular Methodologies

Studies performed prior to the discovery of the causative mutation for FOP revealed overactive BMP signaling in FOP cells through both the canonical SMAD pathway and the p38 MAPK pathway (Ahn *et al.*, 2003; Fiori *et al.*, 2006; Serrano de la Peña *et al.*, 2005; Shafritz *et al.*, 1996).

3.1. BMP signaling in FOP cells

Following the discovery of the FOP ACVR1 mutation, we performed numerous cell-based assays to evaluate the effects of the mutation on BMP signaling (Shen *et al.*, 2009). In these *in vitro* assays, we examined BMP pathway-specific SMAD phosphorylation and downstream transcriptional targets of BMP signaling. We determined that mutant R206H ACVR1/ALK2 activated BMP signaling in the absence of BMP and enhanced BMP signaling in the presence of BMP. We further investigated the interaction of mutant R206H ACVR1/ALK2 with FKBP1A (also known as FKBP12), a glycine-serine domain binding protein that prevents leaky type I BMP receptor activation in the absence of ligand (Kaplan *et al.*, 2007b; Shore *et al.*, 2006). We found reduced FKBP1A binding to the mutant protein suggesting that increased BMP pathway activity in cells with R206H ACVR1/ALK2 is due at least in part to decreased binding of this inhibitory factor (Shen *et al.*, 2009). Relevant methodologies are outlined below.

3.2. Expression plasmid constructs

A human ACVR1/ALK2 expression vector was generated by insertion of the hACVR1/ALK2 protein-coding sequence (GenBank accession number NM_001105.4) into the pcDNA 3.1 D V5-His-TOPO vector (Invitrogen). An FOP mutant ACVR1/ALK2 expression vector was generated by site directed mutagenesis of the wild-type ACVR1/ALK2 sequence at cDNA position 617 (from G to A) using the GeneTailor Site-Directed Mutagenesis System (Invitrogen), and the oligonucleotides: forward 5'-GTACAAAGAACAGTGGCTCaCCAGATTACACTG-3'; reverse 5'-

GTGAGCCACTGTTCTTTGTACCAGAAAAGGAAG-3'. The FKBP1A/FKBP12 expression vector is from Origene. The human *ID1* promoter (−985/+94) luciferase reporter construct was previously described and is a standard BMP early response gene used to monitor downstream BMP transcriptional activity (Katagiri *et al.*, 2002). 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.

3.3. Cell culture methodologies

We transfected a variety of mammalian cell lines to investigate the effects of ACVR1/ALK2 (R206H) on BMP signaling (Shen *et al.*, 2009). COS-7 African green monkey kidney cells, C2C12 mouse myoblastic cells, MC3T3-E1 human osteoblastic cells, and U-2 OS human osteosarcoma cells are obtained from ATCC. Cells are 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 are cultured in a humidified atmosphere of 5% CO₂ at 37°C.

3.4. Cell transfections and luciferase assays

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

3.5. Immunoblot analysis methodologies

COS-7 cells, plated at 70% confluence in 100-mm tissue culture dishes, are transfected with vector alone or pcDNA3 constructs with wild-type or mutant ACVR1. Total proteins are 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 μ g of protein from each total cell lysate is electrophoresed through 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes (iBlot membranes; Invitrogen). Membranes are 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 are washed with PBS and incubated for 1 h with the corresponding secondary antibody conjugated with horseradish peroxidase. The enhanced chemiluminescent Immobilon Western blotting detection system (Millipore) is used to detect the antigen-antibody complex. Similar protocols are 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 is performed according to standard methodology (Shen *et al.*, 2009).

3.6. Immunoprecipitation methodologies

To examine the interaction between FKBP1A and ACVR1/ALK2 in the absence or presence of BMPs, COS-7 cells are cotransfected with normal (c.617G) or mutant (c.617A) ACVR1/ALK2 expression constructs and the FKBP1A expression construct. After 48 h of transfection, cells are starved for 2 h in serum-free medium and then treated for 1 h with 100 ng/ml BMP4 or BMP7 (R&D Systems). Total proteins are isolated, and protein concentration is detected by the Bradford assay.

Immunoprecipitation assays use 500 μ g of protein from each experimental sample and 2 μ g of FKBP1A or ACVR1/ALK2 antibody (both from Santa Cruz Biotechnology, Inc.) at 4°C overnight, followed by treatment with 30 μ l of Protein A/G agarose beads (Pierce) at 4°C for 1 h and centrifugation at 800 $\times$ g for 5 min. The immunoprecipitated complex is dissociated by 12% SDS-PAGE and detected with V5 monoclonal antibody (Invitrogen) or FKBP1A antibody (N19; Santa Cruz Biotechnology, Inc.) (Shen *et al.*, 2009). Additional studies on FOP cells (Fukuda *et al.*, 2009; van Dinther *et al.*, 2009) also describe useful techniques related to the original approaches noted above.

3.7. Connective tissue progenitor cells

While transfected cells (Shen *et al.*, 2009) and immortalized lymphoblastoid cells (Fiori *et al.*, 2006; Serrano de la Peña *et al.*, 2005; Shafritz *et al.*, 1996) have been helpful in deciphering BMP pathway pathology in FOP, the study of FOP is hampered by the lack of readily available connective tissue progenitor cells that reflect the pathophysiology of the disease. In order to overcome this technical difficulty, we isolate such cells from discarded primary teeth of patients with FOP and controls (Billings *et al.*, 2008). Using these primary cell strains, we discovered dysregulation of BMP signaling and rapid osteoblast differentiation in FOP cells compared with control cells. Tissue progenitor cell lines from exfoliated and discarded primary teeth of patients and controls are extremely valuable, and have several advantages for studying activated BMP signaling in FOP over transfected cells because they are obtained directly from FOP patients and thus preserve the normal stoichiometry of BMP receptor copy number and locus fidelity.

3.8. SHED cell isolation and culture

Naturally exfoliated teeth are obtained from children. SHED (stem cells from human exfoliated deciduous teeth) cell strains are established from patients with FOP and unaffected age- and sex-matched controls. Cells are isolated as previously reported (Miura *et al.*, 2003) with minor protocol modifications. The dental pulp is digested with 2 mg/ml type II collagenase for 1 h (37 °C) in serum-free DMEM and filtered through a 100 μ m cell strainer (BD Falcon, Franklin Lakes, NJ, USA). Cells in the filtrate are recovered by centrifugation (400 $\times$ g, 10 min) and plated in DMEM with 10% FCS, GlutaMAX supplement, and antibiotics. The presence of ACVR1/ALK2 mutations in codon 206 (R206H) in FOP cells is confirmed by DNA sequence analysis (as described above).

For experimental treatments, cells are plated in 6-well plates (5×10^4 cells/well) in DMEM/10% FBS and grown for 4–6 days (80–90% confluence). Cells are washed with PBS, incubated for 1 h in serum-free medium, and treated with 100 ng/ml BMP4 in serum-

free medium for 1.5 h. For transfection experiments, SHED cells are seeded into 6-well plates and transfected with expression constructs (1 $\mu\text{g}/\text{well}$) for 48 h using TransIT-LT1 transfection reagent (Mirus, Madison, WI, USA) following the recommended protocol (Billings *et al.*, 2008; Miura *et al.*, 2003).

3.9. SHED cell differentiation assays

Alkaline phosphatase (ALP) activity is detected histochemically with BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBT (nitroblue tetrazolium)-plus substrate (Moss Substrates, Pasadena, MD, USA) at 37°C. To quantify ALP enzyme activity, cells are lysed in 0.1 *M* Tris (pH 7.5) and 0.1% Triton X-100. Cell debris is removed by centrifugation (5000 $\times g$, 5 min, 4°C) and 50 μl aliquots of supernatant are assayed in 0.5 ml of 1 mg/ml Sigma Phosphatase Substrate in 0.1 *M* Tris (pH 9.5), 1 mM MgCl_2 , *p*-nitrophenol (*p*-NP), and detected by spectrophotometry at 405 nm. Protein is determined with a BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL, USA), using BSA as standard. Enzyme activity is expressed as micromoles *p*-NP per minute per microgram protein (Billings *et al.*, 2008).

3.10. SHED cell mineralization assays

To induce mineralization, SHED cells are plated (5×10^4 cells/well, 24-well plates) and after 24 h are treated with osteogenic medium (OM; DMEM/10% FCS, supplemented with 50 $\mu\text{g}/\text{ml}$ ascorbate, 10 mM β -glycerophosphate, and 10 nM dexamethasone) for 14–21 days. The medium is changed twice weekly. To detect calcium mineralization, cells are stained with 1% Alizarin red (LabChem, Pittsburgh, PA, USA) for 30 min. To quantify mineralization, Alizarin red-stained cells are solubilized in 0.5 N HCl and 5% SDS for 30 min and detected at 405 nm using a Bio-Tek Synergy HT microplate reader (Billings *et al.*, 2008).

4. Tissue Methodologies

4.1. Studying FOP in the chicken

Studies using chick assays established ACVR1/ALK2 as a BMP type I receptor (Zhang *et al.*, 2003). ACVR1/ALK2 expression was examined *in vitro* in isolated chick chondrocytes and osteoblasts and *in vivo* in the developing chick limb bud (Zhang *et al.*, 2003).

4.2. Micromass culture system

Micromass cultures are used to investigate the effects of activated BMP signaling on tissue morphogenesis, particularly chondrogenesis. Micromass culture experiments were used to confirm that mutant R206H ACVR1/ALK2 activates BMP signaling in the absence of BMP ligand. It was further determined that expression of early markers of chondrogenesis was enhanced by BMP in cells expressing mutant ACVR1/ALK2 compared to wild type (Shen *et al.*, 2009). We prepare micromass cultures from primary mesenchymal cells isolated from embryonic chicken limb buds. The advantage of using chicken cells is that the gene of interest can be efficiently overexpressed by using an avian-specific replication competent retrovirus as a shuttle.

4.3. Constructs and virus production

The avian retrovirus RCAS (for more information please visit: <http://home.ncifcrf.gov/hivdrp/RCAS/index.html>) is used to overexpress chicken ACVR1/ALK2 or activated variants of it in micromass cultures. Viral constructs were prepared by PCR amplification of the coding sequence of chicken ACVR1/ALK2 from chicken embryo cDNA using the following primer pair: chAcvr1-NcoI-fwd, 5'-accATGGCTCTCCCCGTGCTGCTG-3', and chAcvr1-BamHI-rev, 5'-aggatcc-TCACCAGTCAGCCTTCAGTTT-3'. The PCR product was digested with *NcoI* and *BamHI* and subcloned into the pSLAX-13 shuttle vector. This construct is used to introduce the corresponding human FOP mutation (R206H) and the constitutively active (ca) variant of the receptor (Q207D) by Site-Directed Mutagenesis (QuikChange, Stratagene).

Inserts were subcloned by *ClaI* into the avian-specific viral vector RCASBP(A). To produce the virus, RCASBP plasmids are transfected into DF1 cells, culture medium is harvested, and viral particles are concentrated by ultracentrifugation. Titers of all receptor-expressing RCASBP(A) viruses are determined, and equal concentrations of all constructs are used to infect micromass cultures (Shen *et al.*, 2009).

4.4. Isolation and cultivation of micromass cultures

Micromass cultures are prepared from dissected limb buds from chicken embryos at Hamburger-Hamilton stage HH22-24 (after 4.5 days incubation at 37.5°C). Limb buds are incubated with Dispase II (Roche) in HBSS (3 mg/ml) in a 37°C waterbath for 15 min to remove the ectoderm. Next, limb buds are further digested in digestion solution (0.1% collagenase type Ia (Sigma), 0.1% trypsin, 5% FCS or CS in PBS without Ca/Mg) for 30 min at 37°C. Prewarmed growth media (DMEM:F12, 10% FCS, 0.2% CS, Pen/Strep) is added, and the cell suspension is passed through a Falcon cell strainer (40 μ m) to obtain a single cell solution. The final concentration of the cell suspension is adjusted to 2×10^7 cells/ml. For each culture, 10 μ l of cells (2×10^5) are mixed with the virus and plated in a 24-well plate and incubated for 1 h in a humidified chamber at 37°C and 5% CO₂. After attachment of the cells, 1 ml of growth medium is added to each well and replaced three times weekly.

4.5. Alcian blue staining

Differentiation into cartilage can be evaluated by Alcian blue staining of proteoglycans in the extracellular matrix. Micromass cultures are fixed with Kahle's fixative (28.9% [v/v] EtOH; 0.37% formaldehyde; 3.9% [v/v] acetic acid) and stained with 1% Alcian blue in 0.1 N HCl overnight. Excess dye is removed by washing with water, and cultures are dried before photographs are taken. For quantification of incorporated Alcian blue into the proteoglycan-rich extracellular matrix, cultures are incubated with 6 M guanidine hydrochloride overnight, followed by photometric measurement at OD₅₉₅.

4.6. ALP staining after PFA-fixation

ALP activity will serve as a marker for prehypertrophic chondrocytes. Micromass cultures are incubated with NBT/BCIP. The reaction is stopped with TE buffer, and photographs are taken. For quantification, histomorphometric analysis is performed using Autmess

AxioVision 4.6 software (Zeiss) (Shen *et al.*, 2009). Quantitative determination of ALP activity was previously described (see Section 3.9).

4.7. qPCR for chondrogenic marker genes

Additionally, downstream genes can be analyzed by qPCR. RNA from micromass cultures is extracted using peqGold Trifast (peqLab Biotechnologie GmbH) following manufacturer's instructions. One microgram of RNA is transcribed into cDNA by using the TaqMan Reverse Transcription Kit (Applied Biosystems). Relative expression levels are determined by SYBR Green-based qPCR on a ABIPrism 7900HT cyclor (Applied Biosystems) with primer pairs for chicken collagen type II (marker for early chondrogenesis), chicken aggrecan (marker for extracellular matrix of chondrocytes), chicken *Ihh* (marker for prehypertrophic chondrocytes), and chicken collagen type X (marker for hypertrophic chondrocytes) (Shen *et al.*, 2009). To normalize gene expression, we strongly recommend chicken 18S rRNA or 28S rRNA as reference genes because other standard reference genes such as β -actin, *gapdh*, β -2-microtubulin, *tpb*, or *hpert* are regulated by the constitutively active variant of ACVR1/ALK2 (Q207D) and to a lesser degree by the FOP mutation ACVR1/ALK2 (R206H) (Li *et al.*, 2005). 18S and 28S rRNA are the most abundant RNAs in an mRNA preparation; therefore it is necessary to reduce the amount of cDNA amplified by the primer pairs for the 18S and 28S rRNA relative to other genes. Thus, we use a dilution of 1:10 from the cDNA for qPCR of a standard gene, compared to a dilution of 1:50,000 to detect 18S or 28S rRNA. Relative expression levels normalized to 28S rRNA expression can be calculated using the qBase software (Hellemans *et al.*, 2007).

5. In Vivo Methodologies

Various animal models in different species (including *Drosophila*, zebrafish, chicken, and mice) have been useful in understanding *in vivo* effects of BMPs in ectopic bone formation and have shed light on the pathophysiology of FOP as well as nonsyndromic forms of posttraumatic heterotopic ossification (Fukuda *et al.*, 2006; Glaser *et al.*, 2003; Kan *et al.*, 2004, 2009; Lounev *et al.*, 2009; Shen *et al.*, 2009; Twombly *et al.*, 2009; Urist, 1965; Wozney *et al.*, 1998; Yu *et al.*, 2008b). Here we will focus on zebrafish and mouse models.

5.1. In vivo analysis of zebrafish embryos

In zebrafish embryonic development, a gradient of BMP signaling patterns the dorsal–ventral axis: slight perturbations in this BMP signaling gradient cause easily recognized mutant phenotypes making this system a sensitive assay for increased or decreased BMP signaling (Little and Mullins, 2006). *In vivo* analysis of the ACVR1/ALK2 FOP mutation (R206H) in zebrafish embryos reveals BMP-independent hyperactivation of BMP signaling (Shen *et al.*, 2009). These studies support that the mutant R206H ACVR1/ALK2 receptor in FOP patients is encoded by an activating mutation that induces BMP signaling in both a BMP-independent and a BMP-responsive manner. Zebrafish studies have been helpful in establishing the *in vivo* effects of the FOP mutation during vertebrate embryogenesis (Shen *et al.*, 2009) and in screening small molecule libraries for BMP receptor antagonists (Yu *et al.*, 2008a).

5.2. Zebrafish embryo methodologies

PCR-amplified cDNA encoding control (c.617G) or mutant (c.617A) hACVR1 is inserted into a derivative of pCS2+ vector containing an in-frame C-terminal FLAG epitope. mRNA is *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. Morpholinos against genes of interest are injected at 1 ng per embryo. A morpholino mixture of 2 ng smad5MO1 (5'-ATGGAGGTCATAGTGTGGGCTGC-3') and 2.5 ng smad5MO3 (5'-GCAGTGTGCCAGGAAGATGATTATG-3') per embryo was used to knock down *smad5* translation. All injections are performed at the one-cell stage. For treatment with dorsomorphin (DM), a BMP signaling inhibitor that dorsalizes zebrafish embryos (Yu *et al.*, 2008a), embryos were placed in E3 embryo medium containing DMSO either alone or with 40 μ M DM prior to the first cell cleavage. Embryo imaging is performed on a 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 (Shen *et al.*, 2009).

5.3. Murine BMP overactivity and overexpression models

Models for stimulating BMP-induced heterotopic ossification have traditionally involved the implantation of recombinant BMP into soft connective tissue (Glaser *et al.*, 2003; Urist, 1965; Wozney *et al.*, 1998) or the transgenic overexpression of BMP using the tissue-specific promoters (Kan *et al.*, 2004). These animal models are useful in examining overactivity of the BMP signaling pathway through a ligand-based approach, and the reader is directed to these important *in vivo* studies.

Studies using these models showed that dysregulation of BMP signaling in connective tissue progenitor cells contribute to all cell lineages of FOP-like heterotopic ossification (Kan *et al.*, 2009; Lounev *et al.*, 2009). Further, translational studies in an FOP patient who received bone marrow transplantation for an intercurrent condition established that even wild-type immune cells could induce heterotopic ossification in a genetically susceptible host (Kan *et al.*, 2009; Kaplan *et al.*, 2007a; Lounev *et al.*, 2009).

5.4. A conditional/constitutively active ACVR1/ALK2 model

Generation of a mouse with conditional activation of the BMP type I receptor ACVR1/ALK2 provides a model for investigating activated ACVR1/ALK2 gene function in a tissue-specific manner in a mammalian system (Fukuda *et al.*, 2006). Although the ca mutation (ACVR1/ALK2; Q207D) does not cause FOP (Fukuda *et al.*, 2006), it reproduces some of the features of the disease (Yu *et al.*, 2008b). Use of this animal model to induce heterotopic ossification through adenovirus-mediated Cre induction of a floxed ca ACVR1/ALK2 transgene was recently reported (Yu *et al.*, 2008b). We have found that modifications of the published protocol including using mice younger than 4 weeks of age when inducing heterotopic ossification by adenovirus-mediated Cre induction of caALK2 improve the reproducibility of heterotopic ossification in this model (our unpublished data).

5.5. A knock-in mouse chimera for FOP

The more recent development of a knock-in mouse model of the ACVR1/ALK2 (R206H) mutation in FOP provides the most compelling validation of the role of ACVR1/ALK2 in FOP and its myriad phenotypic effects in development and postnatal life (Chakkalakal *et al.*, 2008). This mouse model is still under development and analysis.

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Table 18.1

ACVR1 primers for human genomic DNA PCR amplification

Protein-coding exon #	Forward primer	Reverse primer	PCR product size (bp)
Exon 1	5'-AAGTAAGGCAATATATCTGAGG-3'	5'-GAGTGTTTTAAGTTTGATAGGC-3'	307
Exon 2	5'-ATATGAACACCACAGGGGG-3'	5'-CCTTCTGGTAGACGTGGAAG-3'	449
	5'-TTTTTCCCCCTTCCTTCTCTC-3'	5'-CAGGGTGACCTTCCTTGTAAG-3'	438
Exon 3	5'-AATTCCTCCCTTTCCCTCCAAC-3'	5'-TAAGAACGTGTCTCCAGACACC-3'	300
Exon 4	5'-CCAGTCCTTCTTCTTCTTCC-3'	5'-AGCAGATTTTCCAAGTTCCATC-3'	350
Exon 5	5'-TCCCAAGCTGAGTTTCTCC-3'	5'-AGAGCAAAGGCAGACAATTG-3'	346
Exon 6	5'-GACATTACTGTGTAGGTCGC-3'	5'-AGAGATGCAACTCACCTAACC-3'	438
Exon 7	5'-TGGGGTTGGTTTAAATCCTTC-3'	5'-AGGTAGCTGGATCAAGAGAAC-3'	337
Exon 8	5'-CACATTATAACCTGTGACACCC-3'	5'-ATACCAGTTGAAACTCAAAGGG-3'	299
Exon 9	5'-GTATTGCTGCTTTTGGCAC-3'	5'-CAGTCCCTACCTTTGCAAC-3'	700

Mutations in *ACVR1* are detected by DNA sequencing following PCR-amplification of genomic DNA corresponding to the nine exons containing protein-coding sequences (ACVR1 Transcript Report, Ensembl v35), using exon-flanking primers. Protein-coding exon 1 contains the ATG protein start codon. The R206H mutation is in protein-coding exon 4. Additional/alternate exons containing 5'UTRs are reported. Ensembl (Gene ID ENSG00000115170; transcript ID ENST00000263640) reports 11 exons for ACVR1 (with the first two exons containing only 5' untranslated sequences), consistent with GenBank BC033867, full-length cDNA clone.

Note: all primers are the same as reported in Shore et al. (2006) Nature Genetics Supplemental Methods except for the Exon 1 primer pair.