Insights from a Rare Genetic Disorder of Extra-Skeletal Bone Formation, Fibrodysplasia Ossificans Progressiva (FOP)

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Abstract

Fibrodysplasia ossificans progressiva (FOP) is a rare human genetic disorder of extensive and debilitating extra-skeletal bone formation. While the challenges of investigating a rare condition are many, the potential benefits are also great – not only for the specific disease under investigation, but also for the unique perspective on how cells normally function and the mechanisms that underlie more common disorders. This review will illustrate some of the many insights that we have gained by studying FOP.

Introduction

William Harvey, the seventeenth century physician who discovered blood circulation, wrote [1]: "Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of Nature by careful investigation of cases of rare forms of disease."

The primary goal of biomedical research is "to advance the proper practice of medicine" by developing reliable diagnostics, efficient treatments, and effective preventions. As William Harvey's quotation illustrates, the value of rare disorders to help us understand common diseases, as well as understand the normal patterns of development and function, has long been recognized.

An increasingly valuable approach to determining the causes of many commonly occurring human diseases is through identifying common genetic variants (polymorphisms) that cause or pre-dispose an individual to a particular disease in combination with other common genetic

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variants and/or environmental influences [2]. Such an approach has great potential to understand many human disorders while also providing an opportunity to elucidate the genes and cellular pathways that regulate normal function. However, special opportunities for insight into how our bodies and our cells function are provided by rare genetic diseases that are caused by mutations in single genes. These conditions are rare because such genes are likely of such critical functional importance that mutations in these genes are rarely tolerated. By identifying the genes that cause these genetic disorders, it becomes possible to reveal key components of cellular function that can be targeted for therapeutic intervention – not only for the rare disease under examination, but also for more common conditions that utilize the same cellular pathways.

In this review, we focus on the rare human genetic disease fibrodysplasia ossificans progressiva (FOP; MIM #135100). While our understanding of FOP remains incomplete, we already have gained tremendous insight into cellular mechanisms that regulate bone formation and that, when mis-regulated, cause this disease.

FOP - Overview

FOP is a rare condition, occurring at a population frequency of about 1 per 2 million [3,4]. Most cases of FOP are sporadic, with a single affected person in a family. FOP can be inherited in an autosomal dominant pattern, however due to the severe disability of FOP, only a few cases of inheritance from one generation of a family to the next are known to have occurred [4,5].

The main characteristic clinical feature of FOP is the formation of extra-skeletal, or heterotopic, bone. In addition, specific skeletal malformations that occur during embryonic development frequently are noted in patients [6]. These clinical observations tell us that the underlying genetic mutation in FOP affects a gene that is important during both pre- and post-natal bone formation.

The clinical features of FOP have been discussed in detail in recent reviews [6,7], and only a brief contextual overview will be described here.

Clinical description and diagnostic criteria for classic FOP

Two major characteristics define "classic" FOP [6,8]. Congenital malformation of the great toes (Figure 1) is the earliest phenotypic feature of FOP [9–11]. This is the most recognizable skeletal feature of FOP, although other subtle skeletal changes (such as cervical spine fusions, short/broad femoral necks, and osteochondromas) also commonly occur [12–14].

The second classic feature is extra-skeletal bone formation (Figure 1) that begins during childhood; formation of heterotopic bone during embryonic development has not been observed [9,10,15]. Heterotopic ossification forms in soft tissues such as skeletal muscle, tendon, and ligaments. The bone occurs independently of the normal skeleton and forms discrete skeletal elements that can fuse with normal skeletal bone. Early stage lesions, particularly those occurring during childhood, are associated with pre-osseous soft tissue swelling and inflammation [9,16,17].

Heterotopic bone formation is episodic with ossification sites generally forming in predictable temporal and spatial patterns, with the first episodes typically occurring along the upper back and neck [9,15,18]. However, the natural progression of the disease can be altered by environmental factors, such as soft tissue injury. Tissue damage and/or the wound healing response that follows are often associated with induction of heterotopic bone formation at the site of injury [10,19–23].

FOP histology

Since surgical trauma to tissues of FOP patients induces additional bone formation, biopsy specimens of developing FOP lesions are extremely rare and have only been obtained before a diagnosis of FOP has been made. The opportunities to observe the stages of lesion formation have therefore been limited, but extremely informative.

FOP heterotopic bone characteristically forms through an endochondral pathway [18,24]. We have recognized several stages of FOP lesion formation leading to mature FOP heterotopic bone: lymphocytic infiltration (inflammation), degradation of muscle tissue, fibroproliferative and highly angiogenic stages, cartilage and finally bone [25,26].

In addition to histological evaluation that revealed tissue changes consistent with endochondral bone formation, extensive analyses determined that the heterotopic bone formed in FOP is normal by several other criteria such as biochemistry, metabolism, radiography, and biomechanics [27,28]. FOP heterotopic bone can form a marrow cavity and also heals normally following fracture [24,29].

POH – A second rare genetic disease of extra-skeletal bone formation

One of the first insights that FOP provided was that all cases of genetic heterotopic ossification do not have the same underlying cause. Clinical referrals of patients with a preliminary diagnosis of FOP soon revealed that these patients segregated into two clearly distinct patterns of type and location of bone formation, leading us to characterize and identify of a second genetic disorder, progressive osseous heteroplasia (POH) [30].

FOP and POH share significant clinical similarities [31,32]. In each condition, patients develop extensive bone formation within the soft connective tissues. The bone formation occurs episodically and is cumulative over time. In both FOP and POH, the progression and stages of heterotopic bone formation appear to be normal, but the induction of bone formation is regulated inappropriately. In other words, normal bone forms in the wrong place at the wrong time, suggesting that these diseases are caused by changes in critical regulators of chondro/osteo-progenitor cells and/or cell fate decisions.

Despite these similarities, POH can be distinguished from FOP by several clinical criteria [31,32]. A key difference is that POH patients characteristically develop ossification within the superficial dermal layer of the skin while this tissue is unaffected in FOP. A diagnostic clinical characteristic of POH is the progression of heterotopic ossification from the dermis to the underlying deep connective tissues, with bone forming through the skeletal muscle and in some cases extending to and fusing with skeletal bone. Unlike FOP, POH is not associated with congenital toe malformations, inflammatory tumor-like swellings, or predictable regional patterns of heterotopic ossification. Also distinct from FOP, POH heterotopic bone forms in an asymmetric mosaic distribution and shows a predominance of membranous rather than endochondral bone formation.

Both FOP and POH are rare conditions, with most cases apparently the result of a *de novo* (spontaneous) genetic mutation. However, examples of inheritance of FOP and POH from one generation to the next occasionally occur, and both diseases are transmitted through an autosomal dominant inheritance pattern. The low frequency of spontaneous mutation cases and the simple Mendelian inheritance pattern suggested to us that each of these diseases is caused by mutations in single genes. Heterozygous inactivating mutations in the GNAS gene, which encodes alpha subunit of the stimulatory G protein (Gsα) of adenylyl cyclase, have been identified in POH patients [33].

FOP experimental approaches

A standard approach to investigating Mendelian genetic diseases is to identify the causative gene through genetic linkage and positional cloning approaches. Although FOP is an inherited disease, through the early stages of our investigations to understand its cause, we were limited in using these genetic strategies by the small numbers of families in which FOP is inherited from one generation to the next.

An alternate approach to studying a rare genetic disease is to identify differences in cell function that reflect the altered cellular activities of the mutated genes. For FOP, we quickly focused on the bone morphogenetic protein (BMP) signaling pathway [34–37]. BMPs are extra-cellular signaling proteins that regulate specific target gene transcription by binding to complexes of type I and type II serine/threonine kinase receptors in the cell membrane. Ligand-bound activated receptors mediate signaling through BMP pathway specific signal transduction factors (Smad1, Smad5, Smad8) and through MAPK signaling pathways.

Bone morphogenetic proteins (BMPs) are members of the TGF-beta family, proteins that regulate cell fate decisions in many different cellular and developmental contexts [38–40]. Many members of the BMP family can specifically induce the complete program of endochondral bone formation [41,42] and BMPs are active both pre- and post-natally in tissues that are affected in FOP.

Identifying a safely obtainable source of cells for gene expression and cell signaling assays was a particular challenge since heterotopic ossification in FOP patients can be triggered by trauma from tissue biopsies. Trauma to deep connective tissues is avoided in peripheral blood sampling, however, and we routinely used circulating lymphocytes from blood to establish lymphoblast cell lines (LCLs), a common source of DNA for genetic analyses. When we initiated our studies there was little precedent for using LCLs for gene expression analyses, however, these cells have proven to be useful for this purpose [43,44].

Although lymphocytes are present in early FOP lesions, no experimental evidence supports a specific role for these cells in FOP bone formation. Therefore, while LCLs have been an important and valuable tool for gene expression studies by revealing differences that reflect the underlying genetic mutation, these studies do not lead us to conclusions regarding the *in vivo* functional relevance of these cells in FOP.

More recently, we have isolated multi-potent progenitor cells from the tooth pulp of discarded primary teeth of FOP children (SHED cells: stem cells from human exfoliated deciduous teeth). These highly proliferative and adherent cells are an important cell system to investigate changes in cell differentiation potential of FOP cells [45,46].

BMP signaling in FOP

We examined several aspects of BMP pathway signaling in FOP cells in order to identify differences in expression of pathway components and pathway activation. Through an extensive series of experiments, we demonstrated significant changes affecting the BMP pathway in FOP cells, suggesting to us that increased BMP pathway signaling plays a role in FOP pathogenesis.

Early studies used several approaches to examine the expression of BMP family members in cells from FOP patients. One of our first discoveries was that BMP4 mRNA and protein are specifically over-expressed [47–50]. Although over-expression was not observed in cell lines from every patient, subsequent experiments revealed a consistent difference among FOP and control cells: while control cell lines regulate BMP4 mRNA levels at relatively steady amounts

in culture over time, FOP cells appear unable to regulate and maintain an even level of BMP4 mRNA instead accumulating steadily increasing levels of BMP4 [51]. Further support for a loss of normal feedback mechanisms in FOP cells was found in studies showing that FOP cells do not appropriately up-regulate mRNA expression of BMP antagonists in response to BMP ligand stimulation [52].

Additional studies further implicated a mis-regulated BMP pathway in FOP, such as impaired receptor internalization and higher levels of cell surface BMPRIA protein (a BMP type I receptor) [51,53] and dysregulated cell surface heparan sulfate proteoglycan (HSPG) modulation of BMP signaling [54] in FOP cells. Increased levels of BMP receptors and altered expression of HSPGs could plausibly lead to increased BMP signaling activity that trigger abnormal bone formation.

BMP receptor signaling is mediated through at least two known pathways: Smad 1/5/8 and p38MAPK. We determined that while LCLs lack BMP-specific Smad mRNA, BMP signaling is activated through ligand-stimulated p38MAPK phosphorylation in control cells [51,53]. FOP LCLs, however, show p38MAPK phosphorylation in the absence of BMP ligand, and in response to ligand, FOP cells show a hyper-phosphorylation of p38MAPK compared to controls cells. Consistent with constitutive signaling in FOP cells that was suggested by the increased levels of basal p38MAPK activation, we found that expression levels of known BMP transcriptional targets are also increased [51,53]. Analyses using SHED cells were consistent with these LCL studies, and also demonstrated ligand-independent BMP signaling and ligand-dependent hyper-responsiveness to BMP in cells from FOP patients [45]. SHED cells, which are amenable to osteogenic differentiation assays, additionally showed that FOP SHED cells expressed higher levels of alkaline phosphatase (ALP) and Runx2 mRNAs and more rapidly differentiated to an osteogenic phenotype.

These data consistently showed enhanced BMP-induced activity in FOP cells, leading us to conclude that increased BMP pathway signaling plays a role in FOP pathogenesis.

Genetics of FOP

Concurrently with investigations of the BMP signaling pathway in FOP cells, we continued to work with colleagues world-wide to identify families with inherited FOP in order to conduct an informative whole-genome linkage analysis. An initial linkage study had supported linkage of FOP to a small number of chromosomal regions with the strongest linkage to a region of chromosome 4 [55], containing the Smad1 gene. However, DNA sequence analysis of Smad1 and other candidate genes within the interval failed to reveal FOP-associated mutations.

As additional families with inherited FOP were identified, a second genome-wide linkage analysis using five two-generation families with classic FOP (malformed toes and patterns of heterotopic ossification) was conducted and identified specific linkage to the 2q23-24 region of chromosome 2 [8]. This relatively large linkage interval contained several candidate genes including ACVR1/Alk2 (Activin A type I receptor) which encodes a receptor for bone morphogenetic proteins (BMPs).

Classic FOP mutations

We examined the ACVR1 gene for mutations in both inherited and sporadic FOP patients with the classic features of FOP by DNA sequencing analysis of ACVR1 coding regions (exons and splice junctions). We discovered that all people with classic features of FOP contain a unique and very specific mutation - the identical heterozygous single nucleotide change at nucleotide position 617 of the cDNA [8] (Figure 2).

This recurrent mutation changes amino acid 206 from arginine to histidine. Amino acid 206 is a highly conserved amino acid in the ACVR1 suggesting its functional importance. Codon 206 occurs within the GS domain of ACVR1, an important functional region of the receptor since phosphorylation of the GS domain is required for downstream signaling [56,57]. The GS domain binds the inhibitory protein FKBP12 to prevent leaky type I receptor activation in the absence of ligand [58–60] and to regulate receptor endocytosis and ubiquitination [61,62].

Although both arginine and histidine are positively-charged amino acids, in silico protein homology modeling of the ACVR1 protein predicts that the shorter histidine side chain at amino acid 206 changes the protein structure of the receptor at the GS domain [8,63] and could disrupt intramolecular interactions that stabilize ACVR1 or that alter interactions between the GS domain and other signaling pathway molecules. Modeling of the ACVR1 structure also predicts that a molecular salt bridge that forms between arginine at residue 206 forms an intramolecular salt bridge with an invariant aspartate in the L45 Smad specificity loop of the protein kinase domain, however histidine at amino acid 206 (as occurs in FOP) would form this interaction only at decreased intracellular pH, effectively acting as a pH-sensitive switch that leads to ligand-independent ACVR1 activation [63].

Atypical and Variant FOP

Following our analysis of individuals with "classic" FOP features, we next turned our attention to more unusual presentations of patients with FOP-like heterotopic ossification [64]. We classified these patients into two groups (Table 1). "Atypical FOP" patients have the characteristic classic FOP features of extensive extra-skeletal ossification and toe malformations, but additionally have one or more features that are not typically associated with FOP. "FOP variants" have heterotopic ossification, but show variations of the classic FOP features, most notably more or less severe malformations of the first digits of the toes and thumbs.

Most atypical FOP patients (those with features not typically associated with FOP) have the classic FOP mutation (c.617G>A; R206H) in the GS domain of ACVR1. This finding suggests that the additional atypical features are independent of FOP and the R206H ACVR1 mutation, however we cannot exclude that these features are unusual variations caused by the ACVR1 mutation. This may be the case for two atypical FOP patients with non-classic ACVR1 mutations. One patient is heterozygous for a mutation within the ACVR1 GS domain, in codon 207, and the second occurs in codon 356 within the ACVR1 protein kinase domain [64].

None of the FOP variant patients had the classic FOP mutation (c.617G>A; R206H) in ACVR1. However, mutations in other amino acid codons of ACVR1 were found in each patient. With one exception (an in-frame 3 base pair deletion in the GS domain), all of the FOP variants had mutations in the protein kinase domain of ACVR1 [64]. This suggested that the classic R206H mutation causes a relatively predictable effect on embryonic skeletal development, at least for first digit formation, but that some ACVR1 mutations induce a greater or lesser signaling defect during skeletal development.

Of particular interest is ACVR1 codon 328 which is mutated in several FOP variants and shows evidence of genotype-phenotype correlations. In some patients, specific codon 328 mutations (c.982G>T; G328W or c.983G>A; G328E) are associated with a more severe developmental phenotype, with nearly complete truncation of the large toes and thumbs, while other mutations (c.982G>A or c.982G>C; G328R) are associated with a significantly milder skeletal phenotype and little or no effects on the development of the thumbs or toes [64].

ACVR1 mutations in FOP - Summary

All patients with FOP-type heterotopic ossification that we examined contain heterozygous mutations in ACVR1 [8,64]. These dominant ACVR1 mutant alleles, affecting the GS or protein kinase domains of the receptor protein, appear to have high penetrance: thus far, the mutations have not been found in any individuals without heterotopic ossification and conversely every person with FOP-like heterotopic ossification has an identified ACVR1 mutation. One immediate benefit of identifying ACVR1 mutation in FOP has been our ability to develop a DNA diagnostic test [65], preventing harmful interventions that frequently have occurred prior to a correct diagnosis.

All of the ACVR1 mutations identified in FOP patients occur in evolutionarily conserved amino acids that are predicted to alter protein conformation by in silico structure modeling. Our data further suggest that the severity of developmental skeletal defects (such as observed in FOP variants) may depend on the specific ACVR1 mutant allele.

ACVR1/ALK2

ACVR1/ALK2 is one of four type I receptors that mediate signaling through BMPs [66–69]. In response to BMP ligand binding, these receptors phosphorylate cytoplasmic signal transduction molecules to regulate target gene transcriptional activation or repression. In vivo, specific functional roles for each of the three type I receptors are likely determined by varying efficiencies of ligand binding and by non-overlapping receptor expression patterns in specific tissues and during development [37,38,40].

The TGF-β/BMP family is among the classes of extracellular signaling proteins that are key mediators of cell differentiation, with essential roles during embryonic development as well as in maintaining and directing cell fate decisions at later stages [38]. Mutations in the ACVR1 type I BMP receptor in patients with FOP confirms that this receptor mediates critical BMP pathway signals that regulate embryonic skeletal bone formation as well as heterotopic ossification in FOP.

Homozygous null mutation of ACVR1/ALK2 in the mouse demonstrated a requirement for ACVR1/ALK2 in extra-embryonic tissues around the time of gastrulation for normal mesoderm formation and showed that ACVR1/ALK2 signaling is essential for later stage embryonic development [70,71]. Conditional ACVR1/ALK2 knockout mouse studies have identified roles for ACVR1 in specific tissues and developmental processes. In order to investigate effects of ACVR1 gain of function during embryonic development in specific tissues, a conditionally regulated mouse model to express a constitutively active form of ACVR1 (caALK2) has also been developed [72].

ACVR1 is expressed in several tissues, including skeletal muscle and cartilage, consistent with the sites of heterotopic ossification in FOP. *In vitro* and *in vivo* assays have shown that ACVR1 induces BMP signaling activities and chick limb culture experiments demonstrated that constitutive activation of ACVR1 signaling expands cartilage elements and stimulates joint fusions [73–76].

Identification of ACVR1 as the mutated gene in FOP provides the foundation for examining the mechanism through which BMP signaling directs cell differentiation leading to cartilage and bone formation. Key importance is the identification of ACVR1 and the BMP signaling pathway as clear targets for developing treatments for FOP as well as for other more common conditions of too much or too little cartilage and bone.

Ongoing and future FOP studies

We are currently examining the functional effects of FOP ACVR1 mutations through both cell signaling studies and animal models in order to understand the mechanism of the altered BMP signaling and to identify the wide range of effects on tissues and cell systems.

In vitro studies [75] using ACVR1 expression constructs for the classic FOP mutation (c. 617G>A; R206H) showed ligand-independent activation of BMP pathway signaling through Smad phosphorylation and promoter reporter assays. We further observed reduced binding of the inhibitory factor FKBP12 to mutant ACVR1, suggesting that increased BMP pathway activity in cells with mutant ACVR1 could be due at least in part to decreased binding of FKBP12 (Figure 3).

In vivo functional studies [75] are examining the ability of ACVR1 to rescue the zebrafish Alk8 (homolog of human ACVR1) knockout, which shows a dorsalized phenotype (*lost-a-fin*) [77]. Transient expression of control ACVR1 RNA in zebrafish embryos showed rescue of the *lost-a-fin* phenotype. However, mutant ACVR1 induced a ventralized phenotype in the embryos, demonstrating an over-compensation of the knockout phenotype and indicating hyper-activation of BMP signaling.

These and additional preliminary investigations using both *in vitro* and *in vivo* assays support that the FOP mutations are activating mutations that enhance BMP signaling pathways and that the mutant receptor stimulates ligand-independent signaling. These data are consistent with our previous observations of increased BMP signaling activity in cells from FOP patients.

Conclusions

FOP is a rare genetic disorder that has given us, and continues to provide, important insight into the cellular and genetic regulation of skeletal and extra-skeletal bone formation. However, the beneficial lessons that FOP has taught us extend far beyond what the relatively small numbers of affected patients might initially cause one to expect. The heterotopic bone that forms in FOP is normal bone by all evaluated criteria; its aberration lies in the lost regulation of cell fate determination that allows inappropriate formation of bone and cartilage in soft connective tissues. Identification of activating mutation of a specific BMP receptor as the cause of FOP reveals a critical regulator of chondro-osseous differentiation potential. However, this receptor and its signaling pathway are not only therapeutic targets for patients with FOP, but also provide an important new focus for developing many wider applications preventing more common nonhereditary forms of heterotopic ossification and for developing tissue engineering strategies for skeletal bone and cartilage repair.

Acknowledgements

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Figure 1. The classic FOP phenotype
Classic fibrodysplasia ossificans progressiva (FOP) is defined by two characteristic clinical features. **(a)** Extensive heterotopic bone formation is seen in this 3-dimensional reconstructed computed tomography (CT) scan of the back of a twelve-year-old child. Flareups of FOP arise and progress in a well-defined spatial pattern that result in ribbons, sheets, and plates of bone that fuse the joints of the axial and appendicular skeleton, entombing the patient in a "second skeleton" of heterotopic bone. **(b)** An anteroposterior radiograph of the feet of a three-year-old child shows symmetrical great toe malformations of metatarsals and proximal phalanges along with microdactyly, fused interphalangeal joints, and hallux valgus deviations at the metatarsophalangeal joints.
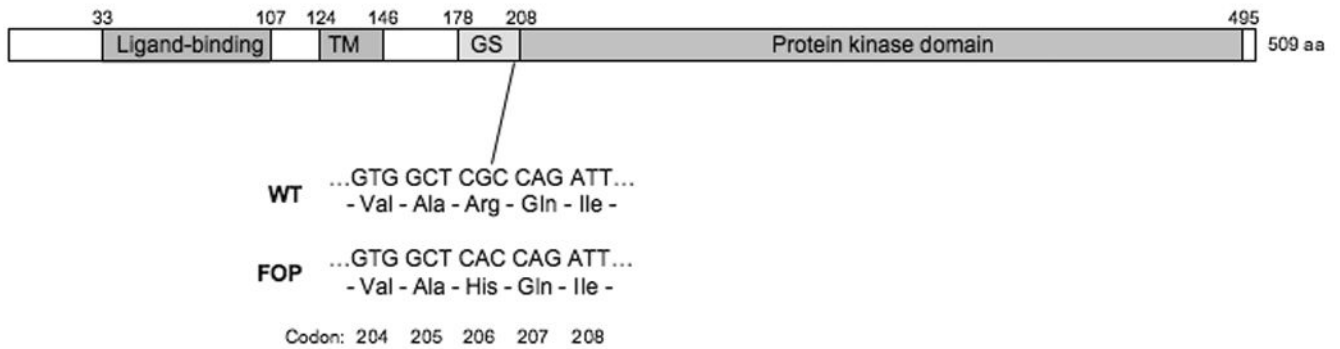
Figure 2. ACVR1 mutation in classic FOP

A schematic of the ACVR1 protein is shown, with key functional domains indicated: the extracellular BMP ligand-binding domain, the single trans-membrane (TM) domain, the glycine-serine (GS) activation domain, and the protein kinase signaling domain. Amino acid positions of each domain boundary are indicated above the schematic. All patients who show the classic characteristic features of FOP have the identical mutation in codon 206 within the GS domain. The nucleotide and amino acid sequences of codons 204–208 are shown for the wild-type and FOP mutation. The classic FOP mutation (a G to A substitution at nucleotide position 617 of the cDNA) causes a substitution of histidine for arginine (Arg206His).

ACVR1
wild-type
(Arg206)

ACVR1
FOP mutation
(His206)
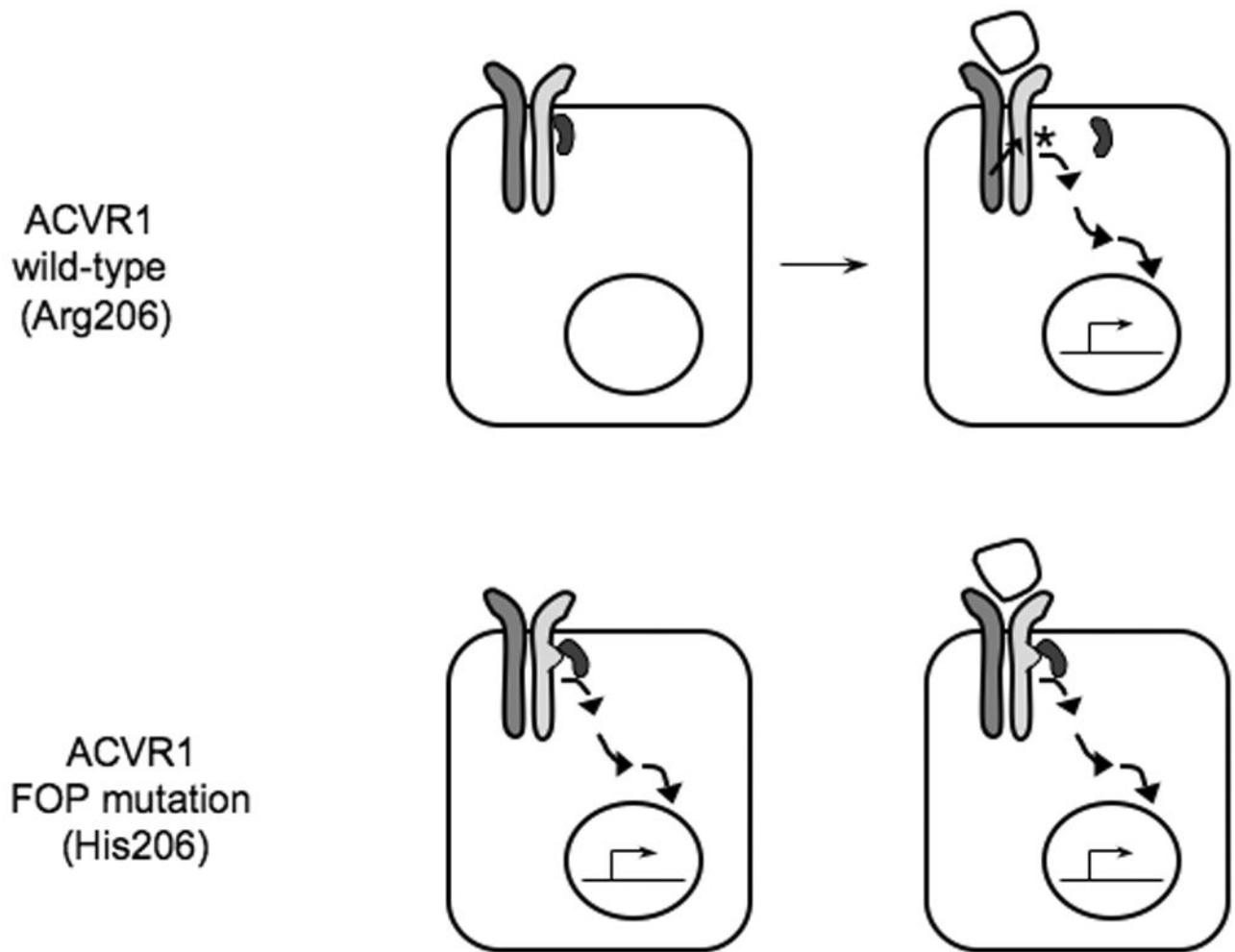
Figure 3. A hypothetical model for ligand-independent activation of BMP signaling in FOP
In the absence of BMP ligand, FKBP12 binds the wild-type type I BMP receptor (with arginine
at codon 206; Arg206) and prevents "leaky" activation of downstream signaling in the absence
of ligand. Upon BMP binding to the type I/type II BMP receptor complex, the type II receptor
phosphorylates (asterisk) the type I receptor within the GS domain and induces the release of
FKBP12 from the receptor and activation of downstream signaling. We hypothesize that
FKBP12 does not correctly bind to the mutant FOP ACVR1 type I receptor (with histidine at
codon 206; His206) either in the presence or the absence of BMP-receptor binding, allowing
increased activation of BMP signaling.

Table 1

FOP Ossification Syndromes

	Classic	Atypical	Variant
Progressive heterotopic ossification	100%	100%	100%
Malformed great toe (hallux valgus)	100%	100%	-
Severe or mild digit reduction deficits	no	no	100%
Unique/unusual FOP-associated clinical feature(s)	no	100%	variable
ACVR1 mutation			
c.617G>A; R206H	yes	yes	no
other ACVR1 mutation	no	yes	yes