RESEARCH HIGHLIGHT

Promiscuous signaling of ligands via mutant ALK2 in fibrodysplasia ossificans progressiva

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Fibrodysplasia ossificans progressiva (FOP) is a rare hereditary disorder characterized by successive heterotopic bone formation, for which at present there is no therapy. Mutations in the bone morphogenetic protein (BMP) type I receptor Activin receptor-like kinase 2 (ACVR1/ALK2) are the main trigger for FOP and inflammation is thought to be the secondary hit. The single nucleotide mutation at position 617 in the cDNA ALK2 sequence, which is found in 98% of FOP patients, results in a R206H change in the intracellular juxtamembrane region of ALK2. Previous studies had revealed that this mutation perturbs the interaction with the negative regulator FKBP12, thereby sensitising cells expressing this mutant receptor to BMPs, which are potent inducers of cartilage and bone formation. Recently, however, a twist in the underlying mechanism of FOP was revealed. Mutant ALK2 was found to respond to Activin-A, whereas wild type ALK2 function is inhibited by Activin-A. The latter cytokine is induced locally upon tissue damage and inflammation. Moreover, therapeutic targeting of Activin-A was found to inhibit heterotopic ossification in a mutant ALK2 knock-in mouse model that is highly reminiscent to human FOP. This review will focus on these latest surprising findings and discuss the implication for treatment of FOP patients.

Keywords: Fibrodysplasia ossificans progressive; heterotopic ossification; Activin receptor-like kinase 2; ALK2-R206H; BMP; TGF-β; Activin-A; inflammation

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Fibrodysplasia ossificans progressiva (FOP; OMIM #135100) is a very rare hereditary disorder, occurring in 1 out of 2,000,000 individuals, and is characterized by congenital deformity of the big toes and progressive ectopic ossification of muscles, ligaments, tendons, and other connective tissues. The ectopic bone formation, also named heterotopic ossification (HO), occurs sporadically through intermittent ossification episodes called flare-ups, which are induced by inflammatory insults, such as physical trauma or viral infections. Whereas FOP is well-known for HO of skeletal muscles, several other tissues, such as ligaments and tendons, can undergo HO [1-3]. Inexplicably, the tongue, the diaphragm, extra-ocular muscles as well as cardiac and smooth muscles are not undergoing HO. In general, in

FOP the progressive HO begins in the first decade in the life and follows a well-established anatomical pattern. Eventually the extra-skeletal bone formed through HO will unite with the normal skeletal bones. The HO progressively impairs their locomotive capacity, so that FOP patients may be unable to walk by adulthood. Due to the eventually fatal pulmonary and dietary insufficiency, FOP is associated with a decreased life expectancy [2, 4].

Mutations in the BMP type I receptor ALK2 underlie FOP

FOP is inherited in an autosomal dominant fashion, and a single point mutation in the gene encoding the bone morphogenetic protein (BMP) type I receptor activin receptor-like kinase 2 (ALK2) was identified in most FOP patients [5-7]. This point mutation is present in all FOP patients with classic clinical characteristics, i.e. displaying as well HO as congenitally deformed great toes, and comprises a conversion of a guanine into an adenine at position 617 of the ALK2 cDNA sequence (c.617G→A), causing the replacement of an arginine for a histidine residue at amino acid position 206 (R206H) [3,7-9]. In non-classically affected patients different mutations in ALK2 have been identified [3,8,10-12]. Thus, ALK2 mutations underlie the development of FOP. It is noteworthy that ALK2 mutations affecting the same residues that characterize FOP, have been recently found in a very rare type of brain tumor named Diffuse intrinsic pontine glioma (DIPG), where such mutations seem to play a secondary role accompaining histone gene alterations [13, 14]. Noteworthy, DIPG patients do not develop HO.

BMPs play a central role in bone homeostasis and remodeling [15]. Under normal conditions, BMPs propagate signals via various combinations of two different classes of serine/threonine kinase receptors, *i.e.* the type-I (ALKs) and type-II receptors. Upon binding of BMPs, the type I and type II receptors form a hetero-oligomeric complex, and the type II receptors phosphorylate serine and threonine residues in the juxtamembrane glycine-serine-rich (GS)-domain of the type I receptor [16]. Phosphorylation of the GS-domain is required for the activation of the type I receptor, which then phosphorylates receptor-regulated Smads (R-Smads), in case of BMPs Smad1, -5 and -8, which subsequently form a complex with Smad4 and translocate into the nucleus where they bind, in combination with additional transcription factors, promoters of BMP-responsive genes and regulate their transcription [16-21]. BMPs are members of a larger family of structurally related cytokines, that includes transforming growth factor-β (TGF-β) and Activin, which signal via their own specific type I (for TGF-β that is ALK5 and for Activin, ALK4) and type II heteromeric receptor complexes, leading to the phosphorylation of R-Smads Smad2 and -3. All TGF-β family members are pleiotropic context-dependent cytokines that have a similar signal transduction mechanism to relay their signal from the plasma membrane to the nucleus via Smad proteins [22, 23].

The R206H mutation is positioned in the ALK2 GS-domain and was found to result in increased receptor activation [7, 24-26]. Besides regulating the initiation of the downstream signaling cascades, the GS-domain controls endocytosis and ubiquitination, and avoids activation of the type I receptor in absence of ligand by binding the inhibitory protein FKBP12 [27-30]. ALK2-R206H appears insensitive to FKBP12-mediated inhibition, and enhances phosphorylation of Smad proteins and basal transcriptional activity of BMP target genes [26, 31]. Moreover, ALK2 R206-expressing cells display a higher sensitivity to undergo differentiation towards the osteoblast lineage in response to BMPs. Thus, ALK2-R206H acts as a mild constitutively active type I receptor, which becomes hyper-activated in presence of BMPs. Notably, by introducing an artificial Q207D mutation, at the position next to the residue mutated in ALK2-R206H, ALK2 becomes constitutively active, i.e. it induces downstream signaling even in the absence of exogenous ligand stimulation, although both ALK2-R206H and ALK2-Q207D still require a type II receptor [32-35].

The contribution of inflammation to FOP

FOP normally progresses through periodic episodes of HO named ‘flare-ups’, which are characterized by painful and inflammatory swelling of soft tissues which then undergo ossification. Flare-ups commonly follow upon minor incidents, like blunt trauma from bruises and falling, influenza virus infection or vaccination [36-38]. Expression of ALK2-Q207D in mice demonstrated that only in the context of inflammation the constitutively active ALK2 receptor can induce HO, analogous to the flare-ups in FOP patients [39]. The lymphocytes, mast cells and macrophages which infiltrate the inflamed tissue are assumed to offer the obligatory conditions for HO to occur [40-42]. To some extent this could include production of BMP4 by the infiltrating activated macrophages and lymphocytes [42, 43]. Notably, bone marrow transplantation showed no significant improvement in one FOP patient, suggesting that the immune system *per se* is not likely to be potentiated by the mutation in ALK2. However, subsequent immunosuppressive treatment successfully ameliorated HO episodes [44]. Moreover, it was demonstrated that in a mouse-model for FOP HO is reduced upon macrophage depletion and that corticosteroid treatment represses HO induced by ALK2 Q207D in mice [39, 42]. Accordingly, FOP patients are administered corticosteroids orally at a high dosage for a

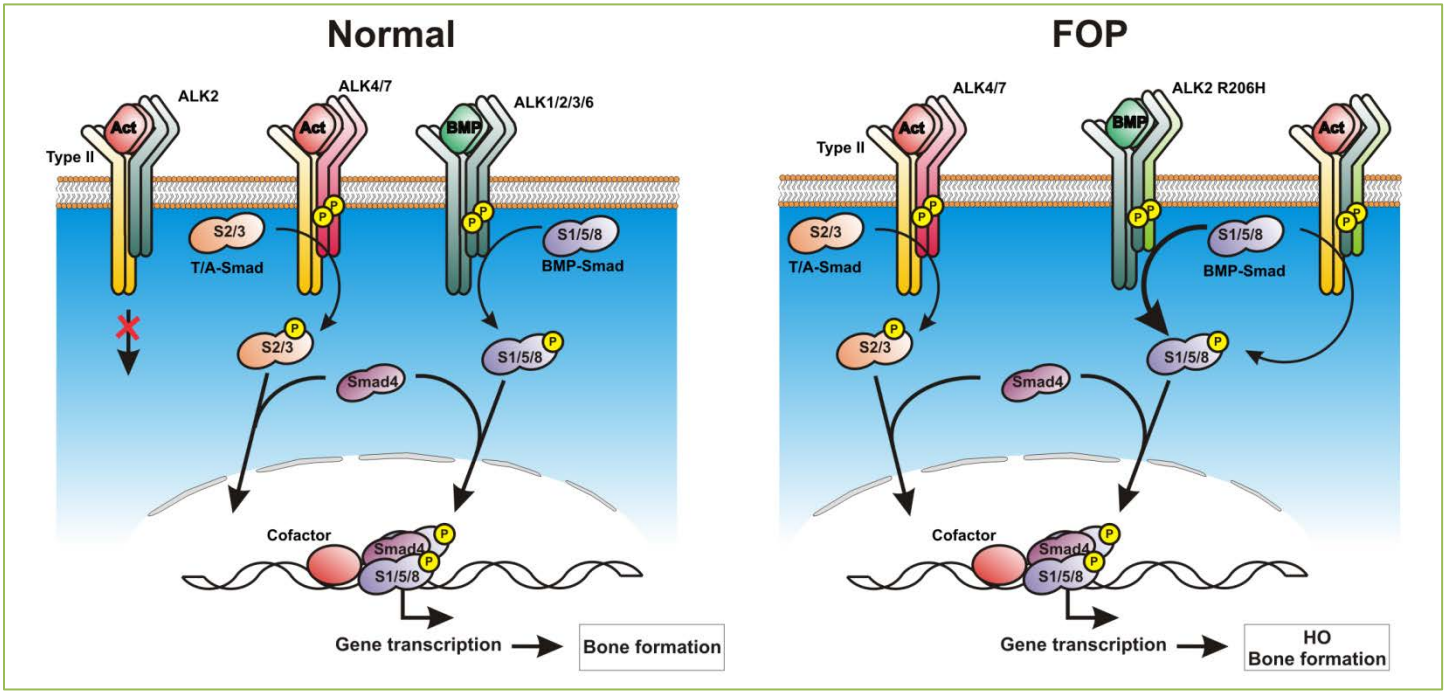

Figure 1. ALK-R206H responds to both BMP and Activin-A. BMPs activate via ALK1/2/3/6 the BMP-regulated Smads (BMP-Smads) Smad1, -5 and -8 (S1/5/8), whereas Activins induce the phosphorylation of the TGF-β/Activin-regulated Smads (T/A-Smads) Smad2 and -3 (S2/3). This occurs in cells of both healthy individuals and of FOP patients. Under normal conditions Activin-A can bind to ALK2, but this does not initiate downstream signaling cascades, and may sequester Activin type II receptors from engaging with BMPs. By sharing Activin type II receptors, BMPs and Activins can mutually antagonise each other for receptor activation. In FOP cells, however, not only is BMP-induced signaling via mutant ALK2 more potent (as this mutant is deficient in binding the negative regulator FKBP12), also Activin-A is now capable of signaling via receptor-complexes containing ALK2-R206H leading to activation of Smad1, -5 and -8, thereby contributing to HO. Not depicted is that Activin/ALK4 signaling results in Smad2/3-Smad4 complexes that also accumulate in the nucleus and regulate gene responses.

brief period of time to decrease the flare-up duration and severity, whereas non-steroidal anti-inflammatory drugs (NSAIDs) can be applied for a long time [45].

ALK2-R206H mediates also TGF-β and Activin signaling

The generation of ectopic bone in FOP occurs via a process called endochondral bone formation, which implies the generation of a preliminary cartilaginous tissue that progressively becomes ossified. This process is mediated by the cooperative activity of chondrocytes and osteoblasts, that are recruited to the HO area, maybe from fibrocyte-like circulating osteogenic precursor (COP) cells of hematopoietic origin [46]. Traditionally, it was considered that such cells are directly derived from mesenchymal progenitor cells in response to osteogenic stimuli [9]. However, by means of lineage tracing techniques and immunostainings on mouse and human tissues, Medici *et al*., showed that cells expressing endothelial cell markers can directly contribute to HO in FOP by undergoing a process named Endothelial-to-mesenchymal-transition (EndMT) [47]. EndMT was first observed in developmental studies of heart formation and it refers to a mechanism characterized by loss of endothelial features (e. g. organization in cell layers, absence of invasiveness and expression of the endothelial cell markers CD31, VE-cadherin, and VEGFR) and acquisition of a mesenchymal phenotype (e. g. absence of cell-cell junctions, highly migratory capacity and expression of specific cell markers: fibroblast specific protein-1, smooth muscle actin, fibronectin) [48]. Noteworthy, EndMT has been shown to partake to a number of human postnatal disorders, and inflammation has been unveiled as a common factor among them [49]. In FOP, endothelial-derived mesenchymal cells would then increase the pool of cells susceptible to differentiate into chondrocytes and osteoblast-like cells. This mechanism was estimated to be responsible for 50% of the HO formed in FOP [50]. Intriguingly, Medici *et al.* also showed that EndMT in FOP not only depends on ALK2-R206H but also on ALK5, which is able to form a complex with ALK2-R206H, and that in response to BMP4 and TGF-β EndMT is induced in an ALK2-dependent fashion [47]. Even though a definitive involvement of TGF-β in FOP still needs to be determined, given that the level of TGF-β is elevated upon muscle damage and that it can stimulate both recruitment of macrophages and EndMT, it could make a significant contribution to HO [47, 51, 52]. A recent publication using cultures of patient-derived subdermal fibroblasts suggests that TGF-β exerts a role in

osteogenic transdifferentiation in FOP [53]. However, it should be noted that the small molecule kinase inhibitor GW788388 used in this study does not interferes exclusively with ALK5 signaling, since it also blocks ALK4 and -7, and therefore also potentially inhibits Activin signaling [54].

Two recent studies demonstrated that ALK2-R206H-expressing cells gained responsiveness to Activin-A, which then, besides the normal activation of Smad2/3, also induced phosphorylation of BMP-Smads and expression of BMP target genes (Figure 1) [55, 56]. In contrast, normally Activin-A binds ALK2 without induction of Smad1/5/8-mediated signaling, thereby sequestering the receptors from binding BMPs [57]. Moreover, Activin-A was shown to enhance chondrogenesis, calcification and HO in an ALK2-R206H-dependent manner, both using FOP patient derived cells and a conditional-on knock-in ALK2-R206H mouse [55, 56]. Furthermore, Activin neutralizing antibodies and Activin (and BMP) ligand traps ActRIIA-Fc were able to inhibit HO in the conditional-on knock-in ALK2-R206H mouse FOP mouse model [55]. Notably, in the cells used in these studies, FOP patient-derived induced mesenchymal stromal cells and HEK293 cells, ALK2-R206H did only enable activation of the classical BMP-Smad pathway in response to Activin-A, not in response to TGF-β [55, 56].

Activin is a critical early mediator of acute inflammation. Several key pro-inflammatory cytokines including lipopolysaccharide (LPS), tumor necrosis factor α (TNFα), and interleukin (IL)-1β induce the expression and secretion of Activin-A by neutrophils, monocytes, macrophages and dendritic cells, whereas this can be inhibited by anti-inflammatory agents such as glucocorticoids and retinoic acid [58-62]. Activin in turn mediates the production of IL-1 and TNFα [61]. Moreover, Activin-A promotes the formation of the pro-inflammatory classical-activated M1 macrophages, and is also secreted by these same M1 macrophages [63]. Thus, Activin-A can mediate an amplification of the inflammatory response, and is abundantly present upon induction of inflammation. This, in combination with the acquired ability of mutant ALK2 to become activated by Activin-A might therefore be the driving force of HO in FOP patients.

It remains to be established how a single point mutation results in the ability of ALK2 to mediate Activin-A signaling. It was shown that the non-mutated ALK2 is able to associate with the Activin type II receptors ACVR2A and ACVR2B, but that in contrary to ALK2-R206H, this does not induce BMP-Smad mediated signaling but rather inhibits it [55]. Moreover, ALK2 Q207D-induced HO was shown to be prevented in the absence of ACVR2A and ACVR2B and, to a lesser extent, the BMP type II receptor BMPR2 [35]. Altogether, this excludes the possibility that due to the FOP mutations ALK2 can form stable complexes with ACVR2A and ACVR2B in response to Activin-A whereas normal ALK2 cannot. The mutations in the GS-domain may alter the interaction of ALK2 with the type II receptors, changing Activin-induced inhibitory complex into an active signaling complex. Interestingly, Hino *et al*. showed an increased binding affinity of radiolabelled Activin-A to ACVR2A, rather than ACVR2B, in ALK2-R206H expressing cells [56]. Of note, the gained Activin-A-responsiveness might occur in a similar manner as described for the epidermal growth factor (EGF) receptor, where a mutation in the intracellular juxtamembrane region was found to increase the EGF binding via an allosteric mechanism [64].

Whereas impaired binding of FKBP12 to ALK2 enhances the amplitude of the signaling response, it does not convert ALK2 in Activin-A-responsive receptor [55]. It should therefore also be considered, that due to the mutations found in ALK2 in FOP patients, this receptor might interact differently with co-receptors compared to normal ALK2. Co-receptors can by binding ligands increase the effective ligand concentration at the cell surface and facilitate the interaction of the ligands with their specific signaling receptors. For example, the GPI-anchored Repulsive Guidance Molecule (RGM) family protein Dragon (also known as RGMb) sensitizes cells to BMPs and enhances intracellular BMP signaling [65]. Whereas in general co-receptors are able to bind multiple ligands, they frequently display a preference in ligand binding and the facilitation of their binding to specific signaling receptors, thereby restricting signaling to occur in cells which express the preferred combination of signaling receptors and co-receptors. Notably, the endothelial-expressed ALK1 is structurally similar to ALK2 and upon activation phosphorylates Smad1, Smad5 and Smad8, but serves besides for BMP-9 also as a type I receptor for TGF-β [66, 67]. In the case of TGF-β-induced ALK1 activation, the TGF-β type I receptor ALK5 mediates recruitment of ALK1 into a TGF-β receptor complex also involving the co-receptor Endoglin [67, 68]. In case of BMPs, Dragon and RGMa were found to mediate signaling of BMP2 and BMP4, but not of BMP-7, whereas hemojuvelin (also known as RGMc) acts mainly as a co-receptor for BMP6 [69,70]. Of interest, Activin-B, but not Activin-A, was recently shown to mediate non-canonical Smad1/5 activation in hepatocytes, which appears to be facilitated by hemojuvilin [71]. Furthermore, the TGF-β family member Myostatin exclusively signals via ALK4 in myoblasts, whereas it has a preference for ALK5 in non-myoblast cells, which is due to expression of the co-receptor Cripto on myoblasts, which is absent on many non-myoblast cells [72]. Whether the acquired responsiveness of mutated ALK2 in FOP to Activin-A is the result of altered

association with co-receptors remains to be determined, but if this is the case it could be that the expression pattern of these co-receptors may explain in part why only specific cell types are able to contribute to HO.

Possible future treatment of FOP

Since the identification of the "FOP gene" in 2006, the obvious target has been the mutated ALK2 receptor. A number of strategies have been developed to normalize ALK2 receptor kinase activity, thereby preventing aberrant signaling causing HO. LDN-193189 is a small molecule inhibitor that inhibits the kinase activity of ALK2 and shown to effectively block HO in ALK2-Q207D mice [39]. Considering the importance of BMP signaling in a wide variety of processes, the use of this or related BMP type I receptor-inhibitors to treat FOP patients could potentially cause undesired side-effects. This might be addressed by developing novel ALK2 inhibitors with little effect on the closely related BMP receptors ALK1/3/6, so BMP ligands can still exert their physiological functions despite the inhibition of ALK2. Nevertheless, the undesired side-effects can be minimalized by giving transient application of the inhibitors in times a flare-up takes place or after elective surgery when heterotopic bone is removed. In addition, siRNA's and antisense oligonucleotides (AONs) have been developed to block specifically ALK2 expression, in order to reduce the possible side-effects [73-75]. Although siRNA's and AONs effectively impaired ALK2 expression in cell culture, it remains to be examined whether they can efficiently be applied to block HO in FOP mouse models or FOP patients. With the recent identification of Activin A as an important ligand for ALK2-R206H in the HO process, Activin neutralizing antibodies and the Activin ligand traps ActRIIA-Fc and ActRIIB-Fc, which potently sequester Activins and weakly BMPs, could be very promising agents to block HO in FOP patients.

Besides targeting the activation or the activity of ALK2, there might be alternative approaches to inhibit HO. Shimono *et al.* argued that since retinoid acid signaling interferes with chondrogenesis, nuclear retinoic acid receptor (RAR) agonists could prevent HO induced by mutant ALK2 [76]. Indeed, they demonstrated that certain RAR-γ agonists, by reducing the Smad protein levels and affecting the cellular differentiation potential, prevent HO in ALK2-Q207D mice. Of additional value might be that retinoic acid has been shown to inhibit expression of Activin in monocytes [59]. This research has led to the development of the only treatment (Palovarotene, NCT02521792) for FOP currently undergoing Phase 2 clinical trials.

Enhanced efficacy might be achieved when potential treatments are being combined. For example, by combining blocking the activation of the mutated ALK2 receptor by means of TGF-β and Activin neutralizing antibodies or anti-inflammatory agents with RAR-γ agonists, the effectiveness of preventing HO to occur upon trauma and inflammation could be maximized. Our current knowledge of the pathology of FOP and the recent development of new mouse models resembling the human disease, as well as patient-derived cell models, *e.g.* induced pluripotent stem cells and subdermal skin fibroblasts, hopefully allows the development of an effective therapy to treat FOP in the near future [53,55,77-79].

Conflicting interests

The authors have declared that no conflict of interests exist.

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Author contributions

DJJdG, GSD and PtD wrote the manuscript. DJJdG made the figure.

Abbreviations

ALK2: Activin receptor-like kinase 2; AONs: antisense oligonucleotides; BMP: Bone morphogenetic protein; FKBP12: 12-kDa FK506-binding protein; FOP: fibrodysplasia ossificans progressiva; GS-domain: glycine-serine-rich domain; HO: heterotopic ossification; NSAIDs: non-steroidal anti-inflammatory drugs; RAR: retinoic acid receptor.

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