Common mutations in ALK2/ACVR1, a multi-faceted receptor, have roles in distinct pediatric musculoskeletal and neural orphan disorders

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Abstract

Activin receptor-like kinase-2 (ALK2), the product of ACVR1, is a member of the type I bone morphogenetic protein (BMP) receptors. ALK2 exerts key and non-redundant roles in numerous developmental processes, including the specification, growth and morphogenesis of endochondral skeletal elements. There is also strong evidence that BMP signaling plays important roles in determination, differentiation and function of neural cells and tissues. Here we focus on the intriguing discovery that common activating mutations in ALK2 occur in Fibrodysplasia Ossificans Progressiva (FOP) and Diffuse Intrinsic Pontine Gliomas (DIPGs), distinct pediatric disorders of significant severity that are associated with premature death. Pathogenesis and treatment remain elusive for both. We consider recent studies on the nature of the ACVR1 mutations, possible modes of action and targets, and plausible therapeutic measures. Comparisons of the diverse – but genetically interrelated – pathologies of FOP and DIPG will continue to be of major mutual benefit with broad biomedical and clinical relevance.

Graphical Abstract

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Conflicts of interest
One of the authors (MP) is a consultant for Clementia Pharmaceuticals.

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1. Introduction

ALK2, the product of ACVR1, is a member of the TGFβ family subgroup of type I bone morphogenetic protein (BMP) receptors that includes ALK1, ALK3 and ALK6 (1). ALK2 partners with type II BMP receptors - including BMPRII and activin receptor type-2A (ACVRIIA) - to form tetrameric complexes that signal mainly via the canonical SMAD1/5/8 BMP signaling pathway in response to ligand binding but can also activate non-canonical signaling such as through p38MAPK (1, 2). ALK2 is able to bind several ligands in vitro such as BMP4, BMP6, BMP7, BMP9 and activins, but the identity and spectrum of its natural ligands in vivo are not clear (3–5). ALK2 exhibits the typical domain structure of ALK receptors that includes a ligand-binding extracellular domain, a transmembrane domain, a juxtamembrane glycine-serine-rich (GS) domain and the large kinase domain (6). Type I receptor kinases are activated by phosphorylation of their GS domain by the type II receptor kinases within the tetrameric complexes. Recent crystallography studies of ALK2 cytoplasmic region (7) have indicated that the kinase domain has a typical bilobal architecture and the GS domain extends from the N-lobe into a helix-loop-helix motif that can bind the endogenous inhibitor FKBP12 (8, 9). Those studies have additionally revealed that FKBP12 prevents access to the regulatory GS loop and inhibits αC movements needed for kinase activation and that the inactive conformation of the region is relatively stable even in the absence of FKBP12 (7), indicating that ALK2 conformation and activity are closely monitored and regulated through multiple mechanisms under normal circumstances.

Acvr1 is expressed in specific spatio-temporal patterns during mouse development (10) and its function appears to be required and non-compensable. Indeed, studies showed that global ablation of Acvr1 arrests mouse embryogenesis at very early stages with a failure of the primitive streak to undergo gastrulation, produce mesoderm and extraembryonic tissues, and express critical mesoderm master genes such as Brachyury (11). Because of such early embryo lethality, conditional mutants were subsequently created to gain insights into ALK2
tissue- and organ-specific function over developmental and postnatal time. For example, Acvr1 ablation in lens-forming head ectoderm via Pax6-Cre was found to lead to undergrowth of lens primordia, abnormal regionalization of lens epithelium, poor expression of lens-characteristic genes, and increased apoptosis (12). Acvr1 ablation in endocardial cushion mesenchyme caused aortic valve defects - including bicuspid aortic valve - that appear to result from substandard specification and differentiation of leaflet precursor cells and an altered balance between canonical (pSMAD1/5/8) BMP signaling and non-canonical p38MAPK signaling (13). By similar approaches, Acvr1 was found to regulate other developmental processes including epithelial-mesenchymal transformation, Mullerian duct regression, and primordial germ cell specification (12, 14), firmly establishing ALK2 as a multi-faceted regulator of embryonic development and growth via direct or indirect action on multiple distinct cell types and tissues and events.

With regard to the musculoskeletal and neural systems that are the focus of this review, several studies have demonstrated that ALK2 regulates several aspects of skeletogenesis and skeletal growth, though less is known about its roles in neural development. Analysis of Acvr1 ablation in neural crest cells via Wnt1-Cre indicated that ALK2 is required for specification and growth of several facial bones, cell proliferation in cartilage, and fusion of palatal shelves, though ALK2 did not appear to regulate neural crest cell migration and survival (15). Acvr1 is strongly expressed in the resting and proliferative zones of the growth plates in developing and elongating skeletal elements, with lower expression in the maturing and hypertrophic zones (5), but the developmental significance and roles of these patterns had remained unclear. Recently, Rigueur et al. ablated Acvr1 in developing mouse embryo cartilage via Col2-Cre (16), and found that although mutants were born at expected Mendelian ratios, they exhibited substandard growth of multiple endochondral skeletal elements including vertebral bodies and the cranial base, accompanied by a reduction in chondrocyte proliferation and lower levels of canonical and non-canonical BMP signaling. Compound Col2-Cre-driven Acvr1/Bmpr1a and Acvr1/Bmpr1b mutants were found to be perinatal lethal and exhibited severe chondrodysplasias (16). These interesting data indicate that ALK2 has a positive, non-redundant and encompassing role in cartilage development, growth and function and exerts coordinated activity with BMPR1A and BMPR1B during initiation, growth and morphogenesis of the endochondral skeleton.

Central and peripheral nervous system development and growth are processes of considerable complexity that involve multipotent progenitors, distinct cytodifferentiation pathways, local and systemic influences, and complex morphogenetic processes (17). Studies dating back over 2 decades have provided clear evidence that BMP ligands and BMP pathway signaling have multiple and dynamic roles in neural stem cell fate and differentiation and in overall CNS development and maintenance (18, 19). Inhibition of BMP pathway signaling is required for specification and formation of the neuroectoderm, while BMP signaling is needed to induce the neural crest (20–22). BMP signaling patterns and gradients establish the dorso-ventral axis of the spinal cord, influence specification and morphogenesis of forebrain and cerebellum and their native cell types, and regulate neuronal subtype identity and neurite outgrowth (23–25). Of particular relevance to this review are studies showing that Acvr1 is expressed in many tissues in mouse embryos including the
cerebral hemispheres, medulla and thalamic regions, with little or no expression in the pons, a region that is rich in Acvr1B (Alk4) transcripts (10, 11, 26). Also relevant are subsequent studies on astrogliogenesis and neuronal cell lineage commitment (27). BMP signaling has been found to promote astroglial cell differentiation via a STAT3-p300/CBP-Smad1 complex cascade involving both canonical and non-canonical signaling pathways (28–30). Oligodendrogenesis and neurogenesis are promoted by basic helix-loop-helix (bHLH) transcription factors including Olig1, Olig2, Mash1, neurogenin and NeuroD (31). In neural precursors, BMP signaling has been found to stimulate expression of Id1, Id3 and Hes-5 that, by virtue of their ability to inhibit bHLH transcription factors, allow BMP signaling to stimulate astroglial commitment and differentiation while inhibiting neural cell differentiation (32).

This brief synopsis makes it clear that BMP proteins, receptors and signaling play important roles in musculoskeletal and brain development, growth, morphogenesis and postnatal function. It is not at all surprising then that aberrations in such key regulatory mechanisms cause developmental problems and pediatric disorders. In this review, we focus on Fibrodysplasia Ossificans Progressiva (FOP) and Diffuse Intrinsic Pontine Glioma (DIPG), distinct pediatric disorders of significant severity that are almost invariably associated with premature death. Intriguingly, many FOP and DIPG patients bear common activating mutations in ACVR1, raising questions about shared pathogenetic mechanisms of these diseases and the effects of, and responses to, common mutations in distinct cell types and populations.

2. Fibrodysplasia Ossificans Progressiva

FOP is a rare and extremely severe genetic musculoskeletal disorder with an incidence of about 1 in 2 million. The disease is characterized by episodic formation of extraskeletal bone tissue – a processed termed heterotopic ossification (HO) - at multiple sites that accumulates over time and eventually involves much of the body (Fig. 1) (33). As a result, the accumulated ectopic tissue causes several health problems and impediments - including skeletal deformities, chronic pain, growth impairment and joint ankylosis - that increasingly interfere with basic daily functions and can lead to breathing difficulties and premature death (34). The HO process in FOP patients can be triggered by injury or small traumas, but is most often spontaneous and initiates with a local “flare-up” characterized by swelling, redness and pain (35). Because of the reactive nature of FOP, surgery to remove the HO lesions or correct skeletal defects is strictly avoided. In addition to excluding surgery as a treatment option, this also limits access to surgical retrieval specimens for diagnostic and prognostic purposes and pathogenesis studies. However, specimens have occasionally become available over the years either from misdiagnosed interventions or autopsies. Analysis of such samples has shown that formation of the heterotopic bone typically involves a recapitulation of the endochondral ossification process (36) by which most skeletal elements including long bones, ribs, vertebrae and cranial base form and grow during embryogenesis (37). As such, the process of HO formation involves the recruitment, condensation and proliferation of skeletal progenitor cells; their differentiation into chondrocytes; organization of growth plate-like structures permitting maturation and hypertrophy of the chondrocytes themselves; recruitment and invasion of blood vessels and
associated osteoprogenitors; and finally replacement of hypertrophic cartilage with endochondral bone (37). Longitudinal x-ray monitoring of the HO lesions suggests that the ectopic bone masses reach a given size and then stop growing, likely following the completion of the cartilage-to-bone transition and/or physically limited for example within the bounds of the fascia surrounding skeletal muscle.

2.1 ACVR1 genetic mutations and FOP clinical presentation

To date, a total of 13 heterozygous ACVR1 missense mutations and one 3-bp deletion (eliminating two amino acids and inserting a different amino acid) at highly conserved positions have been described in FOP patients (Fig. 2) (38–41). About half of the mutations occur in the GS activating domain and the reminder occur in the protein kinase domain, with none reported so far in the transmembrane or extracellular ligand binding domains. Protein modeling and direct functional analyses have indicated that all the mutations are gain-of-function and slightly increase basal ALK2 signaling and downstream transcriptional activity (33, 35). However, the clinical presentation and consequences of these mutations vary, suggesting genotype-phenotype correlations and characterization of FOP patients in distinguishable subgroups (41).

Patients with a “classic” clinical presentation of FOP all bear the identical heterozygous mutation (c.617G > A; p.R206H) and share several clinical features including and early onset of HO before age 10 and malformation of great toes (hallux valgus), together with a frequent incidence of proximal tibial osteochondromas and cervical spine malformations (42). Within this group carrying the R206H mutation, phenotypic variability – such as in age of HO onset and rate of HO progression - has also been noted. Some patients with the R206H mutation and one patient with a Q207E mutation were reported to have additional clinical problems such as significant growth retardation, cataracts and aplastic anemia (41).

Even greater phenotypic variability is seen among patients who have non-R206H ACVR1 mutations. For example, patients with the G328W or G328E mutation have very early onset HO, and instead of the characteristic specific great toe malformation, have reduction of multiple digits and thumb malformations, in addition to mild cognitive impairment and sparse scalp hair (41). Conversely a mild clinical phenotype was found in a patient with a G325A mutation who displayed the great toe malformation, but first developed HO at age 47 (43).

It remains unclear exactly how and why different mutations elicit distinct clinical presentations and trigger different pathological effects on skeletal and non-skeletal tissues and organs. Studies with very rare identical twins with FOP indicate that the environment affects the course of HO in a given patient and is a major influence on disease onset, progression and severity, in addition to the influences of genetic factors and the specific ACVR1 mutation (44).

Because FOP is usually severe and limits reproductive capacity and life expectancy, most cases are sporadic and are thought to arise from germline mutations that would begin to exert their pathogenic effects starting from embryogenesis, as depicted by the great toe malformation visible at birth. However, it is also possible that some patients may have
acquired their ACVR1 mutation at a later embryonic or neonatal point and thus be mosaic; this could potentially be the case for patients without the characteristic skeletal malformations or with late-onset HO and/or very mild forms of the disease.

Protein crystallography and biochemical studies have examined the basis and mode of action of several of the FOP-associated ALK2 mutants, with particular attention given to the R206H mutation seen in classic FOP (7, 45–48). Plasmid-driven protein expression and reporter assays in cultured C2C12 cells for ten ALK2 mutants— including L196P, R206H, Q207E, G328E, P197_F198delinsL, G328R and R375P —were all found to display mildly-elevated basal activity in the absence of exogenous BMP treatment compared to wild type ALK2 (7). Such leaky activity was generally higher in GS mutants than kinase domain mutants. Interestingly, several mutants, including R206H and G328R, were still responsive to, and influenced by, their endogenous inhibitor FKBP12 as indicated by increased signaling activity after treatment with the FKBP12 antagonist FK506. One exception was the GS mutant L196P whose mildly-elevated basal activity was not further enhanced by FK506 treatment (7, 49), consistent with the very mild form of FOP exhibited by patients with this mutation (50). Structurally, the FOP mutations spatially center around the GS domain and the neighboring ATP pocket in the kinase domain (7, 47). The affected residues normally maintain the α-helical structure of the GS domain as well as critical bonds between the GS domain and the kinase N-lobe that are needed to stabilize the inactive conformation of ALK2, thus likely destabilize that conformation, altering its sensitivity to FKBP12 and eliciting an enhanced basal signaling activity. A partial loss of auto-inhibition had been initially proposed to explain the action of FOP ALK2 mutants (41, 48) and reduced binding of mutant ACVR1 to FKBP12 has been experimentally supported (51).

Given the nature of the mutations, it was of interest to ask whether the gain-of-function ALK2 mutants still require cooperation with BMP type II receptors and/or phosphorylation of their GS domain by the type II receptors to express their leaky activity, as is required by wild type ALK2. To approach these questions, Bagarova et al. (43) expressed plasmid-driven wild type ALK2, ALK2R206H or ALK2Q207D, the latter being an engineered constitutive-active mutant (52), in wild type mouse cells and in cells deficient in BmpRII and/or ActRIIa. Reporter assays showed that both GS mutants increased basal signaling activity in both wild type cells and those deficient in either type II receptor, but not in cells deficient in both receptors, supporting the idea that ALK2 mutants require cooperation with type II receptors for activity. Interestingly, however, the ALK2 mutants did not appear to require type II receptor kinase activity nor ligand binding activity to elicit their enhanced basal action, providing further evidence that the GS mutations destabilize the inactive configuration of ALK2 and render it leaky, independently of ligand binding and phosphorylation.

2.2 ACVR1 genetic mutations and endochondral bone formation

As noted above, the HO process in FOP closely mimics endochondral ossification in which progenitor cells undergo chondrogenesis and cartilage formation, become organized into growth plates and are then replaced by bone and marrow (36). Thus, it is pertinent to ask whether the ALK2 mutants have enhanced chondrogenic and osteogenic capacity and
whether an ALK2 mutation is sufficient to cause HO and FOP. A first study addressing these key questions was carried out with the constitutive-active ALK2<sup>Q207D</sup> mutant (52) using a virally-driven over-expression approach in chick embryo limbs (5). ALK2<sup>Q207D</sup> over-expression was found to cause significant over-growth and lateral expansion of the developing cartilaginous elements and led also to fusion of synovial joints, a process previously seen in mutants such as Noggin-null mouse embryos which form excess cartilage at the prospective location of the joints (53). At the cellular level, ALK2<sup>Q207D</sup> over-expression increased the rate of chondrocyte maturation and hypertrophy and caused higher levels of expression of the key growth plate signaling protein Indian hedgehog (54), indicating again that cartilage cells are quite sensitive and respond positively and exuberantly to mutant ALK2 action. In line with these results, van Dinther et al. found that plasmid-driven over-expression of the FOP mutant ALK2<sup>R206H</sup> in COS cells not only enhanced basal BMP/SMAD reporter activity, but also rendered the cells more sensitive to osteogenic cell differentiation and mineralization in response to exogenous BMP treatment compared to cells over-expressing wild type ALK2 (55). They also found that human mesenchymal stem cells that over-express ALK2<sup>R206H</sup> and are implanted subcutaneously in nude mice produced more ectopic bone than cells expressing control ALK2. In good agreement, one of us reported recently that Alk2<sup>R206H</sup> expression in mouse embryo fibroblasts enhanced their chondrogenic differentiation and responsiveness to BMP treatment; when implanted in vivo, the Alk2<sup>R206H/+</sup> cells initiated robust HO and stimulated recruitment and participation of surrounding wild-type cells in the process (56). Interestingly, additional data in the study showed that ablation of Alk2 at the early commitment stage of differentiation compromised the ability of the progenitor cells to undergo chondrogenesis, reiterating Alk2’s importance and non-redundant function.

Though revealing and instructing, in vitro studies cannot address the related question whether an FOP mutation is sufficient to elicit the FOP phenotype. Accordingly, knock-in mice with the classic R206H mutation inserted in the <i>Acvr1</i> locus were developed by one of us recently and were extensively phenotyped for FOP-associated features (57). Radiographic and anatomical analyses of founder generation chimeric <i>Acvr1<sup>R206H/+</sup></i> mice showed that the mutation induced malformation of the first digits in the hind limbs and postnatal extraskeletal bone formation, thus closely recapitulating the key characteristic features of classic FOP. Further histological and molecular analyses showed that the HO process was preceded by local inflammatory cell infiltration, apoptosis of skeletal muscle cells, a robust fibroproliferative response, followed by endochondral ossification, and that cells with a Tie2+ lineage (a marker of endothelial cells and muscle interstitial progenitor cells) participated in the formation of extraskeletal tissue. In addition to extensive spontaneous HO formation, this mouse model also formed HO in response to injury. The study thus provided clear evidence that the classic R206H mutation is sufficient to cause an overall phenotype in mice that very closely resembles FOP, and mimics the pathogenic steps and phases leading to HO. These data and conclusions agree with other studies showing that the engineered constitutive-active Q207D mutation can also provoke robust and even severe HO in mice expressing a conditionally-inducible <i>ACVR1</i><sup>Q207D</sup> transgene (52, 58, 59).
Taken together, these studies have provided strong support for the notion that ALK2 normally acts as an important and direct regulator of commitment of skeletogenic progenitors and their differentiation into cartilage and bone. Its mutant and activated forms can, and do, favor and propel these processes, thus playing an instigating role in HO and a causative and sufficient role in FOP.

2.3 FOP current and potential treatments

Almost invariably, HO in FOP patients is preceded by local inflammation in the form of a spontaneous flare-up or following tissue injury and trauma that involves swelling, pain, erythema, stiffness and accumulation of mast cells and other immune cells (33). Because such an initial inflammatory phase is likely to set the stage for, and instigate the onset of, HO, the current standard of care for FOP patients is a brief 4-day course of high-dose corticosteroids (60). Treatment is normally started within the first 24 hours of a flare-up and aims to reduce the inflammation and edema and in turn, the likelihood of progression to HO. In an analogous manner, non-steroidal anti-inflammatory drugs (NSAIDs) are also considered an option as maintenance medications for FOP patients (60). These drugs inhibit the synthesis of both physiologic and inflammatory prostaglandins (61), and animals pretreated with prostaglandin synthesis inhibitors were originally shown to be resistant to experimental HO induced by intramuscular injection of BMPs (62). Though the steroid and non-steroid anti-inflammatory treatments can and do mitigate inflammation, swelling and pain, they have not been found to consistently reduce the frequency of progression to HO in FOP patients.

The extracellular matrix in musculoskeletal tissues contains and stores a large amount of growth factors, and many studies have indicated that the release of these factors by the action of osteoclasts stimulates osteoblast function and couples bone resorption to bone formation (63, 64). This line of evidence and other considerations have led to the indication that aminobisphosphonates could represent a possible treatment for FOP (60, 65, 66). In addition to interfering with osteoclast function and survival, and in turn bone formation, this class of drugs also has strong anti-angiogenic effects in the context of cancer (67). Because angiogenesis is required during the transition from hypertrophic cartilage to bone (68), the aminobisphosphonates could benefit FOP by blocking this needed developmental process during a similar transition that occurs within the heterotopic cartilaginous-bony tissue mass. Experimental studies showed that systemic treatment with bisphosphonates inhibits HO in trauma- and BMP4-induced mouse models (69). These studies indicated also that the drug treatment reduced presence and activities of monocytes within the lesion area, and there is long standing evidence that monocytes, mast cells and other immune cells may be an integral part of local mechanisms by which HO is instigated and initiated in FOP (70, 71). Potentially then, aminobisphosphonates could interfere with HO at a variety of regulatory levels. Despite these compelling findings and studies, however, the extent to which the drugs represent an effective and safe treatment for FOP remains unclear, even when used in combination therapy to protect the skeleton from the osteopenic effects of high-dose glucocorticoids (72).
The discovery of activating ACVR1 mutations in FOP patients (48) has since propelled research into how such aberrant activity could be blocked or at least reduced, thus counteracting what is undoubtedly the key regulatory pathogenic change in the disease. Conceivably, this could be achieved by blocking the natural BMP ligands for ALK2 or additional ligands possibly utilized by mutant ALK2, interfering with the leaky signaling activity of mutant ALK2 by either suppressing its kinase activity or restoring its normal configuration and interactions via specifically-designed drugs, or counteracting the differentiation steps of progenitor cells recruited to the prospective HO site. In line with the second possibility, Yu et al. reported compelling findings that the selective inhibitor of BMP type I receptor kinases LDN-193189 significantly reduced HO in the limbs of transgenic mice carrying an inducible and constitutive-active ACVR1Q207D transgene (59).

LDN-193189 is a compound optimized for pharmacokinetic characteristics and stability (73) and is a modified version of Dorsomorphin, a previously identified inhibitor of ALK2, ALK3 and ALK6 and their canonical SMAD1/5/8-mediated signaling activity (74). Yu et al. found that the drug treatment reduced phosphorylation and activation of SMAD1/5/8 in the affected tissues expressing ACVR1Q207D and also reduced functional impairment and maintained limb function. In follow-up studies, this group and collaborators developed a more potent inhibitor of the type I receptor kinases – LDN-212854- that displayed a nearly 4-fold higher selectivity for BMP versus related Activin and TGF-β type I receptors compared to previous kinase inhibitors (75, 76). They showed that LDN-212854 exhibited some selectivity for ALK2 compared to ALK1 and ALK3 in cultured cells and as importantly, inhibited HO in the inducible ACVR1Q207D transgenic mouse model. These studies are quite promising and exciting and point to the possibility that type I receptor kinase inhibitors with the necessary specificity and selectivity can be created to be effective against HO and with the appropriate safety profile in FOP patients.

To create a strategy to interfere with cellular processes and differentiation during HO, one of us and collaborators focused on the early developmental steps of HO in which the progenitor cells recruited to the putative HO site are induced to undergo chondrogenesis and cartilage formation by the combined action of mutant ALK2 and other as yet unknown mechanisms (36). We focused on the chondrogenic phase because it is an obligatory early step in endochondral ossification (37) that if blocked, would in turn prevent the subsequent formation of bone and thus halt the overall HO process on its tracks. There are several experimental ways by which chondrogenesis could be inhibited, including genetic and pharmacologic means (37). We targeted the retinoid signaling pathway and its nuclear retinoic acid receptors (RARα, RARβ and RARγ) (77) for the following reasons. Biological and genetic studies had previously demonstrated that chondrogenic cell differentiation in vitro and chondrogenesis in vivo normally require a steep decrease in both endogenous retinoid signaling and RAR expression (78, 79), and that exogenous retinoid agonists are very potent inhibitors of chondrogenesis (80). In two recent studies, we found that synthetic retinoid agonists for RARα and RARγ given systemically by oral administration or injection were effective inhibitors of HO in mouse subdermal and intramuscular injury models and the genetic ACVR1Q207D-inducible transgenic mouse model, with the RARγ agonists being far more effective (58, 81). In vivo and in vitro evidence showed that the retinoid agonists had blocked the chondrogenic phase of HO and showed no significant rebound effect over
time after end of treatment. Mechanistically, the agonists appeared to act by not only blocking expression of master chondrogenic genes such as Sox9, but also greatly diminished canonical pSMAD1/5/8-mediated BMP signaling and promoted SMAD degradation via the proteasome (58). The latter data fit well with previous evidence that there is an antithetical relationship between retinoid signaling and BMP signaling during chondrogenesis and that the normal pro-chondrogenic roles of BMPs in cartilage development and skeletogenesis depend on the attenuation of endogenous retinoid signaling (82, 83). In related studies, we found that retinoid agonists increase the activity of the Wnt/β-catenin signaling pathway (84) which is known for its strong anti-chondrogenic action (85, 86), implying that the retinoid agonists block chondrogenesis and HO by concurrently inhibiting BMP signaling and stimulating Wnt/β-catenin signaling. One of the most effective RARγ agonists was Palovarotene, a drug that was previously tested in a phase 2 clinical trial for a different chronic condition and found to be safe (87). Based on our studies, this repurposed drug is now being tested in a FDA-approved phase 2 clinical trial with FOP patients that was initiated by Clementia Pharmaceuticals in July 2014 (ClinicalTrials.gov identifier NCT02190747). The trial is expected to last 2 years and given its double-blind and placebo design, should provide definitive evidence as to whether Palovarotene is an effective and safe remedy against HO in FOP.

Interestingly also, a just published study has reported evidence of a new aspect of the pathogenesis of HO in FOP (88). Using in vitro systems and a conditional knock-in ACVR1R206H FOP mouse model, the authors found that cells expressing mutant ALK2R206H responded to activin A, a normally antagonistic ligand for wild type ALK2, and signaled via canonical pSMAD1/5/8. Treatment of FOP mice with neutralizing antibodies to activin A reduced the extent of HO formation. Although it remains to be established whether activin A antibodies could stop HO in FOP patients and do so safely, the study is exciting and establishes a concrete link between inflammation and HO, given that activins are often produced by inflammatory cells (89). Note that since Palovarotene and other retinoid agonists block canonical BMP/SMAD1/5/8 signaling (58, 82), Palovarotene is expected to inhibit HO also by blocking BMP signaling induced by activin A via mutant ALK2, supporting its encompassing potency regardless of the nature of HO-inciting stimuli.

3. Diffuse Intrinsic Pontine Glioma (DIPG)

Brain tumors are the most common form of solid tumors affecting children and amount to about 20% of all pediatric cancers (90). The tumors can develop at any site within the brain, have diverse histopathological characteristics and phenotypes, and can remain benign or transition to malignancy over time (91). Brainstem gliomas represent a specific subgroup of brain tumors. About 15 to 20% of them are low-grade astrocytomas that include dorsally exophytic, cervicomedullary and focal brain gliomas, are characterized by specific biological and cellular traits including relatively slow cell growth, and often follow an indolent course with favorable survival for years or even decades (91, 92). The remaining 80 to 85% of brainstem gliomas are diffuse, occupy the ventral pons, have a peak age in middle childhood (6–7 years) and are currently referred to as diffuse intrinsic pontine gliomas (DIPGs) (93). Their histological classification as WHO grade IV reflects the fact that their cells display pleomorphic cyt架构ure and structure, high density and nuclear atypia that
are associated with high mitotic activity in conjunction with microvascular proliferation (94). The tissue also can display areas of necrosis. DIPG occurs at an incidence of approximately 1 in a million with a very poor prognosis, leading to death within 1 to 2 years from diagnosis; DIPG is the main cause of death among all pediatric brain tumor patients (91). The understanding of DIPG biology and pathogenesis has been limited in the past due to the paucity of tumor tissue since biopsies were avoided due to the forbidding anatomical location of the tumors, along with morbidity and mortality concerns; however, the specific peak age of the patients and the specific anatomical location of the tumors hinted to abnormalities in postnatal neuro-developmental mechanisms (95). More recently, biopsy procedures that pose little to no concern have been developed, and rapid retrieval of autopsy material is an effective method to obtain DNA and RNA samples and cells to establish primary cultures and cell lines (96–98). Autopsy samples could harbor additional genetic changes due to exposure to radiation and/or chemotherapy by the patients that would not be present in biopsy samples (99). Nonetheless, these complementary technical, organizational and regulatory changes have paved the way to the recent major advances in understanding on DIPG biology and pathophysiology and to testing functionally defined therapeutics.

3.1 Diverse genetic aberrations in DIPGs, including ACVR1 mutations

A number of studies have demonstrated that DIPGs are characterized by gross chromosomal aberrations and imbalance, including gains at chromosomes 2, 8q and 9q, and by focal gene amplifications and deletions. Amongst the most common amplifications are those affecting PDGFRA and the receptor tyrosine kinases MET, IGF1R and ERBB4 some of which occur simultaneously within the same tumor (99–101). Focal amplifications are also seen in genes involved in the PI3K signaling pathway, including HRAS, AKT1 and PIK3CA, and genes within the retinoblastoma (Rb) pathway such as CCND1, CCND2 and CDK4, with an overall frequency of about 40% in each cohort of patients. Deletions in the tumor suppressors CDKN1C and CDKN2A/2B have been observed in some cases (99–101).

Genome sequencing analyses of biopsy and autopsy samples more closely examined the genomic landscape of DIPG. Somatic heterozygous mutations in histone H3 isoforms, which characterize pediatric high grade gliomas (pHGGs), were found to be more frequent in DIPG tumors, with the specific missense K27M mutation in H3F3A seen in about 60% of the cases (102, 103). Mutations in the same codon were also found in the histone H3.1 gene (HIST1H3B) with a frequency of about 20% of cases. Although comparisons of different cohorts of pHGG and DIPG patients revealed some slight differences in the frequency of H3F3A versus HIST1H3B mutations, the H3.1 mutations were more characteristic of younger children and were largely restricted to DIPG (102–104). Both H3.3 and H3.1 mutations are thought to have a dominant-negative effect on the total histone H3 pool and lead to DNA hypomethylation by inhibition of the H3K27 methylase EZH2 and broad changes in gene expression (105).

Recent whole genome, whole-exome and transcriptome comparisons revealed that, in addition to somatic histone H3 mutations, other genetic changes are characteristically found in 50–60% of DIPG tumors including structural variants generating fusion genes, as well as mutations in genes involved in histone modification, chromatin remodeling and cell cycle
regulation (106). Mutations in the tumor suppressor protein TP53 are also very common in DIPG with an incidence of 40 to 50% of cases (107).

Of particular relevance here are four recent independent reports that collectively examined over 200 DIPG patients and showed that recurrent heterozygous somatic non-synonymous mis-sense mutations in ACVR1 occurred in about 33% of DIPGs (70/209), but were absent in non-brainstem high-grade gliomas (Fig. 2) (106, 108–110). Seven amino acid substitutions were identified: R206H and Q207E in the glycine-serine-rich (GS) domain; and R258G, G328E, G328V, G328W and G356D in the protein kinase domain (Fig. 2). With the exception of G328V, all these mutations are identical to ACVR1 mutation identified in FOP patients (Fig. 2). Of note, the ACVR1R206H mutation occurs in more than 95% of FOP patients but is more rare in DIPG tumors, while the other ACVR1 variant mutations, often associated with more severe forms of FOP, are more common in DIPG tumors.

In the study by Wu et al. (106), the six ACVR1 mutations examined were found to be significantly associated with younger patient age, longer patient survival time, and presence of K27M H3.1 (HIST1H3B) mutation or PIK3CA or PIK3RI mutations, indicating that they may characterize a distinct subset of DIPG patients. As described above for FOP ACVR1 mutations, the affected residues in the mutant DIPG ACVR1 proteins normally maintain the α-helical structure of the GS domain and critical bonds between GS domain and kinase N-lobe that are needed to stabilize the inactive conformation of ALK2. Each ACVR1 mutant was found to increase basal canonical BMP pathway signaling (pSMAD1/5) when over-expressed in mouse primary astrocytes in culture. Because a similar response is seen in skeletal cells (7, 45, 46), the effects of ACVR1 gain-of-function mutants are clearly dominant with regard to signaling and not cell type-dependent, at least for mesenchymal and neural cells. However, the ACVR1 mutants alone failed to impose a tumorigenic phenotype on Tp53-null mouse astrocytes when transplanted in the brain of host mice, indicating that the mutations are not tumorigenic per se but would exert roles in concert with other DIPG mutations.

In the study by Buczkowicz et al. (108), two of the ACVR1 mutants tested (R206H and G328V) elicited increased proliferation when over-expressed in brainstem cell progenitors isolated from Nes-tv-a;Tp53fl mice. Interestingly, while the ACVR1 mutants induced pSMAD1/5 phosphorylation and expression of BMP pathway target genes ID1 and ID2, the latter genes were also induced by over-expression of K27K H3.3 mutant and even more so by K27K H3.3 plus ACVR1 mutant co-expression, suggesting possible additive effects and gene interactions in regulating cell behavior, differentiation and tumorigenesis.

Taylor et al. (110) found that the ACVR1 mutations co-segregated with the histone H3.1 mutant, were twice as frequent in female patients, and correlated with longer overall patient survival times. The authors prepared DIPG case-derived primary cultures (two of which harbor ACVR1 mutations) and found that treatment with the selective ALK2 antagonist LDN-193189 (59) caused marked decreases in both pSMAD1/5-mediated BMP signaling and cell viability in all cultures, indicating that the ACVR1 mutations did not provide any major cell survival advantage at least within such experimental context.
Lastly, Fontebasso et al. (109) also observed an association of $ACVRI$ mutations with $H3.1$ rather than $H3.3$ K27M mutation and detected a strong increase in pSMAD1/5/8 immunostaining in DIPG brain samples compared to controls. They also found that primary DIPG cells carrying $H3.1$ K27M and $ACVRI$ G328V mutations exhibited higher pSMAD1/5/8 levels and higher $ID1$, $ID2$, $ID3$ and $SNAI1$ gene expression than cells with $H3.3$ alteration and wild type $ACVRI$.

### 3.2 Significance and possible consequences of $ACVRI$ mutations

While mutations in $ACVRI$ are necessary and sufficient to cause FOP, it appears that the somatic $ACVRI$ mutations are neither necessary nor sufficient in DIPG, although their high incidence undoubtedly points to possible major roles. As indicated and discussed in the recent DIPG genomic studies (106, 108–110), the $ACVRI$ mutants must be part of far more complex pathogenic mechanisms and genetic interactions that eventually trigger abnormal cell behavior and elicit tumor initiation and growth (see schematic in Fig. 3). Based on the biology of $ACVRI$ and mechanisms known to regulate its function in various developmental processes including brain development, several possibilities can be considered.

The mouse embryo studies cited above indicate that $Acvr1$ expression normally characterizes the developing brain hemispheres and other brain structures but not the pons, indicating that aberrant $ACVRI$ expression in the pons of DIPG patients in conjunction with activating mutations could derange the behavior of pons cells, altering their phenotype – for example via expression of ID genes and broad genome demethylation - and ultimately contributing to their malignant pleiotropic appearance and behavior. The pons normally expresses $ACVRIB$ ($ALK4$), a type I receptor usually associated with TGFβ pathway (pSMAD2/3) signaling (111). If such expression were maintained in DIPG, the combination of mutant $ACVRI$ plus endogenous $ACVRIB$ could alter signaling and shift the balance between BMP and TGFβ signaling. A precedent for this possibility is found in the case of $ALK5$ which normally signals via pSmad2/3 but can signal via pSmad1/5 in association with $ALK1$ (112). BMP pathway signaling normally promotes astroglial cell differentiation via a STAT3-p300/CBP-Smad1 complex cascade involving both canonical and non-canonical signaling pathways (28–30) and thus, an imbalance of those and related pathways could derange the differentiation or differentiated status of local cells and contribute to growth and neoplastic behavior (113).

Genetic cell tracing and tracking have recently become a very popular tool to decipher the origin of progenitor cells and their roles in the formation of distinct tissues and organs during embryogenesis, the mechanisms regulating such complex cell developmental decisions and lineage assignment, and possible aberrations in these fundamental processes in pathologies including FOP (114–117). These powerful approaches have not been used extensively in DIPG research so far, but one notable exception is the study by Monje et al. (118). These authors first analyzed the midbrain, pons and medulla of postnatal human brains from age 0.3 to 18 years and found that immunodetectable Nestin+ progenitor cells (119) were specifically located in the pons in infancy, peaked in number around age 6, but dwindled with time. About 50% of pons-associated Nestin+ cells also expressed the HLH transcription factor $Oligo2$ which is involved in divergence of neural progenitor.
differentiation paths (31). Similar Nestin+/Oligo2+ cells were seen in the ventral pons region in young mice, and the cells exhibited also strong expression of Sox2, a marker of progenitor cells (120), and strong signaling by Sonic hedgehog which is known to regulate Oligo2 expression during neural development (121). Ectopic hedgehog signaling led to a burst of Oligo+ progenitor cell proliferation in the pons, leading the authors to propose that these neural progenitor-like cells may represent a “cancer stem cell population” in DIPG and that hedgehog signaling could be an important (though not sufficient) pathogenic driver as well as a therapeutic target. Numerous studies have shown that the hedgehog signaling pathway works in close concert with the BMP signaling pathway to regulate numerous and major developmental processes and events (122). For instance, Acvr1 induces and regulates expression of Indian hedgehog which in turn controls chondrocyte proliferation and maturation during skeletal development (5). Hedgehog protein gradients have been found to differentially interact with BMP signaling to regulate expression of the homeobox gene engrailed2a and the balance between proliferation and differentiation in myotomal progenitor cells (123). Thus, it is possible that aberrant expression of mutant ACVR1 in DIPG could interact with basal - or in some cases upregulated (124) - hedgehog signaling and contribute to maintaining cells located in the most ventral pons region in a proliferative state, contributing to propagation of their stem/progenitor phenotype and ultimately sustaining tumor growth. Such local and niche-restricted actions and interactions have been observed in other systems as exemplified by Sonic hedgehog-mediated induction of proliferation of primitive hematopoietic cells via BMP signaling (125).

In a similar vein, it is well established that the BMP pathway exerts positive influences on the expression of HOX genes in a variety of developmental systems and processes. For example, dorsal BMP expression and signaling regulate Hox gene expression in the mammalian developing neural tube (126), and BMP action initiates Hox expression during early Xenopus embryogenesis (127). Interestingly, several HOX genes are up-regulated in both DIPG and pHGG, including HOXA9, HOXA10, HOXA2 and HOXA3 (128–130). HOX expression has been suggested to be linked to mutant histone H3-dependent and PI3K-dependent DNA de-methylation, to elicit a glioma stem-like cell phenotype on local cells, and exert anti-apoptotic and pro-proliferative effects (100, 130). Given the usually positive influence of BMP signaling on HOX expression, it is possible that ectopic mutant ACVR1 expression in DIPG may reinforce these aberrant HOX expression patterns and contribute to sustain tumor growth and/or tumor stem-like cell phenotype.

3.3 DIPG therapeutic remedies

Recent comprehensive reviews have provided details and insights on, and critical assessment of, previous and novel therapeutic strategies that are being tested to treat DIPG and improve the currently dismal patient survival rates (91, 93, 100). Radiotherapy has long been used to treat adult HGG with considerable survival success and, in the absence of other options, has become the standard therapy for pHGG and DIPG with, unfortunately, limited success (91). Effectiveness of chemotherapy and combination chemotherapy have also been studied in all these disorders (131). Novel strategies are attempting to exploit the molecular information stemming from the genomic analyses described above. Accordingly, drugs are being tested singly or in combination with other drugs/irradiation to reverse DNA
hypomethylation (a major example being Temozolomide), block receptors including IGF1R, PDGFR and VEGFR, or interfere with signaling pathways such as PI3K/mTOR, Ras/Raf/MEK and CDK (132). Antibody and cell-based therapies are being studied as well. To identify functionally defined therapeutic drugs, a very recent and exciting study screened chemical drug libraries using patient-derived DIPG cell cultures and combined this approach with RNA-seq analysis and informatics and computational modeling (133). This comprehensive study led to the identification of the multi-histone deacetylase inhibitor Panobinostat. The drug markedly reduced viability and proliferation of DIPG cells in vitro, and inhibited growth of DIPG orthotopic xenografts implanted into the skull of host mice and extended mouse survival, particularly in combination with the histone demethylase inhibitor GSK-J4. The recent identification of ACVR1 activating mutations in DIPG has led to the suggestion that interference with BMP signaling could represent an additional therapeutic target and provide for an even more comprehensive and effective combination therapy (106, 108–110).

The task of finding an effective treatment is daunting given the sheer complexity and multiplicity of the genetic alterations in DIPG. The lack of a faithful mouse models is certainly a major hurdle in deciphering the exact pathogenesis and natural history of DIPGs. In particular, what is needed is a better understanding of the temporal and spatial occurrence and sequence of genetic alterations and most importantly, what hierarchical position each alteration occupies with respect to the overall gene mutation landscape and to tissue and local cell pathogenic behavior. Based on differences in genomic landscape within one of the studies above, DIPG patients were suggested to represent three separate molecular subgroups (108). This is certainly possible, but it may also be that these subgroups represent steps along a pathogenic continuum and may have possessed, or would acquire, different genomic traits and signature over time. In this regard, the suggestion noted above that inhibition of excess signaling by mutant ACVR1 could benefit DIPG is understandable, but what is not known yet is exactly what mutant ACVR1 actually does in DIPG and whether its function changes with time, affects different subpopulations differently, and/or is affected by the specific genomic landscape at any given time. It is tantalizing that two of the above four studies actually reported longer survival times of DIPG patients bearing ACVR1 mutations (106, 110). This makes one wonder whether these mutations are indeed pathogenic or may actually have or acquire some protective value.

4. Conclusions and perspectives

FOP and DIPG are diametrically distinct diseases and yet, they share the unfortunate facts that they are not currently preventable, are fatal, have a similar incidence, and become evident in early infancy. FOP children do not have DIPG although neurological symptoms have been observed in some FOP patients including allodynia, neuropathic pain, myoclonus and mild cognitive impairment (134, 135), and a recent report has described an FOP patient with abnormal soft tissue surrounding the brainstem leading to obstructive hydrocephalus and bilateral dentate lesions (136). Likewise, DIPG patients do not have FOP or obvious manifestations of heterotopic ossification. Germline ACVR1 mutations during early development drive skeletal and developmental features of FOP and embryonic and postnatal expression in mesenchymal tissues lead to HO, whereas somatic and local mutations in
ACVR1 add to the multiple genomic alterations and mutation burden in DIPG pathogenesis. As pointed out above, potent and potentially effective drug treatments for FOP have recently been discovered using genetic and injury mouse models of the disease (58, 74, 88), and we ourselves are engaged in the FDA-approved phase 2 clinical trial mentioned above to test the efficacy of the retinoic acid receptor γ agonist Palovarotene in FOP. A main mechanism by which Palovarotene blocks extraskeletal endochondral ossification is by potent inhibition of BMP signaling pathway (58). Thus, should ACVR1 mutations be established as pathogenic in DIPG, one could certainly consider testing Palovarotene to counter the excessive BMP signaling in DIPG. On the other hand, ACVR1 mutations may be determined to be protective in DIPG; for instance, rather than promoting stem cell renewal and tumorigenesis together with HOX genes in the ventral pons, the enhanced BMP signaling from mutant ACVR1 could promote astrogliocytic differentiation and mitotic quiescence (28–30). In such case, retinoid antagonists could be used (137) to boost BMP signaling (82).

It is certain then that continuous efforts to decipher and understand the cellular and molecular pathogenesis of DIPG and FOP will be of significant and mutually beneficial value and critical basic, biomedical and clinical relevance, ultimately providing renewed hope to patients and families alike that effective treatments for these exceedingly severe conditions can be, and are being, developed.

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Biographies

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**Highlights**

- ALK2, the product of *ACVRL1*, is a member of the type I bone morphogenetic protein (BMP) receptors
- ALK2 and the BMP signaling pathway have essential roles in skeletal and neural development and homeostasis
- Common activating *ACVRL1* mutations have been identified in the pediatric disorders FOP and DIPG
- Analysis and comparison of respective pathogenic mechanisms will help to identify therapeutics for these severe and often fatal conditions
Fig. 1.
Anatomical and histological characteristics of FOP and DIPG. (A–B) Images of an FOP patient showing the progression and increased severity of HO lesions along the back at indicated times. Images are from friendswithfop.com. (C) Histological organization of an HO lesion showing the presence of cartilage (arrow) and bone (double arrow) and reflecting the endochondral nature of HO in FOP. Section was stained with hematoxylin & eosin. (D) CT scans from a DIPG patient showing presence of the tumor mass in the pons (arrows). Images are from northwestern.edu/saratsis/.
Fig. 2.
Schematic showing the number and location of missense mutations in ACVR1 reported so far in DIPG and FOP. Note that all mutations found in DIPG tumors have been found in FOP patients with the exception of G328V (in red).
Fig. 3.
Schematics depicting possible roles of mutant ACVR1/ALK2 in the pathogenesis of FOP and DIPG. In FOP, mutations in ACVR1 are known to be necessary and sufficient to cause HO and skeletal problems typical of FOP. The mutations are likely to occur during gametogenesis or very early embryogenesis, and eventually work in concert with local ligands and inflammation (indicated by *) to initiate and sustain a cascade of events that start with recruitment of progenitor cells and culminate with formation of endochondral bone at extraskeletal sites over postnatal life. The various gene regulators controlling each step of the HO process are those previously shown to normally regulate endochondral bone formation in embryos and growing organisms and are assumed to be operating in HO as well. In DIPG, mutations in ACVR1 are currently thought to be not sufficient to cause tumor formation, and possible ligands for the mutant receptors remain unknown as well (indicated by **). The four pathogenic pathways shown here to which mutant ACVR1 could contribute are based on: previous extensive studies on genomic landscapes and transcriptome analyses in cohorts of DIPG patients; and roles that wild type ACVR1 is known to have in other developmental processes and systems. These pathogenic pathways are meant to suggest possible gene interactions that mutant ACVR1 could have with other known DIPG gene mutations/alterations and what specific cell functions and behaviors could in turn be affected.