

Full Length Article

Effects of FKBP12 and type II BMP receptors on signal transduction by ALK2 activating mutations associated with genetic disorders



Aiko Machiya^{a,b}, Sho Tsukamoto^{a,c}, Satoshi Ohte^a, Mai Kuratani^a, Mai Fujimoto^{a,b}, Keigo Kumagai^a, Kenji Osawa^{a,d}, Naoto Suda^b, Alex N. Bullock^e, Takenobu Katagiri^{a,c,*}

^a Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan

^b Division of Orthodontics, Department of Human Development and Fostering, Meikai University School of Dentistry, Saitama, Japan

^c Project of Clinical and Basic Research for FOP, Saitama Medical University, Saitama, Japan

^d Ivy Dental Clinic, Fukuoka, Japan

^e Structural Genomics Consortium, University of Oxford, Oxford, UK

ARTICLE INFO

Article history:

Received 17 November 2017

Revised 8 February 2018

Accepted 14 March 2018

Available online 15 March 2018

Keywords:

Activin receptor-like kinase 2 (ALK2)
Bone morphogenetic proteins (BMPs)
Fibrodysplasia ossificans progressiva (FOP)
Diffuse intrinsic pontine glioma (DIPG)
FKBP12
Signal transduction
Heterotopic ossification

ABSTRACT

Various substitution mutations in ALK2, a transmembrane serine/threonine kinase receptor for bone morphogenetic proteins (BMPs), have been identified in patients with genetic disorders such as fibrodysplasia ossificans progressiva (FOP), diffuse intrinsic pontine glioma (DIPG) and heart defects. In this study, we characterized the ALK2 mutants R258G, G328V and F246Y, which were identified in patients with severe FOP, DIPG and unusual hereditary skeletal dysplasia, respectively. Both R258G and G328V were gain-of-function mutations, but F246Y was equivalent to wild-type ALK2. We also examined the effect of the suppressor FKBP12 on the signal transduction of a further 14 ALK2 mutations associated with FOP and/or DIPG. To varying extents FKBP12 over-expression suppressed the basal signaling induced by thirteen of the ALK2 mutants, whereas PF197-8L was uniquely resistant. In the PF197-8L mutant, the modelled ALK2 residue L197 induced a steric clash with the D36 residue in FKBP12 and dissociated their interaction. The co-expression of BMP type II receptors or stimulation with ligands relieved the suppression by FKBP12 by disrupting the interaction between mutant ALK2 and FKBP12. Taken together, FKBP12 binds to and suppresses mutant ALK2 proteins associated with FOP and DIPG, except for PF197-8L.

© 2018 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Bone morphogenetic proteins (BMPs), members of the transforming growth factor- β (TGF- β) family, were originally identified in demineralized bone matrix during the induction of heterotopic ossification in soft tissues, such as skeletal muscle [1,2]. BMPs induce the osteoblastic differentiation of myoblasts in vitro [3]. Intracellular BMP signaling is activated by binding two types of transmembrane serine/threonine kinase receptors, type I and type II receptors [4,5]. Type I receptors, which have a unique glycine and serine rich domain called the “GS” domain at a juxtamembrane intracellular position, are inactive kinases in their basal state, whereas type II receptors are constitutively active kinases. In response to ligand binding to both receptor types, type II receptors phosphorylate type I receptors on the GS domain and activate type I receptor kinases [6–8]. FKBP12, a cytoplasmic FK506-binding protein 1A (FKBP1A), has been shown to bind the unphosphorylated GS domain of type I receptors to suppress their kinase activity [9,10]. Phosphorylation of the GS domain by type II

receptors in response to ligand stimulation releases FKBP12 and activates type I receptor kinases [9,10].

Patients with fibrodysplasia ossificans progressiva (FOP), which is a rare hereditary disorder, show progressive heterotopic ossification in soft tissues, such as skeletal muscle, tendons and ligaments, similar to the experimental bone induction using BMPs [11–16]. Genetic mutations in the intracellular domain of ALK2 (also known as ACVR1), one of the four types of BMP type I receptors, are associated with FOP. Twelve ALK2 mutations (L196P, P197_F198delinsL (PF197-8L), R202I, R206H, Q207E, R258S, G325A, G328E, G328R, G328W, G356D and R375P) have been identified in patients with typical and atypical FOP who presented with variations in clinical features [11,17–22]. Somatic ALK2 mutations were also identified in patients with diffuse intrinsic pontine glioma (DIPG), a rare childhood brain tumor disorder [23–26]. Mutations in ALK2 associated with DIPG, such as R206H, Q207E, R258G, G328E, G328W and G356D, are shared with FOP, except for G328V, which to date is unique in DIPG [4,27,28]. Additional ALK2 mutations, such as A15G, H286N, R370L and L343P, have also been found in patients with congenital heart diseases [29,30]. Recently, R258G and F246Y in ALK2 were identified in FOP patients with more severe reduction deficits of the hands and feet who exhibited the absence of nails,

* Corresponding author at: 1397-1 Yamane, Hidaka-shi, Saitama 350-1241, Japan.
E-mail address: katagiri@saitama-med.ac.jp (T. Katagiri).

progressive heterotopic ossification, hypoplasia of the brain stem, motor and cognitive developmental delays, atypical facial morphology, small malformed teeth and abnormal hair development and in a family with unusual skeletal dysplasia, respectively [31,32], but the functional protein changes were uncharacterized.

In patients with FOP, acute heterotopic ossification is induced by muscle injury through accidental trauma, biopsies or surgical treatment, suggesting that BMP signaling through mutant ALK2 is activated by a cooperation of the genetic mutations of ALK2 and uncharacterized additional mechanisms [12–15]. Diminished binding of the inhibitory protein FKBP12 has been suggested as a contributing factor in the progression of FOP [33,34], but its impact under different stimuli and across newly identified mutants has been incompletely addressed. Analysis of a crystal structure of the complex between ALK2 and FKBP12 reveals that the phenylalanine and leucine residues at positions 198 (F198) and 199 (L199) in ALK2 are the critical residues responsible for FKBP12 binding [35]. We previously characterized the mutant ALK2 proteins associated with FOP and DIPG except for G328V (twelve) and with heart disease (four) in vitro and classified the mutations as gain of function or loss of function [36]. The former twelve mutants were further activated by co-expressing BMP type II receptors, such as BMPR-II and ActR-IIB, in a phosphorylation-dependent manner. However the latter four mutants did not activate BMP activity even in the presence of type II receptors [36]. In this study, we characterized the recently identified ALK2 mutants and examined the roles of FKBP12 and BMP type II receptors in the signal transduction by ALK2.

2. Materials and methods

2.1. Plasmids

V5-tagged human ALK2 (wild-type, L196P, PF197-8L, R202I, R206H, Q207E, R258S, G325A, G328E, G328R, G328W, G356D and R375P), Myc-tagged murine ActR-IIB, FLAG-tagged human FKBP12, FLAG-tagged murine Smad1 and BMP-specific IdWT4F-luciferase reporter constructs were described previously [36–42]. Human BMPR-II was kindly provided by Dr. K. Miyazono (University of Tokyo, Tokyo, Japan). The other V5-tagged human ALK2 mutants F246Y, R258G and G328V were constructed with standard PCR techniques using KOD FX (Toyobo, Tokyo, Japan) and were confirmed by sequencing with an ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.2. Luciferase reporter assay and ALP assay

Murine C2C12 myoblasts maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesuque, Kyoto, Japan) containing 15% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) as previously described [36]. C2C12 cells were inoculated at 5×10^3 cells/well in 96-well plates one day before transfection. The cells were transfected with 200 ng/well plasmid DNA using 0.5 μ l/well Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions with a change to fresh OPTI-MEM (Thermo Fisher Scientific) with or without ligand [BMP7 (Milteny Biotec, Bergisch Gladbach, Germany) or activin A (Peprotech, Rocky Hill, NJ)] after 2.5 h [36,37]. The luciferase reporter assay was performed using 40 ng/ml IdWT4F-luc [37,41] and 10 ng/well phRL-SV40 (Promega, Madison, WI) for the normalization of transfection efficiency [36,37,41]. The firefly and Renilla luciferase activities were determined using the Dual-Glo Luciferase Assay Kit (Promega).

ALP activity was determined using a substrate solution (0.1 M diethanolamine, pH 10.0, and 1 mM $MgCl_2$ containing 1 mg/ml *p*-nitrophenylphosphate) at room temperature as described previously [36,43]. The reaction was terminated by adding NaOH, and the absorbance was measured at 405 nm.

2.3. Western blot analysis

Western blot analysis was performed as described previously [36,40,42]. Briefly, whole-cell extracts were prepared using a lysis buffer (10 mM Tris-HCl, pH 7.8, and 1% Triton X-100) containing 1 \times complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The extracts were separated by SDS-PAGE and were transferred to polyvinylidene difluoride membranes. Specific proteins were detected by immunoblotting using antibodies against the following proteins: phosphorylated Smad1/5 (rabbit monoclonal; Cell Signaling Technology, Danvers, MA), V5-tag (mouse monoclonal, clone V5005; Nacalai Tesuque), FLAG-tag (mouse monoclonal, clone M2; Sigma-Aldrich, St. Louis, MO), Smad1 (rabbit monoclonal; Cell Signaling Technology), Myc-tag (rabbit monoclonal; Cell Signaling Technology) and α -tubulin (rabbit polyclonal; Cell Signaling Technology). The target proteins were detected using a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). For co-immunoprecipitation assays, whole-cell extracts were incubated with magnetic beads conjugated to an antibody recognizing the FLAG-tag (clone M2, Sigma-Aldrich) for 3 h at 4 °C before separating by SDS-PAGE.

2.4. Statistical analysis

Comparisons were made using an unpaired ANOVA with Turkey-Kramer post-hoc test. The results were expressed as the mean \pm SD ($n = 3$). Statistical significance was indicated as * $P < 0.05$ and ** $P < 0.01$.

3. Results

Characterization of mutant ALK2 alleles R258G, G328V and F246Y, which are associated with severe FOP, DIPG and unusual skeletal dysplasia, respectively.

We characterized sixteen independent ALK2 mutants associated with FOP and heart disorders in vitro using C2C12 myoblasts [36]. In this study, we first characterized the three recently identified ALK2 mutants R258G, G328V and F246Y, which were identified in patients with severe FOP, DIPG and unusual familial skeletal dysplasia, respectively [23–26,31,32]. Transient over-expression of R258G or G328V without the addition of exogenous ligand induced BMP-specific luciferase reporter activity similar to R206H, a mutation associated with both FOP and DIPG (Fig. 1A). In contrast, F246Y showed minimal reporter activity similar to WT ALK2 (Fig. 1A). The basal reporter activities induced by R206H, R258G and G328V increased after treatment with BMP7 or activin A, although F246Y exhibited minimal reporter activity in response to BMP7 but not activin A, similar to WT (Fig. 1A). The expression levels of ALK2 proteins used were equivalent each other (Fig. 1B).

The activity of each ALK2 protein was examined with an alkaline phosphatase (ALP) assay in C2C12 cells. Confirming our previous reports, ALK2 over-expression alone was not sufficient to induce ALP activity in C2C12 cells (Fig. 1C). However, both G328V and R258G, similar to R206H, induced ALP activity when co-expressed with Smad1, BMPR-II or ActR-IIB (Fig. 1D–F). In contrast, F246Y failed to increase ALP activity in any of the examined conditions (Fig. 1C–F). ALP activity was increased by both BMP7 and activin A in the cells transfected with R206H, R258G and G328V but was not induced or slightly decreased in the WT and F246Y cells (Suppl. Fig. 1). These results suggest that both R258G and G328V were gain-of-function ALK2 mutations that were more strongly activated by BMP type II receptors, similar to other ALK2 mutants associated with FOP and DIPG, including R206H.

FKBP12 suppresses the BMP activity induced by mutant ALK2 associated with FOP or DIPG, except for the PF197-8L mutant

Next, we examined the effect of FKBP12, a suppressor of the TGF- β family type I receptor kinases, on the BMP activity induced by the fourteen ALK2 mutants associated with FOP and DIPG, including R258G and

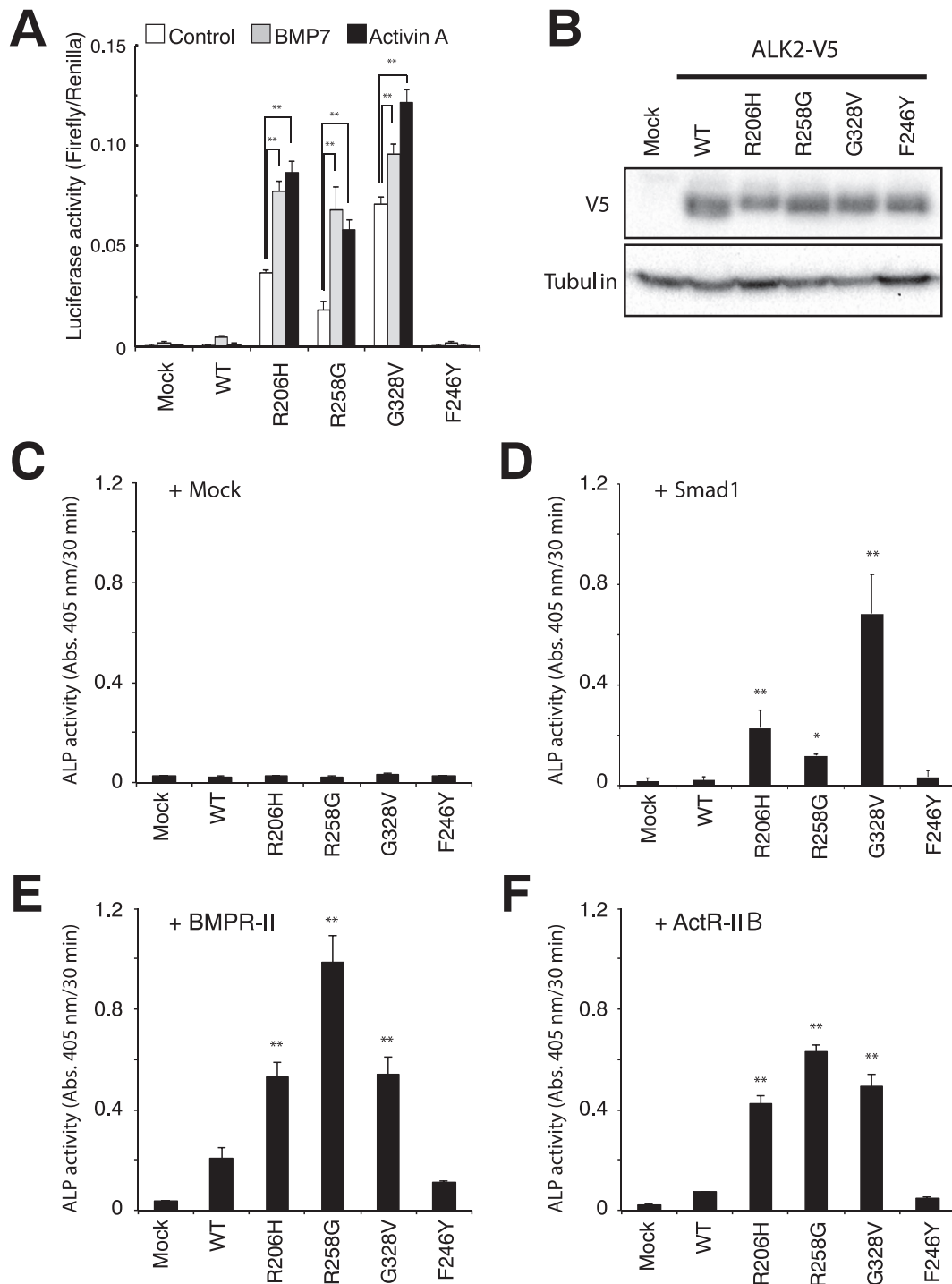


Fig. 1. Characterization of the ALK2 mutants R258G, G328V and F246Y, which are associated with severe FOP, DIPG and unusual familial skeletal dysplasia, respectively. (A) BMP-specific luciferase reporter assay to measure ALK2 induction. C2C12 cells were transfected with a luciferase reporter, *Id1WT4F-luc*, and each of the ALK2 constructs indicated in the figure were assayed without treatment (control, open columns) or with BMP7 (10 ng/ml, gray columns) or activin A (10 ng/ml, black columns) treatment. The reporter activity was determined on day 1. (B) Expression levels of ALK2 proteins in C2C12 cells. V5-tagged ALK2 was analyzed by Western blotting. Values are expressed as the mean \pm S.D. $n = 3$. *, $P < 0.05$ and **, $P < 0.01$. (C–F) ALP activity induced by mutant ALK2 in C2C12 cells. The cells were transfected with each of the ALK2 constructs indicated in the figure and a mock vector (C), Smad1 (D), BMPR-II (E) and ActR-II B (F). ALP activity was measured on day 3. Values are expressed as the mean \pm S.D. $n = 3$. *, $P < 0.05$ and **, $P < 0.01$ vs mock-transfected cells.

G328V. Co-expression of FKBP12 suppressed the all ALK2 mutants in the BMP-specific luciferase assay and the ALP assay in C2C12 cells (Fig. 2A and B). However, the signaling induced by PF197-8L was remained $>83\%$ in the presence of FKBP12 in both assay systems (Fig. 2A and B). R206H was dose-dependently suppressed by FKBP12 at ratios between 0.01 and 1.0 of FKBP12/ALK2 in C2C12 cells (Fig. 2C and D). In contrast, the BMP activity induced by PF197-8L was unchanged at FKBP12/ALK2

ratios of 1 and 0.3 in the luciferase and ALP assay, respectively (Fig. 2C and D). The resistance of PF197-8L to FKBP12 inhibition was further confirmed in an ALK2 kinase assay. The amount of phospho-Smad1 induced by R206H was reduced by FKBP12 co-expression (lanes 6 and 7 in Fig. 2E). However, the amount of phospho-Smad1 induced by PF197-8L was constant even in the presence of FKBP12 (lanes 8 and 9 in Fig. 2E). Overall, these results suggest that ALK2 mutants associated

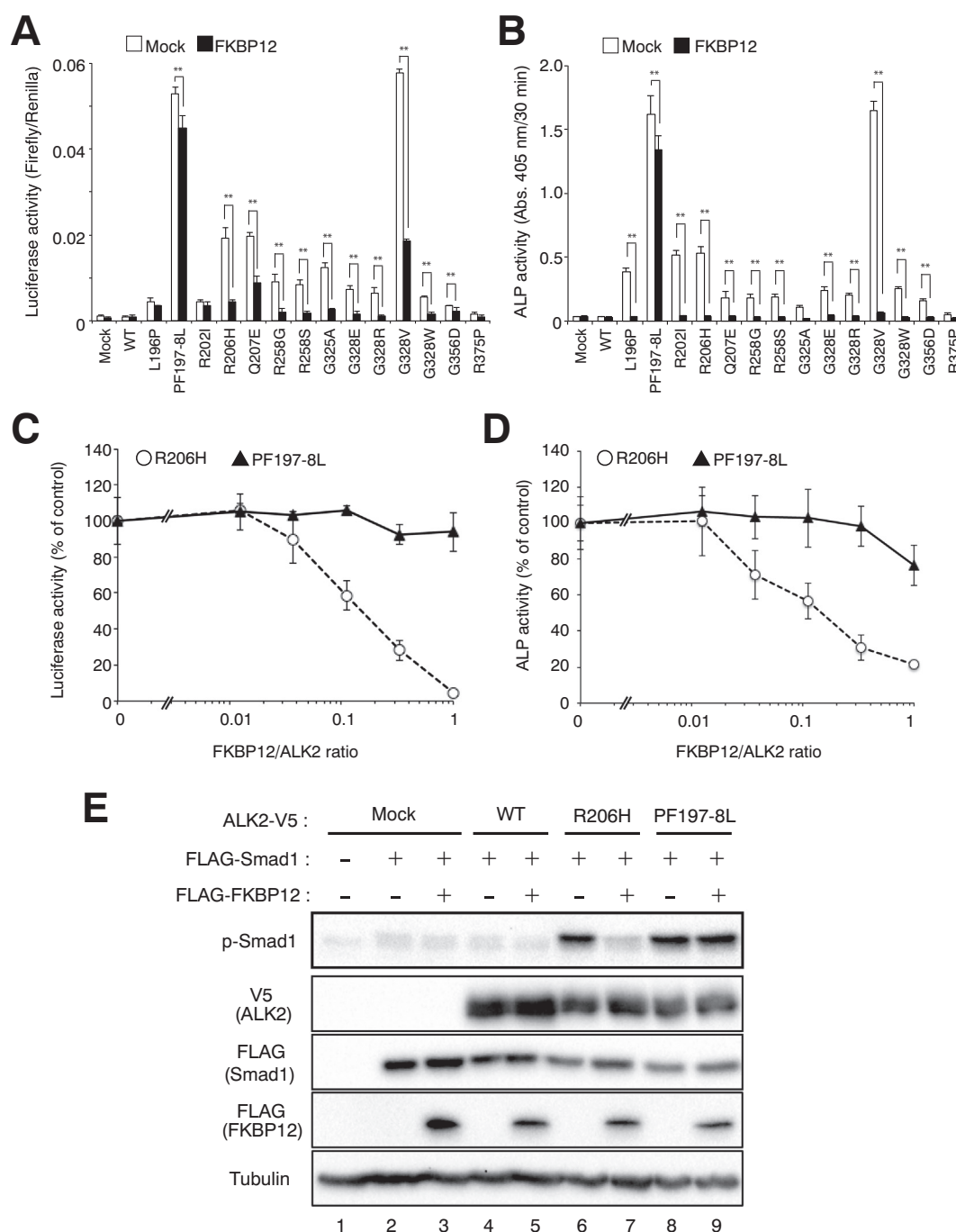


Fig. 2. Co-expression of FKBP12 suppresses the BMP activity induced by mutant ALK2, except for PF197-8L, associated with FOP and DIPG. (A and B) Effect of FKBP12 co-expression on the BMP-specific luciferase reporter (A) and ALP activity (B) induced by WT and mutant ALK2 in C2C12 cells determined on days 1 and 3 after transfection, respectively. Values are expressed as the mean \pm S.D. $n = 3$. *, $P < 0.05$ and **, $P < 0.01$. (C and D) Dose-dependent effects of FKBP12 on R206H and PF197-8L in the luciferase (C) and ALP (D) activity assays in C2C12 cells. C2C12 cells were co-transfected with an expression vector for R206H (open circles) or PF197-8L (closed triangles) with increasing amounts of FKBP12 (diluted with a mock vector plasmid). The total amounts of plasmid DNA transfected were fixed constant with a mock vector. The activities were expressed as a % of the control (without FKBP12). (E) Effect of FKBP12 on the kinase activity of ALK2 by Western blotting. FLAG-Smad1 and mock, WT, R206H or PF197-8L of V5-tagged ALK2 were co-transfected in C2C12 cells with and without FLAG-FKBP12. Phosphorylation levels of Smad1 and the expression levels of each protein were analyzed by Western blotting.

with FOP and DIPG were still suppressed by FKBP12 in vitro, but PF197-8L is resistant to FKBP12 inhibition.

The L197 residue in PF197-8L disrupts direct interaction with FKBP12.

In the PF197-8L mutant, both P197 and F198 residues in ALK2 are replaced with leucine at position 197 by an in-frame 3-bp deletion [18]. Interestingly, Chaikuad et al. [35] found that residues F198 and L199 in ALK2 directly interact with FKBP12. Thus, we examined the ability of WT, R206H and PF197-8L to bind FKBP12 in co-immunoprecipitation

experiments (Fig. 3A). Not only WT but also R206H co-precipitated with FKBP12 from whole-cell lysates (lanes 2 and 3 in Fig. 3A). However, PF197-8L was not detected in the FKBP12 precipitate despite an equivalent expression level to the other ALK2 constructs (lane 4 in Fig. 3A). Furthermore, we analyzed the effect of the PF197-8L mutation on the ability of ALK2 to bind FKBP12 using 3-dimensional structural modelling (Fig. 3B). In contrast to the P197 and F198 residues in WT (yellow sticks in Fig. 3B), the inserted leucine residue at position 197 (purple sticks in Fig. 3B) forms a steric clash with the D36 residue of FKBP12 (green in

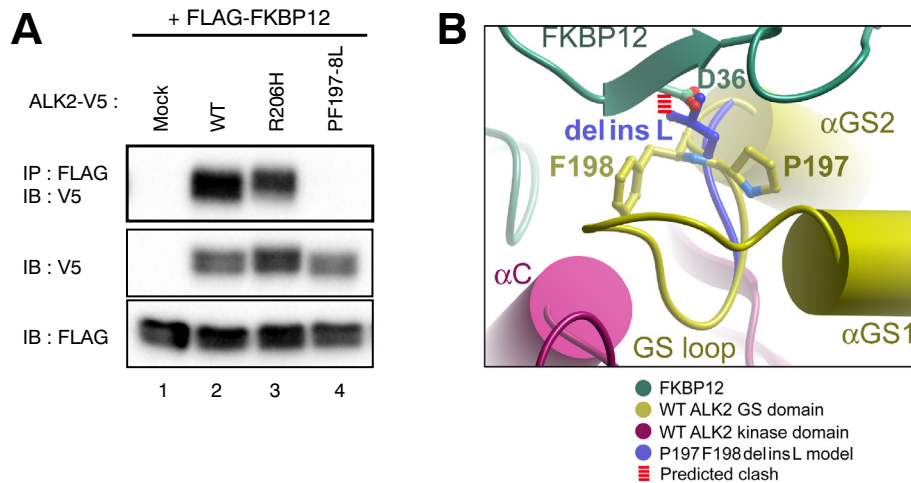


Fig. 3. PF197-8L does not bind FKBP12 due to steric hindrance between the L197 residue in ALK2 and the D36 residue in FKBP12. (A) Co-immunoprecipitation of ALK2 and FKBP12. HEK293A cells were co-transfected with V5/His-ALK2 and FLAG-FKBP12. Complexes formed with FKBP12 were immunoprecipitated from whole-cell extracts using an anti-FLAG antibody, followed by Western blotting analysis using anti-V5 and anti-FLAG antibodies. (B) A three-dimensional structural model of an interaction between WT or PF197-8L ALK2 and FKBP12. Note that, in contrast to WT (yellow), the L197 residue (purple) in PF197-8L disrupts the interaction with the D36 residue in FKBP12 (green). Homology modelling was performed using the ICM software (Molsoft) and PDB 3H9R as the structural template. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3B), thus hindering the interaction of the ALK2 PF197-8L mutant and FKBP12 (Fig. 3B). These findings confirm the importance of ALK2 residues P197 and F198 for FKBP12 binding [35].

Co-expression of the BMP type II receptor or ligand stimulation prevents FKBP12 suppression in mutant ALK2.

Although only PF197-8L was resistant to FKBP12 (Figs. 2 and 3), the patient carrying this mutation had similar clinical features to the other patients with FOP who carried different ALK2 mutations [18]. Thus, we examined the effect of FKBP12 on the fourteen ALK2 mutants, including PF197-8L, in the presence of BMP type II receptors *in vitro*. In contrast to experiments without BMP type II receptor expression (Fig. 2B), co-expression of FKBP12 failed to suppress ALP activity in C2C12 cells induced by any mutant ALK2 construct in the presence of BMPR-II or ActR-IIB (Fig. 4A and B). To examine the molecular mechanism of this phenomenon, we assessed the ALK2 interaction with FKBP12 in the absence or presence of BMPR-II and ActR-IIB (Fig. 4C and D). WT ALK2 bound FKBP12 irrespective of the presence of type II receptors. By contrast, R206H bound FKBP12 only in the absence of type II receptors, with the interaction disrupted upon the co-expression of either BMPR-II (Fig. 4C) or ActR-IIB (Fig. 4D). Again, PF197-8L did not bind FKBP12 in the absence or presence of type II receptors. Taken together, these results suggest that FKBP12 suppression is prevented by BMP type II receptors, which dissociate the ALK2-FKBP12 complex (Fig. 4E). This hypothesis was confirmed by ligand stimulation by BMP7 or activin A, in which both BMP-specific luciferase activity and ALP activity, induced by the mutant ALK2, were kept at high levels even in the presence of FKBP12 (Suppl. Figs. 2–5).

4. Discussion

In this study, we first characterized three novel ALK2 mutations, R258G, G328V, and F246Y, found in patients with severe FOP, DIPG and unusual familial skeletal dysplasia, respectively [23–26,31,32]. Similar to the other fourteen mutations associated with FOP and DIPG, both R258G and G328V are mildly activated gain-of-function mutations [36]. In contrast to R258G and G328V, F246Y was nearly equivalent to WT ALK2 in our experiments. Although F246Y was detected by whole-exome sequencing in a family with unusual skeletal dysplasia, mutations in other genes were also detected in these patients [32]. It is possible that a combination of F246Y in ALK2 and another mutation (s) caused skeletal dysplasia in the family. Further genetic and functional studies will be needed to elucidate this possibility.

In addition, we examined the role of FKBP12, a suppressor of the kinase activity of type I receptors in the TGF- β family, in the activation of fourteen independent ALK2 mutants associated with FOP and/or DIPG, including R258G and G328V. The reduction of the binding affinity to FKBP12 is suggested to be involved in the pathological phenotypes of patients with FOP [33,34]. We found that FKBP12 over-expression *in vitro* still suppressed the BMP pathway activity induced by the thirteen independent ALK2 mutants associated with FOP and DIPG. However, only PF197-8L was almost completely resistant to FKBP12. In this unique mutant, both P197 and L198 residues are replaced by the novel L197 residue [18]. Chaikuad et al. [35] reported that both F198 and L199 residues in ALK2 are critical residues for direct interaction with FKBP12. We confirmed that PF197-8L lost the ability to bind FKBP12 due to steric hindrance between the novel L197 residue and the D36 residue in FKBP12 (Fig. 3). It was interesting to note that the patient with the PF197-8L mutation had normal big toes and heterotopic ossification after a biopsy, which were similar or milder clinical phenotypes of FOP than those observed in patients with other ALK2 mutations [12–16,18]. Moreover, R258G was suppressed by FKBP12 *in vitro*, although this mutation in ALK2 was identified from FOP patients with more severe ankylosis and heterotopic ossification than other patients [31]. These discrepancies between the sensitivity of ALK2 to FKBP12 *in vitro* and the clinical features of FOP patients carrying each mutation *in vivo* suggest that mutant ALK2 activity is regulated not only by FKBP12 but also by an additional mechanism(s).

We previously reported that ALK2 mutants associated with FOP were hypersensitive to BMP type II receptor kinase, and the intracellular signaling of mutant ALK2 was further activated by type II receptors [36]. In this study, we found that the co-expression of BMP type II receptors, such as BMPR-II and ActR-IIB, prevented FKBP12-induced suppression in all the ALK2 mutants examined by disrupting the interaction between ALK2 and FKBP12. Recently, activin A, a member of the TGF- β family, was identified as a ligand that induces heterotopic ossification in patients with FOP in the R206H ALK2 mutant but not WT ALK2 [44,45]. In a mouse model of FOP encoding R206H, both dominant-negative type II receptors and an anti-activin A blocking antibody inhibit heterotopic ossification *in vivo* [44], suggesting that heterotopic ossification in FOP patients is a ligand (activin A)-dependent event. FKBP12 is released from the GS domain of type I receptors through phosphorylation by type II receptors in response to ligand stimulation [9,10]. In agreement with the previous reports, ligand stimulation by BMP7 and activin A prevented the inhibition of ALK2 by FKBP12. Thus, it is suggested that

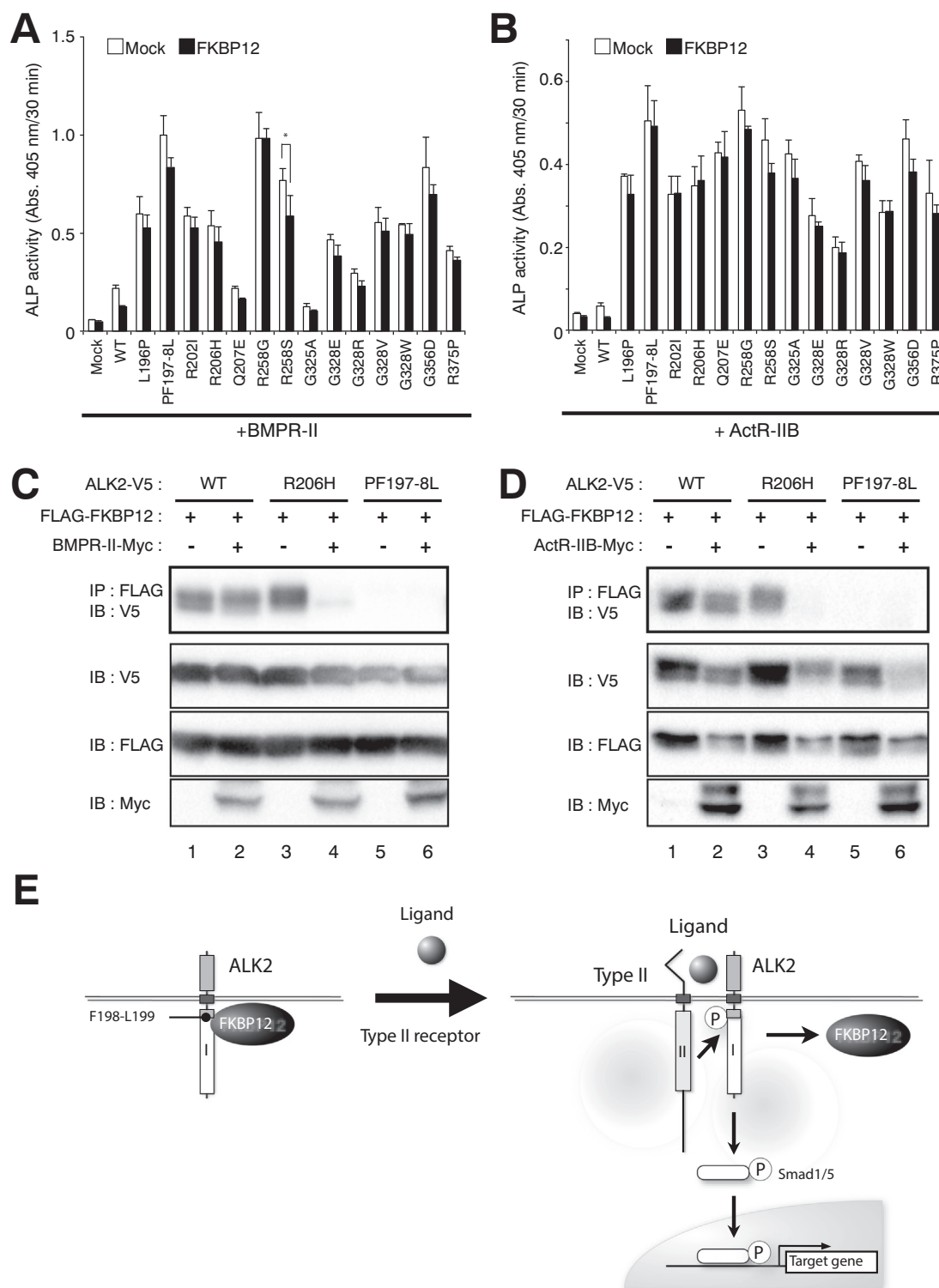


Fig. 4. Expression of BMP type II receptors prevents FKBP12 suppression in mutant ALK2. (A and B) ALP activity was determined in the presence of ALK2, FKBP12 and BMP type II receptor. C2C12 cells were co-transfected with 0.67 μ g of BMP type II receptor, BMPR-II (A) or ActR-IIB (B), 0.67 μ g of mutant ALK2 and 0.67 μ g of mock vector (open columns) or FKBP12 (closed columns). ALP activity was determined on day 3. Values are expressed as the mean \pm S.D. $n = 3$. *, $P < 0.05$ and **, $P < 0.01$. (C and D) The interaction of mutant ALK2 and FKBP12 in the presence of BMP type II receptor, BMPR-II (C) or ActR-IIB (D). HEK293A cells were co-transfected with V5-tagged ALK2 (WT, R206H and PF197-8L), FLAG-tagged FKBP12 and Myc-tagged BMPR-II or ActR-IIB. Whole-cell extracts were immunoprecipitated with an anti-FLAG antibody and were then analyzed by Western blotting. (E) A schema of our hypothesis.

heterotopic ossification is induced in FOP patients by reducing the FKBP12-dependent suppression of ALK2 through phosphorylation by BMP type II receptors in response to ligands.

In conclusion, FKBP12 directly binds and suppresses the BMP activity of mutant ALK2 associated with FOP and DIPG. FKBP12-dependent

suppression of mutant ALK2 was prevented by BMP type II receptors by disrupting the binding between FKBP12 and ALK2. Overall, these data suggest that FKBP12 can reduce basal signaling by mutant ALK2, but not the activin-induced neofunction that appears to drive FOP progression, particularly during episodes of inflammation.

Disclosure statement

TK received research grants from Daiichi-Sankyo, Co., Ltd. The other authors have nothing to disclose.

Funding

This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (17H04317 to TK, 17K11026 to ST, and 16K20067 to MK), a grant-in-aid from “Support Project of Strategic Research Center in Private Universities” from the MEXT to Saitama Medical University, Research Center for Genomic Medicine (S1311002 to TK), Maruki Memorial Award (No. 17-A-1-01 to TK) from Saitama Medical University, and Miyata Research Grant from Meikai University (AM). AM was a recipient of an award from the Iwaware Scholarship Foundation.

Acknowledgments

This work was supported by JSPS KAKENHI Numbers 17H04317 (TK), 17K11026 (ST) and 16K20067 (MK), a grant-in-aid from Support Project for the Formation of a Strategic Center in a Private University from Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) (S1311002 to TK) and the Maruki Memorial Award (No. 17-A-1-01 to TK). The SGC is a registered charity (number 1097737) that receives funds from AbbVie, Bayer Pharma AG, Boehringer Ingelheim, Canada Foundation for Innovation, Eshelman Institute for Innovation, Genome Canada, Innovative Medicines Initiative (EU/EFPIA) [ULTRA-DD grant no. 115766], Janssen, MSD, Merck KGaA, Novartis Pharma AG, Ontario Ministry of Economic Development and Innovation, Pfizer, São Paulo Research Foundation-FAPESP, Takeda and Wellcome [106169/ZZ14/Z].

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bone.2018.03.015>.

References

- [1] V.S. Salazar, L.W. Gamer, V. Rosen, BMP signaling in skeletal development, disease and repair, *Nat. Rev. Endocrinol.* 12 (2016) 203–221.
- [2] M.R. Urist, Bone: formation by autoinduction, *Science* 150 (1965) 893–899.
- [3] T. Katagiri, A. Yamaguchi, M. Komaki, E. Abe, N. Takahashi, T. Ikeda, V. Rosen, J.M. Wozney, A. Fujisawa-Sehara, T. Suda, Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage, *J. Cell Biol.* 127 (1994) 1755–1766.
- [4] T. Katagiri, S. Tsukamoto, M. Kuratani, Heterotopic bone induction via BMP signaling: potential therapeutic targets for fibrodysplasia ossificans progressiva, *Bone* (2017) <https://doi.org/10.1016/j.bone.2017.07.024>.
- [5] T. Katagiri, T. Watabe, Bone morphogenetic proteins, *Cold Spring Harb. Perspect. Biol.* 8 (2016), a021899.
- [6] L. Attisano, J. Cárcamo, F. Ventura, F.M. Weis, J. Massagué, J.L. Wrana, Identification of human activin and TGF type I receptors that form heteromeric kinase complexes with type II receptors, *Cell* 75 (1993) 671–680.
- [7] J.L. Wrana, L. Attisano, R. Wieser, F. Ventura, J. Massagué, Mechanism of activation of the TGF- β receptor, *Nature* 370 (1994) 341–347.
- [8] R. Wieser, J.L. Wrana, J. Massagué, GS domain mutations that constitutively activate T β R-I, the downstream signaling component in the TGF- β receptor complex, *EMBO J.* 14 (1995) 2199–2208.
- [9] T.W. Wang, P.K. Donahoe, A.S. Zervos, Specific interaction of type I receptors of the TGF- β family with the immunophilin FKBP-12, *Science* 265 (1994) 674–676.
- [10] T.W. Wang, B.Y. Li, P.D. Danielson, P.C. Shah, S. Rockwell, R.J. Lechleider, P.K. Donahoe, The immunophilin FKBP12 functions as a common inhibitor of the TGF β family type I receptors, *Cell* 86 (1996) 435–444.
- [11] E.M. Shore, M. Xu, G.J. Feldman, D.A. Fenstermacher, T.J. Cho, I.H. Choi, J.M. Connor, P. Delai, D.L. Glaser, M. LeMerrer, R. Morhart, J.G. Rogers, R. Smith, J.Y. Triffitt, J.A. Urtizberea, M. Zasloff, M.A. Brown, F.S. Kaplan, A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva, *Nat. Genet.* 38 (2006) 525–527.
- [12] J.A. Kitterman, S. Kantanie, D.M. Rocke, F.S. Kaplan, Iatrogenic harm caused by diagnostic errors in fibrodysplasia ossificans progressiva, *Pediatrics* 116 (2005), e654–e661.
- [13] T. Katagiri, Heterotopic bone formation induced by bone morphogenetic protein signaling: fibrodysplasia ossificans progressiva, *J. Oral Biosci.* 52 (2010) 33–41.
- [14] R.J. Pignolo, E.M. Shore, F.S. Kaplan, Fibrodysplasia ossificans progressiva: diagnosis, management, and therapeutic horizons, *Pediatr. Endocrinol. Rev.* 10 (Suppl. 2) (2013) 437–448.
- [15] T. Katagiri, A door opens for fibrodysplasia ossificans progressiva, *Trends Biochem. Sci.* 41 (2016) 119–121.
- [16] Y. Nakashima, N. Haga, H. Kitoh, J. Kamizono, K. Tozawa, T. Katagiri, T. Susami, J. Fukushi, Y. Iwamoto, Deformity of the great toe in fibrodysplasia ossificans progressiva, *J. Orthop. Sci.* 15 (2010) 804–809.
- [17] C.L. Gregson, O. Hollingworth, M. Williams, K.A. Petrie, A.N. Bullock, M.A. Brown, J.H. Tobias, J.T. Triffitt, A novel ACVR1 mutation in the glycine/serine-rich domain found in the most benign case of a fibrodysplasia ossificans progressiva variant reported to date, *Bone* 48 (2011) 654–658.
- [18] F.S. Kaplan, M. Xu, P. Seemann, J.M. Connor, D.L. Glaser, L. Carroll, P. Delai, E. Fastnacht-Urban, S.J. Forman, G. Gillissen-Kaesbach, J. Hoover-Fong, B. Köster, R.M. Pauli, W. Reardon, S.A. Zaidi, M. Zasloff, R. Morhart, S. Mundlos, J. Groppe, E.M. Shore, Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1, *Hum. Mutat.* 30 (2009) 379–390.
- [19] K.A. Petrie, W.H. Lee, A.N. Bullock, J.J. Pointon, R. Smith, R.G. Russell, M.A. Brown, B.P. Wordsworth, J.T. Triffitt, Novel mutations in ACVR1 result in atypical features in two fibrodysplasia ossificans progressiva patients, *PLoS One* 4 (2009), e5005.
- [20] R. Boccardi, D. Bordo, M. Di Duca, M. Di Rocco, R. Ravazzolo, Mutational analysis of the ACVR1 gene in Italian patients affected with fibrodysplasia ossificans progressiva: confirmations and advancements, *Eur. J. Hum. Genet.* 17 (2009) 311–318.
- [21] M.P. Whyte, D. Wenkert, J.L. Demertzis, E.F. DiCarlo, E. Westenberg, S. Mumm, Fibrodysplasia ossificans progressiva: middle-age onset of heterotopic ossification from a unique missense mutation (c.974G>C, p.G325A) in ACVR1, *J. Bone Miner. Res.* 27 (2012) 729–737.
- [22] H. Furuya, K. Ikezoe, L. Wang, Y. Ohyagi, K. Motomura, N. Fujii, J. Kira, Y. Fukumaki, A unique case of fibrodysplasia ossificans progressiva with an ACVR1 mutation, G356D, other than the common mutation (R206H), *Am. J. Med. Genet. A* 146A (2008) 459–463.
- [23] G. Wu, A.K. Diaz, B.S. Paugh, S.L. Rankin, B. Ju, Y. Li, X. Zhu, C. Qu, X. Chen, J. Zhang, J. Easton, M. Edmonson, X. Ma, C. Lu, P. Nagahawatte, E. Hedlund, M. Rusch, S. Pounds, T. Lin, A. Onar-Thomas, R. Huether, R. Kriwacki, M. Parker, P. Gupta, J. Becksfors, L. Wei, H.L. Mulder, K. Boggs, B. Vadodaria, D. Yergeau, J.C. Russell, K. Ochoa, R.S. Fulton, L.L. Fulton, C. Jones, F.A. Boop, A. Broniscer, C. Wetmore, A. Gajjar, L. Ding, E.R. Mardis, R.K. Wilson, M.R. Taylor, J.R. Downing, D.W. Ellison, J. Zhang, S.J. Baker, St. Jude Children's Research Hospital–Washington University Pediatric Cancer Genome Project, The genomic landscape of diffuse intrinsic pontine glioma and pediatric non-brainstem high-grade glioma, *Nat. Genet.* 46 (2014) 444–450.
- [24] P. Buczkowicz, C. Hoeman, P. Rakopoulos, S. Pajovic, L. Letourneau, M. Dzamba, A. Morrison, P. Lewis, E. Bouffet, U. Bartels, J. Zuccaro, S. Agnihotri, S. Ryall, M. Barszczyk, Y. Chornenky, M. Bourgey, G. Bourque, A. Montpetit, F. Cordero, P. Castelo-Branco, J. Mangerel, U. Tabori, K.C. Ho, A. Huang, K.R. Taylor, A. Mackay, A.E. Bendel, J. Nazarian, J.R. Fangusaro, M.A. Karajannis, D. Zagzag, N.K. Foreman, A. Donson, J.V. Hegert, A. Smith, J. Chan, L. Lafay-Cousin, S. Dunn, J. Hukin, C. Dunham, K. Scheinemann, J. Michaud, S. Zelcer, D. Ramsay, J. Cain, C. Brennan, M.M. Souweidane, C. Jones, C.D. Allis, M. Brudno, O. Becher, C. Hawkins, Genomic analysis of diffuse intrinsic pontine gliomas identifies three molecular subgroups and recurrent activating ACVR1 mutations, *Nat. Genet.* 46 (2014) 451–456.
- [25] K.R. Taylor, A. Mackay, N. Truffaux, Y.S. Butterfield, O. Morozova, C. Philippe, D. Castel, C.S. Grasso, M. Vinci, D. Carvalho, A.M. Carcaboso, C. de Torres, O. Cruz, J. Mora, N. Entz-Werle, W.J. Ingram, M. Monje, D. Hargrave, A.N. Bullock, S. Puget, S. Yip, C. Jones, J. Grill, Recurrent activating ACVR1 mutations in diffuse intrinsic pontine glioma, *Nat. Genet.* 46 (2014) 457–461.
- [26] A.M. Fontebasso, S. Papillon-Cavanagh, J. Schwartzentruber, H. Nikbakht, N. Gerges, P.O. Fiset, D. Bechet, D. Faury, N. De Jay, L.A. Ramkissoon, A. Corcoran, D.T. Jones, D. Sturm, P. Johann, T. Tomita, S. Goldman, M. Nagib, A. Bendel, L. Goumnerova, D.C. Bowers, J.R. Leonard, J.B. Rubin, T. Alden, S. Brown, J.R. Geyer, S. Leary, G. Jallo, L. Cohen, N. Gupta, M.D. Prados, A.S. Carret, B. Ellezam, L. Crevier, A. Klekner, L. Bogner, P. Hauser, M. Garami, J. Myseros, Z. Dong, P.M. Siegel, H. Malkin, A.H. Ligon, S. Albrecht, S.M. Pfister, K.L. Ligon, J. Majewski, N. Jhabdo, M.W. Kieran, Recurrent somatic mutations in ACVR1 in pediatric midline high-grade astrocytoma, *Nat. Genet.* 46 (2014) 462–466.
- [27] K.R. Taylor, M. Vinci, A.N. Bullock, C. Jones, ACVR1 mutations in DIPG: lessons learned from FOP, *Cancer Res.* 74 (2014) 4565–4570.
- [28] M. Fujimoto, N. Suda, T. Katagiri, Molecular mechanisms for activation of mutant activin receptor-like kinase 2 in fibrodysplasia ossificans progressiva, *J. Oral Biosci.* 59 (2017) 121–126.
- [29] K.A. Smith, I.C. Joziassie, S. Chocron, M. van Dinther, V. Guryev, M.C. Verhoeven, H. Rehmann, J.J. van der Smagt, P.A. Doevendans, E. Cuppen, B.J. Mulder, P. ten Dijke, J. Bakkers, Dominant-negative ALK2 allele associates with congenital heart defects, *Circulation* 119 (2009) 3062–3069.
- [30] I.C. Joziassie, K.A. Smith, S. Chocron, M. van Dinther, V. Guryev, J.J. van der Smagt, E. Cuppen, P. Ten Dijke, B.J. Mulder, C.L. Maslen, B. Reshey, P.A. Doevendans, J. Bakkers, ALK2 mutation in a patient with Down's syndrome and a congenital heart defect, *Eur. J. Hum. Genet.* 19 (2011) 389–393.
- [31] F.S. Kaplan, J.A. Kobori, C. Orellana, I. Calvo, M. Rosello, F. Martinez, B. Lopez, M. Xu, R.J. Pignolo, E.M. Shore, J.C. Groppe, Multi-system involvement in a severe variant of fibrodysplasia ossificans progressiva (ACVR1 c.772G>A; R258G): a report of two patients, *Am. J. Med. Genet. A* 167A (2015) 2265–2271.

- [32] M. Rafati, F. Mohamhashem, A. Hoseini, F. Hoseininasab, S.R. Ghaffari, A novel ACVR1 mutation detected by whole exome sequencing in a family with an unusual skeletal dysplasia, *Eur. J. Med. Genet.* 59 (2016) 330–336.
- [33] Q. Shen, S.C. Little, M. Xu, J. Haupt, C. Ast, T. Katagiri, S. Mundlos, P. Seemann, F.S. Kaplan, The fibrodysplasia ossificans progressiva R206H ACVR1 mutation activates BMP-independent chondrogenesis and zebrafish embryo ventralization, *J. Clin. Invest.* 119 (2009) 3462–3472.
- [34] J.C. Groppe, J. Wu, E.M. Shore, F.S. Kaplan, In vitro analyses of the dysregulated R206H ALK2 kinase-FKBP12 interaction associated with heterotopic ossification in FOP, *Cells Tissues Organs* 194 (2011) 291–295.
- [35] A. Chaikwad, I. Alfano, G. Kerr, C.E. Sanvitale, J.H. Boergermann, J.T. Triffitt, F. von Delft, S. Knapp, P. Knaus, A.N. Bullock, Structure of the bone morphogenetic protein receptor ALK2 and implications for fibrodysplasia ossificans progressiva, *J. Biol. Chem.* 287 (2012) 36990–36998.
- [36] M. Fujimoto, S. Ohte, K. Osawa, A. Miyamoto, S. Tsukamoto, T. Mizuta, S. Kokabu, N. Suda, T. Katagiri, Mutant activin-like kinase 2 in fibrodysplasia ossificans progressiva are activated via T203 by BMP type II receptors, *Mol. Endocrinol.* 29 (2015) 140–152.
- [37] T. Katagiri, M. Imada, T. Yanai, T. Suda, N. Takahashi, R. Kamijo, Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis, *Genes Cells* 7 (2002) 949–960.
- [38] T. Fukuda, K. Kanomata, J. Nojima, S. Kokabu, M. Akita, K. Ikebuchi, K. Miyazono, A unique mutation of ALK2, G356D, found in a patient with fibrodysplasia ossificans progressiva is a moderately activated BMP type I receptor, *Biochem. Biophys. Res. Commun.* 377 (2008) 905–909.
- [39] T. Fukuda, M. Kohda, K. Kanomata, J. Nojima, A. Nakamura, J. Kamizono, Y. Noguchi, K. Iwakiri, T. Kondo, J. Kurose, K.I. Endo, T. Awakura, J. Fukushi, Y. Nakashima, T. Chiyonobu, A. Kawara, Y. Nishida, I. Wada, M. Akita, T. Komori, K. Nakayama, A. Nanba, Y. Maruki, T. Yoda, H. Tomoda, P.B. Yu, E.M. Shore, F.S. Kaplan, K. Miyazono, M. Matsuoka, K. Ikebuchi, A. Ohtake, H. Oda, E. Jimi, I. Owan, Y. Okazaki, T. Katagiri, Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva, *J. Biol. Chem.* 284 (2009) 7149–7156.
- [40] J. Nojima, K. Kanomata, Y. Takada, T. Fukuda, S. Kokabu, S. Ohte, T. Takada, T. Tsukui, T.S. Yamamoto, H. Sasanuma, K. Yoneyama, N. Ueno, Y. Okazaki, R. Kamijo, T. Yoda, T. Katagiri, Dual roles of smad proteins in the conversion from myoblasts to osteoblastic cells by bone morphogenetic proteins, *J. Biol. Chem.* 285 (2010) 15577–15586.
- [41] S. Ohte, M. Shin, H. Sasanuma, K. Yoneyama, M. Akita, K. Ikebuchi, E. Jimi, Y. Maruki, M. Matsuoka, A. Namba, H. Tomoda, Y. Okazaki, A. Ohtake, H. Oda, I. Owan, T. Yoda, H. Furuya, J. Kamizono, H. Kitoh, Y. Nakashima, T. Susami, N. Haga, T. Komori, T. Katagiri, A novel mutation of ALK2, L196P, found in the most benign case of fibrodysplasia ossificans progressiva activates BMP-specific intracellular signaling equivalent to a typical mutation, R206H, *Biochem. Biophys. Res. Commun.* 407 (2011) 213–218.
- [42] S. Tsukamoto, T. Mizuta, M. Fujimoto, S. Ohte, K. Osawa, A. Miyamoto, K. Yoneyama, E. Murata, A. Machiya, E. Jimi, S. Kokabu, T. Katagiri, Smad9 is a new type of transcriptional regulator in bone morphogenetic protein signaling, *Sci. Rep.* 4 (2014) 7596.
- [43] K. Kodaira, M. Imada, M. Goto, A. Tomoyasu, T. Fukuda, R. Kamijo, T. Suda, K. Higashio, T. Katagiri, Purification and identification of a BMP-like factor from bovine serum, *Biochem. Biophys. Res. Commun.* 345 (2006) 1224–1231.
- [44] S.J. Hatsell, V. Idone, D.M. Wolken, L. Huang, H.J. Kim, L. Wang, X. Wen, K.C. Nannuru, J. Jimenez, L. Xie, N. Das, G. Makhoul, R. Chernomorsky, D. D'Ambrosio, R.A. Corpina, C.J. Schoenherr, K. Feeley, P.B. Yu, G.D. Yancopoulos, A.J. Murphy, A.N. Economides, ACVR1^{R206H} receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A, *Sci. Transl. Med.* 7 (2015), 303ra137.
- [45] K. Hino, M. Ikeya, K. Horigome, Y. Matsumoto, H. Ebise, M. Nishio, K. Sekiguchi, M. Shibata, S. Nagata, S. Matsuda, J. Toguchida, Neofunction of ACVR1 in fibrodysplasia ossificans progressiva, *Proc. Natl. Acad. Sci. U. S. A.* 112 (2015) 15438–15443.