AMPK Downregulates ALK2 via Increasing the Interaction between Smurf1 and Smad6, leading to inhibition of in vitro Osteogenic Differentiation

Hui Lin[1,2], **Ying Ying**[1,2], **Yuan-Yuan Wang**[2], **Gang Wang**[3], **Shan-Shan Jiang**[1,2], **Deiang Huang**[4], **Lingyu Luo**[4], **Ye-Guang Chen**[5], **Louis C. Gerstenfeld**[6], and **Zhijun Luo**[1,2]

[1]Jiangxi Province Key laboratory of Tumor Pathogens and Molecular Pathology and Department of Pathophysiology, Schools of Basic Sciences and Pharmaceutical Sciences, Nanchang University Medical College, Nanchang, China

[2]Department of Biochemistry, Cell Biology and Genomics, Boston University School of Medicine, Boston, MA 02118

[3]Department of Cardiology, Boston Children's Hospital, 300 Longwood Avenue, Boston, MA 02115

[4]The Institute of Digestive Diseases, The First Affiliated Hospital, Nanchang University, Nanchang, China

[5]School of Life Sciences, Tsinghua University, Beijing China

[6]Department of Orthopaedic Surgery, Boston University School of Medicine, Boston, MA

Abstract

Activin A receptor type I or activin receptor-like kinase 2 (ACVRI/ALK2) belongs to type I TGF-β family and plays an important role in bone development. Activating mutations of ALK2 containing the R206 to H mutation, are present in 95% in the rare autosomal genetic disease fibrodysplasiaossificansprogressiva (FOP), which leads to the development of ectopic bone formation in muscle. The effect of AMP-activated protein kinase (AMPK) activation on ALK2R206H-mediated signaling in fibroblast obtained from a FOP patient was assessed in the present study. The activity of the mutated ALK2 was suppressed by pharmacological AMPK activators such as metformin and aspirin, while their actions were blocked by the dominant negative mutant of AMPK and mimicked by the constitutively active mutant of AMPK. Furthermore, activation of AMPK upregulated Smad6 and Smurf1 and thereby enhanced their interactions, resulting in its proteosome-dependent degradation of ALK2. In contrast, knockdown of Smad6 or Smurf1 prevented metformin-induced reduction of ALK2. To evaluate the biological relevance of AMPK action on ALK2 activity, we induced FOP fibroblasts within iPS cells and

Correspondence addressed to: Zhijun Luo, 72 East Concord Street, K302, Boston, MA02118. zluo@bu.edu, zluo559914@gmail.com.

found that their osteogenic differentiation *in vitro* was inhibited by metformin. Our studies provide novel insight into potential approaches to treatment of FOP, since several AMPK activators (e.g. metformin, berberine, and aspirin, etc.) are already in clinical use for the treatment of diabetes and metabolic syndromes.

Keywords

Introduction

Bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily. There are approximately 20 different BMPs found in mammals that are grouped into four subfamilies based on their sequence similarity and functions[1]. Seven type I receptors have been identified termed activin receptor-like kinase 1 to 7 (Alk1 to Alk7), among which, BMPs preferentially bind to the Alk1 to 3 and 6, while other members of TGF-β superfamily such as β1, 2, 3 and activins bind to the Alk5 and Alk4[2]. Four type I (Alk1, BMPR-IA/Alk3, BMPR-IB/Alk6, and Alk2) and three type II BMP receptors (BMPRII, activin type II receptor-ACVR-II or Act-II, and ACVR-IIB or Act-IIB) are able to bind to BMPs. Once BMPs bind to the complex of type I and type II receptors, the type II receptor phosphorylates and activates the type I receptor. The phosphorylated receptors in turn activate Smad-dependent and independent signaling cascades that lead to concerted regulation of the osteogenic program. The inhibitory Smad6 blocks signaling pathways through multiple mechanisms including: (1) competitive binding to the type I receptor or Smad1/5/8 to disrupt their interaction; (2) sequestration of Smad1/5/8 to Smad 4; (3) recruitment of Smurf1, a U3 ubiquitin ligase that would target prosomal degradation of either the receptor I, Smad1/5, or Runx2 [3–5].

Fibrodysplasiaossificansprogressiva (FOP) is a devastating genetic disorder that causes progressive heterotopic ossification (HO) that develops through a process of endochondral bone formation, occurring within muscle, tendon, and ligamentous tissues throughout the body, which results over time in the immobilization of the patient[6, 7]. FOP is attributed to autosomal mutations in activin A receptor type I/activin receptor-like kinase 2 (ACVRI/ALK2), a BMP type I receptor [8]. Mutation rate is very rare, one out of two million people. Most of patients carry a heterozygous germ-line mutation in the *ACVRI* gene with an arginine to histidine substitution at amino acid 206 (R206H) located in the glycine-serine-rich domain of ALK2. Studies have shown that this mutation causes constitutive activation of the receptor. Intriguingly, knock-in studies of activating mutants of ALK2 or transgenic over expression of BMP4 in mice can recapitulate phenotypes of FOP at varying degrees [9–12].

AMPK is an intracellular fuel sensing enzyme consisting of three subunits (α, β, γ). The enzyme is activated under circumstances by which a cell senses energy crisis such as hypoxia and glucose restriction, while it is suppressed in nutrient surplus. When energy is short, AMP or the ratio of AMP to ATP is increased, leading to AMP binding to the γ

regulatory subunit of AMPK, enabling phosphorylation of T172 in the activation loop of the α catalytic subunit by upstream kinases including LKB1 and preventing dephosphorylation of this site, causing full activation of the enzyme [13]. The activation of AMPK inhibits glucose production in the liver, stimulates glucose uptake in the muscle and adipose and reduces lipogenesis [14]. Thus, the net effect of AMPK activation is to alleviate hyperglycemia, hyperlipidemia and hyperinsulinemia. As such, AMPK has emerged as a therapeutic target for type 2 diabetes and metabolic syndromes [14]. Indeed, pharmacological activators of AMPK such as metformin are first line anti-diabetic drug. Several other drugs that are used in human, such as berberine, resveratrol, and salicylate [14] have similar actions on AMPK. In addition, AMPK can be activated by many other activators including hormones, cytokines and especially, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), the first canonical activator that is a cell permeable agent and is phosphorylated by a nucleoside kinase at the plasma membrane and converted to ZMP, an analog of AMP[15].

Previous studies have suggested a role of AMPK in osteogenesis. On one hand, several reports have indicated that AMPK promotes osteogenic differentiation in contrast to its negative effect on adipogenesis[16–22]. On the other hand, there are opposite findings; for instances, one study reported that AMPK activity is reduced during differentiation of osteoblasts and forced activation of AMPK suppressed the differentiation [23]. A most recent study reinforces the negative role of AMPK in osteoblast differentiation[24]. This elegant study has shown that the increase of glucose uptake is prerequisite for the differentiation. This is in part through suppression of AMPK, as the latter induces proteasome-dependent degradation of Runx2 through phosphorylation and activation of Smurf1. Additionally, a few studies reported that a negative effect of AMPK on differentiation of osteoclasts[25–27].

We have recently found that AMPK inhibits TGF-β signaling and EMT in cancer cells [28]. This led us to hypothesis that AMPK would inhibit BMP signaling. Thus, we selected fibroblast cells carrying the ALK2 R206H mutation obtained from a FOP patient and treated the cells with different AMPK activators to assess the effect of AMPK on skeletogenic stem cell differentiation.

Material and Methods

Reagents

Metformin, phenformin, aspirin, 5-amino-4-imidazole carboxamide riboside (AICAR) MG132 and Alizarin red S, and monoclonal antibody against flag epitope were purchased from Sigma-Aldrich (St. Louis, MO). A769962 was from LC Laboratories (Woburn, MA). Human recombinant BMP6 was purchased from BioLegend (San Diego, CA). Customized oligonucleotides, siRNAs, Lipofectamine 2000 and StemPro® Osteogenesis Differentiation Kit were from Life Technologies (Grand Island, NE). Antibodies against phospho-smad1/5 (Ser463/465), total smad1, phospho-AMPKα (T172) and total AMPKα were from Cell Signaling Technology, Inc. (Danvers, MA). Monoclonal antibodies against LKB1 and β-actin and Alkaline Phosphates Detection Kit were from EMD Millipore Corporation (Billerica, MA). Antibodies against ALK2, Smad6 and Smurf1 were from Santa Cruz

Biotechnology (Santa Cruz, CA). Luciferase kit was from Promega Life Sciences (Madison, WI).

Cell culture

Human dermal fibroblast cells (GM00513) were obtained from Coriell Institute (Camden, NJ) and maintained in Eagle's alpha minimal essential medium (αMEM) supplemented with 15% fetal bovine serum (FBS) and penicillin/streptomycin. HEK293T cells and mouse embryonic fibroblast (MEF) cells were cultured in DMEM supplemented with 10% FBS and antibiotics. All cells were cultured in $5\% CO_2$ at $37°C$.

siRNA Transfection

siRNAs for Smad6 (AM16708, 4390771) and Smurf1 (4390824) and scrambled siRNA (AM4611) were purchased from Life Technologies. siRNAs were transfected into the FOP fibroblast cells using Lipofectamine 2000 according to the manufacturer's instructions.

Firefly luciferase complementation assay

Complementation plasmids were constructed according to So et al.[29] In brief, luciferase was divided to aminoterminal (NLuc, aa1-475) and carboxyterminal (CLuc, 265–550) portions, and each of ALK2, Smad6, Smurf1, or Smad1 was fused to NLuc (HindIII and BamHI cloning sites) or CLuc (HindIII and BamHI or KpnI sites). cDNAs for NLuc and CLuc were already in pCDNA3.1. Primers were designed and cDNAs for inserts of interest amplified and cloned into the vectors. The plasmids were then transfected into HEK293T cells by the calcium phosphate precipitation method. Luciferase complementation was assayed on different plasmid pairs to select the optimal pairs to be tested. Luciferase activity was assayed according to protocol provided by manufacture.

Generation of iPSCs derived from human dermal fibroblast cells (HDF)

FOP fibroblast cells (GM00513) at low passages were used for iPS cell reprogramming[30]. One microgram each of three episome plasmids, pCXLE-hOCT3/4-shp53 (expressing OCT3/4 and p53 shRNA), pCXLE-hSK (expressing SOX2 and KLF4), and pCXLE-hUL (expressing L-MYC and LIN28), were electroporated into the cells (1×10^6) with Nucleofector reagent (Lonza) in 100 μl according to the manufacturer's instructions. The cells were trypsinized 6 days after transfection and seeded into 6-well plate coated with growth factor-reduced Matrigel (150–300 μg/ml, 30 min coating) (BD Biosciences). Next day, the culture medium was replaced with standard human iPS medium containing FGF(10ng/ml). The colonies started to appear in about 3–4 weeks after plating, which were picked up based on the morphology and used for further amplification and evaluation.

All pluripotent cell clones were maintained in mTeSR1 medium (StemCell Technologies, Vancouver, Canada) on growth factor-reduced Matrigel-coated plates. The ROCK inhibitor Y-27632 (StemCell Technologies, Vancouver, Canada) dissolved in DMSO was added to mTeSR1 at passage and removed on the following day.

Alkaline phosphatase (ALP) activity

The ALP activity was determined using an Alkaline Phosphatase Detection Kit (EMD Millipore, SCR004) following the manufacturer's instruction. In brief, iPS cells were fixed in 4% paraformaldehyde for 1–2 minutes and washed with PBS. ALP staining solution (Naphthol/Fast Red Violet) was added to cover cells and incubated in dark at room temperature for 15 minutes. The staining solution was removed and washed with distilled water. The cells were examined under a light microscope for ALP activity and images were acquired.

Osteogenic differentiation

Control iPS cells (ALK2 WT) and FOP iPS cells (ALK2 R206H) were cultured in StemPro® Osteogenesis Differentiation medium for different days and the medium replenished every 3 days. The cells were stained in Alizarin red S solution and microscopic photo digital images were taken at 40× magnification. Alizarin deposit was extracted in solution containing 0.5N HCL and 5% SDS, and quantified at OD_{405} absorbance.

Western blot analysis

Cell extracts were prepared in lysis buffer (25 mM Tris–HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 and 25 mM β-glycerol-phosphate, 1 mM DTT, 1% NP-40 and protease inhibitors). The cell debris was removed by centrifugation at $14,000 \times g$ at 4 °C for 15 min and protein concentration measured using a Bio-Rad Protein Assay kit. Protein samples (20 μg) were subjected to SDS-PAGE and electrophoretically transferred to PVDF membranes (EMD Millipore). The membranes were sequentially blotted with the first and second antibodies, and developed by the enhanced chemiluminescence (ECL) method.

Adenovirus Preparation

Adenoviruses encoding the constitutively active mutant, dominant negative mutant of human AMPKα1 subunit, and GFP were prepared as described previously [28].

Statistical analysis

Significance of differences among groups was determined by two tailed student t test. P<0.05 was set for significance.

Results

Inhibition of ALK2 by AMPK

To assess if AMPK plays a role in regulation of BMP-signaling, we obtained fibroblast cells (GM00513) from a FOP patient. Sequencing analysis confirmed heterozygous mutation of ALK2 (R206H). We then treated the cells with different activators of AMPK (metformin, phenformin, A769962, AICAR) at concentrations optimal for activation of AMPK (Figure 1). The data showed that Smad1/5 phosphorylation was inversely related to activation of AMPK (Figure 1A). If the cells were treated with these activators prior to BMP6 incubation, the induction of Smad1/5 phosphorylation was markedly diminished (Figure 1B). Next, we focused on metformin, as it has been used in clinical treatment of type 2 diabetes and

metabolic syndromes and informative outcomes might be insightful for expanding its use in the treatment of FOP. Thus, we examined the dose and time effects of metformin and found that it inhibited Smad1/5 phosphorylation in the FOP fibroblast cells in both dose- and time-dependent fashion, in parallel to activation of AMPK (Figure 1C, 1D). Similar but more robust effects were observed with AICAR (Supplementary Figure 1)

To ascertain if the effect of metformin is mediated by AMPK, we performed the following assays: first, we assessed the effect of metformin on the response of mouse embryonic fibroblast cells (MEF) to BMP6 (Figure 2A); second, we infected adenovirus expressing a constitutively active AMPK mutant (Figure 2B) or dominant negative AMPK mutant (Figure 2C). The results showed that metformin diminished BMP6-induced phosphorylation of Smad1/5 in MEF cells, which was abolished by ablation of LKB1 or AMPK catalytic subunits (Figure 2A). When FOP fibroblasts were infected by constitutively active AMPK α1 subunit, our data revealed that Smad1/5 phosphorylation was progressively inhibited by increasing volumes of adenovirus, concurrently with decreases in abundance of ALK2, whereas no such effects were observed with adenovirus encoding GFP (Figure 2B). We then infected the dominant negative mutant of AMPKα1 and treated the FOP fibroblasts with metformin overnight, followed with BMP6 for 30 min. As shown in Figure 2C, the inhibitory effect of metformin observed with GFP adenovirus was diminished by the dominant negative mutant of AMPK. Hence, the results from Figures 1 and 2 indicate that AMPK inhibits Smad1/5 phosphorylation induced by mutated ALK2 or BMP6.

AMPK upregulates Smad6 and Smurf1

Figure 2B demonstrates that ALK2 abundance is decreased by the active mutant of AMPK. This was not due to inhibition of RNA transcription as we found no differences in qPCR analysis (data not shown). Thus, we tested if AMPK regulates Smad6 and/or Smurf1 abundance as this complex binds to ALK2 and targets it for proteasome degradation. Toward this end, the FOP fibroblast cells were treated with metformin in different doses for 24 hours or different periods of time at 10mM, and alternatively with aspirin which is used as a second AMPK activatorat different doses for 24 hours. Our data revealed that reduction of ALK2 by treatment with metformin or aspirin was associated with increases in Smad6 and Smurf1 (Figure 3).

To assess if increased abundance of Smad6 and Smurf1 leads to changes in their interaction, we engineered two constructs; the first one fusing Smad6 to the aminoterminal portion of firefly luciferase (Smad6-NLuc) and Smurf1 to the carboxy terminal portion of the luciferase (Smurf1-CLuc) (Supplementary Figure 2). The two constructs were cotransfected into HEK293T cells and then treated with or without phenformin, a potent AMPK activator with similar mechanism to metformin (but the latter failed to activate AMPK in HEK293T cells), for 8 hours. The cell extracts were then prepared for the luciferase activity assay. As shown in Figure 4A, the luciferase activity reconstitution assays revealed that the interaction between Smad6 and Smurf1 was enhanced significantly by phenformin ($p<0.05$). Similar assay was conducted with ALK2 and Smad1. Figure 4B shows that the interaction between ALK2 and Smad1 was markedly diminished by phenformin ($p<0.01$).

We transfected Smad6 siRNA into the FOP fibroblast cells in order to examine if knockdown of Smad6 prevented ALK2 degradation triggered by AMPK. The results showed that ALK2 was upregulated and appeared to be resistant to metformin treatment after Smad6 knockdown (Figure 5A). A similar result was obtained with Smurf1 siRNA. To assess if the effect of AMPK is associated with proteosomal degradation of ALK2, we incubated the FOP fibroblast cells with MG132, a proteosome inhibitor, along with metformin for 24 hours. We observed that MG132 increased abundance of ALK2 and abrogated the effect of metformin (Figure 5C). Similarly, MG132 also blunted the effect of the active mutant of AMPKα1 (Figure 5D).

AMPK inhibits osteogenic differentiation of iPS cells derived from FOP fibroblast cells

Weinduced the FOP fibroblast cells to iPS cells by electroporation of episomal plasmids carrying OCT3/4 (plus p53 shRNA), SOX2, KLF4, C-MYC, and LIN28. The iPS clones were isolated, amplified and characterized by Western blot, clonal morphology, alkaline phosphatase staining (Supplementary Figure 3). After confirmation of successful induction and isolation of the iPS clones, they were induced to differentiate into osteogenic cells in the differentiation medium for 21 days. The cells were collected and assayed for mineralization using alizarin red S and expression of osteoblastic markers during the course of induction (Supplementary Figure 3). The results showed that the cells with ALK2 R206H exhibited slightly better differentiation than that with wild type ALK2, as reflected by mineralization assay and Western blot analysis of differentiation markers. To test the effect of AMPK, metformin was added during induction of differentiation. As shown in Figure 6, the data revealed that metformin greatly diminished mineralization. They also showed that metformin significantly inhibited osteogenic differentiation of iPS cells bearing wild type ALK2, which was probably due to the inhibition of BMP signaling, as previous studies reported that BMP2 was induced during osteogenic differentiation [2, 31].

Discussion

FOP is a debilitating disease with the development of extensive systemic HO. Although the incidence is very low, it is incurable and lethal. Current clinical management relies mostly on anti-inflammatory drugs that are at best only partially effective. An ideal treatment strategy would be to target components of the BMP signaling pathway inasmuch as a critical pathogenic factor is the activating mutations of the *ACVRI* allele. However, inhibitors targeting activated ALK2 and downstream components are still in scarcity and at the experimental stage. Therefore, an innovative approach would be to identify clinically available drugs having well defined safety profiles that are used in treating other diseases and re-tasking them to fulfill this clinical need. Toward this end, the present study is designed to assess the impact of some of the clinically used AMPK activators that have proven to be safe, such as metformin and aspirin, on the ALK2-mediated signaling events. We showed that AMPK activated by these agents or genetic manipulations inhibited Smad1/5 phosphorylation in FOP fibroblast cells carrying the ALK2 R206H mutation. Furthermore, we found that the inhibitory effect of AMPK was mediated by upregulation of Smurf1 and Smad6, which leads to increased interaction of these two molecules and subsequent degradation of ALK2. Finally, our data revealed AMPK activation also

suppressed BMP-signaling in iPS cells derived from the FOP fibroblasts and iPS osteogenic differentiation *in vitro*. These data therefore are the first to show that AMPK suppresses BMP signaling, which might have an important implication in the treatment of FOP patients.

Since heterozygous mutation of the *ACVR1* gene was identified to be responsible for FOP in 2006, several attempts have been made to target this signaling pathway in treating this disease using animal models or cell based assays. Thus, two small molecular compounds, dorsomorphin and LDN-193189, have been shown to bind and inhibit ALK2 and reduce the incidence of HO in transgenic mice carrying *ALK2* mutation[32]. Secondly, siRNA specifically targeting the mutated site of ALK2 R206H has been used to suppress osteoblast differentiation [33, 34]. Thirdly, all-trans retinoic acid (RA) and PARγ agonists have also been reported to inhibit HO in transgenic mice [35]. These agents reduce the level of Smads and BMP-induced expression of Id1 [35] All these agents are currently at the experimental stage, and their toxicity, efficacy and pharmacodynamics have not as yet been fully tested. Hence, an alternative approach to the development of totally novel inhibitors to this pathway, would be to identify compounds that are already in use clinically and have proven safety profiles. A recent study of Cappato et al [36] using high throughput screen identified dipyridamole, which was shown to inhibit the expression of ALK2, inhibit osteoblast differentiation *in vitro*, and block heterotopic bone formation *in vivo*. This study could be immediately translated into clinical application as dipyridamole is in clinical use as a platelet anti-aggregate.

It has been known that AMPK can be activated by a variety of pharmacological agents, such as metformin, aspirin, berberine, and resveratrol that are used in treatment of type 2 diabetes and metabolic syndromes. These agents are inexpensive and have been proven to be safe. Hence, our finding that AMPK suppresses BMP signaling would lead to opening of a new avenue for the potential treatment of FOP and other types of heterotopic ossifications. The next step will be to extend our study to animal models. In fulfilling this task, there are several available conditional knock-in or transgenic mouse models in which constitutively active ALK2 is expressed [9, 11]. In addition, induction of iPS cells derived from FOP to osteoblast differentiation and transplantation of the differentiated cells into nude mice has emerged as another type of model that is useful in studying the mechanism of HO, and for testing efficacy of drugs in the treatment of FOP [37, 38]. As such, it will be intriguing to apply clinically available AMPK activators to these FOP models. A positive outcome would prompt fast route of clinical trial.

We have shown that increased Smad1/5 phosphorylation in FOP fibroblasts bearing ALK2 R206H while activated AMPK suppresses ALK2. However, we have not tested if other ALKs are regulated by AMPK in the fibroblast cells used in the present study. It is not surprising if other BMP-regulated ALKs are also downregulated in fibroblast cells, as they are regulated by Smad6/Smurf1. It has been known that different ALKs execute different functions[1]. For example, ALK1 plays an important role in angiogenesis and vascularization, while ALK2 mediates the effect of BMPs on osteogenesis. In fact, our recent publication has shown that AMPK activation inhibits ALK1-mediated angiogenesis via upregulation of Smad6 and Smurf1[39]. Our present study adds new information on AMPK regulation of osteogenic differentiation through induction of ALK2 degradation.

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Conclusion

Our present study has shown that metformin and other AMPK activators can inhibit ALK2-mediated signaling and associated osteogenic differentiation of iPS cells derived from FOP fibroblast cells. The inhibition was ascribed to upregulation of Smurf1 and Smad1 and increased interaction of these two molecules, leading to proteosomal degradation of ALK2. Our finding is of translational potential inasmuch as several pharmacological AMPK activators have been used in clinical treatment of other diseases such as diabetes, obesity and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

ACVRI/ALK2	activin A receptor type I/activin receptor-like kinase 2
ALP	alkaline phosphatase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
BMP	bone morphogenetic protein
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
FGF	fibroblast growth factor
FOP	Fibrodysplasiaossificansprogressiva
HDF	human dermal fibroblast cells
HO	heterotopic ossifications
iPS	induced pluripotent stem cell
LKB1	Liver kinase B1
Smurf1	SMAD Specific E3 Ubiquitin Protein Ligase 1
TGF-β	Transforming growth factor beta
Smad6	SMAD Family Member 6

SOX2	sex determining region Y)-box 2
KLF4	Kruppel-like factor 4
C-MYC	a protooncogene, V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
Lin28	Lin-28 homolog A
RA	retinoic acid
ZMP	5- amino-4-imidazolecarboxamide ribotide

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Highlights

- Fibrodysplasiaossificansprogressiva is a devastating genetic disease which is caused by a R206 to H mutation in ALK2

- AMPK inhibits either mutant ALK2, or wild ALK activated with BMP6

- AMPK causes proteosomal degradation of ALK2 by upregulation of Smurf1 and Smad6

- AMPK inhibits osteogenic differentiation of iPS cells derived from FOP fibroblast cells

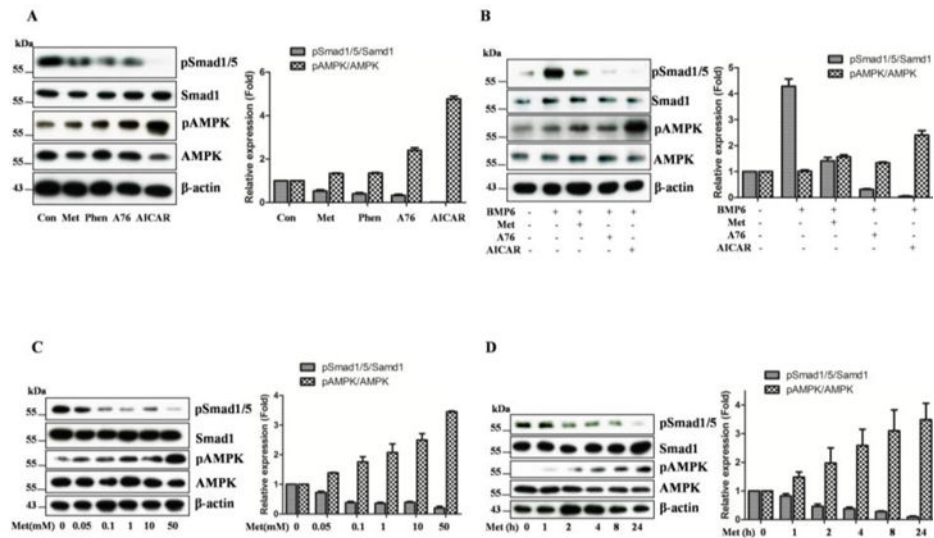- Metformin may be useful in the treatment of FOP patients

Figure 1. Inhibition of ALK2 signaling in FOP fibroblast cells by AMPK activators
A. FOP fibroblast cells were treated with metformin (Met, 10mM), phenformin (Phen, 1mM), A769962 (A76, 10μM), AICAR (1mM) for 8 hours, or left without treatment (Con). B. The fibroblasts were treated with metformin, A769962, or AICAR for 24 hours, followed by BMP6 (50ng/ml) for 30 min. C. The cells were treated with different doses of metformin. D. The cells were treated with metformin (10mM) for different periods of time. Equal amounts of cell extracts (20μg) were blotted with antibodies as indicated. Representative blots were presented and graphs represent scan densitometric ratio of bands from three independent blots (average ratio±SD).
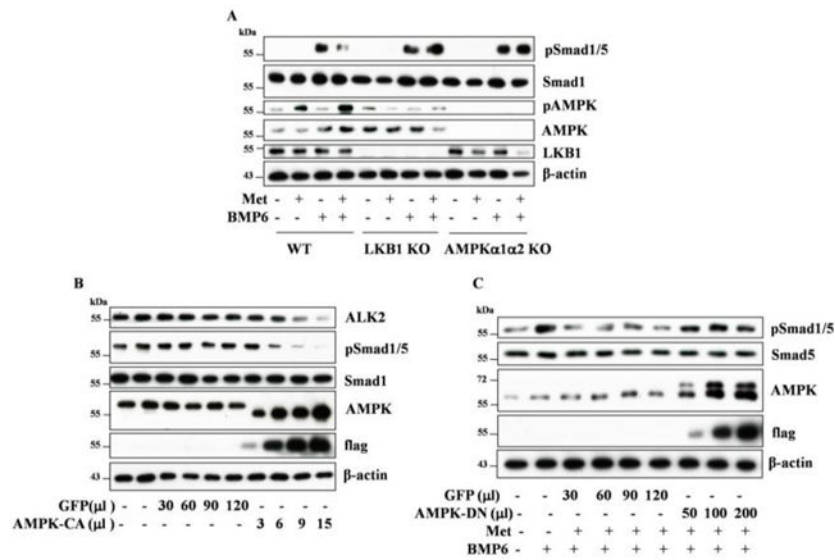
Figure 2. AMPK mediates the inhibitory effect of metformin on ALK2 signaling
A. MEF cells, wildtype, LKB1 KO, or AMPKα1 & α2 KO, were treated with metformin (10mM) for 24 hours, followed by BMP6 (25ng/ml) for 30 min. B–C. FOP fibroblasts were infected with adenovirus in varying volumes for 48 hours. The virus expresses an active mutant of AMPKα1 (AMPK-CA) (B) or a dominant negative mutant of AMPKα1 (AMPK-DN) (C), both tagged with the flat epitope, or GFP as a control. The cells were treated with or without metformin (10mM) for 24 hours and followed with BMP6 (50ng/ml) for 30 min. Equal amounts of cell extracts (20μg) were blotted with antibodies, as indicated.
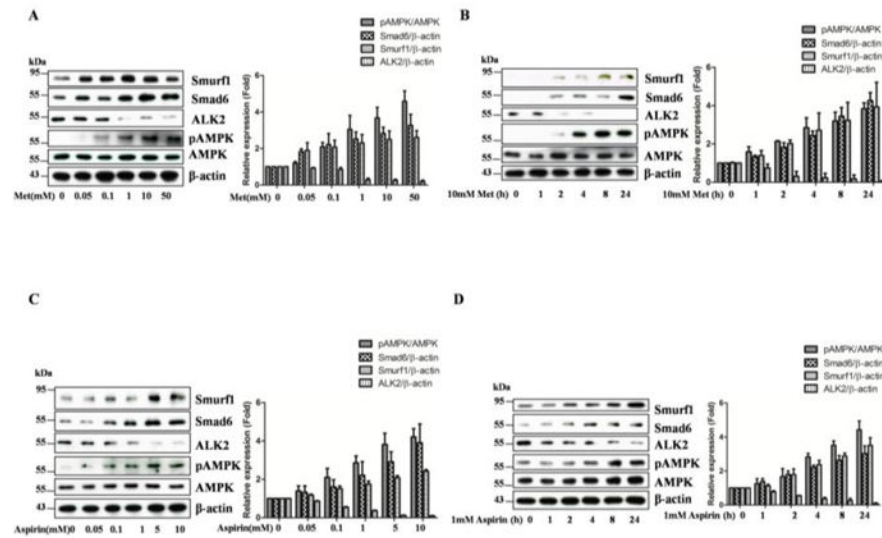
Figure 3. AMPK upregulates Smad6 and Smurf1

FOP fibroblast cells were treated with metformin (A, B) or aspirin (C) at varying concentrations for 24 hours (A, C) or different times (B, D). Equal cell extracts (20µg) were resolved by Western blot with antibodies, as indicated. Representative blots were presented and graphs represent scan densitometric ratio of bands from three independent blots (average ratio±SD).
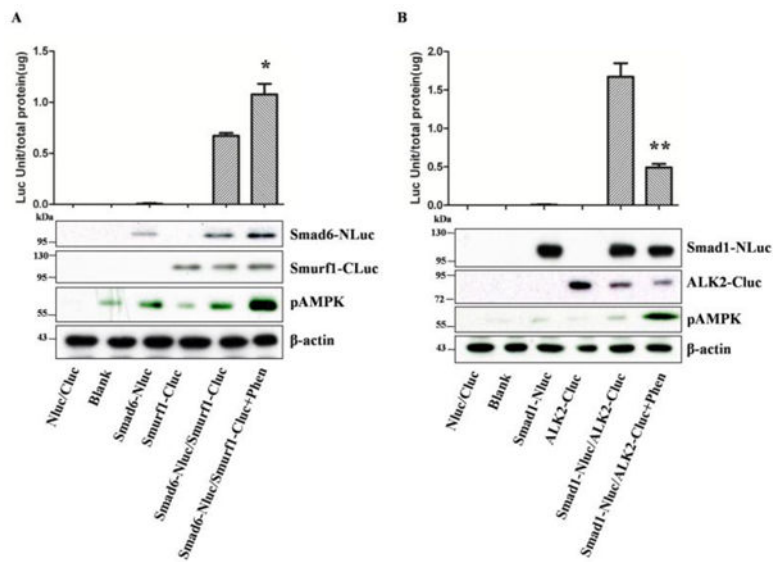
Figure 4. AMPK regulates the interaction of key components of the ALK signaling pathway
Transfection of plasmid pairs as indicated was performed with plasmids into HEK293T cells and 36 hours later, the cells were treated with or without phenformin (1mM) for 8 hours. Luciferase assay was conducted and normalized with total protein (units/μg). A. phenformin enhances Smad6 and Smurf1 interaction. B. phenformmin inhibits ALK2 and Smad1 interaction. The results represent averages of a triplicate experiment (means±SD). *P=0.0482<0.05, **p=0.0007<0.01. Representative blots show expression of recombinant proteins.
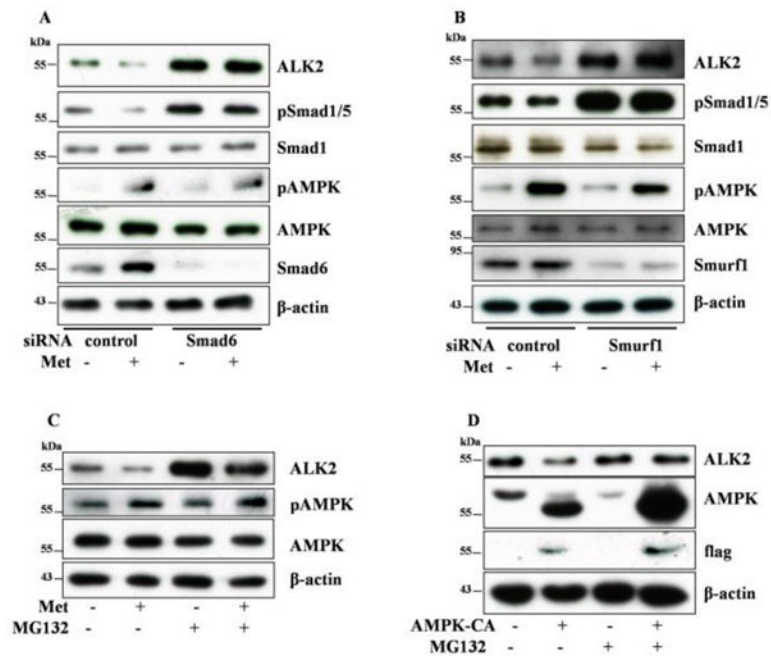
Figure 5. AMPK promotes proteosomal degradation of ALK2

A–B. Scrambled siRNA (control) and siRNAs for Smad6 (A) or Smurf1 (B) were transfected into FOP fibroblast cells and then treated with or without metformin (10mM) for 24 hours. C–D. FOP fibroblast cells were treated with or without MG132 and/or metformin for 24 hours (C), or after infection with adenovirus expressing the active mutant of AMPK (AMPK-CA), the cells were incubated with MG132 for 24 hours (D). Cell extracts were then blotted with antibodies, as indicated.
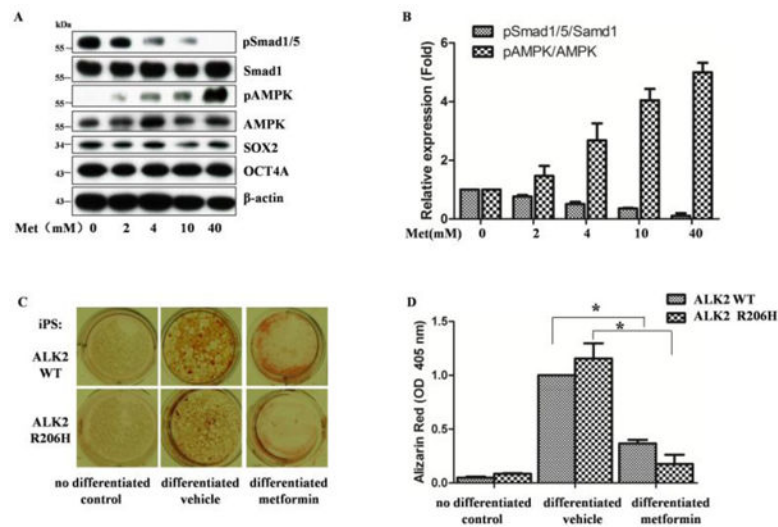
Figure 6. Metformin inhibits osteogenic differentiation of iPS cells
A. metformin inhibits Smad1/5 phosphorylation of iPS cells. The cells were treated with metformin at different doses for 24 hours and blotted with antibodies as indicated. B. Graphs represent scan densitometric unit ratio of related bands in A from three independent experiments (average ratio±SD).C. osteogenic differentiation was induced in the presence or absence of metformin (10 mM). Mineralization is detected as red staining with Alizarin Red S. D. Graph represents alizarin deposit quantitation from three independent experiments using spectrophotometer. ALK2WT (metformin vs vehicle,*p=0.0009), ALK2R206H (metformin vs vehicle, *p=0.0012).